A REGULATORY ROLE FOR PROLINE METABOLISM IN *Arabidopsis thaliana* (L.) Heynh.

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PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, from February 1995 to September 1998, under the supervision of Doctor W.A. Cress and the co-supervision of Professor J. van Staden.

These studies represent work done by the author and have not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it is duly acknowledged in the text.

PDHare

Peter Derek Hare
September 1998

"Missing from a collection of data and theories of individual enzymes is their relationship to one another. This relationship is generated by the fact that enzymes are functionally coupled. The product of one enzymic transformation is the substrate for another. The latter, in turn, has its product, which again, is a substrate for another transformation, and so on. We symbolise these couplings by drawing maps of "pathways" intertwined in complex ways that "begin" and "end" at the edges of the cell. By drawing these sequences in two-dimensional space, however, we are in danger of ignoring the essence from which they were derived. In the living cell, each of the arrows in our map is in fact a *rate* of transformation of substrate(s) into product(s). The map (if complete) shows us all the relationships between the molecules in the cell but is silent about the quantitative effect of such interactions."

H Kacser (1987) Control of Metabolism. *In* Davies DD, The Biochemistry of Plants, Vol. 11 (Biochemistry of Metabolism), pp. 40-41.

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ABSTRACT

Many plants accumulate organic osmolytes in response to the imposition of environmental stresses that cause cellular dehydration. Of these, proline is the most extensively studied. Conclusive demonstration that this imino acid acts as a compatible solute which mediates osmotic adjustment has yet to be achieved, although a causal relationship between increased proline synthesis and plant tolerance of hyperosmotic stresses has previously been demonstrated. It is proposed that in many plants, the metabolic implications of the regulated increase in proline synthesis and/or a decline in proline degradation during stress may play a more important role in acclimation to adverse conditions than the simple accumulation of the end-product of these adjustments. In particular, the stress-induced increase in the transfer of reducing equivalents into proline by Δ^1-pyrroline-5-carboxylate (P5C) synthetase (P5CS) and P5C reductase (P5CR) may be a protective mechanism whereby many species ameliorate shifts in cellular redox potential which accompany all biotic and abiotic stresses which cause proline accumulation, including those that do not cause cellular dehydration.

The presence of several putative stress-regulated promoter elements in the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes of *Arabidopsis thaliana* strongly implicates an adaptive role for stress-induced increases in proline synthesis in this species. Sequence homologies of several regions within the 5' untranslated regions of these genes to promoter elements which have been shown to participate in redox control of gene expression, the actions of phytochrome and hormones, and tissue-specific regulation of gene expression are also identified. These provide useful indicators both of the mechanisms by which proline synthesis is regulated and how these may relate to its importance in maintaining metabolic homeostasis.

In an attempt to resolve the functionality of proline accumulation under stress, chimeric antisense genes comprising 1050 bp and 999 bp fragments of *Arabidopsis* cDNAs encoding AtP5CS1 and AtP5CR respectively were inserted in the reverse orientation between the CaMV 35S promoter and the *GUS* gene (encodes β-glucuronidase) in the plant transformation vector pBI121. These constructs were introduced separately into *Arabidopsis* by cocultivation with *Agrobacterium tumefaciens* strains carrying the pBI-P5CS1(AS) and pBI-P5CR(AS) plasmids. Transgenic plants, which were selected on the basis of kanamycin resistance, regenerated at a low frequency in the presence of 1 mM proline. Transformation of 13 pBI-P5CS1(AS) and 7 pBI-P5CR(AS) lines was confirmed by PCR-mediated amplification of gene fragments within the introduced T-DNA. Segregation ratios for kanamycin resistance indicated that most of the lines have multiple T-DNA insertions. Transformants were characterised with respect to their growth rates and free proline

content. In at least two pBI-P5CS1(AS) transformants and two pBI-P5CR(AS) transformants, a reduction in root growth rates in the presence of inhibitory concentrations of NaCl correlated with reduced β-glucuronidase activity relative to transgenic lines that were no more sensitive to NaCl than were controls. A reduction in root growth rate both in the absence and presence of hyperosmotic stress was noted in two pBI-P5CS1(AS) transformants, designated A5 and B12. In 14 day-old plants of the T_2 generation of both A5 and B12, free proline levels were significantly lower than in wild-type plants both in the absence of stress and following 24 h incubation in either 250 mM NaCl or 550 mM sorbitol or at 5 °C. In both lines, reduced growth rates in the absence of osmotic stress could be restored by exogenous proline, but not by exogenous glutamate. When used at isosmotic concentrations, sorbitol caused a larger reduction in free proline levels in both A5 and B12 than did NaCl. This observation may relate to an ABI1-mediated post-transcriptional effect on *AtP5CS1* gene expression which affects NaCl-, but not sorbitol-mediated proline accumulation in *Arabidopsis*. Post-transcriptional regulation of the expression of the genes involved in proline biosynthesis may account, at least partly, for the absence of dramatic phenotypic effects in any of the pBI-P5CS1(AS) or pBI-P5CR(AS) lines.

Under the premise that regulation of shifts in proline metabolism regulate cellular redox potential under conditions of stress may be mirrored by the involvement of proline metabolism in modulating metabolism during normal growth and development, the effects of exogenous proline on *Arabidopsis* seed germination, seedling growth and *in vitro* shoot organogenesis were investigated. A dose-dependent inhibition of radicle emergence by millimolar concentrations of proline could be overcome by the artificial oxidants methylene blue and phenazine ethosulphate. Assays of the rate-limiting dehydrogenases of the oxidative pentose phosphate pathway (OPPP), as well as changes in the contributions of $^{14}C_1$- and $^{14}C_6$-labelled glucose to respired CO_2 during germination, are consistent with activation of the OPPP during *Arabidopsis* seed germination. An approximately four-fold increase in free proline, which peaked at the time of radical emergence, was not parallelled by changes in other amino acids and could not be ascribed to degradation of seed storage proteins. Delayed radical emergence in T_2 generation seeds of the pBI-P5CS1(AS) lines A5 and B12 correlated with an approximately 35% reduction in the maximal concentration of proline accumulated during germination.

Millimolar concentrations of exogenous proline had a dose-dependent inhibitory effect on *Arabidopsis* seedling growth both in the light and in darkness. This reduction in growth arises at least in part from a decline in cell elongation. Accordingly, exogenous proline increased total extractable peroxidase activity in *Arabidopsis* seedlings through the selective induction of peroxidase isoforms. Histochemical analysis of the hypocotyls of plants grown in the presence of exogenous proline suggested that proline increased the levels of lignin and/or the phenolic

precursors thereof. A dose-dependent decrease in extractable chlorophyll and damage to chloroplastic and mitochondrial ultrastructure was observed in 21 day-old *Arabidopsis* seedlings grown in the presence of millimolar concentrations of exogenous proline. *In vitro* shoot organogenesis from *Arabidopsis* hypocotyl explants was stimulated by 1 mM proline, and to a lesser extent by 5 mM proline, but inhibited by inclusion of 10 mM proline in the hormonally-supplemented regeneration media. The ability of low concentrations of proline analogues (azetidine-2-carboxylate and thioproline) to overcome the stimulatory effect of 1 mM proline, and a slight increase in the stimulative effects of 1 mM proline by D-proline, are consistent with an important role for the interconversions of proline and its precursors in regulating cell division and differentiation.

Together, these data strongly support an important role for the interconversions of proline and its precursors in the regulation of intermediary metabolism under both normal and stressful conditions. These findings draw into question the widely accepted, although poorly investigated, hypothesis that proline is an inert compatible solute that can be accumulated to high levels with minimal effects on cellular metabolism. The novel proposal that stress-induced changes in proline metabolism exert a regulatory effect though an influence on the level of reduction of the cellular NADP pool is discussed in relation to recent evidence that a signal related to proline synthesis and/or degradation selectively increases the expression of stress-induced plant genes.

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ABBREVIATIONS

ABA	-	abscisic acid
ABRE	-	ABA response element
ADH	-	alcohol dehydrogenase
AMP	-	adenosine monophosphate
APX	-	ascorbate peroxidase
AS	-	asparagine synthetase
AuxRE	-	auxin-responsive promoter element
AZC	-	L-azetidine-2-carboxylic acid
BA	-	N^6-benzyladenine
βGlu	-	β-1,3-glucanase
bp	-	base pairs
bHLH-ZIP	-	basic helix-loop-helix leucine zipper
bZIP	-	basic-region leucine zipper
$[Ca^{2+}]_c$	-	concentration of cytosolic free Ca^{2+}
CAB	-	chlorophyll *a*/*b* binding protein (light harvesting complex protein)
cADPR	-	cyclic ADP-ribose
CaM	-	calmodulin
CAM	-	Crassulacean acid metabolism
cAMP	-	adenosine 3':5'-monophosphate
CaMV35S	-	promoter of Cauliflower mosaic virus gene encoding 35S rRNA
cDNA	-	complementary DNA
CDPK	-	Ca^{2+}-dependent protein kinase
cGMP	-	cyclic guanosine monophosphate
CHS	-	chalcone synthase
CHX	-	cycloheximide
CIM	-	callus-inducing medium
CK	-	cytokinin
COR	-	cordycepin
2,4-D	-	2,4-diphenoxyacetic acid
DAG	-	1,2-diacylglycerol
DHAR	-	dehydroascorbate reductase
dH_2O	-	distilled water
DHP	-	3,4-dehydroproline
DRE	-	dehydration response element (C-repeat)
DTT	-	dithiothreitol
EST	-	expressed sequence tag
Fd	-	ferredoxin
FR	-	far-red light
FW	-	fresh weight
GBF	-	G-box binding factor
GDH	-	glutamate dehydrogenase

γ-GK	-	γ-glutamyl kinase
GM	-	germination medium
GOGAT	-	glutamate synthase
GPD	-	glycerol-3-phosphate dehydrogenase
GPR	-	γ-glutamyl phosphate reductase
GPX	-	glutathione peroxidase
G6PDH	-	glucose-6-phosphate dehydrogenase
GR	-	glutathione reductase
GRA	-	GC-rich *RAB* activator
GS	-	glutamine synthetase
GSA	-	glutamic-γ-semialdehyde
GSH	-	*reduced glutathione*
GSSG	-	glutathione disulphide (oxidised form of GSH)
GST	-	glutathione *S*-transferase
GUS	-	β-glucuronidase
HD-ZIP	-	homeodomain-leucine zipper
HPLC	-	high performance liquid chromatography
HR	-	hypersensitive response
HXK	-	hexokinase
IAA	-	indole-3-acetic acid
i.d.	-	inner diameter
INPS	-	*myo*-inositol 1-phosphate synthase
IP_3	-	inositol 1,4,5-trisphosphate
JA	-	jasmonic acid
Kan^r	-	kanamycin resistant
Kan^s	-	kanamycin sensitive
kb	-	kilobase
LB	-	Luria-Bertani (medium)
LUC	-	luciferase
MAPK	-	mitogen-activated protein kinase
MB	-	methylene blue
MDHAR	-	monodehydroascorbate reductase
MJ	-	methyl jasmonate
MS	-	Murashige and Skoog (1962) medium
MS/2	-	half-strength Murashige and Skoog (1962) medium
$NAADP^+$	-	nicotinic acid adenine dinucleotide phosphate
NPTII	-	neomycin phosphotransferase type II
NTR	-	NADPH-dependent TRX reductase
OAT	-	ornithine δ-aminotransferase
OKA	-	okadaic acid
OPPP	-	oxidative pentose phosphate pathway (hexose monophosphate shunt)
ORE	-	OxyR response element (mammalian Y-box)
ORF	-	open reading frame
PAGE	-	polyacrylamide gel electrophoresis
PAI	-	phosphoribosylanthanilate isomerase

PAL	-	phenylalanine ammonia-lyase
PAO	-	phenylarsine oxide
PCNA	-	proliferating cell nuclear antigen
PCO	-	photosynthetic carbon oxidation
PCR	-	polymerase chain reaction
P2C	-	Δ^1-pyrroline-2-carboxylate
P5C	-	Δ^1-pyrroline-5-carboxylate
P5CDH	-	Δ^1-pyrroline-5-carboxylate dehydrogenase
P5CR	-	Δ^1-pyrroline-5-carboxylate reductase
P5CS	-	Δ^1-pyrroline-5-carboxylate synthetase
PDH	-	proline dehydrogenase
PEG	-	polyethylene glycol
PEPCase	-	phospho*enol*pyruvate carboxylase
PES	-	phenazine ethosulphate
PHYA	-	phytochrome A
PKC	-	protein kinase C
PLC	-	phospholipase C
PLD	-	phospholipase D
PPA	-	phosphatidic acid
PP2C	-	protein phosphatase 2C
PR	-	pathogenesis-related
PRK	-	phosphoribulokinase
PSII	-	photosystem II
R	-	red light
RBCS	-	small subunit of RUBISCO
ROI	-	reactive oxygen intermediate
RuBP	-	ribulose 1,5-bisphosphate
RUBISCO	-	ribulose 1,5-bisphosphate carboxylase/oxygenase
SA	-	salicylic acid
SAM	-	*S*-adenosyl methionine
SAR	-	systemic acquired resistance
SDS	-	sodium dodecyl sulphate
SIM	-	shoot-inducing medium
SOD	-	superoxide dismutase
STA	-	staurosporine
TAE	-	Tris acetate electropheresis (buffer)
TCA	-	tricarboxylic acid
TRX	-	thioredoxin
uidA	-	gene encoding β-glucuronidase from *Escherichia coli*
UTR	-	untranslated region
WT	-	wild-type
X-gluc	-	5-bromo-4-chloro-3-indolyl β-D-glucuronide

NUCLEOTIDE BASES

G	-	guanine		
A	-	adenine		
T	-	thymine		
C	-	cytosine		
R	-	purine	-	A,G
Y	-	pyrimidine	-	T,C
M	-	amino	-	A,C
K	-	ketone	-	G,T
S	-	strong interaction	-	C,G
W	-	weak interaction	-	A,T
H	-	not G	-	A,C,T
B	-	not A	-	G,C,T
V	-	not T	-	A,C,G
D	-	not C	-	A,G,T
N	-	any	-	G,A,T,C

AMINO ACIDS

A	Ala	-	alanine
C	Cys	-	cysteine
D	Asp	-	aspartic acid
E	Glu	-	glutamic acid
F	Phe	-	phenylalanine
G	Gly	-	glycine
H	His	-	histidine
I	Ile	-	isoleucine
K	Lys	-	lysine
L	Leu	-	leucine
M	Met	-	methionine
N	Asn	-	asparagine
P	Pro	-	proline
Q	Gln	-	glutamine
R	Arg	-	arginine
S	Ser	-	serine
T	Thr	-	threonine
V	Val	-	valine
W	Trp	-	tryptophan
Y	Tyr	-	tyrosine

1. Introduction

Environmental stresses such as drought, excessive salinity and low temperature have been major selective forces throughout plant evolution and are important factors which limit plant distribution and agricultural productivity (Hare et al. 1996). An understanding of how plants adapt to adverse conditions is thus not only of theoretical interest, but also has considerable practical value. It has long been appreciated that an improved understanding of the biochemical basis of plant stress tolerance is an essential prerequisite for the development of hardier crops. Despite considerable success in increasing the stress tolerance of crops using traditional breeding practices, conventional breeding is widely believed to be of limited value in further attempts to combine complex multigenic traits such as increased stress tolerance and greater productivity (Hare et al. 1996).

Not surprisingly, plant biotechnologists have turned to molecular genetics as a means of specifically targeting single genes in an attempt to increase crop productivity under stressful conditions. The greatest advances in this regard have concerned the identification of a large array of stress-inducible plant genes (Hare et al. 1996). Most of these have been isolated through a differential screening approach where optimal growth conditions and various stress conditions are used to generate different pools of mRNA. Such "shotgun" cloning of stress-inducible genes undoubtably holds considerable promise in terms of elucidating many of the presently enigmatic genetic responses of plants to stress. However, thus far, the success of this approach in identifying *biochemical mechanisms* of stress tolerance has been disappointing. There are at least two shortcomings of the empirical approach to identify promising plant improvement strategies. Firstly, genes which are induced under adverse conditions do not necessarily serve an adaptive function in stressed plants. Secondly, the overexpression of genes which are normally of adaptive value during stress may not be compatible with the requirements of agriculturalists (Hare et al. 1996, 1998). It might thus be argued that the vast amount of effort devoted to empirical approaches concerning the elucidation of genetic responses to stress has been at the expense of the full exploitation of the plant molecular genetic technologies currently at our disposal. In retrospect, it is sobering to consider how seldom these powerful techniques have been used to directly assess the validity of many fundamental tenets in plant physiology which remain controversial.

One such dogma concerns the role played by osmolyte accumulation in plants which have been subjected to water deficit. Following water deprivation and many other stresses which do not necessarily contain an osmotic component, most higher plants accumulate small organic compounds which have been variously referred to as osmolytes, compatible solutes or osmoprotectants. Although the types of osmolytes accumulated differ among species, increases

in the levels of free proline have been reported in species representing most taxonomic families. Since the original report of the phenomenon over forty years ago (Kemble & MacPherson 1954), proline accumulation in stressed plants has been the subject of intensive investigation. Although it has become axiomatic that the primary adaptive role played by proline and other commonly studied osmolytes (e.g. glycine betaine, certain disaccharides and a variety of polyols) in stress tolerance relates to their effects on cellular water potential and the protection of subcellular structure, the evidence in favour of these suggestions is largely circumstantial (Munns 1993; Hare et al. 1998). The accumulation of osmoregulatory solutes has long been perceived as a relatively simple biochemical trait which is amenable to manipulation by the transformation technologies which are currently available. The demonstration by Kavi Kishor et al. (1995) that transgene-mediated increases in proline synthesis improves the tolerance of tobacco plants to drought and salinity stress argues strongly in favour of a specific functionality for proline synthesis in the tolerance of water deficit. However, the mechanism(s) by which protection is conferred is still far from clear (Blum et al. 1996). This re-emphasises the abovementioned shortfall of many contemporary plant molecular genetic studies, *viz.* a failure to test *hypotheses* that might relate changes in gene expression to the resultant phenotypic consequences at the level of the whole plant.

In an attempt to address such issues, the primary aim of this study was to use a combination of both molecular genetic and whole plant physiological approaches to assess whether or not the metabolic changes that support proline accumulation during stress, and its rapid degradation after relief from stress, may provide important clues concerning the functional significance of proline accumulation. The notion that an effect(s) associated with osmolyte accumulation may be more important than the intrinsic value of the osmolyte *per se* is not new (Hanson & Hitz 1982), but it has only recently begun to gain credibility (Bohnert & Sheveleva 1998; Hare et al. 1998). Since deviations from optimal growth conditions are a continuous occurrence for most plants in their natural environments, an important premise on which many of the experiments in this study were based is that the dramatic changes in proline synthesis and degradation under stressful conditions may mirror similar changes in proline metabolism which have also been recruited for the regulation of other physiological processes, such as developmental transitions, throughout the plant life cycle.

As will become evident to the reader, the findings of this study are but a small contribution to the vast and rapidly expanding body of knowledge concerning proline metabolism in higher plants. Nonetheless, together with much supportive evidence from the recent studies of other workers, they appear to be consistent with a long overlooked view which implicates an important regulatory role for proline metabolism in plant growth and development. Investigation of these effects is clearly warranted by the potential applications of the findings to agriculture.

2. LITERATURE REVIEW

2.1. Osmotic adjustment and the compatible solute theory

Many plants accumulate organic osmolytes upon exposure to abiotic stresses which cause depletion of cellular water (drought, high soil salinity and temperature extremes). The most commonly accumulated osmolytes can be broadly categorised as being either polyhydroxylic compounds (saccharides and polyhydric alcohols) and zwitterionic alkylamines (amino acids and quaternary ammonium compounds).

Several recent reviews discuss osmolyte accumulation in plants (Ingram & Bartels 1996; Bohnert & Jensen 1996; Serrano 1996; Hare et al. 1998). It is generally accepted that the increase in cellular osmolarity which results from the accumulation of nontoxic (thus "compatible") osmotically active solutes is accompanied by the influx of water into, or at least a reduced efflux from cells, thus providing the turgor necessary for cell expansion. Since all subcellular structures must exist in an aqueous environment, tolerance to dehydration also depends on the ability of cells to maintain membrane integrity and prevent protein denaturation. Hypotheses that attribute special protective properties of osmolytes to protein structure, dry membranes and liposomes under adverse environmental conditions (dehydration, temperature extremes or denaturants) have been discussed extensively (Csonka 1989; Crowe et al. 1992).

Nonetheless, it has proven difficult to identify the relative importance of these postulated overlapping effects mediated by compatible solutes *in vivo*. Conclusive demonstration that osmotic adjustment contributes to fitness in stressful environments has yet to be achieved (Munns 1993). Likewise, the *in vivo* significance of stabilising macromolecule-osmolyte interactions remains largely a matter of conjecture (Hare et al. 1998). Questions such as whether osmolyte levels are reliable indicators of stress tolerance in breeding programs, or whether the maximum absolute concentrations of compatible solutes are always adequate to account for significant mass-action effects, have been the subject of numerous discussions and are still vigorously debated (Hare & Cress 1997; Hare et al. 1998). All of the hypotheses concerning direct protective effects of organic osmolytes are valid only if the most frequently observed levels of accumulation are confined exclusively to a small subcellular compartment. Although on a *per* mole basis, organic and inorganic electrolytes (when considered as an ion pair such as KCl) are more effective than osmolytes in generating osmolarity, most of the evidence is consistent with the view that concentrations (although not necessarily absolute amounts) of compatible solutes are higher

in the cytoplasm, with energetically less costly electrolytes making the major contribution to osmotic potential within the vacuole and apoplast (Serrano 1996; Stoop et al. 1996; Hare & Cress 1997). Thus, accumulation of compatible solutes has long been considered to equalise the water potentials of the cytoplasm and vacuole.

Traditionally, stress physiologists have used three types of information to identify potential osmolytes : (i) an increase in the abundance of a small organic molecule when there is a decline in the water potential of the cell's environment, (ii) physicochemical properties of the putative osmolyte when studied *in vitro*, and (iii) comparative physiology as a pointer to gain insight into whether osmoprotective solutes confer protection in desiccation-tolerant species or in dehydration tolerant stages of development such as during seed maturation (Drennan et al. 1993; Ingram & Bartels 1996). With advances in enzyme purification and plant molecular genetics, the proposal that osmolyte accumulation plays a critical role in the mitigation of stress has been strengthened by demonstrations that frequently, activity of one or more of the enzymes involved in the biosynthesis of a putative osmolyte, or levels of transcripts encoding these enzymes, is increased following the imposition of stress (Table 2.1). A further argument supporting an adaptive role for osmolyte accumulation in the tolerance of hyperosmotically stressed lines selected for increased osmolyte accumulation frequently (Saneoka et al. 1995; Dörffling et al. 1997), but not always (Maggio et al. 1997), display increased stress tolerance. A similar situation applies for lines genetically engineered to overproduce osmolytes (Table 2.2).

The osmotically-induced increase in levels of a limited number of osmolytes in a diverse set of organisms spanning all of the biological kingdoms argues in favour of the proposal that osmotic adjustment is an evolutionarily ancient trait. Nonetheless, complexity in interpreting this observation arises from the observation that no single compound attributed with special osmoprotective functions accumulates universally throughout the plant kingdom. Ironically, countervailing disadvantages of the accumulation of certain "compatible" solutes have apparently contributed to the evolution of alternative osmolytes in different taxa, although distinctions between the relative abundances of different osmolytes and broad taxonomic divisions are not straightforward. It has often been suggested that accumulation of non-nitrogenous solutes may have implications regarding the nitrogen economy of certain species in particular habitats (Erskine et al. 1996).

Of the organic osmolytes, L-proline (henceforth simply referred to as proline) is by far the most extensively studied. This arises largely from its eminence as the most commonly accumulated osmolyte throughout the plant kingdom. Nonetheless, despite more than forty years of research concerning the functional significance of stress-induced proline accumulation (Delauney & Verma

Table 2.1: Gene products related to osmolyte accumulation which have been shown to increase upon exposure to hyperosmotic stress.

Gene product	Species[1]	Reference
P5C synthetase (proline synthesis)	*Vigna aconitifolia* (T, E)	Hu et al. (1992); Zhang et al. (1995)
	Arabidopsis thaliana (T)	Yoshiba et al. (1995); Savouré et al. (1995); Peng et al. (1996); Knight et al. (1997); Zhang et al. (1997)
	Oryza sativa (T)	Igarashi et al. (1997)
	Lycopersicon esculentum (T)	García-Rios et al. (1997)
P5C reductase (proline synthesis)	*Mesembryanthemum nodiflorum* (E)	Treichel (1986)
	Glycine max (T)	Delauney & Verma (1990)
	Hordeum vulgare (E)	Argandona & Pahlich (1991)
	Pisum sativum (E, T)	Rayapati et al. (1989); Williamson & Slocum (1992)
	A. thaliana (T)	Verbruggen et al. (1993); Hare & Cress (1996)
	Triticum durum (E)	Mattioni et al. (1997)
choline monooxygenase (glycine betaine synthesis)	*Spinacia oleracea* (T)	Rathinasabapathi et al. (1997)
	Beta vulgaris, *Amaranthus caudatus* (E, T)	Russell et al. (1998)
betaine aldehyde dehydrogenase (glycine betaine synthesis)	*H. vulgare* (T)	Ishitani et al. (1995)
	Sorghum bicolor (T)	Wood et al. (1996)
myo-inositol O-methyl transferase (pinitol synthesis)	*Mesembryanthemum crystallinum* (T)	Vernon et al. (1993)
myo-inositol-1-phosphate synthase (*myo*-inositol synthesis)	*O. sativa* (E)	Raychaudhuri & Majumder (1996)
	M. crystallinum (T)	Ishitani et al. (1996)

[1] T, increased abundance of mRNA transcript following hyperosmotic stress; E, increased specific activity and/or abundance of enzyme

Table 2.2: Transgenes involved in osmolyte accumulation already used in attempts to increase osmotic tolerance in plants.[a]
From Hare et al. (1998).

Gene product	Source	Product accumulated	Increased tolerance?	Reference
mannitol-1-phosphate dehydrogenase	*Escherichia coli*	mannitol	yes	Tarczynski et al. (1993); Karakas et al. (1997)
P5C synthetase	*Vigna aconitifolia*	proline	yes	Kavi Kishor et al. (1995)
fructosyltransferase (levan-sucrase)	*Bacillus subtilis*	fructan	yes	Pilon-Smits et al. (1995)
betaine aldehyde dehydrogenase	*Hordeum vulgare*	[b]	no	Rathinasabapathi et al. (1994); Ishitani et al.(1995)
sorbitol-6-phosphate dehydrogenase	*Malus domestica*	sorbitol	ND	Tao et al. (1995)
choline dehydrogenase	*Escherichia coli*	glycine betaine?[c]	yes	Lilius et al. (1996)
trehalose-6-phosphate synthase	*Saccharomyces cerevisiae*	trehalose	yes	Holmström et al. (1996); Romero et al. (1997)
trehalose-6-phosphate synthase, trehalose-6-phosphate phosphatase	*Escherichia coli*	trehalose	no	Goddijn et al. (1997)
myo-inositol-3-phosphate synthase[d]	*Spirodela polyrrhiza*	*myo*-inositol	no	Smart & Flores (1997)
myo-inositol O-methyltransferase	*Mesembryanthemum crystallinum*	ononitol	yes	Sheveleva et al. (1997)
choline oxidase[d]	*Arthrobacter globiformis*	glycine betaine	yes	Hayashi et al. (1997)

[a] all studies involved transformation of tobacco (*Nicotiana tabacum*);
[b] high levels of enzymatically active gene product were detected;
[c] levels were not determined;
[d] introduced into *Arabidopsis thaliana*;
ND, stress tolerance was not investigated.

1993), considerable controversy surrounds the relative importance of this phenomenon in conferring tolerance to adverse conditions (Table 2.3).

The remainder of this brief critique concerning the validity of the osmotic adjustment and compatible solute paradigms will thus focus primarily on studies that have examined the validity of these hypotheses by using free proline as the subject of investigation. Nonetheless, in view of the well documented reciprocal relationship between the accumulation of proline and that of other osmolytes (Hare & Cress 1997), as well as the commonly-held opinion that accumulation of all osmolytes confer stress tolerance by at least some central mechanism(s), reference will also be made to our present understanding of the biophysical implications of the accumulation of other organic solutes attributed with osmoprotective properties.

2.1.1 Biophysical implications of stress-induced proline accumulation

To date, the focus on accounting for the adaptive significance of stress-induced proline accumulation has centred largely on the biophysical effects associated with an increase in cytosolic levels of free proline. Accumulated proline has been proposed to protect enzymes (Schobert & Tschesche 1978; Arakawa & Timasheff 1983, 1985), membranes (Rudolph et al. 1986) and polyribosomes (Kandpal & Rao 1985) during environmental perturbations. However, the mechanism for osmolyte-mediated stabilisation of macromolecular structure remains poorly understood. For example, it is still debated whether stabilisation of the hydration shell of native proteins arises from direct contact between proteins and solutes (Schobert & Tschesche 1978), or more indirectly through changes in the total free energy of the systems comprising proteins, osmolytes and water (Arakawa & Timasheff 1985). It is difficult to distinguish experimentally between these possibilities and they are not mutually exclusive. The "preferential exclusion" model (Arakawa & Timasheff 1985) presently enjoys the most widespread support. According to this hypothesis, stabilisation by compatible solutes is explained as an entropically unfavourable exclusion of osmolytes from the hydration shell of proteins. Exclusion of the compatible solutes from the hydration sphere decreases the entropy of the system. To counteract this thermodynamically unfavourable situation, the solvent system tends to minimise the excluded volume by maintaining proteins in the native state, when they have a smaller surface area than when they are denatured. This may explain why a wide variety of different substances display similar stabilising effects. Nonetheless, there is presently no molecular explanation as to *why* compatible solutes are so effectively excluded from the hydration shells of macromolecules.

Table 2.3: An overview of the contrasting evidence for the value of proline accumulation as a biochemical marker of stress tolerance in higher plants.[1]

Species	Stress	Experimental system	Reference
Positive correlation between proline accumulation and stress tolerance			
Solanum spp.	NaCl freezing	plantlets grown *in vitro* leaf tissue	Martinez et al. (1996) van Swaaij et al. (1985)
Nicotiana spp.	NaCl; mannitol soil drying NaCl; soil drying	cultured cells[2] leaf tissue leaf tissue[3]	Watad et al. (1983) van Rensburg et al. (1993) Kavi Kishor et al. (1995)
Cicer arietinum	NaCl	callus[2]	Pandey & Ganapathy (1985)
Lycopersicon spp.	PEG NaCl	cultured cells cultured cells[2]	Handa et al. (1986) Hassan & Wilkins (1988)
Hordeum vulgare	soil drying	leaf tissue	Singh et al. (1972)
Zea mays	soil drying	leaf tissue	O'Regan et al. (1993)
Oryza sativa	NaCl	seedlings callus[2]	Igarashi et al. (1997) Basu et al. (1997)
Brassica juncea	NaCl; mannitol	callus[2]	Gangopadhyay et al. (1997)
Triticum aestivum	freezing	leaf tissue[4]	Dörffling et al. (1997)
Daucus carota	NaCl	cultured cells[4]	Riccardi et al. (1983)

No unambiguous relationship between proline accumulation and stress tolerance

H. vulgare	PEG	leaf tissue	Hanson et al. (1977, 1979)
Lycopersicon esculentum	soil drying	leaf tissue	Aloni & Rosenshtein (1984)
Brassica spp.	soil drying NaCl	leaf tissue seedlings; leaf tissue	Richards & Thurling (1979) Madan et al. (1995)
Sorghum bicolor	soil drying PEG	leaf tissue callus	Blum & Ebercon (1976) Bhaskaran et al. (1985)
Solanum melongena	NaCl	cultured cells[2]	Jain et al. (1987)
Medicago sativa	NaCl	cultured cells[2]; root, shoot tissue	Petrusa & Winicov (1997)
D. carota	PEG	cultured cells[4]	Maggio et al. (1997)

Negative correlation between proline accumulation and stress tolerance

Gossypium hirsutum	soil drying	leaf and root tissue	Ferreira et al. (1979)
Lycopersicon spp., *S. pennelli*	NaCl; PEG flooding	leaf tissue leaf tissue	Tal et al. (1979) Kuo & Chen (1980)
Brassica napus	mannitol; NaCl; Na_2SO_4	callus[2]	Chandler & Thorpe (1987)
Vigna mungo	NaCl	leaf tissue	Ashraf (1989)
Glycine max	NaCl	leaf tissue	Moftah & Michel (1987)
Manihot esculenta	PEG	excised leaves	Sundaresan & Sudhakaran (1995)
O. sativa	NaCl	root and shoot tissue	Lutts et al. (1996)
Nicotiana sylvestris	NaCl	cultured cells[2]	Dix & Pearce (1981)

[1] unless specified, all comparisons are between stress-tolerant and stress-resistant lines of the same species, or closely related species
[2] cell lines selected for increased salt -tolerance
[3] transgenic plants expressing a proline biosynthetic gene
[4] proline-overproducing plant or cell lines, selected for resistance to proline analogues

Compelling evidence in favour of a direct role for proline accumulation in counteracting the effects of hyperosmotic stress comes from the demonstration that exogenous proline has a protective effect on bacteria and that bacterial proline-accumulating mutants display enhanced osmotolerance (Delauney & Verma 1993). In maize (*Zea mays*) roots grown at low water potentials, it was estimated that proline accumulation may account for approximately 45% of the total osmotic adjustment in the root apex (Voetberg & Sharp 1991). Nonetheless, specificity in the role of free proline in osmotic adjustment is confounded by the demonstration that this osmolyte has been reported to accumulate in many plants in response to a wide range of environmental stresses (Table 2.4).

Many of these stresses (e.g. heavy metal toxicity, anaerobiosis, nutrient deficiency, atmospheric pollution and photooxidative stress) do not have a significant osmotic component. Although Kastori et al. (1992) noted an effect of heavy metal accumulation on the water status of sunflower (*Helianthus annuus*) plants, an increased proline content in fully turgid leaf discs incubated in solutions of Pb^{2+}, Cd^{2+}, Zn^{2+} and Cu^{2+} indicated that the response is probably independent of any effect on water relations. While both chilling and freezing reduce the availability of water, several workers have observed increases in proline levels following cold-hardening experiments in which care was taken to avoid any changes in water status (Draper 1972; Chu et al. 1978; van Swaaij et al. 1985; Naidu et al. 1991). This also indicates that the initiation of proline accumulation by

Table 2.4: Adverse environmental stimuli capable of inducing proline accumulation in plants. Adapted from Hare and Cress (1997).

Stress	References
Water deprivation	Delauney & Verma (1993); Heuer (1994)
Salinisation	Delauney & Verma (1993); Heuer (1994)
High temperature	Chu et al. (1978); Kuo et al. (1986)
Low temperature	Draper (1972); Chu et al. (1978); van Swaaij et al. (1985); Naidu et al. (1991)
Heavy metal toxicity	Alia & Pardha Saradhi (1991); Kastori et al. (1992); Bassi & Sharma (1993)
Pathogen infection	Labanauskas et al. (1974); Meon et al. (1978); Mohanty & Sridhar (1982);
Anaerobiosis	Labanauskas et al. (1974); Kuo & Chen (1980); Aloni & Rosenshtein (1982)
Nutrient deficiency	Göring & Thien (1979); Vaucheret et al. (1992)
Atmospheric pollution	Godzik & Linskens (1974); Anbazhagan et al. (1988)
UV - irradiation	Pardha Saradhi et al. (1995)

cold stress is independent of changes in tissue water balance and other reasons need to be sought to account for the functional significance of this phenomenon.

Using transgenic plants with an elevated capacity for proline synthesis, Kavi Kishor et al. (1995) have provided convincing evidence of a cause-and-effect relationship between proline synthesis and tolerance to drought and salinity stress. However, studies of the water relations of these proline-overproducing plants are not consistent with a role for drought-induced proline accumulation in mediating osmotic adjustment (Blum et al. 1996). Leaf osmotic potentials of the transgenic plants, which produced twice as much proline as comparable controls after water deprivation (approximately 9 μmol g^{-1} fresh weight), were unaffected by soil drying and the plants displayed even less capacity for osmotic adjustment than comparable wild-type tobacco. This finding has highlighted the need for further investigation of the mechanism(s) whereby proline accumulation alleviates the effects of osmotic stress on plant growth. Intriguingly, modest increases in the levels of mannitol (Tarczynski et al. 1993; Thomas et al. 1995b; Karakas et al. 1997), fructans (Pilon-Smits et al. 1995) and trehalose (Holmström et al. 1996; Romero et al. 1997) in tobacco plants engineered to accumulate these solutes were also considered inadequate for osmotic effects to account for the apparent increases in stress tolerance observed. Osmotic adjustment was not assessed in transgenic *Arabidopsis thaliana* plants which accumulate glycine betaine to only 1.0 μmol g^{-1} leaf fresh weight (Hayashi et al. 1997). However, it is unlikely that such a low concentration is adequate for osmotic effects to account fully for the dramatic improvements in stress tolerance observed in these plants. In the leaves of sugar beet (*Beta vulgaris*) plants subjected to a drought stress which decreased the relative water content to less than 60%, the levels of glycine betaine that accumulated were inadequate to make a measurable contribution to osmotic adjustment (Russell et al. 1998). An attempt to account for the protective effects associated with a low level of accumulation of these diverse osmolytes is beyond the scope of this review. The reader is referred to Hare et al. (1998) for an account concerning the importance of light, oxygen and carbohydrate status as important determinants of plant stress tolerance and suggestions as to how an increased capacity for the synthesis of glycine betaine, polyols or non-reducing sugars may confer tolerance through mechanisms not directly related to reduced availability of cellular water.

The induction of proline accumulation by a variety of environmental perturbations can be most easily accounted for by a factor common to all of these stresses. One possible candidate is the redox status of the cell. All organisms living in an aerobic atmosphere are confronted with the challenge of oxygen. While oxygen is itself toxic, reactions which occur under stressful conditions often result in the production of other chemical species e.g. hydroxyl radicals ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$) anions, and hydrogen peroxide (H_2O_2), which have even higher oxidising potentials. All of

the stresses implicated in causing free proline accumulation have been associated with the production of these agents of oxidation (Table 2.5). Oxidative stress is likely to result whenever environmental conditions block the normal dissipation of the light-induced high-energy state. The reduction in the rate of CO_2 assimilation under adverse environmental conditions results in exposure of chloroplasts to excess excitation energy and increases the rate of formation of reactive oxygen intermediates (Smirnoff 1993). While $O_2^{\cdot-}$ and H_2O_2 can inactivate various macromolecules directly, it is their conversion to the $OH^{\cdot}$, catalysed by transition metals (i.e. the Haber-Weiss reaction) that primarily accounts for their toxicity. Hydroxyl radicals react instantaneously with proteins, lipids, and DNA, causing rapid cell damage.

Table 2.5: Adverse environmental stimuli capable of inducing oxidative stress in plants.

Stress	References
Water deprivation	Moran et al. (1994); Zhang & Kirkham (1994); Navari-Izzo et al. (1996)
Salinisation	Hernández et al. (1993); del Rio et al. (1996); Willekens et al. (1997)
High temperature	Mishra & Singhal (1992)
Low temperature	Prasad et al. (1994)
Heavy metal toxicity	Kampfenkel et al. (1995); Caro & Puntarulo (1996); Gallego et al. (1996)
Pathogen infection	Mehdy et al. (1996); Wojtaszek (1997)
Re-aeration after anaerobiosis	Monk et al. (1989); Biemelt et al. (1998)
Nutrient deficiency	Cakmak & Marschner (1988)
Atmospheric pollution	Hippeli & Elstner (1996); Pell et al. (1997)
UV - irradiation	Green & Fluhr (1995)

Besides its stabilising effects on cellular structure, proline is also capable of detoxifying free radicals by forming long-lived adducts with them (Floyd & Nagy 1984; Smirnoff & Cumbes 1989). This is a feature of most, but not all, compatible solutes (Smirnoff & Cumbes 1989). Proline enhanced the photochemical electron transport activities of isolated thylakoid membranes of *Brassica juncea* by arresting photoinhibitory damage (Alia et al. 1991). In the presence of 1 M proline, a reduction in the level of lipid peroxidation during strong illumination suggests that its protective action was mediated via its ability to scavenge free radicals (Alia et al. 1991). In keeping with these findings, proline levels in tobacco cultivars displaying different levels of drought tolerance were positively correlated with the membrane integrity of their chloroplasts (van

Rensburg et al. 1993). The accumulation of free proline in plants exposed to UV radiation has been suggested to confer protection against free radical generation since 1 M proline reduced the time dependent increase in malondialdehyde when linolenic acid micelles were irradiated with UV light (Pardha Saradhi et al. 1995).

However, the validity of many of these arguments rests on the presence of a cytosolic and/or chloroplastic pool of free proline of sufficient size to provide a pronounced osmotic or biophysical influence. This is not found in most glycophytes in which proline accumulation has been reported under conditions of hyperosmotic stress (Hare & Cress 1997). While a number of desirable physiochemical properties are exhibited by proline at molar concentrations (Schobert & Tschesche 1978; Samuel et al. 1997), these are unlikely to play a decisive role when proline is found at the relatively modest concentrations generally observed in plant cells adapted to hyperosmotic conditions. Furthermore, data suggesting a several hundred-fold increase in proline levels accompanying osmotic stress may be misleading since this increase is often based on an initially small proline pool (Hare & Cress 1997).

Although proline concentrations above 5 M were capable of enhancing the solubility of insulin by approximately 170-fold, at concentrations below this, proline had only negligible effects (Schobert & Tschesche 1978). Likewise, although proline concentrations in excess of 4 M could completely prevent the trichloroacetic acid-mediated precipitation of lysozyme, concentrations below 2 M were ineffective (Samuel et al. 1997). These workers proposed that at high concentrations, proline may form higher order aggregates. The formation of amphiphilic supramolecular assemblies involving the stacking of pyrrolidone rings above each other was postulated to account for the protein stabilising properties of proline, since the imino and carboxyl groups on one side of the assembly would provide a polar surface, with the methylene groups of the pyrrolidone ring constituting a hydrophobic surface (Samuel et al. 1997). Despite this intriguing suggestion, the physiological relevance of this model to *in vivo* stress tolerance is questionable. At concentrations below 100 mM, proline exists in a monomeric state (Samuel et al. 1997). Although proline can destabilise the double helical structure of DNA, significant effects on the reduction of the T_m and counteraction of the effect of NaCl on DNA stability are not observed at concentrations less than 1 M (Rajendrakumar et al. 1997). While an adaptive role for proline-DNA interactions might be envisaged in prokaryotes, in which osmoprotectants have direct access to DNA, this would not necessarily apply for eukaryotic cells, which have a nuclear envelope. A physiological role for the levels of proline most frequently accumulated in stressed plants in negating the undesirable effects of NaCl on DNA stability thus seems highly unlikely.

Another important consideration seldom taken into account by many workers is examination of the subcellular location of proline accumulated during stress. More than half of the free proline in *Beta vulgaris* cells may be in the vacuolar compartment, although cytoplasmic concentrations are always greater (Leigh et al. 1981). In PEG-treated *Nicotiana rustica* protoplasts or cells, most of the accumulated proline is sequestered within the vacuole (Pahlich et al. 1983). This suggests that stress-induced increases in proline production in this species is unlikely to have a profound osmotic influence in the cytoplasm. However, although 34% of the total cellular proline in unstressed potato suspension cells was found in the vacuole, following stress-induced proline accumulation, only 16% of the total proline was located in this subcellular compartment (Fricke & Pahlich 1990). When rape (*Brassica napus*) leaf discs were subjected to an osmotic upshock for a period adequate to ensure proline accumulation (20 h), and then transferred to media of higher osmotic potential, only 80% of the accumulated proline was mobilised within 30 h (Trotel et al. 1996). In contrast, when endogenous proline levels were increased to comparable levels by incubation of the leaf discs in 40 mM proline, all of the proline had been metabolised within 10 h after transfer to media devoid of proline (Trotel et al. 1996). These workers suggested that at least 20% of the proline accumulated during the osmotic stress was sequestered in the vacuole and was not metabolised owing to stress-induced changes in properties of the tonoplast.

Irrespective of the precise contribution that stress-induced proline may make to cytosolic osmotic adjustment, it is important to note that several amino acids are excluded from protein surfaces to the same extent as proline (Arakawa & Timasheff 1983, 1985) and are not toxic to enzyme activity at high concentrations. Furthermore, owing to their high reactivity, free radicals react with many compatible solutes other than proline (Smirnoff & Cumbes 1989). Therefore, an exclusively osmotic or biophysical explanation for the accumulation of proline in osmotically stressed plants may be insufficient to account for the high level of conservation of this metabolic response throughout the plant kingdom. Rather, the nature of proline metabolism and its unique position in relation to the rest of intermediary metabolism may explain its eminence as the preferred osmoticum in many plant species.[1]

Furthermore, regulation of the accumulation of organic molecules proposed to have an osmoprotective role in plants is only one aspect of the coordinated changes in plant gene expression in response to water deficit (Table 2.6). Besides the indisputable importance of enzyme systems involved in the scavenging of free radicals in relieving several stresses which have an oxidative effect on plant cells, for salinity stress tolerance, the control over Na^+ and K^+ transport at low K^+ and high Na^+ concentrations apparently plays a major role in tolerance (Rubio et al. 1995; Liu & Zhu 1997b; Zhu et al. 1998). Increasing evidence also implicates the importance of controlling the activity of water channel proteins (aquaporins) under hyperosmotic stress

Table 2.6: Gene products, other than those implicated in osmolyte synthesis, which have been demonstrated to confer tolerance of hyperosmotic stress in plants[1]

Gene product	Mechanism	References
dehydrin-type proteins	protection of protein structure?	Artus et al. (1996); Xu et al. (1996)
glutathione reductase	antioxidative defence	Broadbent et al. (1995)
Mn-superoxide dismutase	antioxidative defence	McKersie et al. (1996)
glutathione *S*-transferase/ glutathione peroxidase	oxidation of cellular glutathione?	Roxas et al. (1997)
catalase[2]	antioxidative defence	Willekens et al. (1997)
K^+ transporters[2]	ion homeostasis	Liu & Zhu (1997b); Zhu et al. (1998)
water channels[3]	facilitated water movement	Chaumont et al. (1997)

[1] unless indicated, functionality was indicated by expression of the gene in transgenic plant tissue
[2] *role for gene product suggested by stress sensitive phenotype in plant mutants*
[3] role for plant gene product demonstrated in *Dictyostelium*

(Yamada et al. 1995, 1997). While water flux across membranes which contain such proteins is dependent on osmotic differences between both faces, opening of the channels is regulated by protein phosphorylation events possibly mediated by growth regulators (Johansson et al. 1998).

Any proposal for a protective effect of osmolyte accumulation on subcellular structure should also incorporate the likely roles of products of the many highly conserved *LEA* (late embryogenesis abundant), *COR* (cold-regulated), *DHN* (dehydrin) and *RAB* (responsive to ABA) genes characterised thus far. Many functions similar to those attributed to osmolytes (e.g. stabilisation of cytoplasmic constituents, renaturation of unfolded proteins, water retention and ion sequestration) have been suggested for these hydrophilic, glycine- or alanine-rich polypeptides which possess no apparent catalytic activity (Close 1997). Nonetheless, their fundamental mode(s) of action and physiological importance during dehydration remain largely enigmatic. Dehydrin- or LEA-like proteins have generally been found to be specifically associated with the cytoplasmic, nuclear or cytoskeletal components of the cell (Egerton-Warburton et al. 1997). It has been suggested that dehydrin-type proteins may act in synergy with osmolytes by increasing the preferential exclusion of solutes from macromolecules, although they are likely to fulfil a number of diverse functions (Egerton-Warburton et al. 1997). Recent demonstrations that constitutive expression of *COR15a* in transgenic *Arabidopsis* increased the *in vivo* freezing tolerance of chloroplasts in nonacclimated plants as well as the *in vitro* freezing tolerance of

protoplasts (Artus et al. 1996) and that expression in rice of *HVA1*, a barley gene encoding a LEA protein, conferred tolerance to water deficit and salinity (Xu et al. 1996), argue strongly for an important role of these proteins in reducing cellular damage caused by severe dehydration.

2.1.2 Conclusion

Although both the osmotic adjustment and compatible solute paradigms are difficult to separate mechanistically, and are usually considered together, incontestable evidence for the viability of either of these hypotheses has yet to be provided. Compatible solutes may protect subcellular structure more indirectly, through their capacity to scavenge free radicals. Bohnert and Jensen (1996) suggested that a distinction be made between osmolytes that they view as osmoprotectants (e.g. glycine betaine) and those that are free radical scavengers (e.g. mannitol). Consistent with the high, diffusion rate limited reactivity of hydroxyl radicals towards most metabolic intermediates, chloroplastic accumulation of mannitol was shown to increase resistance to oxidative stress in tobacco (Shen et al. 1997a). Nonetheless, despite its impressive implications for agriculture, this study contributes little insight into whether compatible solutes normally play a significant role in terminating free radical chain reactions. Although the rate constants for the reactions of mannitol and *myo*-inositol with hydroxyl radicals are similar, increased *myo*-inositol synthesis in *Arabidopsis* did not confer stress tolerance (Smart & Flores 1997). In contrast, the rate constant for the reaction of proline with $OH^{\cdot}$ is four times slower than that for both of these sugar alcohols (Buxton et al. 1988). Whereas 20 mM mannitol inhibited $OH^{\cdot}$ generation in illuminated thylakoids incubated in the presence of Fe^{2+} by approximately 60%, even 80 mM glycine betaine was ineffective in inhibiting the Fenton reaction (Shen et al. 1997b).

Concerns about the role of organic osmolytes in preventing cellular dehydration through direct physical protection of cellular constituents and/or maintenance of turgor pressure equilibrium do not eliminate these proposed effects. Presently, specific osmoprotective functions at low osmolyte concentrations cannot be formally excluded. Perhaps the considerable difficulty experienced in establishing definite causal relationships between the mass action effects of osmolyte accumulation and stress tolerance arises from the likelihood that other parameters which are less easily measured (e.g. developmental, morphological and ultrastructural adaptations) could make an equal or greater contribution to diminishing the debilitating effects of adverse environmental conditions.

As highlighted elsewhere (Hare et al. 1998), it is important to note that many attempts to improve stress tolerance by enhancing osmolyte accumulation are based on the *assumption* that

maintenance of cell turgor is a critical limitation to productivity under stresses that cause dehydration. To date, successful attempts to confer stress tolerance by slight increases in osmolyte levels are not consistent with this premise. While few would argue that osmolyte accumulation can be expected to yield more than marginal protection from damage, it must be recognised that metabolic disruptions that are the inevitable consequence of abiotic stress will limit growth irrespective of turgor status. Since osmolyte accumulation must always be considered in the context of metabolic shifts accompanying stress, hypotheses which suggest that the advantages of osmolyte accumulate arise from an integration of both the osmotic and metabolic requirements for growth under adverse conditions are attractive. The biocompatible features of osmolytes may have selected for the specific upregulation of metabolic processes resulting in their accumulation. While studies involving comparative physiology have focused on the biophysical properties of the osmolytes accumulated by dehydration tolerant species, emphasis on how the overall metabolic balance in these plants is altered by stress relative to stress-sensitive species may be more informative than continued preoccupation with osmotic effects. The energy and cofactor requirements of pathways that support osmolyte biosynthesis may be important determinants of beneficial effects of the process. Thus "compatibility" of osmolytes may be associated as much with metabolic processes important in stress adaptation as with their proposed protective effects on macromolecules, which is commonly attributed to their relatively innocuous biophysical properties. Further justification for this approach in elucidating the functionality of proline accumulation is presented in Section 2.5.

2.2 The importance of cellular redox potential in plant growth and development

2.2.1 Sources of reactive oxygen intermediates and antioxidative defence mechanisms

Despite the efficiency of aerobic metabolism, the presence of oxygen in the cellular environment poses a constant threat to cellular structures and processes. The excess production of reactive oxygen intermediates (ROIs), such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$) is a primary consequence of most, if not all of the environmental stresses which may be experienced by plants (Table 2.5). Substantial evidence suggests that oxidative processes are associated with a wide range of stresses in all living organisms. For example, examination of the tolerance of fourteen strains of *Saccharomyces cerevisiae* to salt, heat, slow and rapid freezing, or high acidity stresses indicated a correlation between tolerance of these stresses and tolerance of high levels of H_2O_2 (Lewis et al. 1997).

Rapid and non-specific reactions of ROIs result in damage to all classes of biomolecules including lipids, proteins and nucleic acids (Bartosz 1997). A dynamic equilibrium between reactions which generate ROIs and antioxidant protective mechanisms exists in all aerobic organisms, even under optimal environmental conditions. In heterotrophic tissues, ROIs are continuously generated by incomplete reduction of oxygen to water during mitochondrial respiration (Bartosz 1997) as well as during the β-oxidation of fatty acids. Oxygen toxicity is a far more threatening challenge to plants than to most other aerobic organisms since chloroplasts have a higher internal oxygen concentration than the surrounding atmosphere (Bartosz 1997). Under conditions of optimal light intensity required for phototrophy, the light harvesting complex antenna and the chloroplastic electron carrier systems are adapted to minimise uncontrolled and inappropriate electron transfer. Nonetheless, because light is continuously absorbed by chlorophyll, exposure to light intensity in excess of the capacity of the plant to use this energy in metabolism results in the over-excitation of chlorophyll molecules and consequently the overproduction of electrons by the water-splitting system. The production of ROIs through the generation of excess electrons is responsible for both the peroxidation of polyunsaturated lipids in the thylakoid membranes as well as for damaging the reaction centre of photosystem II (PSII), in particular the D1 protein, resulting in the perturbation and inhibition of photosynthetic electron transport. This phenomenon is referred to as photoinhibition of photosynthesis (Foyer et al. 1994; Foyer 1997). In addition to these sources, $O_2^{\cdot-}$ is also continuously produced by the univalent reduction of dioxygen by electron donors in reactions catalysed by univalent oxidases, such as xanthine oxidase, NAD(P)H oxidase and aldehyde oxidase. Hydrogen peroxide is produced either by the two-electron reduction of

dioxygen in reactions catalysed by divalent oxidases, such as glycolate oxidase, or by disproportionation of $O_2^{\cdot-}$:

$$2O_2^{\cdot-} + 2H^+ \rightarrow 2H_2O_2.$$

Although in the absence of metal catalysts, H_2O_2 is less reactive than $O_2^{\cdot-}$, H_2O_2 can readily traverse cellular membranes and react at sites distant from the site of generation. Hydrogen peroxide participates in the Fenton reaction to generate $^{\cdot}OH$. This reaction, involving transition metal ions such as Fe^{2+} and Cu^+, is commonly assumed to be a key event in ROI interconversions (Bartosz 1997). Metal ions reduced by $O_2^{\cdot-}$:

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

react with H_2O_2 to generate $^{\cdot}OH$:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + 2\ ^{\cdot}OH \qquad \text{(Fenton reaction).}$$

The net effect of these two reactions involving Fe^{3+} and Fe^{2+} is :

$$O_2^{\cdot-} + H_2O_2 \rightarrow 2\ ^{\cdot}OH + O_2 \qquad \text{(metal catalysed Haber-Weiss reaction).}$$

A change in the balance between ROI generation and antioxidative defence mechanisms in favour of oxidative reactions has been shown to occur in plants exposed to a range of adverse environmental conditions (Table 2.5) which have also been associated with proline accumulation (Table 2.4).

Plants have evolved several non-enzymic protection mechanisms that efficiently scavenge free radicals. Antioxidants such as ascorbic acid, glutathione, α-tocopherols and carotenoids have, like osmolytes (Section 2.1.1) been implicated in the scavenging of ROIs (Inzé & van Montagu 1995). The monothiol tripeptide glutathione (γ-glutamylcysteinylglycine; GSH) is strongly implicated in protection against oxidative stress (Noctor et al. 1998). Glutathione is found in the vast majority of prokaryotic and eukaryotic cells, where it often represents the major pool of non-protein reduced sulphur. All functions thus far described for GSH are related to the cysteine moiety of the tripeptide. In all cells where GSH is found, the reduced tripeptide form exists interchangeably with the oxidised form (glutathione disulphide; GSSG). Various oxidants are able

to oxidise GSH to GSSG e.g.

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O.$$

Enzymatic antioxidative protection mechanisms have also been characterised. Hydroxyl radicals are too reactive to be eliminated enzymatically, but their formation is limited by the scavenging of $O_2^{\cdot-}$ and H_2O_2. Superoxide dismutases (SODs) occur in different isoforms, which can be distinguished on the basis of their metal cofactor. These enzymes eliminate $O_2^{\cdot-}$ radicals (Table 2.7). In general, plants contain a mitochondrial MnSOD, as well as a cytosolic and chloroplastic Cu/ZnSODs. The localisation of SODs in the organelles where large amounts of $O_2^{\cdot-}$ are generated is consistent with their role in suppressing $^{\cdot}OH$ formation at the primary sites of $O_2^{\cdot-}$ production. As will be discussed further in Section 2.2.2.2, "cytosolic" Cu/ZnSOD appears to be restricted to the vicinity of the vacuole, and is also found in the nucleus and in the apoplast (Ogawa et al. 1996). Many plants also contain chloroplastic FeSOD (Inzé & van Montagu 1995).

Hydrogen peroxide is eliminated by catalases and peroxidases. Unlike peroxidases, catalase (EC 1.11.1.6) does not require a reducing substrate for activity. The catalase multigene family in *Arabidopsis* includes three genes encoding individual subunits which associate to form at least six isoenzymes (McClung 1997). They catalyse the reaction :

$$2H_2O_2 \rightarrow 2H_2O + O_2.$$

Despite the ability of GSH to directly reduce most ROIs, the direct reduction of H_2O_2 by GSH is not a major route of H_2O_2 destruction in plants (Noctor et al. 1998). Rather, ascorbate is the primary substrate for the reductive detoxification of H_2O_2 (Figure 2.1). Ascorbate peroxidases (APXs; Table 2.7) are thought to be the most important H_2O_2 scavengers and operate both in the cytosol and chloroplasts (Inzé & Van Montagu 1995). Reduced glutathione participates in the regeneration of reduced ascorbate from its oxidised forms. The Halliwell-Asada cycle (Figure 2.1) thus links the reduction of ROIs to water with a successive cycle of oxidation and reduction reactions involving ascorbate and GSH. Other enzymes involved in this oxidation-reduction cycle are monodehydroascorbate reductase, dehydroascorbate reductase (DHAR) and glutathione reductase (GR).

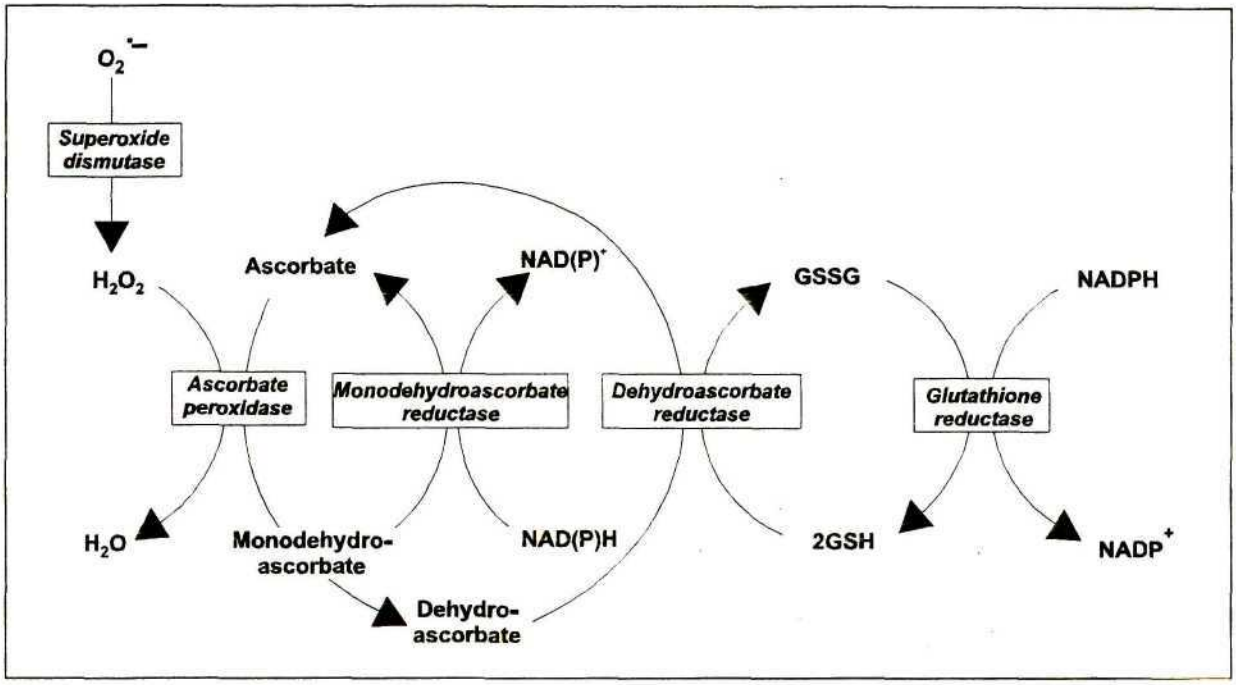

Figure 2.1: The ascorbate-glutathione cycle. Hydrogen peroxide is removed by ascorbate peroxidase and ascorbate is regenerated by the actions of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase and glutathione reductase (GR). Ascorbate is first oxidised to monodehydroascorbate. If monodehydroascorbate is not rapidly reduced again to ascorbate by MDHAR, it spontaneously disproportionates into ascorbate and dehydroascorbate. Dehydroascorbate recycles ascorbate using reduced glutathione (GSH) that is regenerated through the action of GR in an NADPH-dependent reaction. From Inzé and van Montagu (1995).

Table 2.7: Enzymes involved in scavenging of reactive oxygen intermediates.

Enzyme	Reaction catalysed
Superoxide dismutase (SOD; EC 1.15.1.1)	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
Ascorbate peroxidase (APX; EC 1.11.1.11)	2 ascorbate + H_2O_2 → 2 monodehydroascorbate + $2H_2O$
Monodehydroascorbate reductase (MDAR; EC 1.6.5.4)	mondehydroascorbate + NADPH → ascorbate + NADP$^+$
Dehydroascorbate reductase (DHAR; EC 1.8.5.1)	dehydroascorbate + 2 GSH → ascorbate + GSSG
Glutathione reductase (GR; EC 1.6.4.2)	GSSG + NADPH → 2 GSH + NADP$^+$

Glutathione peroxidases (GPXs; EC 1.11.1.9) have been identified in plants (Roxas et al. 1997), and may also play an important role in scavenging H_2O_2, or the products of lipid peroxidation by the reaction :

$$ROOH + 2GSH \rightarrow GSSG + ROH + H_2O.$$

The ratio GSH/GSSG reflects the relative rates of reduction and oxidation and is always greater than 0.9 under non-stress conditions (Noctor et al. 1998). In heterotrophic tissues, the oxidative pentose phosphate pathway (OPPP) which is an alternative to the glycolytic pathway as a means of oxidising glucose, produces the NADPH required to regenerate GSH from its oxidised form, GSSG (Figure 2.2).

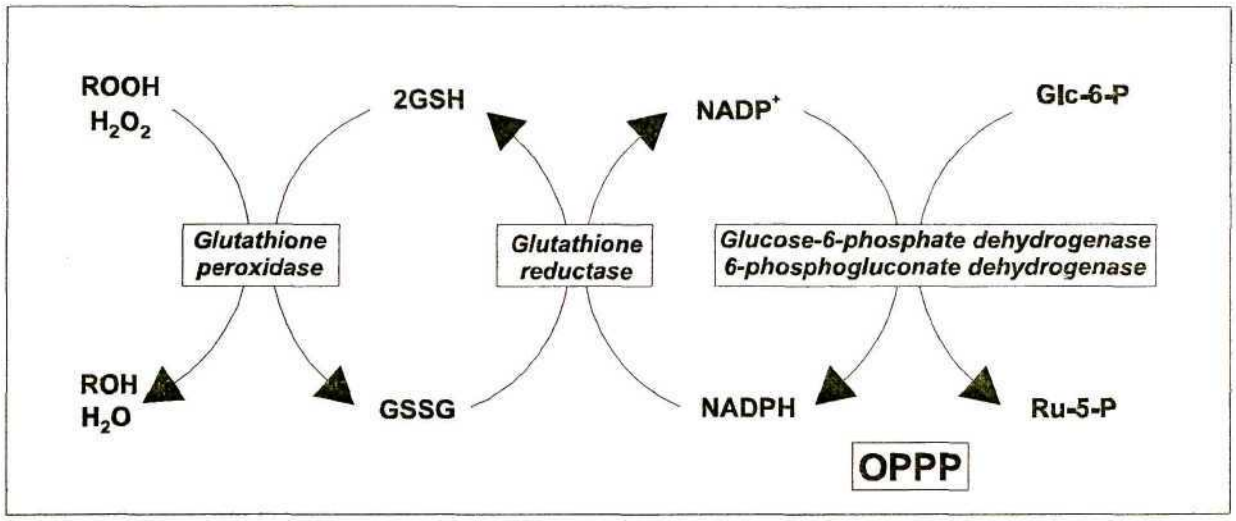

Figure 2.2: The glutathione cycle. In heterotrophic tissues, the two rate-limiting dehydrogenases of the oxidative pentose phosphate pathway (OPPP), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, generate the reductant required by glutathione reductase for the regeneration of reduced glutathione (GSH) which is used by glutathione peroxidase in the scavenging of H_2O_2 or lipid peroxides (ROOH). Glc-6-P, glucose-6-phosphate; Ru-5-P, ribulose-5-phosphate. Adapted from Bartosz (1997).

2.2.2 Regulatory roles of reactive oxygen intermediates

Although frequently viewed only in a destructive light, ROIs are also used by plants in a beneficial way. For example, H_2O_2 is a substrate for peroxidase-dependent reactions such as the synthesis

of lignin and suberin, the decomposition of auxin during development, and for defences against infection by pathogens. Besides playing essential roles in plant defence gene expression as well as programmed cell death and systemic resistance during pathogen infection (Jabs et al. 1996; Alvarez et al. 1998), ROIs have been implicated in mediating cross-tolerance to different abiotic stresses (Bowler et al. 1992; Foyer et al. 1997) and in the regulation of photosynthesis (Hormann et al. 1993). As will be outlined below, cellular reduction/oxidation (redox) status has a major regulatory effect on plant gene expression.

It is therefore not necessarily advantageous to get rid of ROIs as soon as they are generated within plant cells, but rather to control and manage the potential reactions of ROIs. The dual role of ROIs, acting both as destructive metabolic intermediates as well as mediating the induction of stress tolerance mechanisms emphasises the importance of understanding the complexity of the metabolic systems which regulate their abundance.

2.2.2.1 The plant hypersensitive response

When a plant is infected with a pathogen to which it is resistant, induction of a wide range of biochemical and physiological responses provide protection by restricting or even eliminating the pathogen and thereby limiting the damage it causes. Programmed plant cell death in the direct vicinity of infection, an effect commonly referred to as the hypersensitive response (HR), results from an oxidative burst triggered within minutes after contact with an incompatible pathogen (Lamb & Dixon 1997; Wojtaszek 1997). Specifically, the $O_2^{\cdot-}$ and H_2O_2 produced during the oxidative burst participate not only in directly killing both pathogen cells and host cells as part of the HR, but are also used for lignification, phytoalexin production and the rapid cross-linking of hydroxyproline-rich glycoproteins in the cell wall to strengthen the physical barrier against pathogen ingress (Lamb & Dixon 1997; Wojtaszek 1997). Moreover, these ROIs are believed to diffuse to adjacent cells where they cue the induction of a battery of defences based on a massive switch in host gene expression. Prominent among these rapidly induced defence genes are those encoding glutathione *S*-transferase (EC 2.5.1.18; GST) and GPX (Levine et al. 1994), as well as enzymes of phenylpropanoid biosynthesis involved in the production of lignin precursors and a number of phytoalexins (Dixon & Paiva 1995) and also other pathogenesis-related proteins such as chitinase.

The generation of ROIs during the HR strongly resembles the respiratory burst observed in mammalian phagocytes where activation of membrane-bound NADPH oxidase generates ROIs responsible for killing the invading pathogen prior to its ingestion. Not surprisingly, the striking

observation that antibodies raised against components of the neutrophil NADPH oxidase cross-react with polypeptides of similar molecular masses in plants (Levine et al. 1994; Kieffer et al. 1997; Xing et al. 1997) led to the conclusion that ROI production during the HR might be mediated by a plant NADPH oxidase. This enzyme reduces molecular oxygen to $O_2^{\cdot-}$:

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot-} + NADP^+ + H^+.$$

The $O_2^{\cdot-}$ may then dismutate to H_2O_2. The demonstration that selective inhibitors of the human neutrophil oxidase also block an elicitor-induced oxidative burst in plants (Levine et al. 1994; Dwyer et al. 1996) further supports the use of homologous polypeptides by both plant and mammalian systems in mounting a defence response.

Pugin et al. (1997) have presented a model which describes the sequence of early responses to perception of cultured tobacco cells to a fungal elicitor. Well-documented responses to pathogen infection, including ROI production, phytoalexin synthesis, cytoplasmic acidification and extracellular alkalisation were shown to be dependent on the action of the plasma membrane NADPH oxidase, the substrate of which is continuously regenerated by a high rate of OPPP activity (Pugin et al. 1997). Both Ca^{2+} influx and protein kinase activity are essential for these processes. Harding et al. (1997) independently demonstrated an important role for the Ca^{2+}-binding protein calmodulin (CaM) in potentiation of ROI production by an alteration in NAD(H)/NADP(H) homeostasis. Tobacco cell cultures which expressed a mutant CaM that hyperactivates CaM-dependent NAD kinase exhibited a stronger oxidative burst that occurred more rapidly than in comparable control cells challenged with pathogenic stimuli or hypoosmotic stress (an increase in cellular osmotic potential causes mechanical stress). Examination of absolute concentrations and changes in the levels of reduction of both pools of pyridine nucleotides following treatment of both the control and mutant cells with cellulase strongly supports the view that NADPH levels are elevated rapidly through the activation of NAD kinase in response to elicitor or environmental stress, and that the ROI-generating system in these stressed cells preferentially uses NADPH as reductant (Harding et al. 1997).

The development of restricted lesions during the HR has long been known to be accompanied by the development of systemic acquired resistance (SAR) to a broad range of normally virulent pathogens. The mobile signal which induces SAR has long evaded identification. Although numerous studies have indicated that salicylic acid is required both for the HR and the subsequent expression of SAR, conclusive evidence that it is an essential mobile signal for SAR has never been obtained (Lamb & Dixon 1997). Using an ingenious approach, Alvarez et al. (1998) have recently demonstrated that inoculation of *Arabidopsis* leaves with an avirulent

pathogen induces secondary oxidative bursts in discrete cells in distant tissues and uninoculated leaves, resulting in low-frequency systemic micro-HRs which leave no visible lesions. The primary oxidative burst induces these systemic responses, and both the primary burst and the secondary bursts are required for SAR. Hence, the oxidative burst is not confined to the macroscopic HR at the inoculation site and ROIs mediate a reiterative signal network which underlies both systemic as well as local resistance responses (Alvarez et al. 1998).

2.2.2.2 Lignification

Lignin is a complex polymer of aromatic subunits derived from the polymerisation of coniferyl alcohol and other monolignols, which are derived from phenylalanine (Whetten & Sederoff 1995). It provides a matrix around the polysaccharide components of certain cell walls, providing rigidity and compressive strength, as well as rendering the walls hydrophobic and impermeable to water. By far the most attention devoted to the induction of lignin deposition by environmental stresses has concerned the increase in lignin biosynthetic genes following pathogen infection or wounding. As already mentioned, this is believed to strengthen the cell wall at these sites of damage (Vance et al. 1980). However, the physical properties of the cell wall also change at low water potentials (Nonami & Boyer 1990) and accelerated lignification occurs in the roots of salt stressed maize (Azaizeh & Steudle 1991) and water stressed sorghum (Cruz et al. 1992).

Hydrogen peroxide is believed to be necessary for the polymerisation of monolignols into lignin via guaiacol peroxidase (EC 1.11.1.7) activity (Whetten & Sederoff 1995). Fairly recently, simultaneous determinations of "cytosolic" Cu/ZnSOD abundance, lignin and $O_2^{\cdot-}$ formation have led to the proposal that a substantial level of Cu/ZnSOD in the apoplast functions in lignification (Ogawa et al. 1996, 1997). Previous studies had indicated both that guaiacol peroxidases occur in the apoplast and that these lignin biosynthetic enzymes are rapidly inactivated by $O_2^{\cdot-}$ (Ogawa et al. 1996). As has already been mentioned, H_2O_2 which is used in the plant defence against fungal and pathogen infections, is generated by the univalent reduction of oxygen catalysed by NADPH oxidase. Ogawa et al. (1997) have extended the roles of both plant "cytosolic" Cu/ZnSOD and NADPH oxidase by proposing that both enzymes participate in lignin synthesis (Figure 2.3). Inhibitors of NADPH oxidase suppressed both $O_2^{\cdot-}$ and H_2O_2 formation in cross-sections of hypocotyls from spinach (*Spinacia oleracea*) seedlings, and $O_2^{\cdot-}$ accumulated following inhibition of SOD activity (Ogawa et al. 1997). These findings are consistent with the notion that plasma membrane-bound NADPH oxidase generates apoplastic $O_2^{\cdot-}$, which is rapidly disproportionated by Cu/ZnSOD. The "cytosolic" Cu/ZnSOD facilitates lignin biosynthesis both by supplying the substrate H_2O_2 and by suppressing $O_2^{\cdot-}$ -mediated inactivation of guaiacol peroxidase. Inhibition

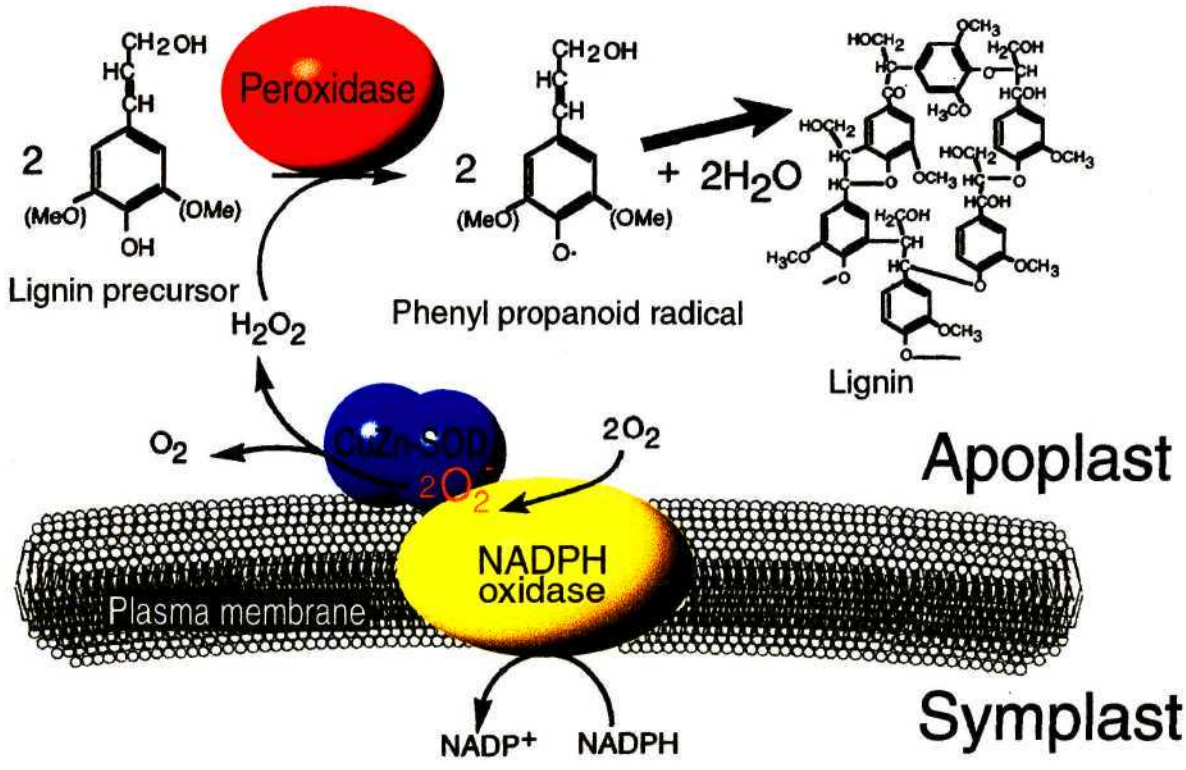

Figure 2.3: Proposed scheme for supplying hydrogen peroxide to lignin biosynthesis. Plasma membrane-bound NADPH oxidase generates apoplastic superoxide ($O_2^{\cdot-}$). Rapid disproportionation of $O_2^{\cdot-}$ by Cu/Zn-containing superoxide dismutase (CuZnSOD) both provides H_2O_2 for the guaiacol peroxidase-catalysed polymerisation of monolignols and prevents $O_2^{\cdot-}$ -mediated inactivation of the peroxidase. From Ogawa et al. (1997).

of lignin accumulation in the secondary cell wall of cultured cells of *Zinnia elegans* by inhibitors of either Cu/ZnSOD or NADPH oxidase substantiates this proposal (Ogawa et al. 1997).

2.2.2.3 Redox-regulation of gene expression

Considerable evidence supports an important role for redox potential in regulating the binding of transcription factors to *cis*-acting elements upstream of the coding regions of certain mammalian genes. Many vertebrate transcription factors (e.g. NF-κB, Fos, Jun, c-MYB and NF-Y) contain critical cysteine residues, modification of which interferes with specific DNA-binding *in vitro* (Myrset et al. 1993; Nakshatri et al. 1996). Certain of these *trans*-acting factors (e.g. Fos, Jun, c-MYB and NF-Y) lose their ability to bind DNA upon oxidation of cysteine residues, but regain this after treatment with sulfhydryl-containing molecules such as the nonphysiological dithiol reagent dithiothreitol (DTT). In other instances, oxidation of cysteine residues augments the DNA binding

activity. For example, in bacteria, the *oxyR* gene product activates a number of genes in response to oxidative stress by binding a specific DNA sequence only when it is in the oxidised state (Storz et al. 1990; Farr & Kogoma 1991). The OxyR response element, which functions as a redox-dependent enhancer in mammalian cells (Duh et al. 1995), appears to be recognised by the redox regulated mammalian transcription factor NF-Y (Nakshatri et al. 1996) and was recently reported to occur twice in the promoter of a maize SOD gene (Guan & Scandalios 1998). Mammalian NF-κB plays an important role in gene activation required for the immune response, malignant transformation, programmed cell death (apoptosis), embryonic development and inflammatory responses to diverse stress stimuli (Baeuerle & Baltimore 1996). This transcription factor is kept in an inactive form by binding to an inhibitory molecule IκB. Like OxyR, NF-κB is inactivated by GSH and indirectly activated by H_2O_2 and other oxidants (Meyer et al. 1993; Ginnpease & Whisler 1996). The activation of NF-κB by ROIs occurs by a post-translational mechanism, which involves dissociation of the inhibitory protein IκB (Pinkus et al. 1996).

The vertebrate transcription factor activator protein-1 (AP-1) couples a variety of extracellular signals to gene activation events associated with growth, differentiation and stress. An important mediator of tumour proliferation, AP-1 is composed of protein products of members of the extensively studied *fos* and *jun* proto-oncogene families, which form homodimeric (Jun/Jun) or heterodimeric (Fos/Jun) nuclear complexes (Abate et al. 1990). *In vitro*, the DNA-binding activity of AP-1 is inhibited by oxidation of a conserved cysteine in the DNA-binding domain of the two proteins (Abate et al. 1990). DNA binding is regained by the presence of reducing agents or by a nuclear factor, denoted redox factor-1 (Ref-1), the activity of which is in turn modulated by various redox-active compounds (Hirota et al. 1997). A homologue of Ref-1, which is identical to a DNA repair enzyme AP endonuclease, has been described in *Arabidopsis* (Babiychuk et al. 1994). Like its mammalian counterpart, the plant homologue is a multifunctional enzyme. It is capable both of activating human Fos and Jun *in vitro* through reduction of a cysteine residue in the DNA-binding domains of these proteins, as well as acting as an apurinic/apyrimidinic class II endonuclease (Babiychuk et al. 1994). The latter function is believed to be of importance in DNA repair following oxidative damage. Taken together, these observations suggest that redox cycles may be important mechanisms for regulating mammalian transcription factors and that similar regulatory mechanisms exist in plants.

Mammalian c-MYB is the prototype of a family of transcription factors which play a central role in controlling cellular proliferation and commitment to development. Like c-MYB, the DNA-binding domain of most of these proto-oncogene products is located near the N-terminus and comprises three imperfect tandem repeats of 51 or 52 amino acids, which are designated R1, R2 and R3. However, only R2 and R3 are required for sequence-specific DNA binding (Howe et al. 1990;

Myrset et al. 1993). In c-MYB, a highly oxidisable cysteine residue functions in turning specific DNA binding on or off by controlling a conformational change in R2 (Myrset et al. 1993).

It is now known that MYB-related proteins are found in all major groups of eukaryotes, including fungi, bryophytes, insects and higher plants (Martin & Paz-Ares 1997). This suggests that proteins with MYB-like DNA-binding domains developed early on in the evolution of eukaryotes. Indeed, it now appears that higher plants have made far more extensive use of the MYB-regulated gene expression than vertebrates. For instance, *Arabidopsis* is estimated to contain more than 100 *R2R3-MYB* regulatory genes (Romero et al. 1998) and at least 20 different MYB domain genes are expressed in *Arabidopsis* flowers alone (Williams & Grotewold 1997). Most notably, most plant MYB domain proteins contain only two repeats, which are equivalent to R2 and R3. Furthermore, their functions appear to be quite distinct from their vertebrate counterparts, which function exclusively in cellular proliferation and differentiation (Martin & Paz-Ares 1997). A detailed account of the many processes in which plant MYB proteins participate is beyond the scope of this review. The reader is referred to the excellent review by Martin and Paz-Ares (1997) for an overview of their roles in the regulation of cellular morphology, the actions of growth regulators (e.g. gibberellins) and the response to environmental signals such as light (Wang et al. 1997). The involvement of an abscisic acid (ABA)-inducible MYB-domain protein from *Arabidopsis* in the regulation of the response to dehydration and salt stress (Urao et al. 1993, 1996; Abe et al. 1997) will be discussed further in Section 2.4.3.2.

Presently, phenylpropanoid metabolism is by far the best characterised plant physiological process in which MYB-domain proteins have been shown to play an important role. The maize MYB-domain proteins P and C1 regulate the accumulation of related, but different, flavonoid pigments through the activation of overlapping sets of flavonoid biosynthetic genes (Lloyd et al. 1992; Grotewold et al. 1994; Sainz et al. 1997). Upregulation of both C1 and R (a maize transcription factor with homology to vertebrate MYC-related transcription factors) results in anthocyanin accumulation, whereas P is required for 3-deoxy flavonoid biosynthesis. The practical implications of ectopic expression of plant MYB-type regulatory genes are exemplified by two recent advances. Tamagnone et al. (1998) demonstrated that expression of two MYB factors from *Antirrhinum majus* affects lignin synthesis in transgenic tobacco, while Grotewold et al. (1998) have shown that ectopic expression of *P* is a viable method for engineering the synthesis of a range of flavonoids in cultured plant cells.

Of particular relevance to this discussion is the high degree of conservation of a cysteine residue in the -Lys-Ser-Cys-Arg- motif of the DNA-binding domain of all eukaryotic MYB-type transcription factors. This cysteine has been implicated in the redox control of c-MYB activity (Myrset et al.

1993) mentioned above. A conserved regulatory mechanism for MYB-domain proteins from all of the biological kingdoms is supported by the demonstration that the maize *P* gene product binds its target DNA sequence only in the reduced state (Williams & Grotewold 1997). Martin and Paz-Ares (1997) reported that at least two additional MYB-domain proteins (AmMYB305 from *Antirrhinum majus* and PhMYB3 from *Petunia hybrida*) are also sensitive to oxidation. Unfortunately, the possible redox regulation of AtMYB2, an *Arabidopsis* gene product that is transcriptionally induced by dehydration, salinity stress and ABA treatment (Urao et al. 1993) and which participates in the transcriptional activation of dehydration-responsive genes (Urao et al. 1996; Abe et al. 1997), has not been reported. Nonetheless, it increasingly seems highly likely that the activities of many, if not all, plant MYB proteins may be subject to redox control. Since a dramatic shift in cellular redox potential is a central feature common to all environmental stresses, this regulatory mechanism may be of considerable significance in mediating stress-related changes in gene expression . For instance, there is already substantial evidence which implicates MYB-class transcription factors in the regulation of lignin synthesis (Section 2.2.2.2) through their control of phenylpropanoid metabolism (Sablowski et al. 1994; Moyano et al. 1996; Uimari & Strommer 1997; Tamagnone et al. 1998). As will be discussed further in Section 2.5.2.2, activation of the shikimate pathway is a widespread response to both biotic and abiotic stresses (Dixon & Paiva 1995; Solecka 1997). In view of the likely importance of proline synthesis in the regulation of intracellular redox potential (Section 2.5.2), MYB-type transcription factors seem likely candidates for mediating the transcriptional induction of genes responsible for proline synthesis.

Several studies have indicated a role for intermediates in the antioxidative stress response in the regulation of stress-related genes. Wingate et al. (1988) demonstrated that exogenous GSH (0.01 - 1.0 mM), but not GSSG, enhanced the expression of genes encoding the phenylpropanoid biosynthetic enzymes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) involved in lignin (PAL) and phytoalexin (CHS, PAL) production. The transcriptional induction of *CHS* expression by either GSH or a fungal elicitor was characterised by Dron et al. (1988). A 429 bp promoter sequence was shown to confer regulation by either of these two stimuli. These studies first suggested a general role for the redox state of the glutathione pool in signalling the response to biotic stress. In an attempt to begin to delineate the signalling events that underlie the redox control of phenylpropanoid biosynthetic genes, Dröge-Laser et al. (1997) identified a factor (G/HBF-1) which binds to the G-box and H-box *cis* elements in a *CHS* promoter. While G/HBF-1 transcript and protein levels do not increase during the induction of phenylpropanoid genes, G/HBF-1 is phosphorylated rapidly in elicited soybean cells. A cytosolic protein-serine kinase which is rapidly and transiently stimulated by GSH with kinetics comparable to those observed after treatment with an avirulent pathogen was shown to phosphorylate G/HBF-1 and to enhance

its binding to the *CHS* promoter (Dröge-Laser et al. 1997). Thus, in this particular case, direct redox-modulation of transcription factor activity seems not to be important.

Besides genes involved in phenylpropanoid biosynthesis, plant genes involved in antioxidative defences are also sensitive to cellular redox status. A variety of sulfhydryl-containing molecules (DTT, GSH and cysteine) increased the expression of a reporter gene coupled to the promoter of the gene encoding cytosolic Cu/ZnSOD from *Nicotiana plumbaginifolia* (Hérouart et al. 1993). Neither GSSG nor the oxidised form of cysteine had any effect on the expression of this gene (Hérouart et al. 1993). Subsequently, Wingsle and Karpinski (1996) demonstrated that changes in the GSH/GSSG ratio regulate the expression of both cytosolic and chloroplastic Cu/ZnSODs in Scots pine (*Pinus sylvestris*), although in this system, a *decrease* in cytosolic Cu/ZnSOD transcript levels which accompanied increases in the GSH/GSSG ratio conflicts with the findings of Hérouart et al. (1993). In pine needles, a decrease in the GSH/GSSG ratio significantly increased GR activity. Since this effect occurred without any change in the levels or isoform population of GR, or of transcripts encoding GR, the GR enzyme itself may be subject to post-translational redox modifications (Wingsle & Karpinski 1997). Exogenous GSH prevented the induction of accumulation of transcripts encoding two cytosolic APXs in *Arabidopsis* following exposure to excess light and halved *APX1* mRNA levels in leaf discs under low-light conditions (Karpinski et al. 1997). The mechanistic basis of the redox regulation of genes encoding SODs or APXs does not appear to have been investigated.

Any discussion of the redox-regulation of plant metabolism should incorporate mention of thioredoxins (TRXs). These ubiquitous small-molecular-weight disulphide proteins (typically 100-120 amino acid residues; approximately 12 kDa) possess a characteristic conserved active site sequence -Cys-Gly(Ala/Pro)-Pro-Cys- (Jacquot et al. 1997). The two cysteines of the active site can form an intramolecular disulphide bond (-S-S-) in the oxidised state of the protein. This disulphide bridge is reduced to the sulfhydryl (-SH) level by either reduced ferredoxin (Fd; an intermediate in the photosynthetic electron transfer chain) or NADPH via one of two specific enzymes. The standard role for TRX is to modulate enzyme activity by reducing disulphide bridges (Besse & Buchanan 1997). While only one class of TRX has been detected in eubacterial or animal cells, three well characterised variants have been identified in plants.

Two of these TRXs (TRX *f* and TRX *m*) are chloroplastic and play a role in regulating photosynthetic enzymes by the Fd/TRX system. Electrons provided by the excitation of chlorophyll in the light are transferred from Fd to either of the two classes of plastidic TRXs through the action of Fd-TRX reductase. After reduction, TRXs *f* and *m* selectively activate or inactivate chloroplastic enzymes through the reduction of intramolecular sulfhydryl groups, thereby directly linking their

activity to the functioning of the photosynthetic electron transfer chain (Buchanan 1991). Enzymes of the reductive pentose phosphate (Calvin) cycle which are activated by the Fd/TRX system include phosphoribulokinase (PRK), fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Several chloroplastic enzymes which do not participate in the Calvin cycle (e.g. NADP-malate dehydrogenase and CF_1-ATPase) are also rendered catalytically competent after light-mediated actuation of the Fd/TRX system (Buchanan 1991). In contrast, plastidic glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), an important regulatory enzyme of the OPPP, is inactivated by Fd/TRX-mediated reduction in the light when the reduction of $NADP^+$ can be accomplished by photosynthesis (Wenderoth et al. 1997). This mechanism also prevents futile cycling that would arise from simultaneous carbohydrate synthesis in the Calvin cycle and its catabolism by the OPPP (Buchanan 1991).

Despite the dominant role played by TRX in regulating the activities of photosynthetic enzymes, it is noteworthy that recent advances (Wedel et al. 1997) have indicated an additional mechanism for the light regulation of the Calvin cycle in spinach chloroplasts that does not implicate the Fd/TRX system. *In vitro*, a 600 kDa complex comprising a small regulatory protein CP12 as well as GAPDH and PRK can be reversibly dissociated by $NADP^+$ and NADPH, but not by NAD^+ or NADH (Wedel et al. 1997). The demonstration that PRK activity of the dissociated complex is inhibited by $NADP^+$ but significantly stimulated in the presence of NADPH, indicates that besides Fd-mediated reduction of TRX, the ratio of accessible $NADP^+$/NADPH may also play an important role in the regulation of photosynthetic capacity. This finding emphasises the likely diversity of the mechanisms that plants may have developed to use the changes in cellular redox potential associated with photosynthetic electron transfer as a means to regulate gene products involved in the assimilation of light energy.

Besides TRXs *f* and *m*, plants also contain a third class of TRX (TRX *h*), which is analogous to that found in heterotrophic organisms. The reduction of TRX *h* is coupled to NADPH by a flavin enzyme named NADPH-dependent TRX reductase (NTR; EC 1.6.4.5). Thus far, plant *h*-type TRXs have been found in the endoplasmic reticulum, cytosol and mitochondria (Besse & Buchanan 1997). Whereas only two TRXs have been characterised in vertebrates, and non-photosynthetic prokaryotes (e.g. *E. coli*) have but a single TRX, eight TRX *h* genes have been identified in *Arabidopsis* (Rivera-Madrid et al. 1995; Jacquot et al. 1997). *Arabidopsis* also contains at least two TRXs *f* and four TRXs *m* (Jacquot et al. 1997). The reactions involved in the Fd/TRX and NADPH/TRX systems are summarised in Figure 2.4.

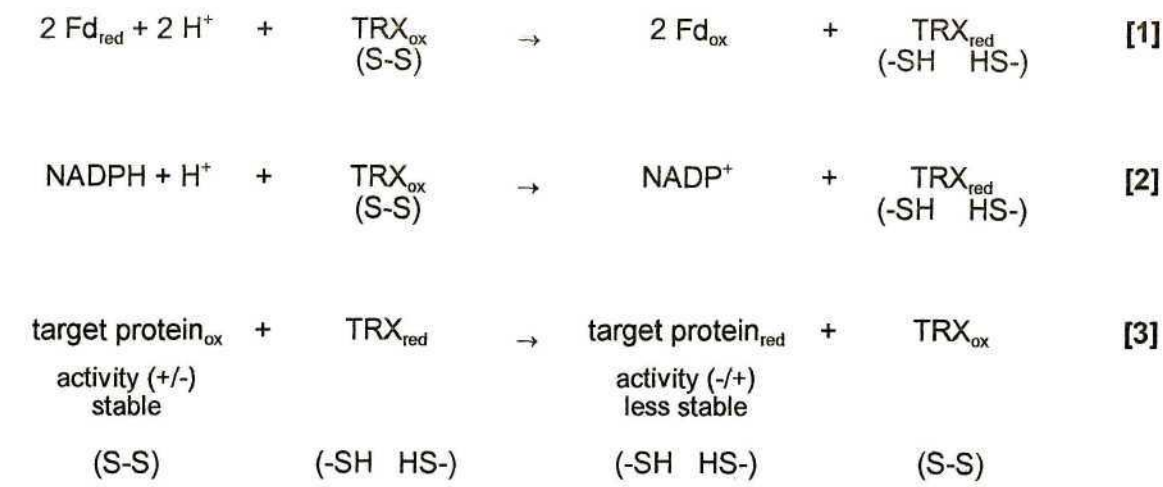

Figure 2.4: The actions of plant thioredoxins. Reaction [1] is catalysed by ferredoxin-dependent thioredoxin reductase and involves thioredoxins *f* and *m*. Reaction [2] involves thioredoxin *h* and is catalysed by NADPH-dependent thioredoxin reductase. However, note that TRX can also be reduced by the nonphysiological dithiol reagent dithiothreitol. As is indicated in reaction [3], depending on the protein targeted, reduction may effect either a loss or an increase in the associated biochemical activity. The demonstration that proteins in the reduced state frequently display an increased susceptibility to proteolysis and heat denaturation indicates that TRX-mediated structural changes affect not only the function, but also the life-expectancies of target proteins (Buchanan et al. 1997). Fd, ferredoxin, -SH, reduced sulfhydryl, S-S, oxidised sulfhydryl; TRX, thioredoxin, +, active protein; -, inactive protein.

Thioredoxin has been implicated in a variety of important physiological roles in mammalian cells. For example, reduced TRX converts the glucocorticoid receptor from a non-binding to a steroid-binding state (Grippo et al. 1985), regulates the DNA-binding activity of NF-κB (Matthews et al. 1992) and mediates the interferon-γ-mediated growth arrest of HeLa cells (Deiss & Kimchi 1991). A human TRX was shown to be identical to the adult T-cell leukemia-derived factor involved in leukaemogenesis, and its redox activity is required for the stimulation of cell division (Gadaska et al. 1994, Oblong et al. 1994, Powis et al. 1994). Although AP-1 is not a direct substrate of TRX, Hirota et al. (1997) demonstrated both association of TRX with nuclear Ref-1 (discussed above) and a requirement for cysteine residues in the TRX catalytic centre for the potentiation of AP-1 activity.

It is thus not surprising that plant TRX *h* has also been proposed to regulate cell division in plants. In *Arabidopsis*, a *cis*-acting promoter element, the TEF1 box, has been implicated in the transcriptional activation of a class of genes related to cell division (Curie et al. 1991). Intriguingly, TEF1 boxes occur within the promoters of genes encoding TRX *h2* from tobacco and a rice TRX *h* gene as well as in other plant genes (e.g. a tobacco GST homologue) which also participate in redox-regulated processes (Regad et al. 1995). Since tobacco TRX *h2* is abundant in growing

tissues, but undetectable in mature nondividing cells (Marty et al. 1993), it was suggested that TEF1-dependent regulation of genes encoding proteins involved in redox regulation may be important in plant cells undergoing the transition from quiescence to growth (Regad et al. 1995).

A recent study concerning the regulation of cell division in the apical meristem of *Arabidopsis* roots strongly suggests that redox-dependent modulation of growth and development may be crucial components in plant adaptation to fluctuating environmental conditions. In this system, exogenous application of micromolar concentrations of GSH increased the number of meristematic cells undergoing mitosis, while depletion of intracellular GSH had the opposite effect (Sánchez-Fernández et al. 1997). A role for endogenous GSH in the control of cell proliferation was supported by the demonstration that in the *Arabidopsis* root apical meristem, high levels of GSH were associated with the actively dividing epidermal and cortical initial cells, with much lower levels in the slowly cycling cells of the quiescent centre (Sánchez-Fernández et al. 1997). The mechanisms controlling cell division could also be activated by other exogenous diffusible reductants such as ascorbic acid and DTT (Sánchez-Fernández et al. 1997). The ability of different antioxidants to elicit the same effects, and alleviation by ascorbate of the decrease in meristematic activity after GSH depletion are consistent with the notion that these effects are based on interference with cellular redox potential. An involvement of GSH in the redox regulation of the cell cycle in human cells (Russo et al. 1995) further corroborates this view. Exogenous GSH, ascorbate and DTT all decreased the average length of trichoblasts in the primary *Arabidopsis* root. Treatment with either GSH or DTT caused significant, dose-dependent increases in both root hair density and root hair length, although treatment with ascorbate had no effect on these parameters (Sánchez-Fernández et al. 1997). It was thus concluded that growth of the primary *Arabidopsis* root and trichoblast cell length are influenced by nonspecific redox couples, whereas root hair abundance and root hair tip growth have specific requirements for sulfhydryl groups (Sánchez-Fernández et al. 1997). Unfortunately, the direct involvement of TRX-mediated changes in eliciting these effects was not investigated.

Notably, TEF1 elements appear to be a common feature not only of the promoters of many genes which participate in the redox-control of the plant cell cycle, but also of the upstream regions of genes encoding components of the cellular translational apparatus (Regad et al. 1995). Allen et al. (1995) reported ^{35}S-methionine incorporation studies which indicated that isolated chloroplast and mitochondria from pea leaves synthesise specific subsets of proteins when incubated in the presence of a variety of different redox reagents, electron donors and electron transport inhibitors. Their results were interpreted to indicate that expression of genes within the chloroplast and mitochondrial genomes is regulated by photosynthetic and respiratory electron transport respectively. However, the approach used by Allen et al. (1995) could not discriminate between

whether these effects are primarily mediated at transcriptional or post-transcriptional levels.

In an elegant study, Danon and Mayfield (1994) provided evidence that reducing power generated by photosystem I activates the translation of a light-regulated chloroplast mRNA from *Chlamydomonas reinhardtii* through a change in the redox state of chloroplastic TRX. These workers investigated the light-mediated translational regulation of *psbA* mRNA, which encodes the D1 protein of PSII. Based on the previous knowledge that the binding of several proteins to a stem-loop structure in the 5'-untranslated region of *psbA* transcript correlates with translation of this mRNA, Danon and Mayfield (1994) tested whether the formation of this mRNA-protein complex could be affected by treatment with either reducing or oxidising agents. Addition of the oxidant dithionitrobenzoic acid completely abolished the RNA-binding capacity of the protein complex, while its reduction with DTT restored binding. The ability of reduced TRX purified from *E. coli* to activate mRNA-binding better than DTT alone implicates TRX as the *in vivo* factor required for the reduction of a regulatory disulphide bond in the complex, which facilitates its binding to the mRNA (Figure 2.5; Danon & Mayfield 1994). The obvious appeal of this model is that it provides a simple regulatory mechanism by which the chloroplast can coordinate the synthesis of components of PSII with the availability of light.

Redox-regulated changes in the transcription of other light-regulated plant genes have recently been reviewed (Durnford & Falkowski 1997) and the reader is referred to this excellent account of our current knowledge of the mechanisms by which components of the chloroplast electron transport chain regulate both plastidic and nuclear gene expression. Most notably, in contrast to redox-mediated translational (Danon & Mayfield 1994) and post-translational (Buchanan 1991) mechanisms, a role for the Fd/TRX system in controlling transcriptional initiation is presently not evident. Pharmacological studies using the unicellular alga *Dunaliella tertiolecta* strongly suggest that light intensity is sensed through the redox status of the plastoquinone pool (Escoubas et al. 1995). The signal initiated by an abundance of reduced plastoquinone in the chloroplast may be transduced through a redox-regulated protein kinase that (in)directly interacts with the nuclear transcriptional apparatus, which modulates the expression of genes that encode components of the light harvesting complexes (Escoubas et al. 1995). A similar scenario seems valid for higher plants. Using inhibitors of photosynthetic electron transport, Karpinski et al. (1997) concluded that a signalling pathway which is associated with photoinhibition under conditions of exposure to excessive light intensity and rapidly induces increases in the levels of mRNAs that encode two genes encoding cytosolic APXs in *Arabidopsis*, is initiated at plastoquinone and is regulated, at least in part, by the redox status of the plastoquinone pool. Treatment of leaves with exogenous GSH abolished this signal, thus indicating that the redox status of the glutathione pool has a regulatory impact on this signalling pathway (Karpinski et al. 1997).

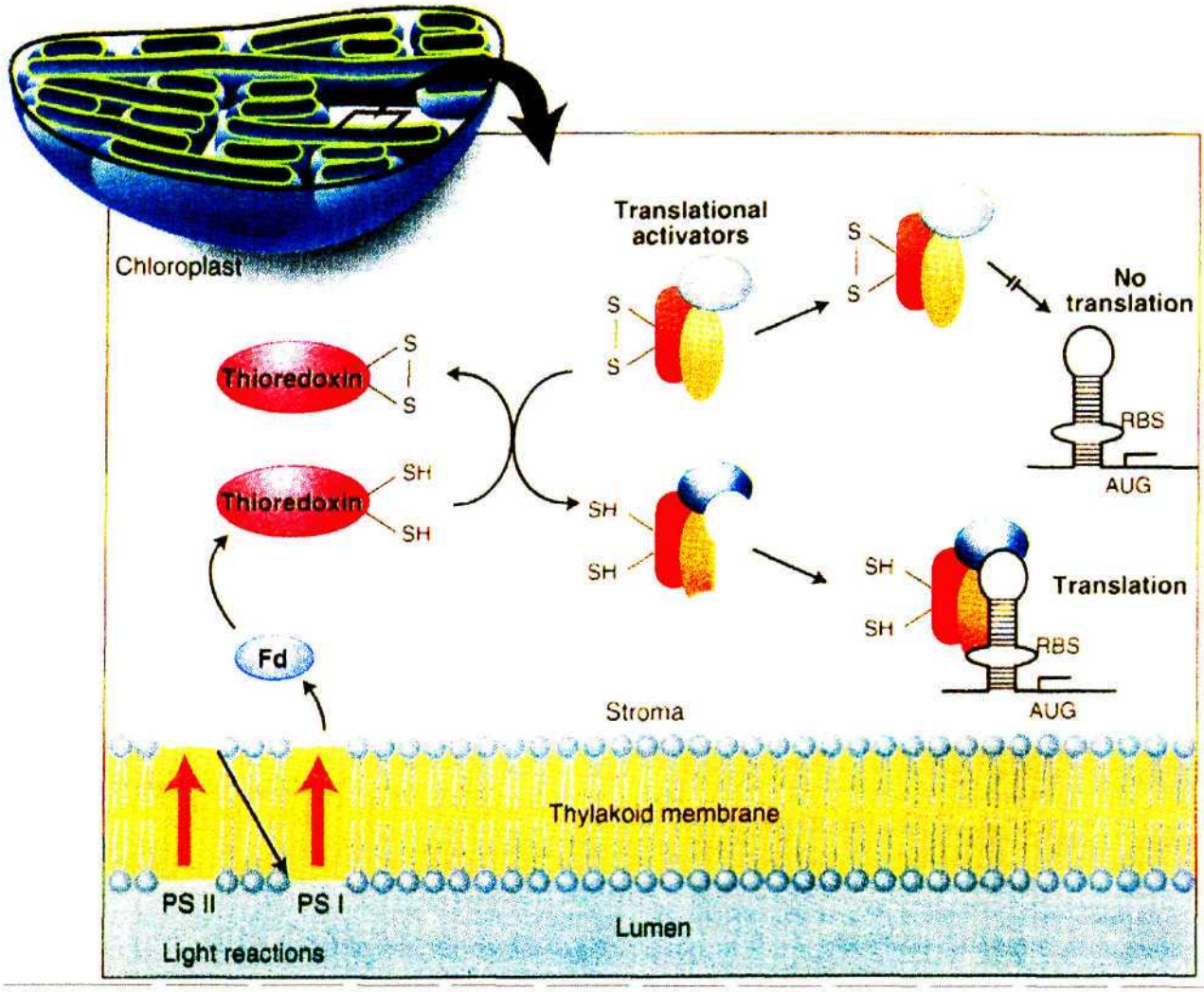

Figure 2.5 : Thioredoxin-mediated modulation of translation of *psbA* transcript in *Chlamydomonas reinhardtii*. Two reducing equivalents provided by reduced ferredoxin (Fd) generated during photosynthesis convert the disulphide bond of thioredoxin to dithiols. The reduced thioredoxin activates the RNA binding of translational activators by reducing a regulatory disulphide bond of the RNA-binding protein complex. Only the reduced translational activator protein complex can bind to the 5'-untranslated region of the *psbA* mRNA (Danon & Mayfield 1994). Binding of the reduced translational activator protein complex to the *psbA* transcript permits translation of the D1 protein of photosystem II. AUG, start codon; RBS, ribosome-binding site. From Levings and Siedow (1995).

It is also worth mentioning that at least two other hypotheses besides feedback regulation by a signal derived from the photosynthetic electron chain seem valid for modulating the expression of gene involved in the acclimation to excessive light intensity (Durnford & Falkowski 1997). The first postulates the likely involvement of photoreceptors such as phytochromes, which sense variations in photon flux density and are known to cause changes in nuclear gene expression (Section 2.4.5.1; Thomas et al. 1997). A second hypothesis invokes feedback from light regulated chlorophyll synthesis, a process which is restricted to the plastids. All three hypotheses have merit and are not mutually exclusive (Durnford & Falkowski 1997). Using *C. reinhardtii*, Kropat et al.

(1997) have provided convincing evidence that plastid-derived chlorophyll precursors Mg^{2+}-protoporphyrin IX and its dimethyl ester can replace light in the induction of two nuclear heat-shock genes. This extends into the importance of "cross-talk" between seemingly unrelated signal transduction pathways. This subject, in particular the likely overlap between redox sensing pathways and phytochrome signal transduction, will be dealt with in Section 2.4.5.

As outlined above, our present appreciation of the molecular mechanisms by which redox potential influences plant gene expression in response to environmental changes appears to be limited to the findings of investigations into photoacclimatory responses to excessive irradiance. Nonetheless, Rey et al. (1998) recently reported that a potato (*Solanum tuberosum*) chloroplastic protein which is transcriptionally induced by water deficit displays typical features of TRXs in its C-terminal region and possesses TRX activity. Further studies are needed to investigate the precise role of this protein in preserving the thiol/disulphide redox potential of the chloroplastic stroma during water deficit.

2.2.3 The role of cellular redox potential in seed germination

Seed germination and post-germinative events constitute critical transitions in the life cycle of higher plants (Bewley 1997). During this period, nutrient reserves which have been stored throughout the dormant phase, must be rapidly mobilised to support the initial growth of the germinating seedling. These changes occur simultaneously with the establishment of the basic architecture of the mature plant from the pattern established during embryogenesis. By definition, germination incorporates those events that commence with imbibition of the quiescent dry seed and terminate with the protrusion of the embryo (in *Arabidopsis*, the radicle) through the seed coat (Bewley 1997). The stimulation of embryo growth is the consequence of a signal transduction chain that is induced after perception of the required environmental signals by nondormant seeds. In light-requiring seeds such as *Arabidopsis*, the active form of phytochrome (P_{fr}) is believed to be an important trigger of this pathway, although plant hormones, particularly ABA and the gibberellins, are also involved. The absolute levels of hormones and the responsiveness of plant tissues to these growth regulators is also controlled by natural environmental cues such as light, temperature and nutrient availability. Nonetheless, how these diverse factors are integrated in a signalling network that initiates germination is currently not well understood (Thomas et al. 1997). Although many hypotheses have been proposed, a fundamental regulatory principle underlying seed dormancy or the triggering of germination under appropriate environmental conditions has never been established (Li & Foley 1997).

An extensive body of evidence now suggests that TRX *h* plays a central role in coordinating several regulatory steps during seed germination. Target proteins with intramolecular disulphide bonds that are specifically reduced by the NADPH/TRX system are summarised in Table 2.8. Where tested, GSH was not effective in altering the biochemical properties of any of these proteins.

Table 2.8: Germination-related proteins regulated by thioredoxin *h*.

Target protein	Source[1]	Reference
α-amylase/trypsin inhibitors	*Triticum aestivum*	Kobrehel et al. (1991)
Kunitz and Bowman-Birk trypsin inhibitors	*T. aestivum*	Jiao et al. (1992)
gliadins/gluteneins (seed storage proteins)	*T.aestivum*	Kobrehel et al. (1992)
2S seed storage proteins	*Ricinus communis*	Jiao et al. (1993)
α-amylase/subtilisin[2] inhibitors	*Hordeum vulgare*	Shin et al. (1993)
thiocalsin (a Ca^{2+}-dependent serine protease)[3]	*T. aestivum*	Besse et al. (1996)
limit-dextrinase (pullulanase)[4] inhibitors	*H. vulgare*	Wong et al. (1996)
α-amylase[5]	*Populus x canadensis*	Witt & Sauter (1996)

[1] unless indicated, all of the proteins characterised are from seeds
[2] a serine protease
[3] activation by Ca^{2+} occurs only after reduction by TRX
[4] catalyses the cleavage of α-(1-6)-glucosidic linkages of amylopectin
[5] purified from mature poplar leaves

As is represented in Figure 2.6, TRX-mediated reduction of regulatory disulphide bonds in all of these proteins appears to be important in the mobilisation of carbon and nitrogen reserves for seedling growth. Whereas enzymes involved in nutrient mobilisation are activated by reduction of disulphide bonds, reduction of proteinaceous inhibitors of these enzymes is accompanied by a loss of their activities. Maintenance of seed storage proteins in the oxidised state appears to further stabilise seed amino acid reserves since TRX-mediated reduction of seed storage proteins renders them more susceptible to proteolysis (Figure 2.6).

Experiments conducted using wheat seeds have indicated that the endosperm fraction contains the enzymes (hexokinase, G6PDH and 6-phosphogluconate dehydrogenase) necessary to reduce NADP by OPPP activity (Figure 2.6). Parallel studies indicated that activities of the two rate-limiting dehydrogenases of the OPPP increased within 24 after imbibition. At this time, TRX of wheat endosperm was converted from an oxidised to a partially reduced state (Lozano et al.

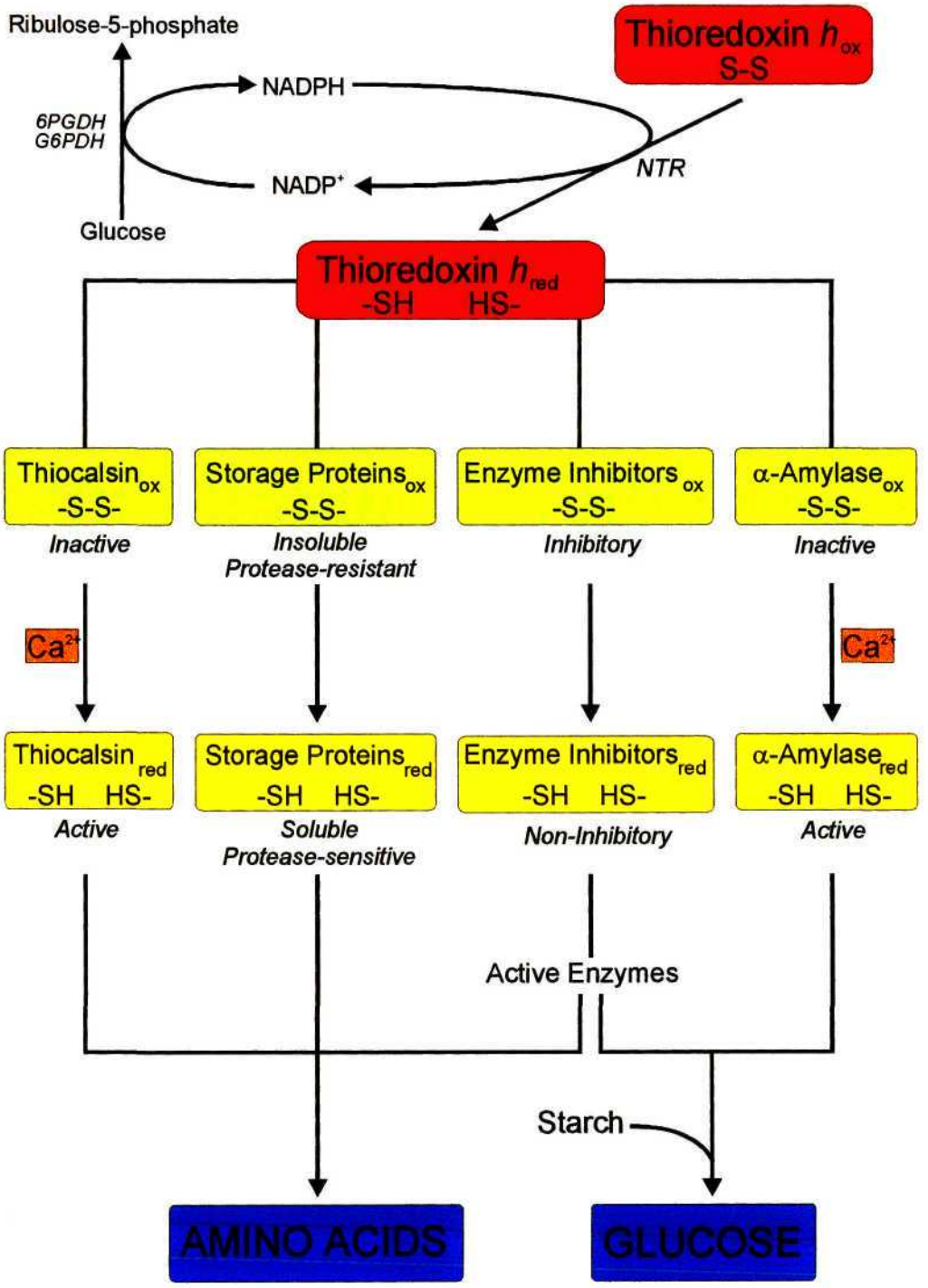

Figure 2.6: The roles of thioredoxin *h* in seed germination. Thioredoxin *h* is converted from an oxidised to a reduced state by the NADPH-dependent thioredoxin reductase (*NTR*), which uses NADPH obtained from oxidative pentose phosphate pathway activity. Thioredoxin (TRX)-mediated reduction of seed storage proteins renders them more susceptible to proteolysis (Kobrehel et al. 1992; Jiao et al. 1993). In wheat seeds, a new type of serine protease, thiocalsin, which is dependent both on reduced TRX and Ca^{2+} but not calmodulin, catalyses the degradation of the reduced forms of gliadins and gluteneins (Besse et al. 1996). Thioredoxin *h* also reduces several proteinaceous inhibitors of enzymes participating in the mobilisation of carbon and nitrogen reserves, thus rendering them inactive (Table 2.8; Kobrehel et al. 1991; Jiao et al. 1992; Shin et al. 1993; Wong et al. 1996). Additionally, a regulatory role for TRX, in combination with Ca^{2+}, in the direct activation of an amylopectic enzyme from mature poplar leaves has been suggested (Witt & Sauter 1996). *G6PDH*, glucose-6-phosphate dehydrogenase; *6PGDH*, 6-phosphogluconate dehydrogenase. Adapted from Besse et al. (1996).

1996). The NADPH formed by OPPP activity can reduce TRX *h* owing to the presence of NTR in the endosperm (Lozano et al. 1996). Although the level of TRX later declined, the available reducing equivalents of TRX (defined as the product of the relative abundance of TRX and the percent reduction) increased in accord with the level of reduction of storage proteins (Lozano et al. 1996; Wong et al. 1996). Interestingly, the abundance of TRX *h* in wheat endosperm appears to be controlled by the embryo via ABA and gibberellin action (Lozano et al. 1996).

Although it has apparently never been explicitly stated, these findings may relate to a long-held view that the OPPP plays a special role in triggering seed germination in a wide range of species. Roberts (1973) provided a detailed account of evidence in favour this proposal. In short, classical studies have indicated that :

i) seed dormancy is frequently relieved by the removal of structures surrounding the embryo, or exposure of seeds to elevated oxygen levels;

ii) respiratory inhibitors which block terminal oxidation and the tricarboxylic acid (TCA) cycle in mitochondria (e.g. cyanide, azide, malonate) or glycolytic flux (e.g. fluoride), but which do not affect OPPP activity, also break dormancy in several species;

iii) dormancy breaking agents such as methylene blue, nitrate and nitrite are electron acceptors and activate the OPPP most probably through preferential oxidation of $NADP^+$ (rather than NAD^+);

iv) low-temperature treatment of imbibed seeds (stratification), a dormancy-breaking treatment in many species, apparently increases carbon flux through the OPPP relative to glycolysis;

v) the early respiration of nondormant imbibing seeds from many species primarily involves OPPP activity.

The stimulatory effects of electron acceptors and inhibition of conventional respiration on germination are consistent with the notion that carbon flux through glycolysis and the TCA cycle may suppress activity of the OPPP, the continued operation of which requires the oxidation of the NADPH which is generated by this pathway. The stimulatory effects of elevated oxygen on seed germination were proposed to arise from a requirement for oxygen to oxidise NADPH to relieve feedback inhibition of the two dehydrogenases of the OPPP (Roberts 1973). The consumption of oxygen by conventional respiration would restrict this process. Although the existence of such an oxidation system in plants has long remained contentious, as discussed above (Sections 2.2.2.1, 2.2.2.2), there is now a wealth of evidence to support important regulatory roles for plasma membrane-bound NADPH oxidase(s). Thus, while Roberts (1973) conceded that the stimulatory effects of elevated oxygen concentrations on germination seem paradoxical in view of the postulated significance of OPPP activity in triggering germination, it now seems feasible to propose that O_2-mediated oxidation of NADPH may provide one mechanism by which OPPP

activity may be activated in nondormant seeds. In this regard, it is worth noting that the NADP pool in most dormant seeds is predominantly in the reduced state and would have to be oxidised in order to relieve feedback inhibition by NADPH of the two rate-limiting dehydrogenases of the OPPP (Botha et al. 1992). Once activated, continued OPPP activity would be dependent on reoxidation of the NADPH generated. Interestingly, Roberts (1973) suggested that an inadequate NADPH oxidising system may be the primary restraint to germination in dormant seeds.

During the two decades following Roberts' proposal, a number of studies aimed at assessment of the role of the OPPP in the germination of a range of species. These involved measurement of the activities of the two dehydrogenases involved in the OPPP as well as monitoring changes in the relative contributions of [^{14}C]-6-glucose and [^{14}C]-1-glucose to CO_2 evolved by seeds prior to, during and after dormancy breaking (Botha et al. 1992). The C_6/C_1 labelling technique is based on the fact that during glycolysis, glucose is split into two 3-carbon units, with both carbon-1 and carbon-6 being converted to pyruvate and subsequently decarboxylated in an identical fashion at equal rates. Hence, if glucose is respired exclusively via glycolysis, the C_6/C_1 ratio is unity. Any participation of the OPPP in respiration decreases the C_6/C_1 ratio, since only carbon-1 of the original glucose molecule is decarboxylated when 6-phosphogluconate is converted to ribulose-5-phosphate (Roberts 1973). Although much of the data obtained from these studies supports the proposal of Roberts (1973), owing to the failure of certain investigators to correlate dormancy release with activation of OPPP activity, support for an important role for the pathway in the regulation of germination has never been universal (Botha et al. 1992).

As pointed out by Roberts (1973), the apparent significance of the OPPP during early germination is enigmatic. The traditional role of the OPPP, *viz.* the generation of reductant for anabolic reactions, was suggested to be unlikely in view of the evidence that agents which maintain the NADP pool in the oxidised state stimulate loss of dormancy. In view of our increasing awareness of the importance of TRX-mediated changes in the activities of germination related proteins (Table 2.8) as well as redox control of plant gene expression (Section 2.2.2.3), it is tempting to suggest that activation of the OPPP may influence transcriptional or post-transcriptional activities important in triggering germination. Nonetheless, if we are to assume that the dormancy-breaking activities of electron acceptors emulate the chain of events which normally precede radicle emergence, then this view is once again confounded for the same reason as for the metabolic rationale outlined above.

Irrespective of these complications, it may be significant to note that in oilseed plants such as *Arabidopsis*, where lipids are the primary storage reserve, considerable amounts of H_2O_2 and toxic by-products of lipid peroxidation are likely to accumulate during triacylglycerol breakdown

upon germination. Accordingly, three of the six *Arabidopsis* catalase isoforms are detectable in freshly imbibed seeds, and an additional two isozymes become evident in two-day-old seedlings (McClung 1997). All three of the mRNAs which encode the subunits which associate to form these isoenzymes are detectable in newly imbibed *Arabidopsis* seeds, although the relative abundances of the three mRNAs varies during early germination (McClung 1997). Furthermore, a cDNA clone with extensive sequence homology to GPX was isolated by differential screening of dormancy and nondormancy associated libraries of *Avena fatua* (Johnson et al. 1995). Therefore, an important although seldom considered role for the OPPP during radical emergence and the early stages of seedling establishment, may be the provision of NADPH at a time prior to photosynthetic competence but when extremely high metabolic activity (including mitochondrial electron transport) is likely to cause considerable oxidative stress if an effective antioxidant defence system is not operative. Furthermore, the ability of NADH, but not NADPH to reduce Fe^{3+} (Farr & Kogoma 1991) suggests that NADPH production yields an electron donor for NTR and GR without facilitating ·OH production by the Fenton reaction.

Consistent with the likely importance of the OPPP in providing reductant required for antioxidative defence, four *Arabidopsis* NADPH oxidoreductases were shown to confer tolerance of yeast to the thiol-oxidising drug diamide (Babiychuk et al. 1995). It was proposed that the protective effects of these enzymes may arise from their participation in $NADP^+$/NADPH redox cycling, which in turn is coupled to up-regulation of OPPP activity (Babiychuk et al. 1995). This is consistent with the demonstration that yeast mutants deficient in any of five enzymes participating in the OPPP display increased sensitivity to H_2O_2 (Juhnke et al. 1996) and that mouse embryonic stem cells rendered deficient in G6PDH by targeted homologous recombination are extremely sensitive to even mild oxidative stress (Pandolfi et al. 1995). Although an essential role for the OPPP in mediating antioxidative defence responses is less likely in autotrophs which are capable of generating NADPH by photosynthesis, heterotrophic stages of plant development such as seed germination and seedling establishment represent exceptional systems.

2.2.4 Conclusion

Consistent with its importance as an indicator of their overall energy status, all living organisms need to continuously monitor cellular redox potential. While the incomplete reduction of oxygen to water poses a threat to all aerobic organisms, the evolution of efficient mechanisms to monitor redox status is likely to have been a particularly important selective factor for phototrophs. Although studies of the mechanisms by which plants use redox potential to effect appropriate changes in gene expression are still in their infancy, recent advances clearly demonstrate that the

plant cell is capable of monitoring the level of reduction of pools of important intermediates such as NADP, glutathione and plastoquinone. Thioredoxins and redox-sensitive transcription factors, such as those from the large family of plant MYB-domain DNA-binding proteins, presently appear to be the most likely candidates for translating environmentally-induced fluctuations in redox potential into changes in gene expression.

As will be elaborated in Section 2.5, the interception of light energy in excess of what can be assimilated by the plant through metabolism is a major, if not the primary unified cause of stress-induced damage in plants. Mechanisms which ensure the continuous assessment of cellular redox potential, the maintenance of redox homeostasis and the coupling of changes in redox potential with altered gene expression patterns are likely to play key roles in plant acclimation to all adverse environmental conditions. Physiologists interested in plant responses to abiotic environmental stresses have only recently begun to investigate the effects of cellular redox potential *per se* on the gene regulation in higher plants exposed to abiotic stresses (Karpinski et al. 1997; Rey et al. 1998). Thus far, no single overriding mechanism has emerged in studies involving redox-regulated plant gene expression and it seems likely that plants may have evolved several different approaches to alter genomic responses to changes in the cellular prooxidant/antioxidant ratio during stress. Nonetheless, the likelihood that NADPH availability is a primary indicator of the oxidative state of the plant cell warrants emphasis. This coenzyme functions not only in reductive biosynthesis, but also in the reduction of GSSG by GR and oxidised TRX *h* by NTR. By virtue of the role of $NADP^+$ as the normal electron acceptor in photosynthetic electron transport, the level of reduction of the NADP pool is also likely to impact on the Fd-dependent reduction of TRXs *f* and *m*. The involvement of flux through the proline biosynthetic pathway in regulating the $NADP^+$/NADPH ratio and the notion that this may account for the effects of proline and proline biosynthetic intermediates in the regulation of gene expression (Garcia et al. 1997; Iyer & Caplan 1998) will be discussed in Section 2.5.

2.3 Proline biosynthesis, degradation and transport in plants

Proline accumulation induced by stress conditions is mediated both by increased synthesis and reduced oxidation of the imino acid (Figure 2.7). Although proline may also be synthesised from ornithine (Delauney et al. 1993; Roosens et al. 1998), metabolic labelling and molecular genetic studies indicate that most of the proline accumulated in the vegetative tissues of mature plants in response to stress is the result of enhanced synthesis from glutamate (Rhodes et al. 1986; Delauney et al. 1993; Roosens et al. 1998). A decrease in proline oxidation frequently accompanies prolonged stress (Stewart et al. 1977; Rayapati & Stewart 1991; Madan et al. 1995), although this in itself is unlikely to account for the levels of proline often accumulated (Elthon & Stewart 1984; Chiang & Dandekar 1995). The same intermediates, glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C), which are in tautomeric equilibrium with each other, are involved in both proline biosynthesis and catabolism. The final step in proline synthesis and initial step in proline oxidation are catalysed by different enzymes (P5C reductase and proline dehydrogenase, respectively), as are the formation and catabolism of the intermediate P5C (P5C synthetase or ornithine δ-aminotransferase for the former and P5C dehydrogenase for the latter). A detailed historical perspective of the elucidation of proline biosynthetic and degradative pathways in plant, microbial and mammalian systems was provided by Hare (1995). The sections which follow emphasise progress made over the past four years. The reader is referred to Hare (1995) for further details concerning the enzymology of proline metabolism.

2.3.1 Proline biosynthesis from glutamate

Proline is synthesised from glutamate via two successive reductions. Whereas in *Escherichia coli*, γ-glutamyl kinase (γ-GK; EC 2.7.2.11; encoded by the *proB* gene) first phosphorylates glutamate into γ-glutamyl phosphate which is then reduced to glutamate semialdehyde (GSA) by γ-glutamyl phosphate reductase (GPR; EC 1.2.1.41; encoded by the *proA* gene), in higher plants, the committing reaction in the biosynthetic route from glutamate (reduction of glutamate to its semialdehyde) is catalysed by a single bifunctional enzyme named P5C synthetase (P5CS). As in microorganisms, the tautomer of GSA, P5C, is reduced to proline by P5C reductase (P5CR).

2.3.1.1 P5C synthetase

Genes and cDNAs encoding P5CS have been cloned from *Vigna aconitifolia* (Hu et al. 1992), *Arabidopsis* (Savouré et al. 1995; Yoshiba et al. 1995; Peng et al. 1996; Strizhov et al. 1997),

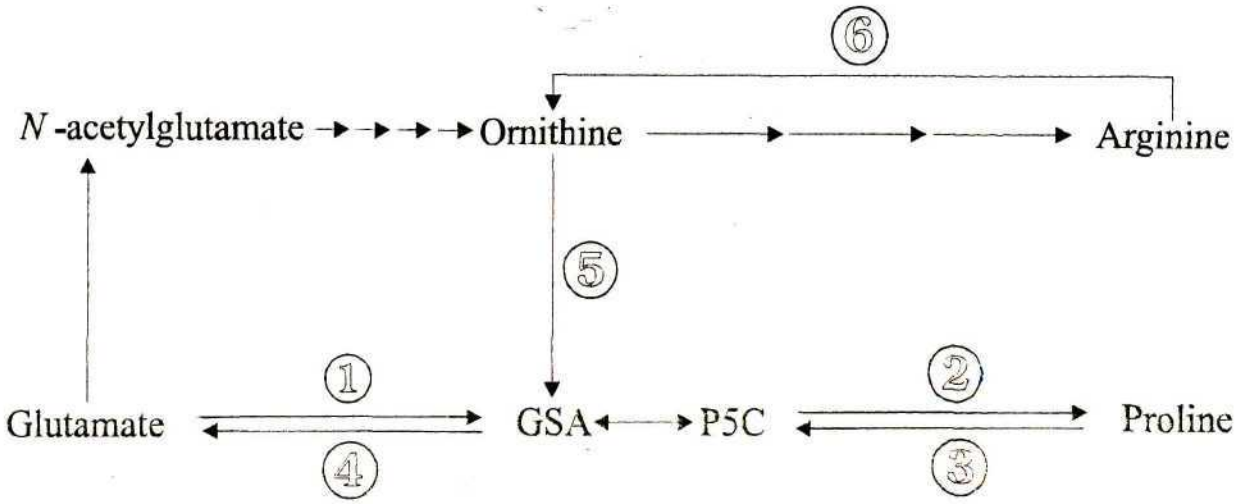

Figure 2.7: Pathways of proline biosynthesis and degradation in plants. The pathways of proline biosynthesis from glutamate and ornithine converge at the point of formation of glutamic-γ-semialdehyde (GSA), with the subsequent spontaneous cyclisation of GSA to Δ^1-pyrroline-5-carboxylate (P5C) and the reduction of P5C to proline being common to both pathways. Numbered enzymes mediating the various steps are listed in Table 2.9. From Hare and Cress (1997).

Table 2.9: Enzymatic reactions of proline biosynthesis and degradation in plants.

Enzyme		Reaction catalysed
No.	**Name**	
①	Δ^1-pyrroline-5-carboxylate synthetase [EC (2.7.2.11)(1.2.1.41)][2] (P5CS)	a) Glutamate + ATP → glutamyl γ-phosphate + ADP b) Glutamyl γ-phosphate + NADPH + H^+ → GSA (↔P5C) + $NADP^+$
②	Δ^1-pyrroline-5-carboxylate reductase (EC 1.5.1.2; P5CR)	P5C + NAD(P)H + H^+ → proline + $NAD(P)^+$
③	proline dehydrogenase (EC 1.5.99.8; PDH)	Proline + $^1/_2O_2$ + FAD → P5C + H_2O + $FADH_2$
④	Δ^1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12; P5CDH)	P5C + $NAD(P)^+$ → glutamate + NAD(P)H + H^+
⑤	ornithine δ-aminotransferase (EC 2.6.1.13; OAT)	ornithine + 2-oxoglutarate → GSA (↔ P5C) + glutamate
⑥	*arginase* (EC 3.5.3.1)	arginine + H_2O → ornithine + urea

[1] GSA, glutamic γ-semialdehyde; P5C, Δ^1-pyrroline-5-carboxylate

[2] An EC number for the bifunctional P5CS characterised in plants has not previously been assigned. The designation provided here is consistent with the recommendations of the Nomenclature Committee of the International Union of Biochemistry for the reporting and describing a multienzyme polypeptide [Eur J Biochem 185: 485-486 (1989)].

tomato (*Lycopersicon esculentum*; García-Rios et al. 1997), rice (*Oryza sativa*; Igarashi et al. 1997), kiwifruit (*Actinidia deliciosa*; Walton et al. 1998), alfalfa (*Medicago sativa*; Genbank Accession No.'s 98421, 98422) and *Mesembryanthemum crystallinum* (Genbank Accession No. AF067967). In all of the cases characterised thus far, plant P5CS is a fused protein with two separate catalytic domains. In eubacteria, γ-GK and GPR form a multimolecular complex that facilitates direct and protected transfer of the extremely labile γ-glutamyl phosphate (Baich 1969; Deutch et al. 1984; Omori et al. 1991). The C-terminal GSA dehydrogenase domain interacts with γ-GK to effect the release of this intermediate. It seems likely that the evolution of a hybrid γ-GK and GPR protein in plants arose as consequence of the selective advantage of better proline synthesis.

Interestingly, a conserved region in both the eubacterial γ-GK and GPR proteins has been suggested to have facilitated gene fusion during plant evolution through homologous recombination (Hu et al. 1992; Savouré et al. 1995). The tom*PRO1* locus, which encodes one of the two P5CS isoforms found in tomato, contains two open reading frames (ORFs) and directs the synthesis of separate γ-GK and GPR products when expressed in *E. coli* (García-Ríos et al. 1997). In tomato tissues, the TAA stop codon at the end of the ORF which encodes γ-GK is ignored through a translational mechanism, thus making tom*PRO1* the first example of a plant nuclear genetic element that encodes two functional enzymes in two distinct ORFs (García-Ríos et al. 1997). A putative leucine zipper motif in each of the two enzymatic domains of the P5CS from *Vigna aconitifolia* was postulated to facilitate protein-protein interaction to maintain inter- or intra-molecular interaction of the two enzymatic domains within plant P5CS (Hu et al. 1992). Nonetheless, in an *Arabidopsis* P5CS, these motifs do not occur in an α-helix and do not match the general consensus of four contiguous heptad repeats with a leucine residue at every seventh position forming a ridge on one side of an α-helix that permits interdigitation with a complementary helix (Savouré et al. 1995).

In *Arabidopsis* (ecotype Columbia), two differentially regulated genes, which will be referred to as *AtP5CS1* and *AtP5CS2* throughout this document, have been shown to map to different chromosomes (Strizhov et al. 1997). *AtP5CS1* is located at position 78.5 centimorgans of chromosome 2, whereas *AtP5CS2* maps close to 101.3 centimorgans on chromosome 3 (Strizhov et al. 1997). A P5CS gene from the *Arabidopsis* ecotype Landsberg *erecta* has more than 98% nucleotide homology to the *AtP5CS2* gene from ecotype Columbia in both the exon and intron regions (Zhang et al. 1997). Divergence of the biochemical function(s) of P5CS isoforms, which have also been observed in alfalfa (GenBank entries X98421; X98422) and tomato (García-Ríos et al. 1997), is suggested by the observation that whereas the *AtP5CS1* gene occurs in differentiated tissues, but cannot be detected in dividing cell cultures in the absence of stress

stimuli, the recently identified *AtP5CS2* gene from *Arabidopsis* is solely responsible for the synthesis of abundant P5C synthetase mRNA in rapidly dividing cell cultures (Strizhov et al. 1997). Consistent with the ability of a cDNA which encodes P5CS in *Vigna aconitifolia* to complement an *E. coli* mutant disrupted in the *proBA* operon (Hu et al. 1992), an *AtP5CS1* cDNA complemented *proBA* mutants of *E. coli* and expression of the GSA dehydrogenase domain of *AtP5CS2* complemented an *E. coli proA* mutant (Strizhov et al. 1997). Several cDNAs corresponding to the tom*PRO1* locus of tomato complement both *proB* and *proA* mutants of *E. coli* (García-Ríos et al. 1997).

With the exception of the kiwifruit gene encoding P5CS, which was not tested (Walton et al. 1998), all of the plant P5CS genes characterised thus far are induced by hyperosmotic stress at the level of transcript accumulation (Table 2.1). In addition, salt stress increased the abundance of P5CS protein in roots of *Vigna aconitifolia* (Zhang et al. 1995). The kinetics of induction of *Arabidopsis P5CS* genes by hyperosmotic stresses and their control by growth regulators is discussed in Section 2.4.3 of this document.

A recombinant P5CS encoded by the *Vigna aconitifolia* cDNA characterised by Hu et al. (1992) has been expressed in *E. coli*, and the enzyme purified to homogeneity (Zhang et al. 1995). The enzyme exhibited both γ-GK and GSA dehydrogenase activities and appears to comprise six identical subunits (Zhang et al. 1995). Interestingly, addition of plant root extract to the purified P5CS inhibited the γ-GK activity, but boiling the extract prior to its addition, eliminated the inhibition of P5CS (Zhang et al. 1995). It was suggested that the presence of a protein inhibitor of P5CS in plant cells may account for the failure to demonstrate a reaction synthesising P5C from glutamate in plants for over 25 years, despite concerted efforts by several research groups. Consistent with this interpretation, a factor(s) in tobacco leaf extract which is inhibitory to P5CS activity could not be removed by dialysis of the crude plant extract (Kavi Kishor et al. 1995). The recombinant P5CS has a K_m of 3.6 mM for glutamate, while the K_m for ATP was 2.7 mM (Zhang et al. 1995). The γ-GK activity of the enzyme was competitively inhibited by proline, with 50% inhibition occurring at 5.0 mM proline. The GSA dehydrogenase activity of the enzyme was insensitive to proline (Zhang et al. 1995). The *in vivo* activity of plant P5CS also appears to be responsive to the cellular energy status, since ADP is a mixed competitive inhibitor of γ-GK activity (Zhang et al. 1995). Site-directed mutagenesis of the *Vigna aconitifolia* enzyme revealed that substituting an alanine residue for a phenylalanine (position 129) practically eliminated feedback inhibition of γ-GK activity by proline, without affecting the other properties of the mutant P5CS. The 50% inhibition value of γ-GK activity in the mutant P5CS was 960 mM proline (Zhang et al. 1995). Feedback inhibition of P5CS action appears to be restricted to the enzyme level, since a 24 h treatment of transgenic *Arabidopsis* which expressed an *AtP5CS2::GUS* fusion with 10 mM

proline did not affect GUS activity in these plants (Zhang et al. 1997). The γ-GK activity of one tomato P5C synthetase isoform is 70- to 250-times more sensitive to feedback inhibition by proline than the recombinant P5C synthetase characterised from *Vigna aconitifolia* (García-Ríos et al. 1997). Feedback-inhibition of any of the other P5CS enzymes identified in plants has not been reported.

2.3.1.2 P5C reductase

Owing to the relative ease with which P5C reductase (P5CR) may be assayed in comparison with P5CS, the enzymology of P5C reduction is far better characterised than that of P5C synthesis from glutamate. Plant P5CR activity has been assayed from a diverse range of sources (Hare 1995) and the enzyme has been purified to apparent homogeneity from barley (*Hordeum vulgare*; Krueger et al. 1986), tobacco (LaRosa et al. 1991) and soybean (*Glycine max*; Chilson et al. 1991; Szoke et al. 1992). Additionally, cDNA clones encoding P5CR have been isolated from soybean (Delauney & Verma 1990), pea (*Pisum sativum*; Williamson & Slocum 1992), *Arabidopsis* (Verbruggen et al. 1993) and kiwifruit (Walton et al. 1998). The tissue-specific expression of the *AtP5CR* gene has been characterised in *Arabidopsis* (Hare & Cress 1996; Hua et al. 1997).

Expression of a soybean P5CR cDNA in transgenic tobacco resulted in a 200-fold increase in the P5CR activity, although the proline levels in the transgenic plants were not significantly altered. Together with the observation that P5CR activity in cultured tobacco cells is at least 1000-fold in excess of the requirements for proline synthesis (LaRosa et al. 1991), this indicates that P5CR does not limit proline accumulation in plants. Consistent with this interpretation, transgenic tobacco plants with elevated P5CS activity did not accumulate detectable levels of P5C (Kavi Kishor et al. 1995). Furthermore, in contrast to the results obtained from overexpression of P5CR (Szoke et al. 1992), a direct correlation between P5CS expression level and proline accumulation was noted by Kavi Kishor et al. (1995). Although levels of mRNA transcript encoding P5CR increase concomitant with proline accumulation during flower development in kiwifruit, investigation of the effects of the dormancy breaking chemical hydrogen cyanamide on proline levels and P5CR activity indicated that the reduction of P5C does not limit proline synthesis in this system (Walton et al. 1998).

While these findings collectively tend to discredit an important role for P5CR in regulating stress-induced proline accumulation, several workers have reported increases in extractable P5CR activity in plants in which proline accumulates under hyperosmotic stress. Activity of P5CR

increased approximately four-fold in response to salt stress in both the halophyte *Mesembryanthemum nodiflorum* (Treichel 1986) and the halophytic alga *Chlorella autotrophica* (Laliberte & Hellebust 1989). A four-fold increase in activity of P5CR from the epidermal cells of water stressed barley leaves has also been reported (Argandona & Pahlich 1991). Both KCl and $MgCl_2$ increased P5CR activity in pea chloroplasts by at least two-fold (Rayapati et al. 1989). Furthermore, levels of P5CR transcript increased six-fold in the roots of soybean seedlings (Delauney & Verma 1990) and approximately five-fold in the roots of pea seedlings (Williamson & Slocum 1992) in response to short-term salinisation. Although in *Arabidopsis*, salt-stress was reported to induce a five- and two-fold increase in the level of *AtP5CR* transcript in the leaves and roots respectively (Verbruggen et al. 1993), Yoshiba et al. (1995) failed to observe any enhancement in levels of this mRNA following dehydration. Prolonged drought selectively increased *AtP5CR* transcript levels in certain tissues of the flowering stalk of mature *Arabidopsis* plants (Hare & Cress 1996). Conversely, in seedlings of *Triticum durum*, both dehydration and NaCl treatment caused an approximately 100% increase in extractable P5CR activity, although use of an *Arabidopsis* P5CR cDNA probe did not indicate any changes in the level of the P5CR transcript in durum wheat (Mattioni et al. 1997). This may indicate the importance of post-translational activation of the enzyme under hyperosmotic stress conditions in this system. On the basis of an absence of cysteine residues in soybean and pea P5CRs, and the poor conservation of cysteine residues in other P5CRs characterised (Hare 1995), it is unlikely that sufhydryl groups are involved in post-translational modification of plant P5CR activity (Hare 1995).

Proline synthesis from glutamate appears to be cytosolic. Although chloroplastic P5CR has been reported (Rayapati et al. 1989; Szoke et al. 1992), the absence of a convincing transit peptide in any of the genes encoding products in proline biosynthesis from glutamate (Delauney & Verma 1990; Hu et al. 1992; Verbruggen et al. 1993; Savouré et al. 1995; Williamson & Slocum 1992; Yoshiba et al. 1995; Walton et al. 1998) and genomic copy number determinations of proline biosynthetic genes from several species do not provide strong support for the proposal that a significant proportion of proline biosynthesis is plastidic. Nonetheless, as has been pointed out recently (Hare et al. 1998), the absence of a convincing transit peptide in any of the gene products involved in proline synthesis from glutamate does not preclude a plastidic site for proline synthesis. Despite the absence of a recognisable transit peptide, spinach and beet betaine aldehyde dehydrogenases were targeted to the chloroplast in leaves of transgenic tobacco plants (Rathinasabapathi et al. 1994). Similarly, although a *myo*-inositol 1-phosphate synthase (INPS) from *M. crystallinum* does not contain a transit peptide for chloroplast import, salt-induced enhancement of INPS activity was noticeable in chloroplasts from light-grown salt-tolerant varieties of rice (Raychaudhuri & Majumder 1996). The observation that inhibitors of photosynthetic electron transport and chloroplast differentiation had no effect on salt-induced

proline accumulation in the cotyledons of radish (*Raphanus sativus*) seedlings led Hervieu et al. (1995) to conclude that plastids do not participate in proline synthesis in this system. Nonetheless, Rayapati et al. (1989) reported that P5CR in chloroplasts can account for all of the pea leaf P5CR activity. These workers also detected P5CR activity within the etioplasts of etiolated seedlings. On the basis of its kinetic parameters, this enzyme appeared to be different from the P5CR found in chloroplasts (Rayapati et al. 1989). Convincing immunological evidence for the existence of at least two P5CR isoforms in soybean has been presented (Chilson et al. 1991, 1997; Szoke et al. 1992), although genomic Southern analysis suggests the existence of only a single gene encoding P5CR in *Arabidopsis* (Verbruggen et al. 1993; Hare & Cress 1996). No evidence of multiple peaks of activity was observed in chromatographic profiles throughout the purification of tobacco P5CR (LaRosa et al. 1991).

2.3.2 P5C synthesis from ornithine

Two homologous cDNAs encoding ornithine δ-aminotransferase (OAT) have been isolated from *Vigna aconitifolia* (Delauney et al. 1993) and *Arabidopsis* (Roosens et al. 1998). Enzymatic studies (Taylor & Stewart 1981), and the presence of a mitochondrial transit peptide in the products encoded by these OAT cDNAs strongly suggest that in plants, transamination of ornithine to P5C occurs in mitochondria.

Controversy surrounds the relative importance of the two biosynthetic pathways involved in P5C synthesis. The pathway to P5C from arginine and ornithine does not contribute significantly to stress-induced proline accumulation in barley (Boggess & Stewart 1976; Boggess et al. 1976b), but a different situation applied in wilted bean (*Phaseolus vulgaris*) leaves (Stewart & Boggess 1977) and osmotically stressed Jerusalem artichoke (*Helianthus tuberosus*) tuber tissues (Wrench et al. 1977). Activity of OAT increased three-fold and two-fold in water stressed leaves of drought susceptible and drought tolerant cultivars of cassava (*Manihot esculenta*) respectively (Sundaresan & Sudhakaran 1995). An increase in OAT activity was observed as early as 2 h after PEG treatment and reached a maximum level after 12 h, which was sustained throughout the rest of the stress period. An increase in OAT activity, and a decrease in the apparent energy of activation of the enzyme was observed during cold acclimation of wheat (Charest & Phan 1990). Unfortunately, synthesis of P5C from glutamate was not assessed by either of these groups of workers.

Molecular studies using seedlings of *Vigna aconitifolia* suggest that the choice of pathway for P5C synthesis depends on the nitrogen status of the plant. High nitrogen input induced the

accumulation of transcript encoding OAT possibly via an accumulation of ornithine or arginine, but decreased levels of mRNA encoding P5CS (Delauney et al. 1993). The demonstration that drought-stressed tobacco plants accumulate more proline in nitrogen-rich soil than under nitrogen-limited conditions (Kavi Kishor et al. 1995) led Zhang et al. (1997) to investigate whether nitrogen status has any effects on transcription of the *AtP5CS2* gene in *Arabidopsis*. Nonetheless, a 24 h treatment with 5 mM glutamine did not activate transcription of the *AtP5CS2* promoter. The possible involvement of a signal(s) related to nitrate abundance (Scheible et al. 1997) in mediating the reciprocal regulation of genes encoding OAT and P5CS in *Vigna aconitifolia* is discussed in Section 2.4.5.3 of this document.

In 7 d-old *Arabidopsis* seedlings grown in a nitrate-replete nutrient medium, ^{14}C-isotope tracer experiments established that glutamate, ornithine and arginine all contribute to increased proline biosynthesis following a PEG-mediated reduction in water potential. More than four times the amount of proline was made from L-[^{14}C(U)]-glutamate as from L-[^{14}C(U)]-ornithine or L-[^{14}C(U)]-arginine (Chiang & Dandekar 1995). After 24 h of PEG-induced water stress, the radioactivity incorporated into proline from L-[^{14}C(U)]-glutamate increased 5.7-fold, whereas the respective increases in ^{14}C incorporation in plants incubated in L-[^{14}C(U)]-ornithine and L-[^{14}C(U)]-arginine were 9.7- and 33.3-times greater than unstressed controls (Chiang & Dandekar 1995). Thus, although proline synthesis from glutamate during stress may still be quantitatively more important than synthesis from ornithine, these data emphasise the poor appreciation of the relative contributions of both pathways of P5C synthesis under optimal and adverse conditions.

A recent study indicates that the contribution of OAT to proline synthesis in *Arabidopsis* is developmentally regulated (Roosens et al. 1998). In young plants, free proline content, levels of *AtP5CS1* and *AtOAT* transcripts and OAT activity all increase upon salinisation. However, in 4-week-old plants after salt stress, *AtOAT* transcript levels were not detectable, OAT activity was unaffected and *AtP5CS1* mRNA levels increased concomitant with proline accumulation (Roosens et al. 1998). Accordingly, application of gabaculine, a powerful and irreversible inhibitor of OAT, reduced salt-induced proline accumulation of radish cotyledons (Hervieu et al. 1995). This correlated with a dose-dependent increase in OAT with increased salinity. However, the contribution of the ornithine pathway to salt-induced proline synthesis was less at a later stage of seedling development. The finding that OAT activity declined over the period from bud-break to flower development in kiwifruit, thus indicating that it does not contribute to proline accumulation during the floral transition in this species (Walton et al. 1998), is consistent with the view that the relative importance of the two pathways of P5C synthesis from glutamate and ornithine is developmentally regulated.

2.3.3 Proline degradation

In plants, the oxidation of proline to P5C and the oxidation of P5C to glutamate is restricted to the mitochondria (Boggess et al. 1978; Huang & Cavalieri 1979; Elthon & Stewart 1981, 1982; Sells & Koeppe 1981). These two reactions are catalysed by the sequential actions of proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) respectively (Figure 2.7). Proline oxidation is believed to be responsible for transferring electrons into the first portion of the electron transport chain (Elthon & Stewart 1981, 1982). The oxidation of both proline and P5C in maize mitochondria is inhibited by rotenone (Elthon & Stewart 1982). This indicates that electrons from these substrates enter the respiratory chain prior to at least one of the rotenone sensitive iron-sulphur proteins.

2.3.3.1 Proline dehydrogenase

Plant PDH is an oxygen-dependent flavoprotein (Huang & Cavalieri 1979; Elthon & Stewart 1982). Although frequently referred to as proline oxidase, the more appropriate classification of the enzyme as a dehydrogenase (Elthon & Stewart 1982) is now generally accepted (Kiyosue et al. 1996; Peng et al. 1996; Hare & Cress 1997). In plant cells, PDH is bound to the matrix side of the inner mitochondrial membrane (Boggess et al. 1978; Elthon & Stewart 1981, 1982). *Arabidopsis* PDH has recently been characterised at the molecular genetic level (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al 1996a). The *Arabidopsis* PDH gene maps to approximately 69.4 centimorgans on chromosome 3 (Verbruggen et al. 1996a). The amino acid sequence of AtPDH contains a putative mitochondrial targeting sequence (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al 1996a). Accordingly, the gene product was detected immunologically in a mitochondrial fraction from cultured *Arabidopsis* cells (Kiyosue et al. 1996). When expressed in *Saccharomyces cerevisiae*, a cDNA encoding AtPDH complemented the yeast *put1* mutation and yielded PDH activity in the mutant (Peng et al. 1996).

Activity of PDH is reduced after prolonged exposure to hyperosmotic stress (Stewart et al. 1977; Rayapati & Stewart 1991; Madan et al. 1995). Although it was long proposed that this may arise exclusively from a loss in the integrity of mitochondrial membrane structure, examination of *AtPDH* expression has demonstrated that levels of transcript encoding PDH decline after prolonged air-drying (Kiyosue et al. 1996) as well as exposure to NaCl (Peng et al. 1996) or polyethylene glycol (Verbruggen et al. 1996a). Upon relief from hyperosmotic stress, *AtPDH* transcript levels increase rapidly (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996a), concomitant with a decline in *AtP5CS1* transcript abundance (Kiyosue et al. 1996; Peng et al.

1996). This tightly regulated control of PDH activity at the genetic level is consistent with the long-proposed importance of the proline accumulated during stress as a rapidly mobilisable reserve of carbon, nitrogen and energy during recovery from stress (Hare & Cress 1997). Levels of *AtPDH* transcript are substantially increased in *Arabidopsis* plants that have been incubated in a medium that contains proline (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996a). However, the induction of *AtPDH* mRNA accumulation by proline is inhibited by salt stress (Peng et al. 1996), thus indicating that a signal related to hyperosmotic stress can override induction of the gene by the substrate of its product. The kinetics of changes in *AtPDH* transcript levels and their relation to levels of free proline and *AtP5CS1* mRNAs during dehydration and rehydration of *Arabidopsis* plants are discussed in Section 2.4.4.

2.3.3.2 P5C dehydrogenase

The isolation of a cDNA encoding plant P5CDH has yet to be reported. Indeed, plant P5CDH has only recently been purified to electrophoretic homogeneity and characterised with respect to its structural, kinetic and biochemical properties (Forlani et al. 1997a). The enzyme from cultured potato cells is a homotetramer. Although NAD^+ is the preferred electron acceptor (K_m = 0.11 mM with 2 mM P5C), $NADP^+$ also yielded a high rate of catalysis. At 2 mM $NADP^+$, the enzyme exhibited more than 60% of the maximal activity obtained using NAD^+ (Forlani et al. 1997a). A similar conclusion concerning the relative affinities for both pyridine nucleotide cofactors was reached by Stewart and Lai (1974) using mitochondrial preparations from root and shoot tissue of pea and maize, castor bean (*Ricinus communis*) endosperm and pumpkin (*Cucurbita maxima*) cotyledons. A 50% inhibition of P5CDH by chloride ions at a concentration of 213 mM strengthens the notions that hyperosmotic stress negatively modulates proline oxidation to glutamate *in vivo* and that this may arise at least in part through a post-transcriptional effect. Interestingly, in contrast to previous reports which indicated a requirement for detergents in order to achieve maximal recovery of P5CDH activity from plant tissues (Stewart & Lai 1974; Boggess et al. 1975), inclusion of detergents in the extraction buffer did not improve yield of the enzyme recovered from cultured potato cells (Forlani et al. 1997a). Thus, in contrast to earlier suggestions that plant P5CDH is bound to the matrix side of the inner mitochondrial membrane (Stewart & Lai 1974; Boggess et al. 1975; Elthon & Stewart 1981, 1982), the enzyme may be a soluble protein found in the mitochondrial matrix (Forlani et al. 1997a), as has been shown to be the case in mammalian systems (Small & Jones 1990; Hu et al. 1996).

Although Forlani et al. (1997a) found no evidence of isoforms of P5CDH in their study using cultured potato cells, two P5CDHs have been detected in maize mitochondria. An isoform with

an acidic pH optimum oxidises P5C derived from proline and transfers electrons and protons directly into the respiratory chain, while a second activity with a basic pH optimum oxidises P5C synthesised from ornithine (Elthon & Stewart 1982). The latter enzyme forms a complex with mitochondrial OAT (Elthon & Stewart 1982). The inability to detect P5CDH isoforms in suspension cultured potato cells may be ascribed to the use of tissue harvested only during the early exponential growth phase of the cultures (Forlani et al. 1997a). In a subsequent study, the same workers demonstrated that two P5CDHs are found in suspension cultured cells of *Nicotiana plumbaginifolia* (Forlani et al. 1997b). The relative activities of both isoforms, which have similar molecular masses and a broad maximum activity around pH 7.4, is differentially regulated throughout the cell growth cycle. One activity increased in exponentially growing cells and declined rapidly in the late logarithmic phase, while the second activity, which had lower affinities for P5C and NAD^+, was found at a substantial level only in cells which were entering the stationary phase (Forlani et al. 1997b). Whether these two isoforms differ in their abilities to oxidise P5C derived from ornithine or proline remains to be established. Addition of detergents to the extraction buffer did not significantly enhance the yield of either enzyme (Forlani et al. 1997b), thus further corroborating the notion that plant P5C dehydrogenase activity may not be membrane-associated.

2.3.4 Proline transport

In higher plants, the assimilation of nitrogen absorbed from the soil occurs mainly in the mesophyll cells of mature leaves, from where amino acids may be exported to sink tissues via the phloem. Although a number of studies have shown the rate of exudation of amino acids to the phloem to be independent of the concentration of free amino acids in the leaves, the mechanisms that regulate amino acid export to the phloem sieve tubes are presently not well characterised (Caputo & Barneix 1997). Nonetheless, there are indications that loading of sucrose into the phloem involves transport against a concentration gradient, probably driven by a proton symport, and that a similar mechanism may be involved in the uptake of amino acids into the sieve tubes (Rentsch & Frommer 1996).

Several genes which resemble those encoding broad specificity amino acid permeases that have been characterised in yeasts, are expressed in mature leaves of *Arabidopsis* (Rentsch & Frommer 1996; Fischer et al. 1998). The influx of proline into roots of *Arabidopsis* can be dissected into two components (Verbruggen et al. 1996b). One, which is at least partially defined by the *RAZ1* locus, possesses a high affinity and low capacity for the imino acid, while the other component has low affinity but high capacity for proline uptake. Two proline-specific transporters in *Arabidopsis*,

PROT1 and *PROT2*, have been cloned (Rentsch et al. 1996). Neither appear to encode the *RAZ1* gene product, since the toxic proline analogue azetidine-2-carboxylate did not compete for proline uptake mediated by these transporters. In keeping with evidence that proline synthesis and degradation play an important role in flowering and seed set (Verbruggen et al. 1996a; Hare & Cress 1997; Hua et al. 1997), expression of *PROT1* is highest in open flowers, particularly in the phloem of the medial vascular strands in the carpels, and declined following fertilisation. In contrast to *PROT1*, which was unaffected, and two broad specificity amino acid permeases, which were repressed, *PROT2* was strongly induced under water or salt stress (Rentsch et al. 1996). Consistent with the proposal that roots are important sites of proline synthesis, but export most of the product to shoot tissues (Hua et al. 1997), both *PROT1* and *PROT2* are actively expressed in roots (Rentsch et al. 1996). The relatively rapid induction of *PROT2* transcript accumulation under saline conditions strongly suggests that proline translocation is of importance during stress. Unfortunately, the effects of relief from stress on transport capacity were not assessed.

Together with the recent advances in understanding the environmental, developmental and tissue-preferential control of genes involved in proline synthesis and degradation, the findings of Rentsch et al. (1996) emphasise the importance of tight regulation of the genes involved in proline metabolism and translocation in facilitating an integrated response to environmental extremes and developmental transitions both at the subcellular and whole-plant levels.

2.4 Signals involved in mediating stress-induced shifts in proline metabolism

2.4.1 General stress response signalling in plants

The many environmental stresses that plants may encounter (drought, flooding, temperature extremes, nutrient deficiencies, heavy metal toxicity, herbivory, excessive irradiance or infection by pathogens) seldom occur in isolation. Frequently, the level of any single stress factor (e.g. moisture availability, temperature, light intensity or threat from pathogens) may not be adequate to constitute stress by itself, but in combination with other equally marginal factor(s), plant vitality may be considerably reduced. Since plants must continuously adapt to new combinations of stresses they encounter, understanding how various stresses are coupled is an important requisite for future strategies to improve the performance of field-grown crops.

Increasing evidence suggests that there may be a basic physiological framework involved in the regulation of plant responses to environmental stress. Not only do environmental stresses frequently occur in concert, but they are often linked by common aspects of their effects, the signal systems whereby they are detected, or the response to them. Ecologists have noted that reduced growth rate, a low capacity to capture resources and a high investment in reserve storage are consistently found amongst plants indigenous to hostile environments (Chapin 1991). Furthermore, the multitude of adverse stimuli that impact on plant growth can induce, besides very specific responses, many similar effects at the cellular level. Widespread consequences of the imposition of different stresses include osmotic challenge, changes in membrane chemistry and ion transport, alterations in cellular redox potential and protein denaturation (Hare et al. 1996). Accordingly, gene products commonly induced following imposition of a diversity of suboptimal conditions include enzymes involved in the synthesis of putative osmoprotectants, enzymes associated with detoxification of reactive oxygen intermediates, proteins involved in the uptake and compartmentation of ions, lipid desaturases for membrane modification, proteases and cyclophilins (Hare et al. 1996, 1997). The frequent association of these effects with the induction of common subsets of genes and the activation of specific metabolic pathways has led to the proposal that different stress responses may be regulated by overlapping and interacting cellular signal transduction pathways (Hare et al. 1996; Figure 2.8). Mechanistic similarities between different stresses are seen both in the types of injuries they cause as well as cellular protective mechanisms or ways of repairing the damage that is sustained. The well-documented phenomenon of cross-tolerance to stresses is consistent with this proposal. Activation of appropriate responses to one stress have frequently been shown to ameliorate the impact of subsequent imposition of a different stress. For example, previous exposure to water stress,

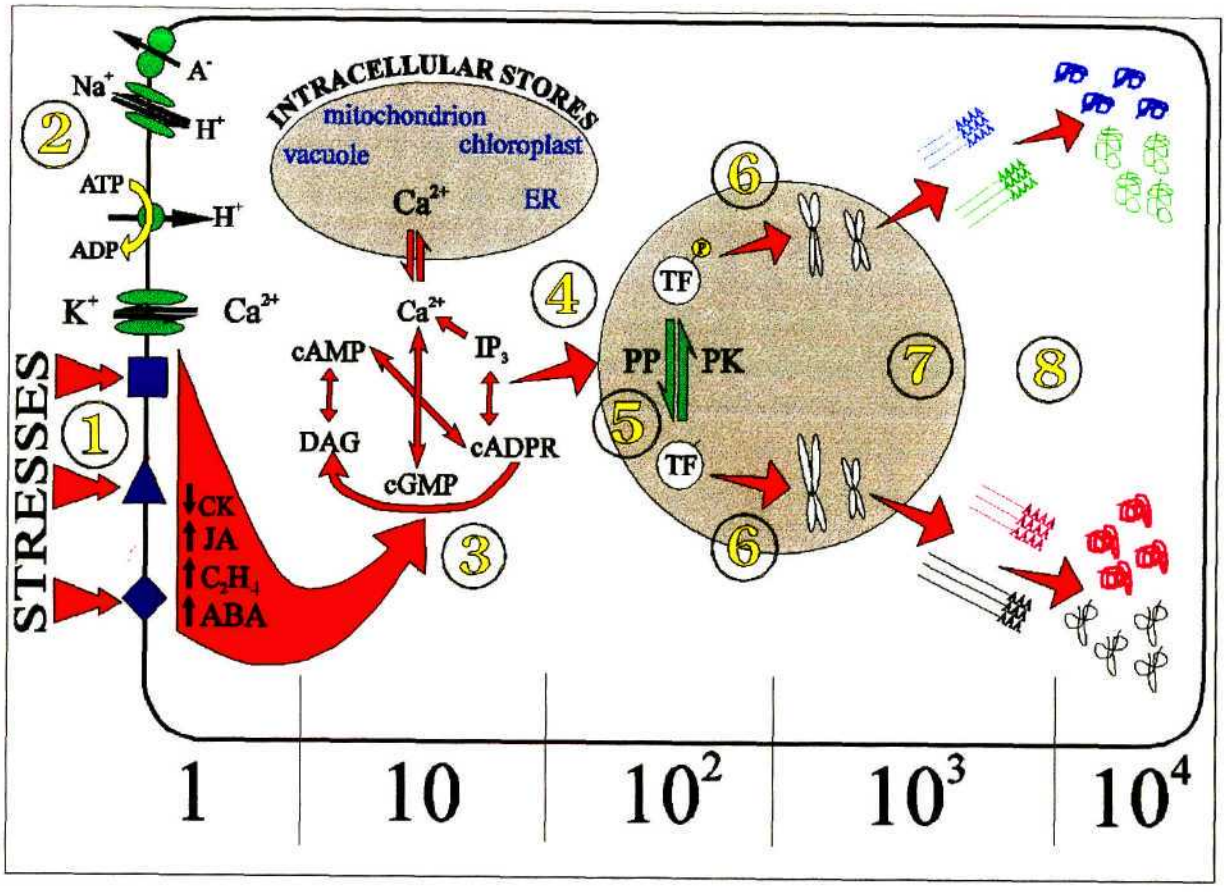

Figure 2.8: A hypothetical representation of stress-related signal transduction in plant cells. Following the perception of stress at the cell membrane [①], there is a change in the activity of membrane-bound ion channels and other transporters [②]. Changes in the relative concentrations of hormones such as abscisic acid (ABA), cytokinins (CK), ethylene (C_2H_4) and jasmonic acid (JA) are also likely to be effected. Secondary messengers, such as Ca^{2+}, inositol 1,4,5-trisphosphate (IP_3), diacylglycerol (DAG), cyclic ADP ribose (cADPR) and the cyclic nucleotides cAMP and cGMP, themselves likely to interact extensively via "cross-talk" [③], activate protein kinases (PK) and/or phosphatases (PP) [④], which act *inter alia* on stress-related phosphoprotein transcriptional factors (TF) [⑤]. Transcriptional activation of stress-related genes [⑥] causes accumulation of mRNA transcripts [⑦] encoding protein products that initiate the chain of events leading to specific cellular responses to stress [⑧]. The use of such as hierarchical system introduces the possibility both of signal complexity and signal amplification. The numbers below the figure, corresponding to each stage in the chain of events, illustrate the immense amplification potential of such a mechanism. If an amplification factor of only 10 is assumed for each stage in the signalling cascade, a single triggering event may elicit thousands of transcriptional events. From Hare et al. (1996).

salinity or abscisic acid (ABA) at non-hardening temperatures increases subsequent cold-hardiness (Mäntylä et al. 1995; Ryu et al. 1995) and irradiance of tobacco plants with UV light or fumigation with ozone induced resistance to a subsequent challenge with tobacco mosaic virus (Yalpani et al. 1994).

Several recent studies of stress-induced gene induction augment this idea. For example, the pattern of gene expression observed in ozone-treated *Arabidopsis* plants overlaps significantly with the pattern of gene expression observed during a hypersensitive response to pathogen attack (Sharma et al. 1996). Transcripts of several maize genes induced by treatment with $HgCl_2$ also accumulated in response to heat stress, NaCl, UV-irradiation, polluted rainwater, wounding, cold stress and pathogen infection (Didierjean et al. 1996). Despite their responsiveness to several abiotic extremes, these did not always elicit similar transcriptional changes in the genes studied. This confirms the conclusions of others (Vernon et al. 1993) that several interacting signalling pathways are likely to regulate plant responses to biotic and abiotic stresses. While overlap between genetic responses to distinct stress conditions most likely arises from their common effects on cellular physiology, different stresses must also elicit unique genetic responses in order to enable the plant to distinguish between stress treatments.

It has long been appreciated that the ability of plants to withstand stressful environments is controlled by a number of genes. The multigenic character of stress tolerance is a primary limitation to its manipulation using both traditional breeding approaches as well as more recent transformation technologies. Recent studies of signalling cascades in higher plants have identified ion channels, intracellular signalling proteins and second messengers as critical components which mediate early events in signal transduction. Reversible protein phosphorylation by kinases and phosphatases is now recognised to play a fundamental role in the transduction of extracellular signals into intracellular biochemical changes. In most cases, both the phosphorylation and dephosphorylation reactions are active at the same time and their relative speed determines the steady-state level. In the light of the notion that overlapping responses to different environmental stresses may be mediated by common cellular signal transduction pathways (Hare et al. 1997), it seems feasible to propose that in the long term, targeting the genes encoding components of stress-related signal transduction pathways may be more profitable than the manipulation of individual genes at the termini of these cascades. With refinement, this approach should permit regulated alterations in the expression of several genes implicated in stress tolerance. At least three recent reports exemplify its potential. Overexpression of a putative intermediate in ABA signal transduction conferred desiccation tolerance to callus tissue of *Craterostigma plantagineum* in the absence of ABA and resulted in the constitutive expression of several genes which have been associated with dehydration tolerance in differentiated tissues of this resurrection plant (Furini et al. 1997). Furthermore, increased levels of different transcription factors which bind to the promoters of many drought- and cold-inducible genes increased both the expression of these genes as well as the freezing tolerance of non-acclimated *Arabidopsis* plants (Jaglo-Ottosen et al. 1998; Liu et al. 1998). Thirdly, overexpression of the *Arabidopsis NPR1* gene, a key regulator of acquired resistance responses which induces

expression of a battery of pathogenesis-related genes, has indicated that this single gene appears to be a workable target for genetic engineering of nonspecific disease resistance in plants (Cao et al. 1998). Although these studies have not examined cross-tolerance of the transgenic lines to different stresses, the targeting of genes involved in more general aspects of plant stress responses may open the way for the engineering of crops with an increased ability to adapt to several stresses experienced concurrently in the field (Hare et al. 1996). Nonetheless, better characterisation of the molecular signals involved in stress perception and the molecular events that specify the expression of stress tolerance will be necessary to provide a sound basis on which such strategies to improve agricultural productivity can be founded.

The likely complexity of such a system of partially overlapping stress response patterns has led to the suggestion that the integration of plant stress responses is dependent on an overall shift in the relative activities of several growth regulators. Of these, ABA and cytokinins (CKs) have been proposed to be the most likely candidates (Chapin 1991), although other growth regulators are also likely to play a crucial role in controlling plant responses to adverse environmental conditions (Figure 2.8). While the mechanisms of hormonal balance in plants remain poorly understood, growth regulators are known to mutually affect their absolute concentrations at the levels of their synthesis and metabolism as well as interacting at the level of differential induction or repression of gene expression (Hare & van Staden 1997).

Further complexity arises from the plasticity of plant development. Since the pattern of development is to a large measure determined by environmental factors, vascular plants must not only adapt appropriately to changing conditions on a rapid time scale, but also coordinate developmental processes to produce physiological and morphological preparations in anticipation of and in response to environmental eventualities. Thus under adverse conditions, changes in gene expression patterns modified by extrinsic cues need to be superimposed upon intrinsic plant developmental programs. Signal transduction pathways downstream of the perception of adverse conditions must thus be integrated with other environmental cues, such as light, as well as with endogenous signals derived, for example, from plant hormones and the circadian oscillator. The resultant modifications of flexible gene expression patterns thus ensure a response that is quantitatively appropriate, correctly timed, and coordinated with other activities of the cell.

This section concerns our current understanding of the events that occur between the perception of stress and changes in the expression of the genes which control proline accumulation. The consequences of the actions of both plant hormones and photoreceptors on proline metabolism will also be considered, and the implications of these findings will be reviewed in relation to how the proline metabolic system may provide a useful paradigm in efforts to improve our overall

understanding of stress-related signal transduction. In view of the causal relationship between proline synthesis and tolerance of drought and salt stress demonstrated in tobacco plants which express a transgene encoding P5CS (Kavi Kishor et al. 1995), characterisation of the signalling events that regulate proline accumulation is justified not only by the possibility that an understanding of the molecular basis of regulated proline accumulation may further enhance the stress tolerant phenotype observed by these workers, but also by the known sensitivity of proline levels to a wide range of adverse biotic and abiotic conditions, many of which do not contain a significant osmotic component (Table 2.4; Hare & Cress 1997). As reviewed below, only limited information exists on the nature of the signal transduction pathway which links the perception of osmotic stress to proline accumulation. Nonetheless, several recent advances provide a foundation on which further details of the stress-induced signalling process may be built.

2.4.2 Stress-induced proline accumulation is dependent on cycloheximide-sensitive gene activation

An important factor in the protection of metabolism against stress is the rapidity with which a plant can activate or ameliorate its defensive strategies. During stress, genes may be induced either as part of a primary response to minimise damage or as a result of secondary effects (e.g. generalised protein denaturation or inappropriate expression resulting from inactivated regulatory proteins) which arise from stress-induced damage. Clearly, any effective physiological response to adverse environmental conditions must be invoked before the onset of significant damage to the stressed tissues. Furthermore, the more rapidly a gene is induced, the less likely it is to have arisen from secondary effects. Therefore, independence of the stress-induced modulation of levels of transcripts encoding proline metabolic enzymes from changes in *de novo* protein synthesis is an important criterion for establishing both whether these events are primary responses to stress and whether the response is likely to be of adaptive significance. The protein synthesis inhibitor cycloheximide (CHX) is most often used as an indicator of the distance of any downstream event from the initial signal perception event. If the observed effect on transcript level occurs both in the presence and absence of CHX, it is assumed that the reaction does not depend on *de novo* synthesis of proteins, but instead depends on signal transduction elements that are constitutively present.

The use of transcriptional and translational inhibitors in studies involving 10 d-old *Arabidopsis* plantlets indicated that both *de novo* transcription and translation are required during the first 4 h of salt stress before proline begins to accumulate (Verbruggen et al. 1993). Transcriptional and translational inhibitors also inhibit proline accumulation in barley leaves that have wilted or been

treated with ABA (Stewart et al. 1986). In salt-shocked barley leaves, cordycepin (COR)-mediated inhibition of transcription prevents proline accumulation when added after salinisation but before the onset of proline accumulation, but not when added after proline begins to accumulate (Stewart et al. 1986). A conflicting situation was found in *Arabidopsis*, where COR adversely affected proline accumulation even 12 h after the imposition of salt stress (Verbruggen et al. 1993). Cycloheximide delayed proline accumulation in salt-shocked barley leaves, although with time, proline accumulated in CHX-treated leaves at rates comparable to NaCl-treated controls (Stewart et al. 1986). This delay and subsequent accumulation was observed irrespective of whether CHX was added before, during or after salt treatment. Nonetheless, the earlier in the salt treatment that CHX was applied, the longer was the observed delay (Stewart et al. 1986). The genetic regulation of proline accumulation may vary between species. For example, in the unicellular alga *Chlorella autotrophica*, proline accumulation begins immediately after osmotic shock and is not dependent on protein synthesis (Ahmad & Hellebust 1984). Therefore, although it is difficult to make a generalised conclusion concerning all plant species, at least certain aspects of the process(es) which signal proline accumulation under salt stress in higher plants are dependent on the synthesis of new proteins.

In *Arabidopsis*, detectable accumulation of *AtP5CS1* transcript has been observed within 1 h after the imposition of osmotic stress (Yoshiba et al. 1995; Savouré et al. 1997; Strizhov et al. 1997). Pretreatment of seedlings with CHX caused only a slight (25%) reduction in *AtP5CS1* transcript accumulation during the first h after exposure to 200 mM NaCl, but prevented a further increase in transcript levels, which peaked 6 h after NaCl addition in seedlings not treated with CHX (Strizhov et al. 1997). This may be interpreted to indicate that two phases of *AtP5CS1* induction occur upon salt shock and that only the mechanism which operates during the first h of stress is independent of protein synthesis. Induction of *AtP5CS2* mRNA accumulation is slower and reaches a lower maximum than that observed for *AtP5CS1* under identical salt stress conditions (Strizhov et al. 1997). Induction of *AtP5CS2* mRNA accumulation displays absolute dependence on protein synthesis (Strizhov et al. 1997). In transgenic *Arabidopsis* plants which express a fusion of the *AtP5CS2* promoter to the β-glucuronidase (GUS) reporter gene, an approximately five-fold increase in GUS activity was observed within 3 h of dehydration and increased to almost seven-fold of control levels within 24 h (Zhang et al. 1997). Thus, *AtP5CS2* transcript accumulation following osmotic stress apparently arises predominantly from transcriptional activation and the factors that facilitate this process are not constitutively present in unstressed cells.

2.4.3 Signal transduction events upstream of P5CS gene induction

Overwhelming evidence supports the conclusion that P5C synthesis is the rate-limiting step in proline synthesis from glutamate (La Rosa et al. 1991; Szoke et al. 1992; Kavi Kishor et al. 1995; Walton et al. 1998). Although levels of transcript encoding P5CR have been reported to increase under stress in at least two legumes and P5CR enzyme activity has been shown to increase under stress in several plant species (Section 2.3.1.2), controversy has arisen concerning whether or not *Arabidopsis AtP5CR* transcript abundance is sensitive to salinisation. Whereas one group has reported induction of *AtP5CR* mRNA levels in response to osmotic stress (Verbruggen et al. 1993; Savouré et al. 1997), others have contested a significant accumulation of transcripts encoding P5CR in osmotically-stressed *Arabidopsis* (Yoshiba et al. 1995). Using an *Arabidopsis* P5CR cDNA as a probe, Mattioni et al. (1997) concluded that an increase in P5CR activity in *Triticum durum* seedlings during dehydration and salt stresses is unrelated to levels of the corresponding transcript.

In contrast, there is a general consensus that levels of transcripts encoding P5CS are rapidly induced to high levels upon dehydration and exposure to high NaCl concentrations (Yoshiba et al. 1995; Peng et al. 1996; Igarashi et al. 1997; Savouré et al. 1997; Strizhov et al. 1997). Limited accumulation of *AtP5CS1* transcript is observed after 24 h exposure of *Arabidopsis* plants to 4°C, although no detectable increase was observed 10 h after commencement of this stress (Yoshiba et al. 1995). In contrast to dehydration stress, 48 h incubation at 4°C did not induce transcriptional activity of the *AtP5CS2* promoter in transgenic *Arabidopsis* plants which expressed an *AtP5CS2::GUS* fusion (Zhang et al. 1997). Collectively, these data suggest that different pathways regulate *Arabidopsis* P5CS transcript accumulation under chilling and osmotic stresses. However, Xin and Browse (1998) have subsequently reported a three-fold increase in *AtP5CS1* level and an approximately ten-fold increase in proline content after a two day exposure of wild-type (WT) *Arabidopsis* plants to 4°C. In rice, *OsP5CS* transcript levels increased within less than 2 h of transfer to 4°C, which was more rapid than induction by salt or dehydration (Igarashi et al. 1997). Heat treatment (40°C for at least 24 h) does not induce *P5CS* gene expression in either *Arabidopsis* (Yoshiba et al. 1995) or rice (Igarashi et al. 1997). Although on the basis of these studies, induction of *P5CS* transcript accumulation does not appear to be a general stress response, further investigation of the response in a range of species with different levels of tolerance of temperature extremes may be warranted.

Arabidopsis is a chilling- and freezing-tolerant plant which is capable of cold acclimation (Mäntylä et al. 1995). Whereas non-acclimated *Arabidopsis* is killed at - 3°C, a two day exposure to 4°C has frequently been shown to increase the freezing tolerance to - 10°C (e.g. Leyva et al. 1995,

Jaglo-Ottosen et al. 1998; Xin & Browse 1998). Besides several studies which have examined the molecular basis of this response to low temperature, responses to drought and high salinity have also been well characterised in this species. Like *AtP5CS1*, many commonly studied *Arabidopsis* genes are responsive to low temperatures, desiccation and salinity. These have been variously designated as *RD* (responsive to dehydration), *KIN* (kykna-indusoitu, Finnish for cold-induced), *RAB* (responsive to ABA), *COR* (cold regulated), *LTI* (low-temperature induced) and *AtDi* (*Arabidopsis thaliana* drought-induced). In an attempt to synthesise the available data and suggest how recurrent themes of stress-regulated signal transduction which have emerged from the study of these genes may relate to elucidating the pathways that control proline accumulation, this discussion will focus primarily on *RD29A* (also referred to as *COR78*, *LTI78*, *LTI140*, *COR67* and *COR160* by various workers), *KIN2* (*COR6.6, pHH29*) and *RAB18* (*AtDi8*).

2.4.3.1 The involvement of ABA in P5CS gene expression

For many years, ABA has captured the attention of most hormone physiologists interested in plant responses to biotic and abiotic stresses. Many morphological and physiological adaptations to stress, including stomatal closure, are under the control of ABA. In addition to mediating stress responses of vegetative tissues, ABA also participates in developmental processes such as embryogenesis and seed dormancy. Plant ABA levels increase both during embryo development, shortly before the onset of seed desiccation (King 1976), as well as in tissues subjected to dehydrative stresses such as drought, salinity and freezing (Chandler & Robertson 1994), heat stress (Abass & Rajashekar 1993; Cheikh & Jones 1994), pathogen infection (Tuomi et al. 1993) and soil flooding (Else et al. 1996). All of these stresses are capable of inducing proline accumulation (Table 2.4). Observations that exogenous application of ABA to unstressed plants can induce biochemical changes similar to those observed under stress, and that treating plants with ABA frequently hardens them against stress, have long suggested that ABA may normally be used to control at least some aspects of the acclimation to stress (Chandler & Robertson 1994).

Nonetheless, like all adaptive traits which have been associated with plant stress tolerance, the relationship between ABA content and stress resistance is not absolute and many exceptions to the generalisation have been noted. For example, the dominant role played by ABA in mediating responses to water deprivation has often been called into question by investigations which failed to correlate inhibition of shoot growth or leaf turgor pressure with ABA levels (Walker & Dumbroff 1981; Trejo & Davies 1991). As will be discussed below, ABA levels frequently peak shortly after the imposition of stress, but thereafter decline to unstressed levels even under sustained stress.

Several studies have indicated that accumulation of ABA under stressful conditions often lags behind stomatal closure (Beardsell & Cohen 1975; Trejo & Davies 1991; Else et al. 1996) and upon relief from stress, a decline in leaf ABA levels preceded stomatal opening (Beardsell & Cohen 1975; Ludlow et al. 1980; Else et al. 1996). In keeping with these reservations, considerable evidence indicates the existence of ABA-independent dehydration- (Gosti et al. 1995; Rey et al. 1998) and cold-induced (Gilmour & Thomashow 1991; Nordin et al. 1991; Capel et al. 1997) signal transduction pathways. Many of the genes which are induced by these stresses in an ABA-independent manner are nonetheless responsive to applied ABA. Dissecting the interactions between ABA-dependent and ABA-independent signalling cascades is currently the focus of extensive investigation (Ishitani et al. 1997; Shinozaki & Yamaguchi-Shinozaki 1997).

A causal link between ABA and proline accumulation has been suggested for some, but not all plant species investigated (Stewart & Voetberg 1987; Finkelstein & Somerville 1990; Chou et al. 1991; Dallmier & Stewart 1992; Xin & Li 1993; Fedina et al. 1994; Ober & Sharp 1994; Savouré et al. 1997). In *Arabidopsis*, exogenously applied ABA increases the levels of *AtP5CS1* (Yoshiba et al. 1995; Savouré et al. 1997; Strizhov et al. 1997) and *AtP5CS2* (Strizhov et al. 1997) transcripts. In rice, rapid accumulation of *OsP5CS* transcript is observed within 2 h of treatment with ABA (Igarashi et al. 1997). Analysis of stress-responses in ABA-related mutants of *Arabidopsis* provides a powerful means of assessing the functional significance of ABA-mediated processes in acclimation to stress. These mutants can be broadly categorised as those with abnormally low levels of ABA in all tissues (*aba*) and those which are impaired or deficient in various responses that are regulated by ABA (ABA-insensitive, *abi*). Investigation of *aba1* (formerly *aba*), an *Arabidopsis* mutant which is defective in ABA synthesis, indicated that induction of the accumulation of both free proline and *AtP5CS1* transcripts by low temperature and sorbitol-mediated dehydration is independent of the endogenous ABA level (Savouré et al. 1997). Somewhat contradictory evidence exists concerning the necessity for ABA synthesis in mediating the increases in *AtP5CS1* expression following salt stress (Table 2.10). Whereas the results of Strizhov et al. (1997) indicate an absolute requirement for ABA synthesis in mediating the accumulation of both *AtP5CS1* and *AtP5CS2* transcripts, Savouré et al. (1997) did not observe a decreased abundance of *AtP5CS1* transcript in NaCl-stressed *aba1* plants relative to WT controls. Salt-stressed *aba1* plants accumulated proline, albeit to lower levels than comparable WT plants (Table 2.10).

Table 2.10: Overview of the effects of various mutations characterised in *Arabidopsis* on selected responses to abiotic stresses. Where tested, an *abi3* mutation did not affect any of the responses listed.

	WT	*aba1*	*abi1*	*abi2*	*aux1*	*axr1*	*axr2*	Reference
***AtP5CS1* transcript levels**								
Control	-	-	-/+	-/+	ND	ND	ND	
50 µM ABA	++	++	++	-/+	ND	ND	ND	
250 mM NaCl	+++	+++	+++	+++	ND	ND	ND	Savouré et al. (1997)
550 mM sorbitol	++	++	++	++	ND	ND	ND	
cold (4°C)	-/+	-/+	-/+	-/+	ND	ND	ND	
Control	-	-	red.	-	-	ND	-	Strizhov et al. (1997)
200 mM NaCl	+++	-	+	ND	+++	ND	++	
***AtP5CS2* transcript levels**								
Control	-	-	red.	-	-	ND	-	Strizhov et al. (1997)
200 mM NaCl	++	-	-	ND	+	ND	-/+	
***P5CR* transcript levels**								
Control	-	-	-	-	ND	ND	ND	
50 µM ABA	-/+	-/+	-/+	-/+	ND	ND	ND	Savouré et al. (1997)
250 mM NaCl	++	++	++	++	ND	ND	ND	
550 mM sorbitol	+	+	+	+	ND	ND	ND	
Proline accumulation								
Control	-	-	-	-	ND	ND	ND	
50 µM ABA	++	++	-	++	ND	ND	ND	
250 mM NaCl	+++	-/+	++	+++	ND	ND	ND	Savouré et al. (1997)
550 mM sorbitol	++	++	++	++	ND	ND	ND	
cold (4°C)	+	+	-/+	+	ND	ND	ND	
Drought rhizogenesis	+++	-/+	-/+	+++	+++	ud	ND	Vartanian et al. (1994)
***RD29A* transcript levels**								
Control	ud	ud	ud	ND	ND	ND	ND	Nordin et al. (1991)
60/100 µM ABA	+	+	-/+	ND	ND	ND	ND	
desiccation	++	++	++	ND	ND	ND	ND	Yamaguchi-Shinozaki
cold (4°C)	+++	+++	+++	ND	ND	ND	ND	& Shinozaki (1993)
RD29A protein levels								
Control	ud	ud	ud	ND	ND	ND	ND	
60 µM ABA	+	+	+	ND	ND	ND	ND	Mäntylä et al. (1995)
desiccation	+	-/+	+	ND	ND	ND	ND	
cold (4°C)	+++	+++	+++	ND	ND	ND	ND	
***KIN2* transcript levels**								
Control	-	red.	red.	-/+	ND	ND	ND	Hajela et al. (1990)
50 µM ABA	++	++	-/+	++	ND	ND	ND	
desiccation	++	ND	ND	ND	ND	ND	ND	Gilmour &
cold (4°C)	+++	+++	+++	+++	ND	ND	ND	Thomashow (1991)

	WT	*aba1*	*abi1*	*abi2*	*aux1*	*axr1*	*axr2*	Reference
***RAB18* transcript levels**								
Control	ud	ud	ud	ud	ND	ND	ND	
10/60 µM ABA	+++	+++	ud	ud	ND	ND	ND	Lång & Palva (1992)
desiccation	+++	ud	-/+	+++	ND	ND	ND	Gosti et al. (1995)
cold (4°C)	-/+	ud	ud	ND	ND	ND	ND	
RAB18 protein levels								
Control	ud	ud	ud	ND	ND	ND	ND	
60 µM ABA	++	+++	-/+	ND	ND	ND	ND	Mäntylä et al. (1995)
desiccation	++	ud	-/+	ND	ND	ND	ND	
cold (4°C)	+	ud	-/+	ND	ND	ND	ND	
***ATHB-7* transcript levels**								
Control	-	ud	-/+	-	ND	ND	ND	
1 µM ABA	+++	++	+	+++	ND	ND	ND	Söderman et al.
desiccation	+	ud	-/+	++	ND	ND	ND	(1996)
100 mM NaCl	+	ud	-/+	+++	ND	ND	ND	
cold (4°C)	-	ud	-/+	-/+	ND	ND	ND	
***ADH* transcript levels**								
Control	-	-/+	-	-	ND	ND	ND	
100 µM ABA	++	++	+	-	ND	ND	ND	de Bruxelles et al.
dehydration	++	+	+	-	ND	ND	ND	(1996)
cold (4°C)	+++	+++	+++	+++	ND	ND	ND	

-, constitutive level; -/+, slight induction; +++, maximal response; ND, not determined; red., reduced level relative to unstressed wild-type (WT) control; ud, not present at a detectable level.

Unlike *aba1*, *Arabidopsis abi* mutants do not have reduced endogenous ABA levels and their phenotypes cannot be reversed by exogenous application of ABA. The *Arabidopsis ABI1* and *ABI2* genes encode homologous serine/threonine phosphatases of the 2C class (PP2C) which are intermediates in ABA signal transmission primarily in vegetative tissues (Leung et al. 1997). Recent studies indicate that together with at least a third PP2C, ABI1 and ABI2 function redundantly as repressors of ABA signal transduction (Sheen 1998). Both the *abi1-1* and *abi2-1* mutations involve conversion of a conserved glycine residue to an aspartate (Leung et al. 1997). This alteration in ABI1 constitutes a gain-of-function, rather than a dominant loss-of-function mutation, despite the elimination of PP2C activity (Sheen 1998). However, although the ABI1 (wild-type) and abi1 (mutant) proteins inhibit ABA action by different mechanisms, it is likely that abi1 and ABI1 act at the same level in the ABA signal transduction pathway (Sheen 1998). Many well characterised stress responses, including drought rhizogenesis, a morphological response associated with proline accumulation (Vartanian et al. 1992), as well as ABA-mediated induction of *KIN2* mRNA accumulation and ABA- or stress-induced increases in levels of transcript encoding a homeodomain-containing leucine zipper (HD-ZIP) protein ATHB-7, are compromised in *abi1* but not in *abi2* (Table 2.10). In contrast, induction of transcript encoding alcohol

dehydrogenase (ADH) by exogenous ABA and dehydration appears to require the action of ABI2, but not ABI1 (de Bruxelles et al. 1996). The *abi1* and *abi2* mutations have identical effects on the induction of *RAB18* transcript by ABA (Leung et al. 1997; Table 2.10), although the ABA-dependent accumulation of the same transcript by desiccation is severely inhibited in *abi1*, but not *abi2* (Table 2.10).Thus, analysis of ABA response mutants permits assignment of different stress responses to particular branches of the signalling network downstream of ABA perception. However, it is worth noting that although ABA-regulated expression of many genes (e.g. *RD29A*, *KIN2*, *ADH*) may be impaired in an *abi1* mutant, their induction by cold or desiccation may not be affected by mutation at this locus (Table 2.10).

Based on their analysis of the effects of isosmotic concentrations of NaCl and sorbitol on *AtP5CS1* abundance and proline levels in *abi1* and *abi2* (Table 2.10), Savouré et al. (1997) concluded that an increase in proline upon salinity stress is not controlled at the level of *AtP5CS1* transcript abundance, but possibly at a post-transcriptional level regulated by a pathway disrupted by mutation of *ABI1*. Little is known about post-transcriptional effects governed by ABA, although their importance in fine-tuning of the salt-stress response has recently been emphasised (Moons et al. 1997). Translational effects mediated by pathway(s) affected by mutations in *ABI1* and *ABI2* have been implicated in the expression of both *RD29A* and *RAB18* (Table 2.10). Nuclear run-on transcription assays indicated that cold-induced increases in *KIN2* transcript levels are primarily due to post-transcriptional control mechanisms (Hajela et al. 1990). Zhang et al. (1997) analysed the promoter activity of the *Arabidopsis AtP5CS2* gene by its fusion to the GUS reporter gene in transgenic *Arabidopsis* and tobacco plants. Although expression of the chimeric gene was induced by dehydration and NaCl stresses, ABA failed to induce GUS activity. Whereas these workers concluded that induction of *AtP5CS2* gene expression is a specific response to salt and water stresses, Strizhov et al. (1997) observed induction of *AtP5CS2* transcript by ABA in *Arabidopsis* leaves and suspension cultured cells, although not in roots of 28 d-old seedlings grown in liquid culture. The most obvious way to reconcile these findings is to conclude that ABA is involved in post-transcriptional regulation of *AtP5CS2* gene expression at the level of stabilisation of *AtP5CS2* transcript. Interestingly, in the leaves and roots of *Arabidopsis* seedlings, but not in suspension cultured cells, induction of *AtP5CS1* mRNA accumulation by ABA is far more rapid and extensive than that of *AtP5CS2* (Strizhov et al. 1997).

Returning to the study of Savouré et al. (1997), it is worth emphasising that there is no definitive evidence that the role of the signalling pathway affected by mutation in *ABI1* is confined exclusively to action downstream of ABA perception. Indeed, after treatment with ABA, induced *AtP5CS1* transcript levels were not lower in *abi1* than in the WT or *aba1*, although interestingly, ABA-mediated induction of *AtP5CS2* was less effective in *abi2* (Savouré et al. 1997). This mirrors

the induction characteristics of *RAB18* for which *abi2* inhibits transcript accumulation by exogenous ABA but not by progressive drought (Gosti et al. 1995; Table 2.10). The findings of both Savouré et al. (1997) and Strizhov et al. (1997) do not implicate a role for *abi2* in salinity- or dehydration-induced *AtP5CS1* transcript accumulation. In contrast to Savouré et al. (1997), Strizhov and co-workers found reduced levels of both *AtP5CS1* and *AtP5CS2* transcripts in unstressed *abi1* relative to the WT. This suggests that the pathway disrupted by mutation in *ABI1* may normally affect the basic level of expression of these genes. However, control of *P5CS* gene expression by this pathway during stress is not absolute, since a significant accumulation of both transcripts was noted in NaCl-stressed *abi1* plants (Strizhov et al. 1997; Table 2.10). Surprisingly, Savouré et al. (1997) observed a slight *induction* of constitutive *AtP5CS1* transcript levels in unstressed *abi1* seedlings! The basis of this phenomenon is uncertain, although it has been observed for other ABA-responsive genes *e.g. KIN2, ATHB-7* and *ADH* (Table 2.10). It may be related to the participation of ABI1 and ABI2 in an autoregulatory circuit that coordinates ABA content and flux through ABA signalling pathways with levels of the intermediates that transmit the primary stimulus (Leung et al. 1997). Although this effect was not observed in *aba1*, another explanation is that these *abi* mutants constantly suffer from increased water loss from their aerial parts owing to an inability to regulate stomatal aperture.

In this regard, characterisation of proline accumulation and *AtP5CS1* expression in the *Arabidopsis sos1* (salt overly sensitive) mutant has suggested an important role for turgor reduction in signalling both of these responses. The *sos1* mutant, which is deficient in both Na^+ and K^+ uptake, accumulates more proline and two- to three-fold higher levels of *AtP5CS1* transcript than the WT under saline conditions (Liu & Zhu 1997a). These workers proposed that Na^+ and K^+ are the predominant osmolytes in salt-stressed *Arabidopsis*, and that the higher levels of proline synthesis in *sos1* may arise from a reduced capacity for the mutant to reduce its internal osmotic potential by ion uptake. Both of the studies conducted thus far which have concerned *P5CS* expression in *abi1* have been conducted *in vitro*. This decreases the likelihood that reduced control over stomatal aperture may account exclusively for the constitutively higher *AtP5CS1* transcript levels observed by Savouré et al. (1997). Nonetheless, investigation of the response in undifferentiated tissues may help to establish whether or not the effects of the *abi1* mutation on stress-induced *AtP5CS1* transcript levels and proline accumulation are exercised exclusively at the cellular level or via a more indirect effect on leaf water status. Furthermore, both studies involved examination of responses in whole seedlings. Thus, the effects of *abi1* on these responses in root tissue cannot yet be assessed.

The proposal by Liu and Zhu (1997a) that reduced turgor may signal proline accumulation is not consistent with the demonstration by Savouré et al. (1997) that NaCl is a more effective inducer

of both free proline and *AtP5CS1* mRNA accumulation than an isosmotic concentration of sorbitol (Table 2.10). However, subsequent workers reported that mannitol induces higher steady state levels of *AtP5CS1* transcript than does an isosmotic concentration of NaCl (Knight et al. 1997). This was observed at both high (0.666 M) and low (0.333 M) concentrations of mannitol and isosmotic concentrations of NaCl. Mannitol was also a better inducer of *RD29A* and *RAB18* transcript accumulation (Knight et al. 1997). In assessing the involvement of inorganic ions in controlling proline accumulation, it is worth mentioning that differences have been noted in the time courses of *AtP5CS1* induction by desiccation and salt treatments. Whereas *AtP5CS1* mRNA levels start to accumulate within 1 h after treatment with 250 mM NaCl and continue to accumulate for a further h, levels subsequently decrease constantly over a 24 h period (Yoshiba et al. 1995). In contrast, desiccation caused a slower accumulation of *AtP5CS1* mRNA, but the maximum level reached 5 h after commencement of the stress was sustained for at least a further 20 h (Yoshiba et al. 1995). Comparison of the effects of these treatments on cell turgor is difficult, although they suggest that osmotic adjustment resulting from ion uptake under salinity stress may account for the more transient accumulation of *AtP5CS1* transcripts after exposure to NaCl. Accordingly, sorbitol is more effective than an isosmotic concentration of NaCl in reducing *Arabidopsis* root growth (Cramer & Jones 1996). However, a contrasting situation was found for rice seedlings: an increase in *OsP5CS* levels was also evident 5 h after the start of dehydration, but reached a maximum after 10 h and then decreased to pre-stress levels by 24 h (Igarashi et al. 1997). Moreover, induction of *OsP5CS* was evident only 10 h after treatment with 250 mM NaCl, but a maximum level attained within 24 h was sustained until at least 72 h after imposition of salt stress (Igarashi et al. 1997). Certainly, characterisation of *sos1* has eliminated the likelihood that Na^+ accumulation alone signals proline synthesis in *Arabidopsis*. Furthermore, whereas proline and betaine accumulation in gram-negative bacteria is affected by the intracellular concentration of K^+, elevated levels of proline synthesis in *sos1* indicate that depletion of intracellular K^+ is not a sufficient signal for *AtP5CS1* induction in *Arabidopsis* (Liu & Zhu 1997a). By examining the effects of the K^+ channel blocker tetraethylammonium chloride on mannitol-induced *AtP5CS1* expression, Knight et al. (1997) independently reached the same conclusion that K^+ uptake is not essential for *AtP5CS1* expression.

Any attempt to assess the importance of ABA in mediating stress-induced changes in P5CS activity should consider whether the kinetics of transcript accumulation match changes in the level of endogenous ABA during stress. Unfortunately, changes in ABA have not been monitored in any of the studies which have investigated the induction of genes encoding P5CS. In keeping with the transient induction of *AtP5CS1* transcript levels in response to salinisation (Yoshiba et al. 1995) and *OsP5CS* levels in response to dehydration (Igarashi et al. 1997), dehydration-, salt- and cold-induced transients in ABA concentrations have frequently been reported (Cowan et al.

1997). In the roots of rice seedlings, salt shock (150 mM NaCl) caused an approximately ten-fold increase in ABA content within 8 h of the imposition of the stress, but these decreased to near control levels by 12 h and remained stable for the next 60 h (Moons et al. 1997). Dehydration stress (0.6 M mannitol) induced an almost 30-fold increase in the level of ABA in roots and an approximately five-fold increase in shoots of *Arabidopsis* plants within 12 h, although by 24 h after the imposition of the stress, the ABA levels had dropped to about half of the respective maxima (de Bruxelles et al. 1996). Low-temperature treatments (0°C or 4°C) apparently had no effect on ABA levels in 28 d-old *Arabidopsis* plants (de Bruxelles et al. 1996), although Lång et al. (1994) reported a two- to four-fold transient increase in shoot ABA content from plants grown at 4°C. In axenically-grown *Arabidopsis* plants, levels peaked between 6 h and 24 h of exposure to cold, since at these times, ABA content was comparable to that of controls (Lång et al. 1994). Although it is difficult to draw conclusions, comparison of these data with the time courses of P5CS gene induction outlined above, which were obtained in separate studies, suggests that ABA content may not be the primary regulator of P5C synthesis under dehydration, salt or cold stresses, at least in rice and *Arabidopsis*. It may be of relevance to note that detectable induction of both *AtP5CS1* and *OsP5CS* within at least 2 h after treatment with ABA is sustained for at least 24 h in the case of *Arabidopsis* (Yoshiba et al. 1995), and 48 h in the case of rice seedlings (Igarashi et al. 1997). However, within 72 h after treatment with 1 mM ABA, *OsP5CS* levels in rice seedlings had declined to pre-treatment levels (Igarashi et al. 1997). In cultured *Arabidopsis* cells, both *AtP5CS1* and *AtP5CS2* transcripts accumulate within less than 1 h of exposure to 1 µM ABA, but levels of both transcripts had declined to pre-treatment levels within 48 h (Strizhov et al. 1997).

2.4.3.2 Transcriptional activation of stress-regulated genes

Recent studies have begun to unravel some of the mechanisms whereby water stress elicits its effects on gene expression by transcriptional activation. Analyses of the promoters of several dehydration-inducible genes which do not require protein synthesis for induction have revealed a *cis*-acting element that is involved in their induction by ABA (Shinozaki & Yamaguchi-Shinozaki 1997). This ABA responsive element (ABRE, YACGTGGC) resembles the G-box, a ubiquitous *cis*-acting element comprising a hexameric core with internal dyad symmetry (CACGTG) which is present in the promoters of many inducible genes which are responsive to apparently unrelated environmental stimuli such as red light, hypoxia, UV irradiation, heat shock, pathogen infection and wounding, as well as growth regulators and developmental cues (Daugherty et al. 1994; Dröge-Laser et al. 1997). A large family of proteins called G-box binding factors (GBFs), which are bZIP proteins (contain a conserved basic domain followed by a minimum of three leucine or other small hydrophobic residues located at intervals of seven amino acids), exhibit a relaxed

DNA binding specificity for sequences containing the ACGT core, although none has yet been shown unambiguously to mediate induction by ABA *in vivo*. Sequences flanking the ACGT core affect the binding specificity and affinity of GBF interactions. Coupling DNA elements, located upstream or downstream from the ABRE, are required in conjunction with an ABRE to generate an ABA-responsive complex (Shen & Ho 1997), although the mechanism(s) by which ABA initiates the binding of bZIP proteins to ABREs is not yet established.

Shinozaki and Yamaguchi-Shinozaki (1997) have proposed the existence of at least four independent signal transduction pathways that regulate drought-, salt- and cold-inducible genes (Figure 2.9). Of these, only two are dependent on ABA action. Whereas genes containing ABREs are induced by the action of a signal transduction chain that uses intermediates which are constitutively present, a second class of ABA-dependent genes, best exemplified by *RD22*, requires the synthesis of protein factors before osmotically-induced gene expression (Shinozaki & Yamaguchi-Shinozaki 1997). The region of the *RD22* promoter which confers ABA responsiveness lacks ABREs, but contains recognition sites for MYC- and MYB-type transcription factors. Dehydration- and ABA-inducible MYC and MYB homologues have been shown to bind and activate the *RD22* promoter (Abe et al. 1997). Cold-, dehydration- and ABA-responsive bZIP transcription factors (Kusano et al. 1995; Nakagawa et al. 1996), have also been proposed to act in this ABA-dependent pathway which requires protein synthesis upstream of ABA-mediated gene induction (Figure 2.9; Shinozaki & Yamaguchi-Shinozaki 1997).

A further two pathways were proposed to mediate ABA-independent gene expression (Figure 2.9). The postulated bifurcation in the ABA-independent signalling mechanism arises from the observed differential expression of genes that are induced by cold, salinity and drought, or by salinity and drought only. Abscisic acid-independent gene expression under dehydration, salinity and cold stress conditions has been shown to frequently be dependent on a *cis*-acting element named the dehydration-responsive element (DRE, TACCGACAT). The core motif CCGAC (also referred to as the C-repeat) occurs in the promoters of several cold- and drought-inducible genes including *RD29A*, *RAB18* and *KIN2* (Shinozaki & Yamaguchi-Shinozaki 1997).

2.4.3.3 Stress- and ABA-related signal transduction

The identification of stress-responsive promoter elements has opened the way for characterisation of the events near the termini of ABA-dependent and -independent stress-induced signalling pathways. Several stress-responsive gene products which bind to the ABRE, DRE/C-repeat and MYB or MYC target sequences have been identified (Abe et al. 1997;

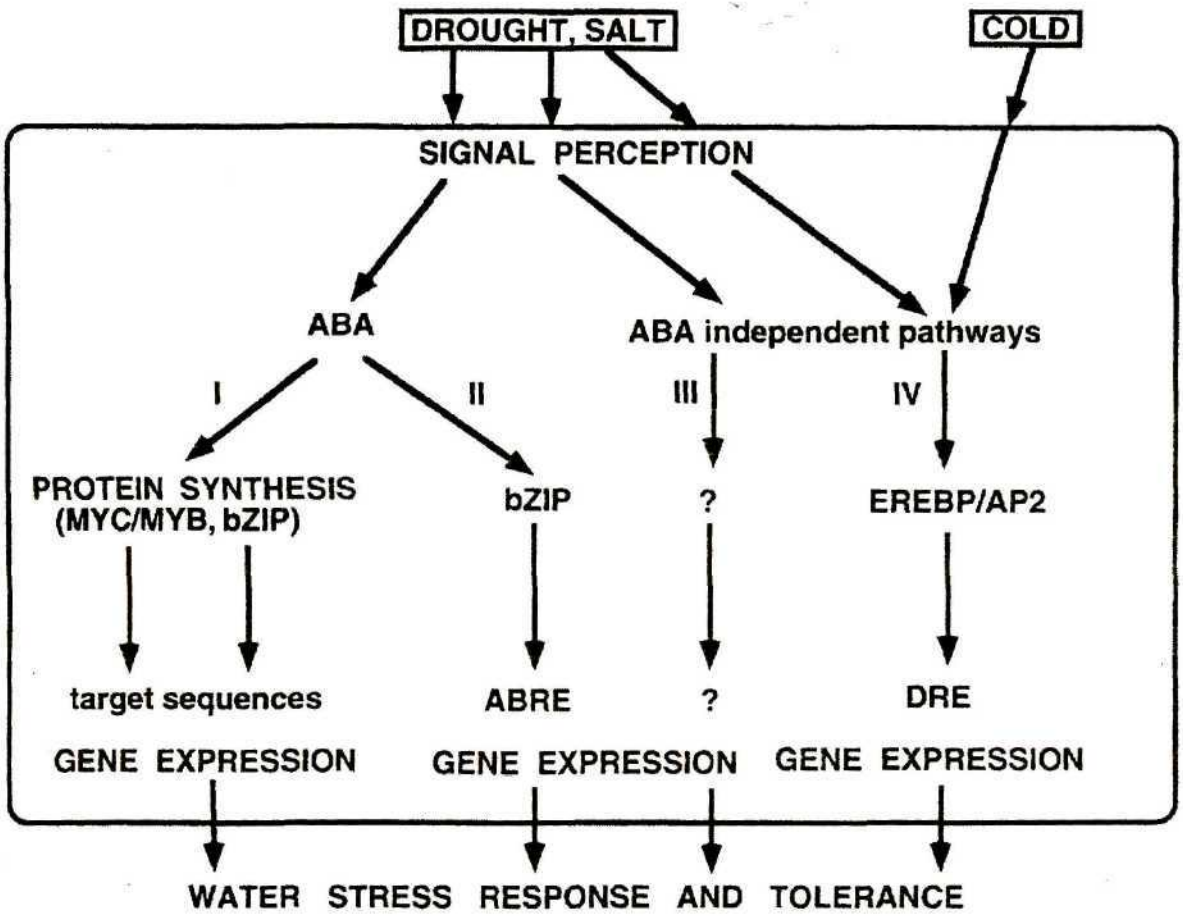

Figure 2.9: Putative signal transduction pathways between the perception of hyperosmotic stress and gene expression. At least four signal transduction pathways (I-IV) are proposed: two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein biosynthesis is required in one of the ABA-independent pathways (I). The action of another ABA-dependent pathway (II), which activates the ABRE, is not affected by CHX. In one of the ABA-independent pathways (IV), the DRE is involved in the induction of genes not only by drought and salt, but also by cold stress. All of the DRE-binding proteins characterised thus far contain a conserved DNA-binding motif that has also been reported in EREBP and AP2 proteins (EREBP/AP motif) that are involved in ethylene-responsive gene expression and floral morphogenesis, respectively (Shinozaki & Yamaguchi-Shinozaki 1997; Liu et al. 1998). Another ABA-independent pathway is controlled by drought and salt but not by cold (III). From Shinozaki and Yamaguchi-Shinozaki (1997).

Shinozaki & Yamaguchi-Shinozaki 1997; Stockinger et al. 1997; Liu et al. 1998). Overexpression of CBF1, one of the transcriptional activators known to bind to the DRE (Stockinger et al. 1997), was recently shown to induce the expression of several cold-regulated *Arabidopsis* genes, including *RD29A* and *KIN2*, and increased the freezing tolerance of nonacclimated *Arabidopsis* plants (Jaglo-Ottosen et al. 1998). Two additional DRE-binding proteins, DREB1A and DREB2A, are induced by low-temperature stress and by dehydration, respectively (Liu et al. 1998). Overexpression of DREB1A in transgenic *Arabidopsis* plants induced strong expression of DRE-

regulated genes under optimal growth conditions and increased freezing and dehydration tolerance, although the plants were dwarfed (Liu et al. 1998). As will be discussed below, several studies have indicated the power of single cell analyses in deciphering the events closer to the site(s) of stress perception. Since the mechanisms whereby plants translate physical parameters such as temperature extremes, water availability or ionic strength into physiological responses are largely unknown, approaches involving mutant isolation are likely to be particularly valuable in defining the primary biochemical events in stress-related signalling.

Ishitani et al. (1997) reported the use of misexpression of a luciferase (*LUC*) transgene under the control of the *RD29A* gene promoter to identify 103 *Arabidopsis* mutants with aberrant transcriptional responses to low temperature, ABA and NaCl stress. The *RD29A* promoter contains the DRE/C-repeat and ABRE motifs. Examination of the separate effects of low temperature, osmotic stress and ABA on *RD29A* expression in these *cos*, *los* or *hos* (constitutive, low or high expression of osmotically responsive genes, respectively) mutants indicated that certain of the mutations only affect *RDA29::LUC* expression in response to low temperature or NaCl. This conforms with the view that ABA-independent signal transduction pathways act in parallel to ABA-dependent cascades (Figure 2.9; Shinozaki & Yamaguchi-Shinozaki 1997). However, many of the mutations enhance or reduce the response to more than one of the three stimuli. These must define shared components in the cold-, NaCl- and ABA-regulated signalling pathways, the existence of which cannot be reconciled with the view that osmotic and cold signalling invoke parallel ABA-dependent and ABA-independent pathways (Figure 2.9). Thus, mutational analysis suggests that rather than acting in parallel by operating separately on discrete DNA sequence motifs, ABA-dependent and ABA-independent pathways interact extensively before converging on the promoters of stress-related genes.

Characterisation of proline synthesis in the range of *cos*, *hos* and *los* mutants is warranted, since like *RD29A*, *AtP5CS1* is subject to control by both ABA-dependent and ABA-independent mechanisms. Thus far, only *hos1-1* has been characterised in any detail. This mutation caused superinduction of a range of cold regulated genes besides *RD29A*, although its effects on genes involved in proline synthesis were not reported (Ishitani et al. 1998). Surprisingly, it appears that HOS1 is a negative regulator of a low temperature-specific pathway, but that it acts as a positive factor for the induction of the same genes by PEG-mediated osmotic stress or ABA (Ishitani et al. 1998). Xin and Browse (1998) recently reported on the proline biosynthetic capacity of a constitutively freezing tolerant *Arabidopsis* mutant named *eskimo1* (*esk1*). Measurement of freezing tolerance using an ion leakage assay indicated that the temperature which caused 50% ion leakage was - 7.9°C for *esk1* compared with - 2.8°C for non-acclimated WT plants. The demonstration that non-acclimated *esk1* plants have an approximately 35-fold higher level of free

proline and eight-fold higher level of *AtP5CS1* transcript than non-acclimated WT plants implicates a role for proline biosynthesis in mediating cold tolerance. Previously, Savouré had interpreted their finding that cold-induced proline accumulation and *AtP5CS1* mRNA levels in an ABA-deficient mutant incapable of cold acclimation are comparable to the levels in WT plants (Table 2.10) to indicate that proline biosynthesis does not play an important role in the tolerance of low temperatures. Although the *ESK1* gene has not yet been cloned, ESK1 is believed to be a repressor of *AtP5CS1* expression. Like *AtP5CS1*, *RAB18* is constitutively expressed at a higher level in *esk1* than in the WT, although mutation of *ESK1* did not cause constitutive expression of either *RD29A* or *KIN2* (Xin & Browse 1998). These workers have extended the proposal by Ishitani et al. (1997) that it is not appropriate to consider the events that result in cold acclimation as a simple linear pathway such as is depicted in Figure 2.9. Despite the value of this model (Figure 2.9) in an heuristic sense, note also that it does not incorporate ABA-dependent gene expression in response to low temperature. Based on their observation that *esk1* does not affect genes such as *RD29A* and *KIN2* (which have been implicated in all previous models of both ABA-dependent and ABA-independent signalling), and their presently unpublished observations that other freezing tolerant mutants are affected in neither proline biosynthetic capacity nor *RD29A*/*KIN2* expression, Xin and Browse (1998) proposed that at least four separate and possibly overlapping pathways participate in cold acclimation.

In view of the evident complexity in the network of signalling chains that control genetic responses to stress, further investigation of the events that regulate proline accumulation may shed some light on how these chains are intertwined at the molecular level to coordinate a single response to stimuli such as a change in endogenous ABA concentration or cell turgor. As will be elaborated below, an important role for Ca^{2+} is now well established for ABA-dependent gene induction. Stretch-activated Ca^{2+} channels, which are responsive to changes in turgor potential, have also been identified (Cowan et al. 1997). Tightly regulated changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) have been identified as important components of signalling pathways induced in response to the imposition of several environmental stresses associated with proline accumulation (Hare et al. 1997). Increases in $[Ca^{2+}]_c$ are observed in response to the imposition of anoxia (Sedbrook et al. 1996), cold-shock (Monroy & Dhindsa 1995; Knight et al. 1996), chemical treatments that simulate stress-induced enhancement of oxygen activation (Price et al. 1994) and elicitor-stimulated oxidative burst, defence-related gene activation and phytoalexin production (Zimmermann et al. 1997). A disruption of cellular Ca^{2+} homeostasis has long been proposed to be the primary response to salt stress (Rengel 1992).

Characterisation of the *sos3* mutant of *Arabidopsis* has confirmed the long-postulated importance of Ca^{2+} in tolerance of NaCl, through its effects on K^+/Na^+ selectivity (Liu & Zhu 1997b). Increased

extracellular Ca^{2+} completely suppresses the growth defect of *sos3* plants on media that contains low K^+ and partially suppresses the hypersenstive phenotype of *sos3* plants grown in the presence of 50 mM NaCl (Liu & Zhu 1997b). The ameliorative effect of Ca^{2+} on *sos3* growth is not observed for *sos1*. Nonetheless, because both *sos1* and *sos3* exhibit similar phenotypes, the two genes probably function in a common pathway that regulates salt tolerance (Liu & Zhu 1997b). These workers subsequently reported that the *SOS3* product shares significant sequence similarity with a subunit from yeast calcineurin and neuronal Ca^{2+} sensors from animals (Liu & Zhu 1998). Calcineurin is a Ca^{2+}/calmodulin (CaM)-dependent phosphatase which is known to play an essential role in NaCl tolerance in yeast cells through its regulation of Na^+ efflux and K^+ uptake. Pardo et al. (1998) recently confirmed the importance of intracellular Ca^{2+} signalling through a calcineurin-like pathway in mediating the beneficial effect of Ca^{2+} on plant salinity tolerance through their demonstration that overexpression of constitutively activated yeast calcineurin in tobacco plants substantially increased their tolerance of saline conditions. Since genetic evidence suggests that *sos1* is epistatic to *sos3*, the *SOS1* gene product may encode a component upstream of SOS3 (Liu & Zhu 1997b). In view of the higher levels of NaCl-induced *AtP5CS1* expression and free proline accumulation observed in *sos1* relative to WT *Arabidopsis* (Liu & Zhu 1997a), it would be interesting to discover whether *sos3* has an elevated capacity for proline biosynthesis. Recently, Zhu et al. (1998) reported the characterisation of a third salt tolerance locus, *SOS2*. Double mutant analysis indicates that *sos1* is probably also epistatic to *sos2*, although the epistatic relationship between *sos2* and *sos3* has not yet been established. Like *sos1* (Liu & Zhu 1997a), *sos2* is characterised by a higher level of *AtP5CS1* expression than the WT, although constitutive levels of *AtP5CS1* transcript are lower in *sos2* than in *sos1* (Zhu et al. 1998). Unlike *sos3*, but like *sos1*, elevated exogenous Ca^{2+} concentrations cannot restore growth of *sos2* seedlings on low K^+-containing culture medium (Zhu et al. 1998).

Increased exogenous Ca^{2+} not only increased the K^+/Na^+ ratio, but also enhanced proline content in alfalfa callus cultures (Shah et al. 1990). However, since extracellular Ca^{2+} exists at millimolar concentrations, and cells must thus constantly remove Ca^{2+} to keep the $[Ca^{2+}]_c$ at levels where it can function effectively in signal transduction, such studies are difficult to interpret in the context of a role for Ca^{2+} in mediating the molecular events associated with proline accumulation and other stress responses. With the advent of more sophisticated approaches, substantial progress has recently been made in identifying some of the biochemical changes that are involved in the induction of *AtP5CS1*, *RD29A*, *RAB18* and *KIN2* genes. These data, which are summarised in Table 2.11, provide a sketchy framework on which to base further investigations.

Knight et al. (1997) used intact transgenic *Arabidopsis* seedlings that express the soluble Ca^{2+}-sensing luminescent protein aequorin in the cytosol to demonstrate that a transient increase in

Table 2.11: Features of ABA and/or stress-related signalling events that lead to induction of *AtP5CS1*, *RD29A* and *RAB18*.

	AtP5CS1	*RD29A*[1]	*RAB18*	Reference
induction by stress/ABA requires new protein synthesis	✔	✖[2]	ND	Strizhov et al. (1997); Wu et al. (1997)
induction by both ABA-dependent and -independent signalling pathways	✔	✔[2]	✖[3]	Savouré et al. (1997); Yamaguchi-Shinozaki & Shinozaki (1994)
ABA-mediated induction involves ABI1	✔	✔	✔	Strizhov et al. (1997); Nordin et al. (1991); Lång & Palva (1992)
overexpressed in *sos1*	✔	✖	ND	Liu & Zhu (1997a)
overexpressed in *sos2*	✔	✖	ND	Zhu et al. (1998)
overexpressed in *esk1*	✔	✖[2]	✔	Xin & Browse (1998)
overexpressed in CBF1[4] overexpressing transgenic lines	ND	✔[2]	ND	Jaglo-Ottosen et al. (1998)
induction by mannitol inhibited by La^{3+}[5]	✔	✔	✔	Knight et al. (1997)
induction by NaCl inhibited by La^{3+}[5]	✔	✖	✖	Knight et al. (1997)
induction by mannitol inhibited by Gd^{3+}, verapamil and EGTA[5]	✔	ND	ND	Knight et al. (1997)
sufficiency of $Ca^{2+}$for induction	✖	✔[2]	ND	Knight et al. (1997); Wu et al. (1997)
induction by ABA blocked by EGTA	ND	✔[2]	ND	Wu et al. (1997)
induction by cADPR (blocked by EGTA)	ND	✔[2]	ND	Wu et al. (1997)
response to Ca^{2+}, ABA and cADPR abolished by K252a / diminished by STA	ND	✔[2]	ND	Wu et al. (1997)
induction by OKA in absence of ABA	ND	✔[2]	ND	Wu et al. (1997)
induction by IP_3	ND	✔[2]	ND	Wu et al. (1997)

✔, yes; ✖, no; ND, not determined

[1] also known as *COR78*, *LTI78*, *LTI140* , *COR67* and *COR160*

[2] an identical response shown for *KIN2* (*COR6.6*)

[3] induction is ABA-dependent (Lång et al. 1994; Gosti et al. 1995)

[4] CBF1 is a transcriptional factor which binds to the DRE (Stockinger et al. 1997)

[5] induction of *KIN2* expression by cold is inhibited by EGTA, La^{3+}, Gd^{3+}, ruthenium red and W7, an antagonist of CaM and CDPK action (Tähtiharju et al. 1997)

cADPR, cyclic ADP-ribose; OKA, okadaic acid; STA, staurosporine

$[Ca^{2+}]_c$ upon mannitol or NaCl treatments can be substantially inhibited by pre-treatment with the Ca^{2+}-channel blocker lanthanum (La^{3+}) and to a lesser extent, the Ca^{2+}-chelator EGTA (Knight et al. 1997). This increase in $[Ca^{2+}]_c$ participates in NaCl- and dehydration-mediated upregulation of proline biosynthesis, since the induction of *AtP5CS1* transcript by either stressor can be inhibited by La^{3+} (Knight et al. 1997). Mannitol-, but not NaCl-induced *RD29A* and *RAB18* transcript accumulation was also inhibited by La^{3+} (Table 2.11). Induction of expression of *AtP5CS1* by mannitol was also inhibited by EGTA and the Ca^{2+}-channel blockers gadolinium (Gd^{3+}) and verapamil (Knight et al. 1997).

The observation that Ca^{2+} transients were of a similar magnitude and duration in response to isosmolar concentrations of mannitol and NaCl, yet mannitol induced a greater abundance of *AtP5CS1* transcript than an isosmolar concentration of NaCl, led Knight and co-workers to conclude that a factor(s) other than $[Ca^{2+}]_c$ participates in the discrimination between dehydration and salinity signals in *Arabidopsis*. Furthermore, a substantial transient in $[Ca^{2+}]_c$ obtained by adding external Ca^{2+} was not adequate for full induction of *AtP5CS1* transcript accumulation. This indicates that additional signalling factor(s) are required for the response. Although the demonstration that both La^{3+} and EGTA inhibit the NaCl- and mannitol-induced elevations in $[Ca^{2+}]_c$ in *Arabidopsis* seedlings indicates the involvement of extracellular Ca^{2+} in this response, the observation that neither of these inhibitors abolish the response totally led these workers to investigate the additional involvement of intracellular Ca^{2+} stores.

In animal systems, many extracellular stimuli are known to activate phosphoinositide-specific phospholipase C (PLC), which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate to generate two second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). While DAG activates protein kinase C (PKC), IP_3 activates receptors coupled to Ca^{2+} channels to induce release of Ca^{2+} from intracellular stores. The Ca^{2+} in turn activates enzymes responsible for the ensuing cellular response. Since previous studies had indicated that osmotic stress enhances the competence of IP_3-sensitive vacuolar Ca^{2+} channels to respond to IP_3, Knight et al. (1997) assessed the change in $[Ca^{2+}]_c$ in the microdomain adjacent to the tonoplast in *Arabidopsis* plantlets exposed to mannitol. In contrast to what had previously been found for low temperature treatment, where the peak height of the Ca^{2+} response at the cytosolic face of the vacuolar membrane was ca. 70% of that observed in the cytosol, the $[Ca^{2+}]_c$ in the vacuolar microdomain was equal to or higher than that observed in the cytosol. It also exhibited a faster rate of increase and was of longer duration (Knight et al. 1997). Despite these differences in cold- and dehydration-induced Ca^{2+} transients, it is interesting to note that previous researchers, using the same approach as Knight and co-workers, had demonstrated that increases in $[Ca^{2+}]_c$ in cold-shocked *Arabidopsis* seedlings can be blocked by La^{3+} and Gd^{3+} as well as by an extracellular Ca^{2+} chelator (Polisensky & Braam 1996).

Use of inhibitors of PLC and *myo*-inositol-1-phosphatase suggested that a significant release of vacuolar Ca^{2+} following dehydration of *Arabidopsis* seedlings occurred through IP_3-dependent Ca^{2+} channels (Knight et al. 1997). Nonetheless, a direct role for the activation of a phosphoinositide-sensitive vacuolar Ca^{2+} channel in contributing to the induction of *AtP5CS1* gene expression by mannitol and salt treatments was not investigated (Knight et al. 1997). It is worth noting that interpretation of the effects of Li^+, the inhibitor of *myo*-inositol-1-phosphatase used by Knight et al. (1997) may be complicated by the fact that this K^+ analogue, which has been traditionally used as an antagonist in salt uptake studies, may have metabolic effects unrelated to phosphoinositide signalling. It would be interesting to know whether or not the effects of inhibition of phosphoinositide signalling can be reversed by Ca^{2+} or intermediates of the phosphatidylinositol cycle as well as whether any effects on stress-inducible gene expression might be mimicked or suppressed by protein kinase and/or phosphatase inhibitors. Whereas NaCl-stressed *sos1* displays elevated *AtP5CS1* expression relative to comparable WT plantlets, accumulation of transcript encoding a *AtPLC1S*, a PLC which is responsive to ABA, dehydration, salt and cold stresses (Hirayama et al. 1995), was not affected in *sos1* (Liu & Zhu 1997a). However, a *sos2* mutant which also has elevated *AtP5CS1* mRNA levels relative to the WT, although not as high as those found in *sos1*, is characterised by higher levels of *AtPLC* expression than comparable salt-stressed WT plants (Zhu et al. 1998). The expression of *RD29A* is unaffected in *sos1* and *sos2* (Table 2.11; Liu & Zhu 1997a; Zhu et al. 1998). The involvement of phosphoinositide signalling in the regulation of *AtP5CS1* expression thus requires further investigation, although transcriptional activation of the gene encoding a stress-inducible PLC does not appear to be required for *AtP5CS1* expression. A role for PKC in plant stress tolerance is suggested by the demonstration that H7, a preferential PKC inhibitor, slightly reduces freezing tolerance in *Arabidopsis* (by about 0.5°C) and inhibited *KIN1* and *KIN2* transcript accumulation in cold-treated plants (Tähtiharju et al. 1997).

A further significant advance in characterising the biochemical events which trigger the induction of stress-responsive genes has arisen through single-cell microinjection experiments using tomato hypocotyls (Wu et al. 1997). Separate co-injection of either ABA or Ca^{2+} together with fusions of the GUS gene to the promoters of *RD29A* and *KIN2*, activated expression of both reporter genes. The ABA-mediated induction of both genes was blocked by EGTA. This is consistent with the demonstration that inclusion of Ca^{2+} ionophores in Ca^{2+}-containing medium can induce the expression of an ABA-responsive gene in maize protoplasts (Sheen 1996). Thus, in contrast to the apparent situation for *AtP5CS1*, at least part of the cellular apparatus responsible for *RD29A* or *KIN2* expression appears to be constitutively primed to respond to increases in a universal signalling intermediate with minimal intrinsic informational specificity (Table 2.11).

Based on a previous demonstration that Ca^{2+} release from plant vacuoles may be triggered by cyclic ADP-ribose (cADPR), Wu et al. (1997) further demonstrated that cADPR can substitute for ABA or Ca^{2+} in stimulating *RD29A* and *KIN2* expression. Prevention of cADPR-mediated induction of both genes not only by EGTA, but also by an analogue of cADPR and a specific inhibitor of cADPR activity, strongly implicate cADPR as an intermediate in ABA signal transduction which elicits its effects via intracellular Ca^{2+} release. Furthermore, a bioassay indicated that amounts of cADPR in *Arabidopsis* plants increased in response to ABA treatment and before ABA-induced gene expression (Wu et al. 1997). Microinjection of ADP-ribosyl cyclase activated expression of both reporter genes in the absence of ABA. This enzyme not only synthesises cADPR from NAD^+, but also catalyses the synthesis of nicotinic acid adenine dinucleotide ($NAADP^+$) from $NADP^+$ (Graeff et al. 1998). The effectiveness of only nanomolar concentrations of $NAADP^+$ in activating Ca^{2+}-release in animal cells is well documented. Since cADPR and $NAADP^+$ mobilise intracellular Ca^{2+} stores by totally independent mechanisms, and these are pharmacologically distinct from those activated by IP_3 (Graeff et al. 1998), differential regulation of ADP-ribosyl cyclase potentially holds an important clue concerning how cells may differentiate between different Ca^{2+}-mobilising signals.

Importantly, the experiments of Wu and co-workers have demonstrated that ABA-induced *RD29A* and *KIN2* expression is cell autonomous and the primary stimulus does not necessarily need to act downstream of Ca^{2+} release in order to induce expression of *RD29A* and *KIN2*. Nonetheless, in view of the findings of Ishitani et al. (1997) concerning the regulation of *RD29A* by ABA, as well as by cold and salt stresses, there may be considerable modification of the signal upstream of cADPR-mediated Ca^{2+} release. Regarding the findings of Knight et al. (1997), it is noteworthy that IP_3 also induced expression of *RD29A::GUS* and *KIN2::GUS*, and that this could be blocked by heparin, a competitive antagonist of the IP_3 receptor (Wu et al. 1997). However, since heparin had no effect on cADPR- or ABA-activated gene expression, and *AtPLC1S* transcript accumulates in response to treatment with ABA (Hirayama et al. 1995), it was concluded that IP_3 may be involved in a secondary, rather than a primary response involved in transcriptional activation of both genes (Wu et al. 1997). The effects of agonists and antagonists of ryanodine receptors, the putative targets of cADPR action, were not reported by Wu et al. (1997). However, it is interesting to note that others have independently shown that such an inhibitor, ruthenium red, partially inhibits *KIN2* transcript accumulation and freezing tolerance in cold-stressed *Arabidopsis*, albeit not as effectively as La^{3+} or Gd^{3+} (Tähtiharju et al. 1997).

Besides changes in $[Ca^{2+}]_c$, protein kinase and phosphatase actions have been implicated in ABA signal transduction, although the relationship of kinase/phosphatase balance to $[Ca^{2+}]_c$ is not yet well characterised. Plants possess at least four distinct types of serine/threonine-specific protein

phosphatases, which can be distinguished on the basis of their substrate specificity, metal requirements and inhibitor sensitivity. Further micro-injection experiments using inhibitors of protein kinases (K252a and staurosporine) and phosphatases (okadaic acid, OKA) suggested that an OKA-sensitive phosphatase(s) may act upstream of cADPR, Ca^{2+} release and protein kinase action in the induction of *RD29A* and *KIN2* expression (Table 2.11). Induction of ABA-responsive gene expression appears to be positively regulated by protein kinases and negatively regulated by protein phosphatases. Accordingly, Sheen (1996) has demonstrated that in maize leaf protoplasts, the ABA-responsive barley *HVA1* promoter can be activated in the absence of ABA by overexpressing the catalytic domain of certain Ca^{2+}-dependent protein kinases (CDPKs). Five CDPKs are differentially upregulated by cold stress in *Arabidopsis* and W7, an inhibitor of CDPKs and CaM, prevented cold acclimation as well as the induction of *KIN1* and *KIN2* expression (Tähtiharju et al. 1997). The WT ABI1, and two mutant abi1 proteins all significantly blocked CDPK-mediated *HVA1* expression (Sheen 1998). The action of ABI1 and abi1 proteins downstream of the positively-acting CDPK action in ABA signal transmission is also consistent with the insensitivity of ABI1 and ABI2 to OKA (Leung et al. 1997). Despite several different approaches, no evidence has been obtained for a direct role for Ca^{2+} in regulating ABI1 activity (Sheen 1998). Recent findings implicate the CHX-independent activation of phospholipase D (PLD) in ABA signalling in barley aleurone cells (Ritchie & Gilroy 1998). Application of phosphatidic acid (PPA), the product of PLD-mediated hydrolysis of phospholipids, simulates ABA-regulated processes and these can be overcome by inhibition of PLD activity (Ritchie & Gilroy 1998). The involvement of PLD activity in stress-regulated gene expression remains to be investigated. Regarding the findings of Knight et al. (1997), it is noteworthy that DAG and PPA are readily interconvertible (Ritchie & Gilroy 1998). These workers presented evidence that it is unlikely that a classical DAG/IP_3 signalling pathway operates in the ABA response of barley aleurone protoplasts.

The findings of Knight et al. (1997) and Wu et al. (1997) clearly provide a valuable basis for further detailed characterisation of second messengers involved in stress-related signalling in plants. Nonetheless, the response of $[Ca^{2+}]_c$ to osmotic stress does not appear to be uniform and may vary with species, cell type, tissue or developmental stage. Using ratiometric fluorescent imaging, Cramer & Jones (1996) observed a rapid *reduction* of $[Ca^{2+}]_c$ in cells from the meristematic region of *Arabidopsis* roots exposed to NaCl. Isosmotic concentrations of $NaNO_3$, KCl and sorbitol reduced $[Ca^{2+}]_c$ to the same extent, thus indicating that this was primarily an osmotic effect. A concentration effect was noted for ABA treatments, although $[Ca^{2+}]_c$'s always ultimately declined in response to ABA. Use of an *aba1* mutant indicated that the reductions in $[Ca^{2+}]_c$ elicited by osmotic stress in root cells were unlikely to be mediated by changes in endogenous ABA (Cramer & Jones 1996). However, ABA-mediated PPA release apparently

reduces $[Ca^{2+}]_c$'s in gibberellin-treated barley aleurone protoplasts (Ritchie & Gilroy 1998). It appears that of the many different Ca^{2+}-regulated pathways that occur in a single cell, some may override others depending on the cell type or developmental stage.

2.4.3.4 Cytokinins and auxins

Cytokinins are generally considered to be antagonists of ABA, with the two hormones having opposing effects in several developmental processes including stomatal opening, cotyledon expansion and seed germination (Hare et al. 1997). In contrast to ABA, we still have a poor understanding of the precise role of CKs in plant stress responses. Even less well characterised is the role that auxins may play in adaptation to environmental stresses (Dunlap & Binzel 1996; Leymarie et al. 1996; Lopez-Carbonell et al. 1996).

Cytokinins were reported to act independently of osmotic stress in mediating proline accumulation and accumulation of an isoform of phospho*enol*pyruvate carboxylase (PEPCase; EC 4.1.1.31) involved in the transition from C_3 photosynthesis to Crassulacean acid metabolism (CAM) in the facultative halophyte *M. crystallinum* (Thomas et al. 1992). In contrast, exogenous ABA was a poor substitute for NaCl in inducing these responses, neither of which were prevented by an inhibition of stress-induced ABA accumulation (Thomas et al. 1992). Many workers have exploited the use of the *ipt* gene from *Agrobacterium tumefaciens*, which encodes an enzyme that catalyses the rate-limiting step in CK biosynthesis. Several studies involving the constitutive or regulated expression of *ipt* have demonstrated that the resulting plant phenotypes are similar to those associated with exogenous application of CKs (Hare & van Staden 1997). Subsequent investigation of whether CK signalling might overlap with stress-induced response pathways in a glycophyte involved the study of stress responses in tobacco transformed with *ipt* under the control of a light-inducible promoter. Under inducing conditions, a ten-fold increase in levels of zeatin-type CKs was accompanied by increased levels of proline (Thomas et al. 1995a).

Interestingly, these findings related to CK-induced proline accumulation contrast with earlier reports (Wample & Bewley 1975; Stewart et al. 1986), where it was shown that exogenous application of higher CK concentrations prevented stress-induced proline accumulation in barley and sunflower respectively. Similarly, the conclusions concerning a stimulative effect of CK on PEPCase levels in *M. crystallinum* conflict directly with those presented by others studying the same system (Schmitt & Piepenbrock 1992). These paradoxical findings concerning the involvement of CKs in stress responses are parallelled by many other apparently contradictory effects of this poorly understood class of phytohormone (Hare et al. 1997). A recent report (Peters

et al. 1997) may have resolved much of the conflict surrounding whether CKs may act as positive or negative effectors of stress responses in *M. crystallinum*. Exogenous N^6-benzyladenine (BA) either stimulates or inhibits induction of PEPCase gene expression depending on the organ to which it is applied. Transcript encoding PEPCase and the activity of the enzyme were strongly induced in root-treated plants, although an additional application of BA to the leaves overcame this induction. When BA was applied to shoot tissue, PEPCase levels were decreased relative to those found in untreated plants (Peters et al. 1997). Although application of BA to roots induced an approximately 50% increase in free proline levels in leaves of *M. crystallinum*, application of the CK to roots caused a 150% increase, while application to both roots and shoot tissue increased proline levels by more than 200% (Peters et al. 1997). It is pertinent to note that at the concentrations used, BA applied to the roots decreased leaf water content, while application to the shoots had no effect on this parameter. Thus, as has already been suggested above for the effects of the *abi1* mutation, a component of the higher induction of proline accumulation in leaves from plants treated with BA at the roots may arise as a secondary effect related to a loss of cell turgor.

In *Arabidopsis*, CK does not affect the accumulation of *AtP5CS1* mRNA in roots, but caused some reduction of *AtP5CS1* transcript levels in leaves at least after 6 h treatment (Strizhov et al. 1997). Levels had apparently recovered to those found in untreated leaves within 24 h. In contrast, BA caused a significant induction of *AtP5CS2* mRNA transcript in leaves, but not in roots (Strizhov et al. 1997). Transfer of cultured cells to CK-supplemented medium after they had been washed in hormone-free medium, had no effect on levels of either transcript encoding P5CS when monitored throughout a 48 h period (Strizhov et al. 1997).

Auxin (2,4-D) caused a slight (approximately three-fold) induction of *AtP5CS1* mRNA accumulation in leaves and roots of 28 d-old *Arabidopsis* seedlings. Application of 2,4-D strongly stimulated *AtP5CS2* mRNA accumulation in leaf tissue, but had no effect on, or caused even slight suppression of, the abundance of this transcript in roots (Strizhov et al. 1997). To further investigate a role for auxin in the regulation of *AtP5CS1* and *AtP5CS2*, Strizhov et al. (1997) studied their expression in two mutants which are affected in auxin action. The *Arabidopsis axr2* mutant accumulates lower levels of both *AtP5CS1* and *AtP5CS2* transcripts after a 6 h exposure to NaCl (Strizhov et al. 1997). However, interpretation of this effect is complicated by the likelihood that this is a neomorphic (gain-of-function) mutation and that AXR2 may not normally function in hormone action (Estelle & Klee 1994). Moreover, *axr2* is resistant not only to auxin, but also to ethylene and ABA (Estelle & Klee 1994). In contrast to *abi1* (Strizhov et al. 1997, but see Savouré et al. 1997 for a different conclusion regarding *AtP5CS1*), the *axr2* mutation does not affect the basic level of both *AtP5CS1* and *AtP5CS2* transcripts in the absence of stress

(Strizhov et al. 1997). It was suggested that *axr2* and *abi1* may disrupt a common signalling pathway downstream of ABA perception, which is not affected by *abi2* or *abi3* mutations. The *aux1* mutant, which in all likelihood is deficient in an auxin transporter (Bennett et al. 1996), is resistant to auxin, ethylene and CK but displays normal sensitivity to ABA in a root elongation assay (Estelle & Klee 1994). After salt stress, *aux1* accumulated *AtP5CS1* and *AtP5CS2* transcripts to levels similar to those observed in WT *Arabidopsis* (Strizhov et al. 1997).

2.4.4 Signal transduction events upstream of PDH gene induction

Following prolonged dehydration or salt stress, when expression of the proline biosynthetic genes is activated, expression of the gene encoding PDH is suppressed (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996a). Upon rehydration after desiccation (Kiyosue et al. 1996) or PEG-mediated dehydration (Verbruggen et al. 1996a), as well as after relief from salinisation (Peng et al. 1996), *AtP5CS1* transcript levels decline and expression of the *AtPDH* gene is rapidly induced to high levels (Figure 2.10). Although P5CDH has yet to be characterised at the gene level, and the relative importance of *P5CR* gene induction is questionable (Yoshiba et al. 1995; Hare & Cress 1997), P5CS and PDH appear to catalyse the rate-limiting steps in proline synthesis and degradation respectively. The reciprocal regulation of the genes encoding these enzymes introduces the possibility that their relative levels may be coordinated by the same signalling cascade. Recent studies of phytochrome A signal transduction suggest that the same pathway,

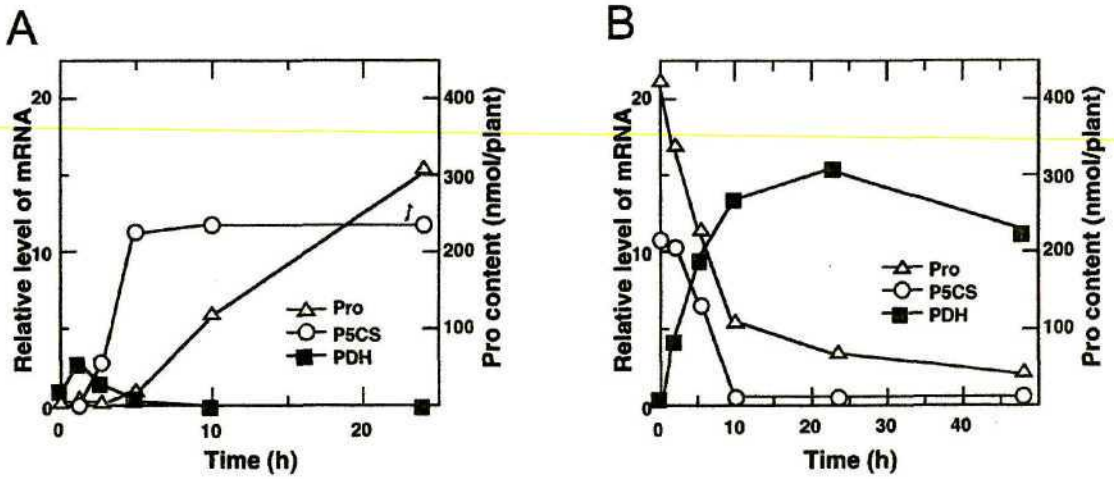

Figure 2.10: Changes in levels of free proline (Pro) and *AtP5CS1* (P5CS) and *AtPDH* (PDH) transcripts in 4 week-old *Arabidopsis* plants during dehydration (A) and after rehydration of plants which had been dehydrated for 10 hours (B). From Kiyosue et al. (1996).

involving both Ca^{2+} and cGMP, both activates a gene encoding ferredoxin $NADP^+$ oxidoreductase and mediates repression of an asparagine synthetase gene in the light (Neuhaus et al. 1997). Furthermore, specific PP2C activity apparently negatively regulates a single ABA signalling pathway that controls both gene activation and repression (Sheen 1998). Although abiotic stresses are known to cause both an increased and reduced abundance of discrete gene products, repression of gene expression by adverse conditions is seldom investigated. Investigation of the regulation of *PDH* expression may not only provide insight into the mechanisms of stress-mediated gene repression, but may also shed light on the interesting question of whether plants possess rehydration-specific signalling pathways, or whether physiological responses that follow relief from stress simply involve derepression of genes which are downregulated or silenced under stress. The possible involvement of rehydration-related signalling pathways is not indicated in the graphical representation of our current knowledge of the factors which control proline synthesis and degradation provided in Figure 2.11.

In view of the downregulation of PDH activity following prolonged stress, it was surprising that Kiyosue et al. (1996) isolated a PDH cDNA clone (ERD5, early responsive to dehydration) by differential screening of a library prepared from *Arabidopsis* plants which had been dehydrated for 1 h. Nonetheless, Northern analysis confirmed that the abundance of *ERD5* transcript (henceforth referred to as *AtPDH*) increased transiently 1 h after desiccation, before decreasing to nondetectable levels within 10 h after the commencement of the stress (Figure 2.10). Although *AtPDH* levels are rapidly induced by exogenously applied proline (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996a), the transient induction of *AtPDH* mRNA accumulation precedes detectable proline accumulation by at least 4 h and occurs simultaneously with the accumulation of *AtP5CS1* mRNA (Kiyosue et al. 1996). A transient increase in *AtPDH* transcript levels was also observed within 2 h after exposure to 4°C, and within 1 h of incubation at 40°C, with levels continuing to increase for at least a further h in the case of heat treatment (Kiyosue et al. 1996). Neither of these temperature extremes were as effective as dehydration in inducing *AtPDH* expression. Interestingly, the same anomalous induction of *AtPDH* expression was also observed in both WT and *esk1 Arabidopsis* plants after incubation at 4°C for 2 days (Xin & Browse 1998), although once again these workers specifically stated that they had no rationale for a stress-induced *induction* of *AtPDH* expression. As will be discussed more extensively in Section 2.5.2.2, these findings together with the rapid induction of *AtP5CS* transcripts discussed above, have been interpreted to indicate an important role for cycling between proline and its precursors in ensuring metabolic homeostasis under moderate environmental fluctuations or as an early response to severe stress (Hare et al. 1998). This may be accomplished through associated effects on adenylate charge as well as the level of reduction of pyridine nucleotides, particularly NADP.

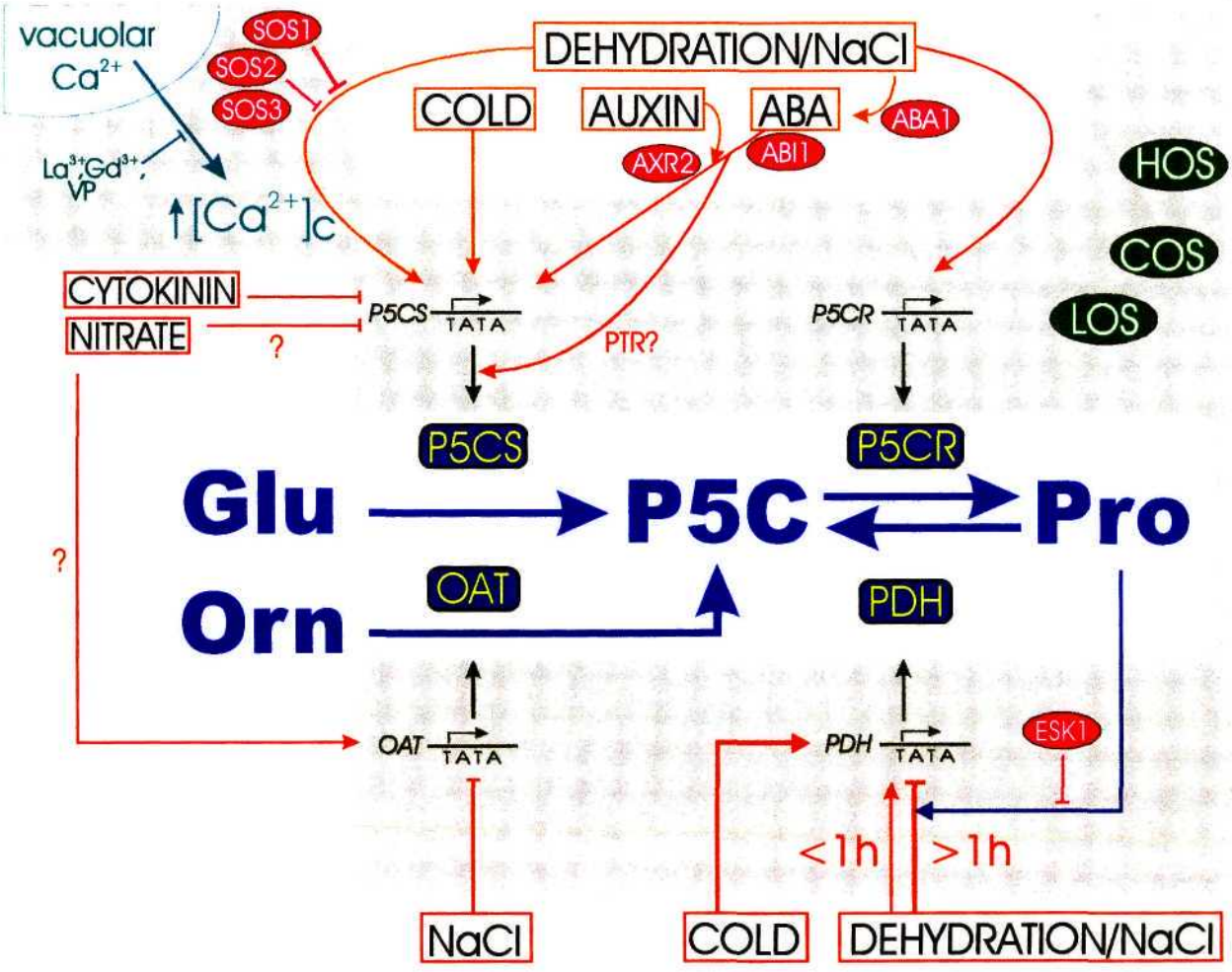

Figure 2.11: An heuristic model of signalling events associated with stress-regulated changes in proline synthesis and degradation. Although not indicated, the pathways downstream of the perception of desiccation, salinity stress, low temperature stress and ABA are likely to share only certain signalling intermediates. Induction by these stimuli are postulated to occur against the background of the signalling network defined by the *COS*, *LOS* and *HOS* (Ishitani et al. 1997) gene products (shaded). Data used to reflect the regulation of *P5CS* transcript abundance applies for the *AtP5CS1* gene only. In the interests of simplicity, pathways that mediate induction of *PDH* transcript accumulation and repression of *P5CS* transcript accumulation upon rehydration after dehydrative stresses (Kiyosue et al. 1996), are not shown. Whether or not these processes are simply mediated by suppression of stress-induced signalling remains to be established. No data is available concerning the regulation of genes encoding P5C dehydrogenase (P5CDH).

Neither feedback inhibition of the *P5CS* gene product by proline (Zhang et al. 1995) nor constitutive repression of *AtP5CS1* expression by ESK1 (Xin & Browse 1998) are indicated in the figure. The postulated repressive effect of nitrate on *P5CS* transcript abundance may not be direct. This, together with the repressive effect of NaCl stress on *OAT* transcript abundance, is based on proline synthesis in *Vigna aconitifolia* (Delauney et al. 1993). The relative positions of ABI1 and AXR2 cannot be defined with certainty. Since the *abi1* and *axr2* mutations which abrogate *P5CS* gene expression are neomorphic mutations, ABI1 and AXR2 may not necessarily normally participate in the regulation of proline synthesis in *Arabidopsis*. Glu, glutamate; Orn, ornithine; Pro, proline; PTR, post-transcriptional regulatory event that does not affect transcript abundance; VP, verapamil; →, positive action; ⊣, negative action; ?, uncertain.

Strong induction of *AtPDH* expression upon rehydration after desiccation occurs within 2 h and reaches a maximum level between 24 h and 48 h after rehydration (Figure 2.10). Within 10 h after rehydration, free proline levels declined to approximately one-fifth of the level accumulated after dehydration, and continued to decline until 48 h after rehydration, when *AtPDH* transcript abundance is still more than ten-fold higher than at the end of the dehydration stress (Kiyosue et al. 1996). It is thus tempting to speculate that induction of *AtPDH* transcript accumulation upon relief from dehydration is mediated by a rehydration-related signal transduction pathway and not simply by proline accumulated during stress. Irrespective of whether or not *AtPDH* expression upon rehydration is primarily induced by free proline after suppression by dehydration has been relieved, the demonstration that repression of *AtPDH* expression by salt stress overrides induction by exogenous proline (Peng et al. 1997) indicates some interaction between the signalling events downstream of stress perception and assessment of free proline levels (Figure 2.11). The recent characterisation of *esk1*, which has elevated levels of *AtP5CS* expression (Section 2.4.3.3) substantiates this proposal. The observations that mutation of *ESK1* causes a 30-fold higher level of free proline without any effects on *AtPDH* transcript levels led Xin and Browse (1998) to investigate whether the *esk1* mutation acts to prevent the induction of *AtPDH* by proline. Ten h after watering WT plants with a 100 mM proline solution, proline levels were about one-third of the constitutive level found in *esk1* and *AtPDH* transcript levels were significantly increased. In contrast, the *AtPDH* level in *esk1* was only slightly increased (Xin & Browse 1998). Thus, further characterisation of *esk1* may open the way for dissecting the reciprocal control of proline synthesis and degradation under sustained stress. Note however, that exogenous proline does not seem to affect the expression of the *AtP5CR* (Verbruggen et al. 1993) and *AtP5CS2* (Zhang et al. 1997) genes, although the effects of proline on *AtP5CS1* expression do not appear to have been investigated.

Hormonal regulation of *AtPDH* expression is presently not well characterised. A 10 h treatment with ABA, BA and 2,4-D had no apparent effects on *AtPDH* transcript levels in 28 d-old *Arabidopsis* (Kiyosue et al. 1996). A previous study indicated that ABA is unlikely to significantly reduce extractable PDH activity in well-watered maize seedlings, at least not to the levels observed after drought treatment (Dallmier & Stewart 1992).

The CHX-independence of the transient induction of *AtPDH* expression following dehydration or temperature extremes, the repression of *AtPDH* expression upon prolonged (more than 5 h) exposure to these stresses, and the rapid induction of *AtPDH* mRNA accumulation upon relief from dehydration, have not been reported. Nonetheless, Trotel et al. (1996) have examined the effects of a variety of transcriptional and translational inhibitors on proline degradation in rape leaf discs after transfer from a medium of low osmotic potential to one of higher osmotic potential. The

significant inhibition of proline mobilisation by α-amanitin and actinomycin D (transcriptional inhibitors), as well as CHX and to a lesser extent chloramphenicol (translational inhibitors), indicated the involvement of gene expression at the transcriptional level in mediating the degradation of proline accumulated at a low osmotic potential.

2.4.5 Signal cross-talk

A primary limitation to facile elucidation of the processes that regulate free proline levels under both optimal and stressful conditions is the realisation that the multitude of signals which regulate plant growth and development are not transduced *via* linear pathways operating in parallel. Instead, plant responses to environmental stimuli are integrated with endogenous developmental programs by a complex network which is characterised by extensive ramification and redundancy. Since the various inputs into this network modulate each other, both positively and negatively, the overall context of this web of interacting components, and no single factor in isolation, is likely to regulate any of the physiological changes that accompany the imposition of hyperosmotic stress. Determining how the multitude of individual chains downstream of the stimuli that regulate proline synthesis and degradation are interwoven is thus an important consideration in interpreting the effects of hyperosmotic stress on these processes.

Investigation of the factors which regulate the expression of genes involved in proline synthesis and degradation is complicated by the fact that their products have important housekeeping functions in the absence of stress. In contrast, many well characterised stress inducible genes, including *RD29A* and *RAB18* (Table 2.10) appear to be induced *de novo* under adverse conditions. The roles of proline synthetic and degradative enzymes in stress-related responses must be superimposed upon the plant's continuous requirements for protein synthesis during growth. For instance, it may be of interest to note that the *AtP5CS2* promoter contains two transcription start sites. Dehydration stimulates transcription predominantly from the downstream site (Zhang et al. 1997). As will be discussed in Section 2.5.2.4, considerable circumstantial evidence supports an important role for proline synthesis in regulating several physiological responses, including developmental transitions, even in the absence of stress (Hare & Cress 1997).

One can therefore anticipate considerable flexibility in the signalling events which induce the genes involved in proline biosynthesis. Indeed, informational and functional redundancy, superimposed upon subtle modulation by developmentally regulated processes, may account for much of the contradictory evidence outlined above regarding the involvement of ABA levels and

ABI1 in mediating *AtP5CS1* expression (Savouré et al. 1997; Strizhov et al. 1997), as outlined above. Such redundancy is also consistent not only with the existence of plant isoforms of P5CS, but also with the increasingly accepted view that the accumulation of organic solutes such as proline may fulfill multiple roles in facilitating acclimation to stress (Hare et al. 1998). Thus, although complete dissection of the signalling events that contribute to the regulation genes involved in proline metabolism may be more challenging than for more widely studied "stress-specific" genes (e.g. *RD29A*, *KIN2* or *RAB18*), it may provide a useful perspective on how a diversity of factors can be integrated to ensure a response that is appropriate not only to the prevailing environmental conditions, but also to endogenous developmental cues.

2.4.5.1 Light signal transduction

Light quality and quantity are perhaps the most significant extrinsic cues which affect plant growth and development. Plants contain at least three photoreceptor systems involved in modulating growth and development, which differ in the wavelengths of light to which they are most sensitive. These are the phytochromes, blue light/UV-A cryptochromes and an as-yet unknown UV-B receptor(s). These multiple photoreceptors have discrete as well as partially overlapping roles and under certain conditions they also interact with each other. Together, these photosensory molecules provide plants with the capacity to continuously track the presence, absence, spectral quality, fluence rate and duration of incoming light signals, and to adjust their growth and development toward optimal radiant energy capture and survival. Light perception activates signalling pathways that modulate changes in gene expression, which can be translated into a range of physiological and developmental responses (Chory et al. 1996).

The phytochrome system senses the relative intensities of photosynthetically active red light (R) and non-photosynthetic near far-red (FR) light, thus sensing information about light quality and the suitability for photosynthesis. Phytochrome genes encode a small family of photoreceptors. In *Arabidopsis,* the apoprotein is encoded by at least five genes, designated *PHY-A, PHY-B, PHY-C, PHY-D* and *PHY-E*, with related sequences occurring in a diversity of other plants across the phylogenetic spectrum. The photosensory functions of phytochromes are based on their capacity for reversible interconversion between the R-absorbing P_r form (λ_{max}=666-668 nm) and the FR-absorbing P_{fr} form (λ_{max}=730-734 nm) when R and FR are absorbed sequentially (Chory et al. 1996). Phytochrome A (PHYA) is necessary for continuous reception of FR. Its conversion to the P_{fr} form by absorption of R causes PHYA to rapidly aggregate in the cytoplasm and degrade. Phytochrome B (PHYB) is necessary for continuous R perception. It is the principal phytochrome responsible for the classical R/FR reversible response and for the responses of

light-grown plants to low R/FR ratios. It is present in low levels in both the dark and light, being very stable in the P_{fr} form. Little is known about the *PHY-C, -D* and *-E* gene products (Quail et al 1995).

Since many stress-related responses display a strong dependence on light (Cockburn et al. 1996; Hare et al. 1997), establishing the most important sites of crosstalk between phytochrome and stress-related pathways is likely to be of prime importance in dissecting the intricate network of interactions that regulate plant responses to adverse conditions. The importance of light in mediating proline accumulation is well documented (Larher et al. 1993; Chiang & Dandekar 1995; Sanada et al. 1995; Sheveleva et al. 1997) and recent studies implicate the involvement of phytochrome in the response (Pesci 1996). Exposure of light-adapted *Arabidopsis* plants to darkness caused an approximately 50% reduction in *AtP5CS1* mRNA levels, but did not significantly affect *AtP5CS2* mRNA abundance (Strizhov et al. 1997). Phytochrome effects are also inextricably intertwined with the action of hormones. For example, light differentially affects the absolute levels of both ABA and CKs, while CK downregulates *PHYA* gene expression (Thomas et al. 1997).

While much is known about the phytochrome photoreceptor itself, the molecular nature of the primary transduction processes by which the photoreceptors relay their sensory information to downstream signalling components is largely unknown. The P_{fr} form of PHYA is believed to initiate a signal cascade which ultimately results in developmental processes, including a specific set of genes, cell elongation and cell differentiation. Changes in protein phosphorylation appear to be involved in phytochrome-regulated gene expression (Sheen 1993; Harter et al. 1994) Biochemical and microinjection experiments ("single cell assays") analogous to those used by Wu et al. (1997) to elucidate ABA signal transmission, have demonstrated the involvement of heterotrimeric G-proteins, cGMP, Ca^{2+} and CaM in the signalling cascade downstream of PHYA activation (Neuhaus et al. 1993, Bowler et al. 1994). Heterotrimeric G-protein(s), which change activity upon GTP binding, are the most upstream component of the PHYA signal transduction machinery. By monitoring the expression of specific marker genes, three different branches of PHYA signalling downstream of G-protein action were defined (Figure 2.12). It is important to note that this model has yet to be confirmed and extended by results obtained with other approaches, since it reflects only the induction of phytochrome-responsive gene expression in a single cell.

One pathway involves Ca^{2+}/CaM and regulates expression of the genes encoding components of PSII such as the chlorophyll a/b binding proteins (*CAB*) and small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*RBCS*), another route involving cGMP results in the expression of the chalcone synthase gene (*CHS*) and the production of anthocyanins, and the third involving a combination of both second messengers is necessary for the production of mature chloroplasts and regulates the expression of genes encoding components of PSI such as ferredoxin $NADP^+$ reductase (*FNR*). Subsequent studies (Neuhaus et al. 1997) indicated that the

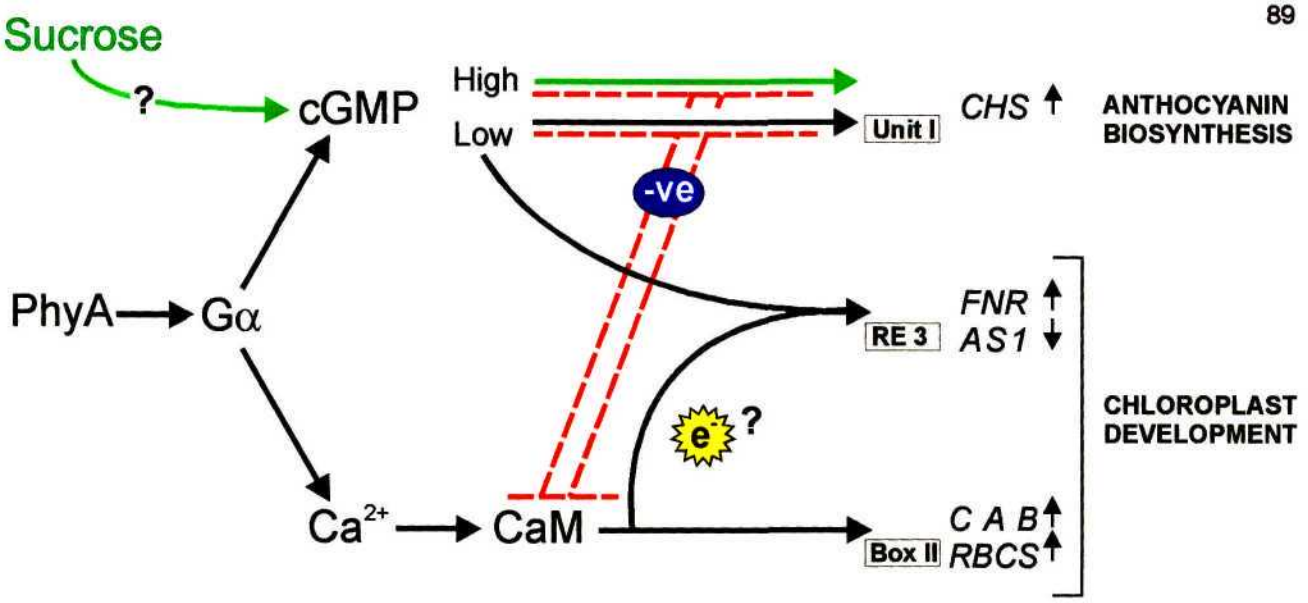

Figure 2.12: Signalling pathways controlling photoregulated gene expression in plants. Phytochrome A (PhyA) signal transduction (black) is mediated by heterotrimeric G proteins (G_α) and subsequently by three pathways dependent on either Ca^{2+}, cGMP or both Ca^{2+} and cGMP together (Bowler et al. 1994). The cGMP-dependent pathway regulates the gene encoding chalcone synthase (*CHS*) and anthocyanin production. Sucrose (green) also induces *CHS* expression. The Ca^{2+}-dependent pathway involves Ca^{2+}-activated calmodulin (CaM) and induces the expression of genes encoding the chlorophyll a/b binding proteins (*CAB*) and genes encoding components of photosystem II, such as the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (*RBCS*). The Ca^{2+}/cGMP-dependent pathway induces the expression of the gene encoding ferredoxin $NADP^+$ oxidoreductase (*FNR*) and genes encoding components of photosystem I. It is also responsible for the repression of an asparagine synthetase gene (*AS1*). Both the Ca^{2+} and Ca^{2+}/cGMP-dependent pathways are necessary for the development of mature chloroplasts. The light-responsive promoter elements Box II (TGTGTGGTTAATATG) from pea *RBCS-3A* and Unit I (GATCCTTATTCCACGTGGCCATCCGGTGGCCGTCCCTCCAACCTAACCTCCCTTGA) from parsley *CHS* are termini of the Ca^{2+}- and cGMP-dependent pathways respectively (Wu et al. 1996). Repression of *AS1* expression by the Ca^{2+}/cGMP-dependent pathway is mediated by RE3 (GATCTGGTGGGAGCTAG), a promoter element found in many genes that are repressed by phytochrome action (Neuhaus et al. 1997). Negative regulation (interpathway reciprocal control; denoted -ve) between the Ca^{2+}- and cGMP-dependent pathways is indicted by dashed lines in red. Redox signals (denoted e^-) arising from plastoquinone are proposed to regulate the relative activities of the two Ca^{2+}-dependent pathways. Adapted from Barnes et al. (1997) and Mustilli & Bowler (1997).

pathway requiring both Ca^{2+} and cGMP can also mediate repression of the asparagine synthetase (*AS1*) gene (Figure 2.12).

This model is substantiated by the demonstration that previously characterised light-responsive *cis*-elements selectively respond to Ca^{2+} and cGMP. Microinjection studies indicated that fusions of Box II (a light-responsive element from the pea *RBCS-3A* promoter) and Unit I (a light-responsive element from the parsley *CHS* promoter) to the *GUS* reporter gene were transcriptionally induced by Ca^{2+} and cGMP respectively (Wu et al. 1996). Using the same

approach, Neuhaus et al. (1997) identified a 17 bp *cis*-element (RE3) within the pea *AS1* promoter that is both necessary and sufficient for PHYA-mediated gene repression (Figure 2.12). This sequence is most likely the binding site for a PHYA-generated repressor whose activity is regulated by both Ca^{2+} and cGMP. An interesting feature of PHYA signalling, which was also confirmed by studies concerning the inductions of *BoxII:GUS* and *UnitI::GUS* fusions by Ca^{2+} or cGMP respectively (Wu et al. 1996), is that flux through the Ca^{2+}-dependent branch is repressed by high concentrations of cGMP, while cGMP-mediated induction of *CHS* expression is abrogated by Ca^{2+} (Figure 2.12).

Mustilli and Bowler (1997) have used this basic framework to propose mechanisms whereby signals downstream of the interception of light, such as sucrose accumulation and activity of the photosynthetic apparatus, may control the expression of photoregulated genes. As has already been outlined (Section 2.2.2.3), a signal(s) arising from perception of the level of reduction of the plastoquinone pool appears to be capable of modifying the expression of nuclear-encoded photosynthetic (Escoubas et al. 1995) as well as antioxidant (Karpinski et al. 1997) genes. Redox signals were proposed to interfere with the two Ca^{2+}-related phytochrome signalling pathways which control chloroplast functions, in order to regulate activation of genes encoding components of the two photosystems (Figure 2.12; Mustilli & Bowler 1997). Owing to the central role played by sugars in the coordination of the plant genetic response to stress, and the involvement of exogenous carbohydrate in stimulating proline accumulation, the participation of sucrose in light signal transmission will be discussed further in the section which follows.

2.4.5.2 Carbohydrate sensing mechanisms

In plants, sugars serve not only as essential substrates for the growth of sink tissues, but they also regulate the expression of a variety of genes involved in the coordination of photosynthetic carbon fixation with carbon use, mobilisation and allocation throughout development (Koch 1996; Jang & Sheen 1997; Smeekens & Rook 1997). Although exceptions to this broad generalisation have been noted (Koch 1996), genes involved in sink formation (affecting storage, respiration and biosynthesis) are induced by sugars, while the expression of those concerned with source establishment (involving photosynthesis, mobilisation of reserves and export) are repressed by sucrose or its hexose degradation products (Hare et al. 1998). Since activation of cellular responses to adverse conditions requires energy and thus the induction of sink metabolism, one might anticipate that sugars play an important role in regulating the induction of stress-responsive genes. Presently, our appreciation of the effects of sugar sensing on genes involved in plant defence against biotic stresses far exceeds that regarding abiotic stresses. Sugar-induced expression has been demonstrated for several genes involved in the response to pathogen-infection (Herbers et al. 1996; Ehness et al. 1997). The possible involvement of hexose sensing in mediating plant responses to adverse abiotic conditions has been discussed recently,

specifically in the context of metabolic and signalling effects associated with stress-induced accumulation of osmolytes such as trehalose and fructans (Hare et al. 1998).

Complexity in elucidating the functional significance of sugar-mediated changes in the expression of stress-related genes arises from the demonstration that expression of genes regulated by carbohydrates is invariably affected by other factors, including phytochrome-mediated light perception (Dijkwel et al. 1997) and hormonal action (DeWald et al. 1994; Jang et al. 1997; Perata et al. 1997). Sugars feedback inhibit photosynthesis not only at the level of enzyme activity, but also through the repression of phytochrome-responsive nuclear genes involved in photosynthesis e.g. *CAB* and *RBCS* (Jang et al. 1997). In contrast, other phytochrome-inducible genes e.g. *CHS* are induced by sucrose (Tsukaya et al. 1991). Hexose sensing has been proposed to interact with the cGMP-dependent branch of PHYA signalling (Figure 2.12; Mustilli & Bowler 1997). This is consistent with reciprocal control mechanisms between the phytochrome-activated cGMP- and Ca^{2+}-dependent pathways (Figure 2.12).

Since sugar levels do not always correlate with carbohydrate-regulated gene expression, it is suggested that metabolic flux, rather than absolute sugar content, triggers sugar-responsive gene expression (Koch 1996). Nonetheless, some controversy surrounds the origin(s) of hexose sensing pathways in plants. Antisense and ectopic expression studies with genes encoding hexokinase (HXK) have indicated that this enzyme is a key regulator of sugar responses in *Arabidopsis* (Jang et al. 1997). Accordingly, certain data indicate that the repression of genes involved in photosynthesis and glyoxylate metabolism of glucose displays an absolute requirement for its phosphorylation by HXK. Glucose-6-phosphate and other glycolytic intermediates are incapable of repressing these genes (Jang & Sheen 1997). Nonetheless, it is widely accepted that other sugar-sensing mechanisms also exist (Jang & Sheen 1997; Smeekens & Rook 1997). For instance, hexose- and sucrose-transporters have also been proposed to enable plants to sense carbohydrate status and initiate signal transduction (Smeekens & Rook 1997).

Herbers et al. (1996) used three classes of transgenic tobacco plants which express yeast invertase in different subcellular compartments to assess the site of perception of hexose abundance prior to sugar-induced expression of pathogenesis-related (PR) protein transcripts and repression of photosynthetic gene transcripts. Ectopic expression of invertase in the vacuole, cell wall or cytoplasm results in elevated hexose levels in all three lines. However, the observation that lines which express vacuolar and apoplastic invertase, but not those with elevated cytosolic invertase levels, displayed induction of PR gene expression and repression of photosynthetic gene expression led to the conclusion that cytosolic HXK does not mediate these responses. Rather, Herbers et al. (1996) proposed that the sugar sensing mechanism is associated with the secretory membrane system, possibly at the endoplasmic reticulum or Golgi apparatus. The observation that induction of PR transcripts and repression of *CAB* levels required the same

threshold level of hexoses was suggested to indicate a common mechanism of sugar sensing in mediating the inverse regulation of both classes of genes (Herbers et al. 1996). A subsequent study (Ehness et al. 1997) has assessed these two proposals. The ability of a non-phosphorylatable analog of glucose, 6-deoxyglucose, to mimic glucose in the repression of *RBCS* and the induction of PR transcripts encoding phenylalanine ammonia lyase (*PAL*) and extracellular invertase is consistent with the view that HXK does not mediate the response of these genes to sugar availability (Ehness et al. 1997). However, different effects of the protein kinase inhibitor staurosporine on the induction of genes encoding extracellular invertase and *PAL* by glucose and a fungal elicitor indicated that carbohydrate and stress-related stimuli act independently of each other, although the different signalling pathways are ultimately integrated to coordinate source and sink metabolism and activate defence responses (Ehness et al. 1997).

Whether there is any connection between sugar sensing and signal-response mechanisms in mediating the response to hyperosmotic stress remains to be determined. The importance of integrating proline synthesis with the energy assimilating capacity of the plant will be emphasised in Section 2.5.2. Sucrose and its hexose degradation products have been shown to stimulate proline accumulation independently of any osmotic effect (Larher et al. 1993). While purely metabolic explanations for this phenomenon seem feasible (Section 2.5.2.2), the possibility of a direct effect of sugars on the expression of proline biosynthetic genes cannot be dismissed. The relatively poor stimulatory effect of mannose (a sugar which is phosphorylated by HXK, but not metabolised further by glycolysis) on free proline accumulation in rape leaf discs (Larher et al. 1993) tends to argue against the likelihood that proline accumulation may be mediated through enhanced HXK activity (Hare et al. 1998). Thus far, plant carbohydrate status does not appear to have been considered as an important parameter influencing the expression of genes involved in proline biosynthesis. In this regard, it is tempting to speculate that contrasting observations regarding NaCl-mediated induction of *AtP5CR* transcript levels discussed above may arise from differences in the carbohydrate status of the stressed plants. Whereas one group (Verbruggen et al. 1993, Savouré et al. 1997) included sucrose in the growth medium, Yoshiba et al. (1995) omitted an exogenous carbohydrate source and did not observe transcriptional induction of the gene by salinisation.

2.4.5.3 Is nitrate another signal which regulates proline synthesis?

In *Vigna aconitifolia*, expression of P5CS appears to be responsive not only to osmotic stress, but also to plant nitrogen status (Delauney et al. 1993). Salt stress and nitrogen starvation induced *P5CS* mRNA levels and depressed levels of transcript encoding OAT. Conversely, plants fed with NH_4NO_3 had considerably higher *OAT* transcript levels, while *P5CS* mRNA levels were reduced (Delauney et al. 1993). Subsequent studies by this group indicated that a 24 h treatment with 5 mM glutamine did not affect the expression of an *AtP5CS2::GUS* fusion (Zhang et al. 1997),

although the effects of nitrogen metabolites on *AtP5CS1* promoter activity have not been investigated.

Recent studies of transgenic tobacco plants with very low nitrate reductase activity have indicated an important role for nitrate, and not metabolites thereof, in signalling coordinated changes in several enzyme activities and levels of transcripts involved in carbon and nitrogen metabolism (Scheible et al. 1997). Nitrate may directly affect *P5CS* gene expression through its contribution to nutrient assessment pathways that appear to interact extensively with both light and hormonal action (Thomas et al. 1997; Figure 2.11). It may also be of relevance to note that transgenic tobacco plants which express an antisense mRNA encoding nitrite reductase accumulate proline to levels almost 5-fold in excess of those found in the WT (Vaucheret et al. 1992). This line had reduced ammonium and glutamine levels and enhanced nitrate reductase activity. Unfortunately, nitrate levels in these proline-overproducing lines were not assessed.

2.4.5.4 How might the signals be integrated?

The involvement of $[Ca^{2+}]_c$ and protein phosphorylation in the regulation of almost all cellular signal transduction pathways makes them obvious candidates for mediating interactions between various stimuli. Although the redox-regulation of gene expression has already been discussed separately (Section 2.2.2.3), recall that Ca^{2+} and CaM have been implicated in mediating the regulated oxidative burst (Harding et al. 1997; Pugin et al. 1997) which increasingly appears to be a central component in the signalling cascades which lead to protection against various abiotic and biotic stresses (Prasad et al. 1994; Foyer et al. 1997; Hare et al. 1997; Dat et al. 1998). Chemical treatments that increase the cellular prooxidant/antioxidant ratio (H_2O_2 as well as inhibitors of APX and GSH synthesis) increase $[Ca^{2+}]_c$ and effect changes in gene expression which can be blocked by La^{3+} (Price et al. 1994). A detailed account of the central role played by salicylic acid (SA), an important regulator of plant stress responses, in coordinating the transient increase in ROIs under adverse conditions is beyond the scope of this review, since its effects on proline metabolism do not appear to have been investigated. Nonetheless, both SA and H_2O_2 appear to mediate not only incompatible plant-pathogen interactions (Section 2.2.2.1; Lamb & Dixon 1997), but also plant responses to ozone and UV-irradiation (Yalpani et al. 1994; Sharma et al. 1996) as well as heat shock (Dat et al. 1998; Lopez-Dalgado et al. 1998). Based on the ability of CKs to induce both catalase and FeSOD expression, it was proposed that CK action, which is also known to be mediated by changes in $[Ca^{2+}]_c$ and protein phosphorylation (Hare & van Staden 1997), may also potentiate stress-induced ROI production (Hare et al. 1997). It is becoming increasingly apparent that the plant antioxidative defence system should be viewed not merely as a metabolic response capable of annihilating ROIs, but perhaps even more importantly, as a means of permitting regulated adjustment of the cellular redox state to alert metabolism to the presence of both biotic and abiotic threats (Foyer et al. 1997; Hare et al. 1998).

Considerable evidence implicates both $[Ca^{2+}]_c$ and protein phosphorylation in the response to biotic and abiotic stresses (Hare et al. 1997) and the involvement of changes in $[Ca^{2+}]_c$ (Knight et al. 1997), most probably mediated through a calcineurin-like pathway involving SOS1, SOS2 and SOS3 (Liu & Zhu 1997a, 1997b, 1998; Zhu et al. 1998) in the regulation of free proline levels and *AtP5CS1* transcript abundance has already been outlined in detail. Although the effects of inhibitors of protein kinases and phosphatases on proline biosynthetic capacity have not yet been assessed, two independent groups of workers have implicated the involvement of ABI1, a PP2C participating in ABA signalling, in regulating P5CS gene expression (Savouré et al. 1997; Strizhov et al. 1997).

To date, very little is known about the molecular events which link the different stimuli (hyperosmotic stress, hormones, light and carbohydrate status) which affect proline biosynthetic capacity. In particular, we presently have a poor understanding of the mechanisms of how the various ubiquitous signalling components identified using pharmacological approaches interact in the context of a signalling pathway comprising macromolecular intermediates. It is becoming increasingly apparent that many of the gene products which have been characterised as transducers of one signal also participate downstream of the perception of other signals. For instance, extensive hypocotyl elongation in the light has been widely used in screens for mutants disrupted in light signalling. The observation that all known *Arabidopsis* long hypocotyl (*hy*) mutants, with the exception of *hy5* (deficient in a bZIP protein likely to be a transcriptional regulator; Oyama et al. 1997), render photoreceptors nonfunctional, suggests that few positively acting components downstream of the photoreceptors are specific for this particular light response (Thomas et al. 1997). A set of gene products defined by the *COP* (constitutively photomorphogenic), *DET* (de-etiolated) and *FUS* (fusca) loci were originally characterised in *Arabidopsis* as central regulators in light signalling (Chory et al. 1996). However, subsequent studies have indicated that the proteins affected in these mutants function in multiple signalling pathways (Thomas et al. 1997). For instance, DET1, COP1 and COP9 participate not only in phytochrome signal transduction, but also mediate developmentally-regulated and stress-induced gene expression (Mayer et al. 1996). The pleiotropic nature of the *det*, *cop* and *fus* mutants introduces considerable difficulty in dissecting the individual signal transduction pathways that participate in the global regulatory network that controls the plants response to environmental conditions. However, on the positive side, these intermediates potentially provide a central framework from which to establish relationships to well characterised parts of more specific signalling pathways (Hare et al. 1997). Although the mechanism whereby an array of inputs is integrated remains unclear, it seems feasible that they may converge on a single downstream target defined by DET/COP/FUS members and then branch further downstream.

Signal pathways known as mitogen-activated protein kinase (MAPK) cascades have recently emerged as central components in plant responses to a variety of adverse conditions, including dehydration, excessive salinity, low temperature, wounding and elicitor treatment (Hare et al.

1997). The alternative name for MAPKs, namely ERKs (extracellular signal-related protein kinases) describes the role that these ubiquitous eukaryotic proteins often play in trans-cytoplasmic signalling from the cell surface to the nucleus, where they induce transcription of specific genes through phosphorylation and activation of transcription factors (Figure 2.13). Several parallel MAPK-type pathways may operate simultaneously in a single cell type. Furthermore, since any single MAPK may have multiple substrates, it can set in motion a wide range of events. Ehness et al. (1997) recently demonstrated the induction of MAPK activity in suspension cultured *Chenopodium rubrum* cells after transfer to medium containing 40 mM glucose. The failure of an isosmotic concentration of mannitol to induce these activities strongly implicates MAPK action not only in mediating stress responses, but also in hexose signal transduction.

Each MAPK has a regulatory kinase, MAPK kinase or MEK (for MAPK/ERK kinase), necessary for its activation by phosphorylation on tyrosine and threonine residues. This enzyme (MAPKK) is in turn regulated through serine phosphorylation by another kinase termed MEKK (for MEK kinase). In *Arabidopsis*, more than nine genes encode MAPKs, and phylogenetic analysis indicates at least four families of MAPK (Mizoguchi et al. 1996; Shinozaki & Yamaguchi-Shinozaki 1996).

Although MAPK action was not included in the heuristic model of stress-induced signal transduction proposed by Shinozaki and Yamaguchi-Shinozaki (1997) (Figure 2.9), the inability of ABA to induce accumulation of transcripts encoding stress responsive MAPK and MAPKKK homologues from *Arabidopsis* and alfalfa (Jonak et al. 1996; Mizoguchi et al. 1996) suggests that stress-induced MAPK activation may be independent of ABA action. Nonetheless, in barley aleurone protoplasts, the ability of a tyrosine phosphatase inhibitor to completely block ABA-induced MAPK activation and induction of *RAB16A* gene expression led to the conclusion that ABA-mediated induction of this gene requires activation of a MAPK and that this occurs *via* the action of a tyrosine phosphatase(s) (Knetsch et al. 1996). Meskiene et al. (1998) recently identified a PP2C which negatively regulates the drought, cold and wounding-inducible MAPK chain which has been characterised in alfalfa (Jonak et al. 1996). While the involvement of MAPKs in mediating proline accumulation has apparently not been investigated, two hallmark features of MAPK cascades justify the potential involvement of this class of pathway in regulating the process. Firstly, characterisation of MAPK cascades in animal systems has indicated their ability to integrate multiple signals transmitted by various second messengers. Thus different signalling pathways often converge at MAPKs, which distribute the varying signals to different downstream targets, thereby permitting the integration of multiple signals. Secondly, MAPK cascades display considerable potential for signal amplification (Hare & van Staden 1997).

Microinjection technology also offers a powerful approach to dissect the individual components of separate pathways. For example, in contrast to what was found using *RD29A* and *KIN2*

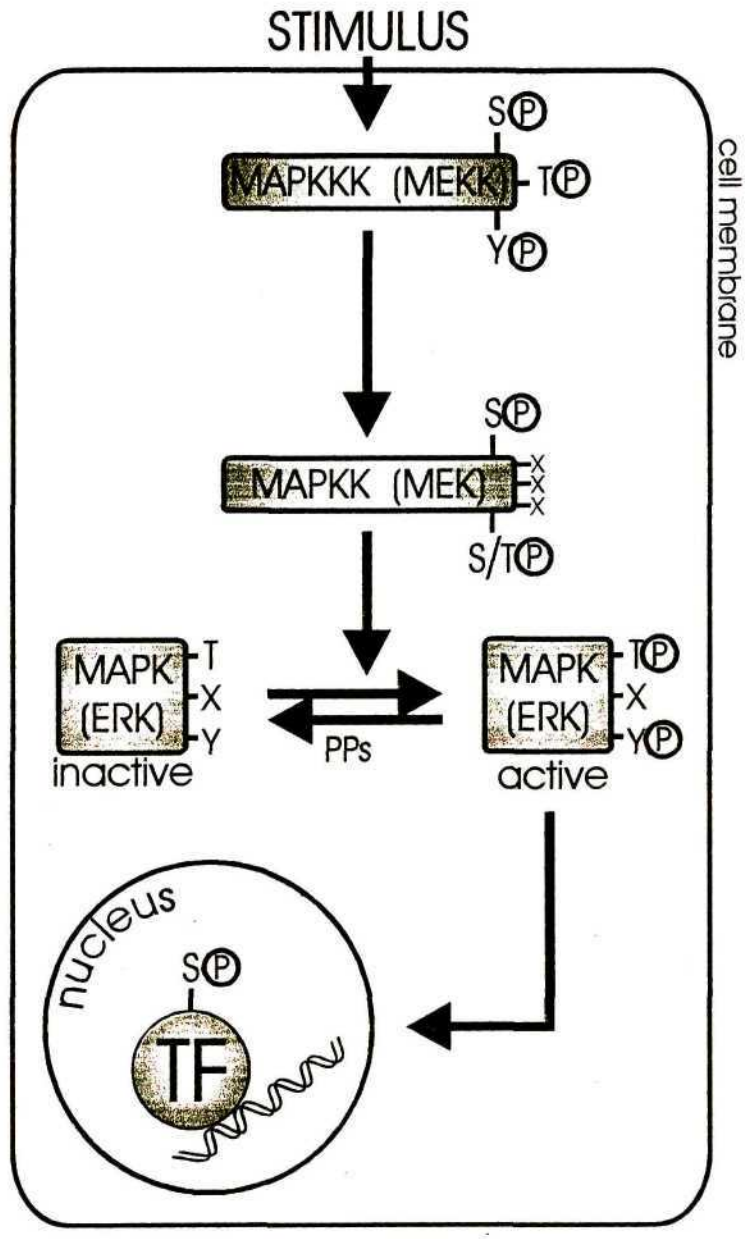

Figure 2.13: Generic MAP kinase module. Following activation by the appropriate stimulus, the signal is transmitted to the canonical MAPK module comprising three protein kinases which phosphorylate and thereby activate the next member in the sequence. The progression of events for all MAPK-type cascades is identical, although specific isoforms of each enzyme type and possibly the physical association of components related to the propagation of a single signal confer a degree of specificity on individual pathways. MAPK kinase kinase (MEKK) homologues are serine(S)/threonine(T)-specific protein kinases that activate MAPK kinase (MEK) enzymes by dual phosphorylation on two S or T residues with a S-X-X-X-S/T motif. Activated MAPK kinase homologues, which are mixed function S/T/Tyr(Y) protein kinases, phosphorylate MAPK(ERK) enzymes on both T and Y residues within the T-X-Y consensus sequence. Phosphorylation at only one of the two positions does not activate the enzyme, but may prime the kinase domain for receipt of the second phosphorylation event. Specific protein phosphatases (PPs) can theoretically downregulate the signal by dephosphorylating either one or both of the phosphorylated residues. MAPK phosphorylation activates one or more nuclear transcriptional factors (TFs). From Hare et al. (1997).

promoters, expression of a reporter gene under the control of a *CAB* promoter (*CAB::GUS*) is induced by Ca^{2+}, but not by cADPR (Wu et al. 1997). Thus, although phytochrome can induce $[Ca^{2+}]_c$ transients, stress-induced cADPR apparently elicits a unique cytoplasmic Ca^{2+} transient that results in activation of at least a subset of ABA-responsive genes, but not certain PHYA-responsive genes. Furthermore, the inability of IP_3 to activate *CAB::GUS* suggests that it too does not participate in the Ca^{2+}/CaM -dependent branch of PHYA signal transduction despites its involvement in transcriptional activation of *RD29A* and *KIN2* (Wu et al. 1997). Contrariwise, although heterotrimeric G-proteins have been implicated in the short-term regulation of stomatal aperture (a classical ABA-mediated response), and are known to be the most upstream component of the PHYA signal transduction machinery (Mustilli & Bowler 1997), a lack of any effect of microinjection of either an activator or antagonist of G-protein action on *RD29A* and *KIN2* transcriptional activation negates a role for G-proteins in ABA signal transduction events that control the expression of these genes (Wu et al. 1997). Neither *RD29A* nor *KIN2* were activated when PHYA was microinjected into hypocotyl cells of a tomato mutant in which PHYA-regulated processes are severely compromised (Wu et al. 1997).

2.4.6 Conclusion

A vast amount of evidence now indicates that stress responses are elicited through several pathways and that these pathways are cross-wired in order to ensure that the overall response to the signal triggered by the primary stressor (e.g. water deficit or ion toxicity) is appropriate, given other environmental (e.g. light) and physiological (e.g. nutrient status) parameters which control growth. Since plant receptors that sense drought, salinity or temperature (assuming that they exist!) have yet to be identified, we presently lack understanding of the origin of the signals downstream of the perception of these abiotic stresses. Nonetheless, the recent advances which have been made in identifying several stress-responsive promoter elements, which must represent the termini of these signal transduction chains, provide the opportunity to begin dissection of the web of interactions that determines the level of expression of any gene. By itself, application of this approach is unlikely to provide a complete explanation of the functionality of the regulation by stress of genes involved in proline metabolism. However, as will be demonstrated by certain findings of this study, it provides some insight into the factors that regulate free proline levels. This knowledge needs to be interpreted in the context of additional physiological data if we are to gain an integrated view of the functional significance of regulated proline metabolism.

2.5 Metabolic implications of stress-induced proline accumulation[1]

As discussed in Section 2.1, many plants accumulate organic osmolytes in response to the imposition of environmental stresses that cause cellular dehydration. Although an adaptive role for these compounds in mediating osmotic adjustment and protecting subcellular structure directly or through their capacity to scavenge harmful ROIs has become a central dogma in stress physiology, the available evidence in favour of these hypotheses is largely correlative. Marginal improvements in drought and salinity tolerance have been observed in transgenic plants engineered to accumulate several osmolytes to levels considered unlikely to mediate osmotic adjustment (Table 2.2). These studies have enabled re-assessment of the functional significance of compatible solute accumulation (Hare et al. 1998). Multiple benefits of organic solute accumulation besides mediation of osmotic adjustment have been proposed to augment the classically accepted roles of these compounds. There is no *a priori* reason why any end product of metabolic adjustment resulting in the accumulation of any osmolyte is the primary effector of stress tolerance. The metabolic implications of an increase in osmolyte synthesis and/or a decline in osmolyte degradation have been suggested to warrant at least equal attention as any osmotic consequences of these processes, since the maintenance of turgor or protection of subcellular structure alone is unlikely to enable continued growth under adverse conditions (Hare et al. 1998).

In establishing why in comparison with other proteogenic amino acids, proline metabolism appears to be extremely sensitive to adverse environmental conditions, it may be of value to compare its metabolism with that of other amino acids. In comparison with most other amino acids, proline has the metabolic advantage of being the terminal product of a relatively short and highly regulated pathway. Proline accumulation therefore affects fewer metabolic reactions than the buildup of multi-use substrates such as glutamate, which are participants in many equilibrium reactions central to intermediary metabolism. As pointed out by Phang (1985), proline and its immediate precursor P5C are not interconverted by a single reversible enzyme, but by two distinct enzymes with different mechanisms and in different subcellular compartments. Although a low level of "proline dehydrogenase" activity has been reported for P5CRs from barley (Krueger et al. 1986) and soybean (Szoke et al. 1992), under specific buffer conditions and at high pH, this is unlikely to be of physiological significance (Szoke et al. 1992). Therefore, since proline and P5C are not linked by a single equilibrium reaction, the final product of the proline biosynthetic pathway is not necessarily in equilibrium with its immediate precursor. Because its α-nitrogen is a secondary amine, proline cannot participate in the transamination or decarboxylation reactions

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[1] **The contents of this section are reproduced in part from:**
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common to other amino acids (Phang 1985).

The proline biosynthetic pathway from glutamate, although short, involves an extremely high rate of consumption of reductants. Furthermore, proline degradation is capable of high energy output. The accumulation of proline appears to be an excellent means of storing energy since the oxidation of one molecule of proline can yield 30 ATP equivalents (Atkinson 1977). These two features may have contributed substantially to a role for proline in plants as a resource of value either in the acclimation to stress or in recovery upon relief from stress. The benefit of possessing a metabolic system displaying extreme sensitivity to stress may derive more from its regulatory effects on apparently unrelated pathways than on accumulation of the end product itself. Accordingly, it has been suggested that the positive effect of proline accumulation is that it augments growth upon relief from stress, rather than serving any direct function during the period of exposure to stress (Blum & Ebercon 1976; Itai & Paleg 1982).

Possible metabolic implications of alterations in proline biosynthesis and degradation in plants during and after relief from stress, as discussed below, are represented diagrammatically in Figure 2.14. The hypothesis comprises two major aspects:

i) During stress, proline synthesis may ameliorate the effects of the concomitant reduction of the pyridine nucleotide pools, particularly the accumulation of excessive amounts of NADPH. The oxidation of NADPH accompanying proline synthesis may assist in restoration of the terminal electron acceptor of the photosynthetic electron transport chain. By enabling a steady rate of photochemical de-excitation of reaction centres, proline synthesis may provide some protection against photoinhibition under adverse conditions. Furthermore, since the cellular pool of NADPH is mostly reduced under normal conditions while the NAD^+ pool is primarily in the oxidised form (Gibon & Larher 1997), a small change in the ratio $NADP^+$/NADPH may have a large effect on flux through a redox-sensitive pathway such as the OPPP, which is dependent on $NADP^+$ availability and inhibited by NADPH. The alternating oxidation of NADPH by proline synthesis and reduction of $NADP^+$ by the two oxidative steps of the OPPP serve to link both elements of the proposed scheme and thereby facilitate the continuation of high rates of proline synthesis during stress.
ii) Upon relief from stress, the rapid catabolism of proline generates reducing equivalents that support mitochondrial oxidative phosphorylation. This process is independent of NADH oxidation because electrons enter the electron transport system directly at the level of a flavoprotein (Section 2.3.3).

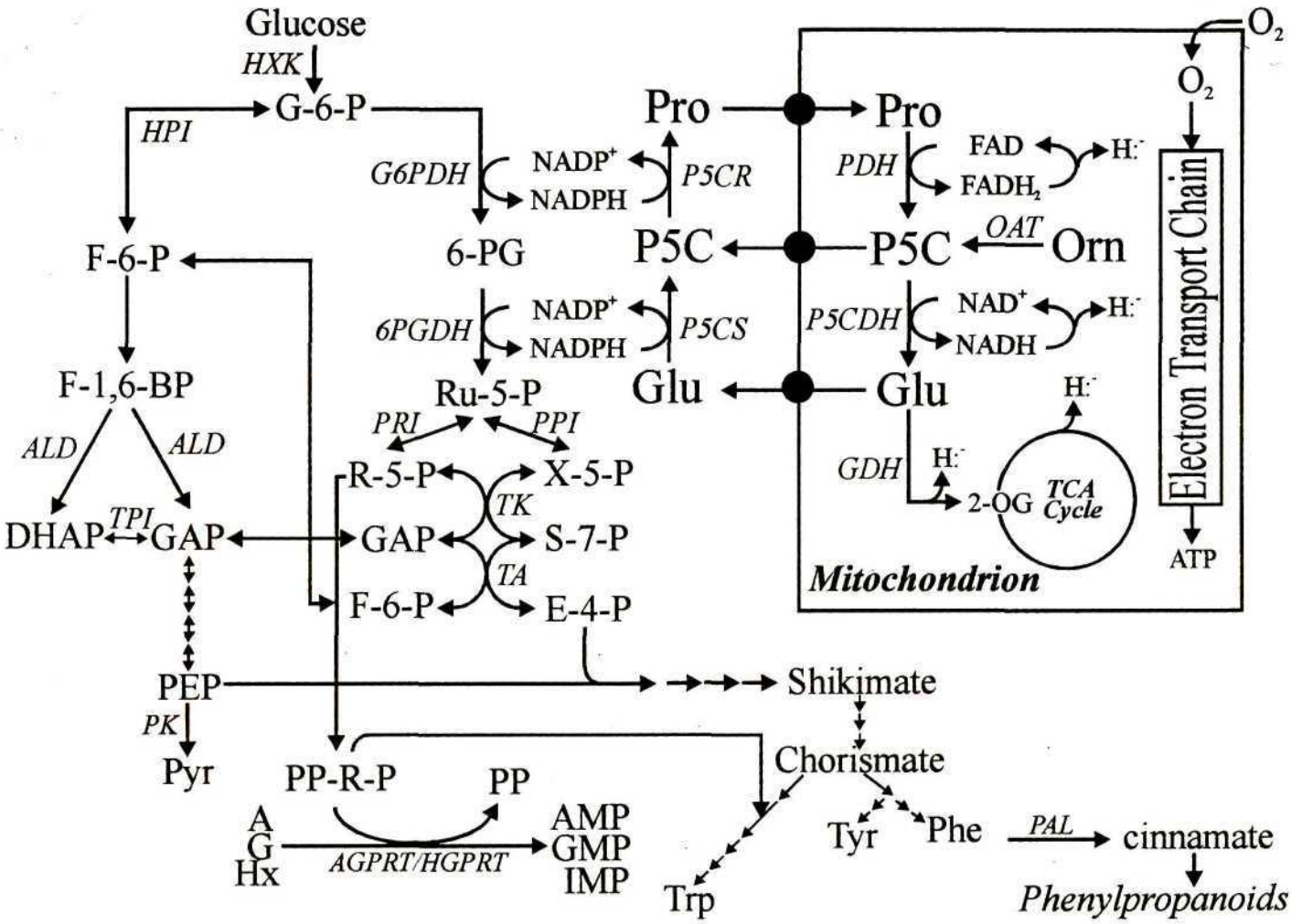

Figure 2.14: Possible metabolic functions of proline biosynthesis and degradation in plants during and after relief from stress. Processes occurring in the mitochondrion are likely to be of importance only during recovery from stress. Abbreviations of intermediates are: A, adenine, AMP, adenosine monophosphate; DHAP, dihydroxyacetone phosphate; E-4-P, erythrose-4-phosphate; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6-bisphosphate; G, guanine; G-6-P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; Glu, glutamate; GMP, guanosine monophosphate; Hx, hypoxanthine; IMP, inosine monophosphate; 2-OG, 2-oxoglutarate; Orn, ornithine; 6-PG, 6-phosphogluconate; P5C, Δ^1-pyrroline-5-carboxylate; PEP, phospho*enol*pyruvate; Phe, phenylalanine; PP-ribose-PP, phosphoribosylpyrophosphate; Pro, proline; Pyr, pyruvate; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate, S-7-P, sedoheptulose-7-phosphate; Tyr, tyrosine; Trp, tryptophan, X-5-P, xylulose-5-phosphate. Abbreviations of enzymes (italicised) are : *AGPRT*, adenine phosphoribosyltransferase; *ALD*, aldolase; *G6PDH*, glucose-6-phosphate dehydrogenase; *GDH*, glutamate dehydrogenase; *HGPRT*, hypoxanthine, guanosine phosphoribosyl transferase; *HXK*, hexokinase; *HPI*, hexose phosphate isomerase; *OAT*, ornithine δ-aminotransferase; *6PGDH*, 6-phosphogluconate dehydrogenase; *PAL*, phenylalanine ammonia-lyase; *P5CDH*, P5C dehydrogenase; *P5CR*, P5C reductase; *P5CS*, P5C synthetase; *PDH*, proline dehydrogenase; *PK*, pyruvate kinase; *PPI*, phosphopentose epimerase; *PRI*, phosphoriboisomerase; *TA*, transaldolase; *TK*, transketolase; *TPI*, triose phosphate isomerase. Also indicated is the tricarboxylic acid (TCA) cycle. Reducing equivalents produced in the oxidation of Pro, P5C, Glu and 2-OG are represented by H:$^-$. Solid lines represent reactions and broken lines, transport. The presence of a putative mitochondrial membrane-bound proline transport system (Cavalieri & Huang 1980) is also indicated. From Hare and Cress (1997).

As will be elaborated below, there is also no reason why proline synthesis and degradation need to be temporally separated. Although proline accumulation accompanies the onset of stress and there is a rapid degradation of the imino acid upon relief from stress, both processes may occur simultaneously under either condition, albeit not at the same level. In this way stress-related shifts in proline metabolism might primarily be of regulatory value to plant cellular metabolism. Accompanying the biosynthesis and catabolism of proline, unrelated metabolic pathways that involve none of the products or intermediates in proline metabolism are activated, and might thereby confer an adaptive advantage to the plant either under adverse conditions or during recovery from stress.

Proline synthesis has also been implicated as a mechanism of alleviating cytosolic acidosis, a condition often associated with stress (Kurkdjian & Guern 1989). A decrease in intracellular pH has been implicated as a factor capable of eliciting proline accumulation in plants (Chou et al. 1991) and removal of H^+ excess due to proline synthesis may prevent a depression in respiration in salt- or water-stressed soybean seedlings (Krackhardt & Guerrier 1995). The excellent review of Venekamp (1989) should be consulted for a detailed discussion of this aspect of the regulatory effect of proline biosynthesis on cellular metabolism under conditions of water deficit. In contrast to the model outlined in Figure 2.14, which involves a continuous cycling of proline and its precursors and would thus have no net effect on removal of H^+ excess, it was proposed that NADPH needed for continued proline synthesis may be regenerated without the concurrent production of H^+ by the mitochondrial oxidation of glycine (Venekamp 1989).

2.5.1 Contribution of proline to oxidative respiration

A role for proline in recovery from stress is consistent with the observation that the extensive accumulation of proline in stressed tissues is usually followed by its rapid disappearance when the stress is removed (Handa et al. 1986). This is primarily by oxidation via P5C to glutamate and 2-oxoglutarate. For example, in *Chlorella emersonii*, a visible decrease in proline levels is evident within ten minutes after relief of hyperosmotic stress (Greenway & Setter 1979). In this regard, proline differs from glycine betaine which is apparently maintained at stable levels long after relief from stress (Goas et al. 1982; Naidu et al. 1990).

The mitochondrial location of proline degradation and presence of glutamate dehydrogenase (GDH; EC 1.4.1.2) in the mitochondrial matrix suggests that this process may contribute carbon to the TCA cycle. Although the importance of GDH in nitrogen metabolism has long been equivocal, Melo-Oliviera et al. (1996) demonstrated that the *Arabidopsis GDH1* gene product

functions in the direction of glutamate catabolism under carbon-limiting conditions. Glutamate dehydrogenase activity was reported to increase in bean leaves which had been water stressed for several days (Jäger & Meyer 1977). On the basis of its thermostability and higher homology to GDH from archaebacterial species than those from other eubacteria and other eukaryotes, it was proposed that plant GDH has an important function under conditions of environmental stress (Syntichaki et al. 1996). Since the rate of protein turnover in plants is likely to be small in comparison with the rate of respiration, it is generally considered unlikely that amino acids make a substantial contribution to plant respiration. However, in certain instances, the catabolism of proline following relief from stress may represent an exception to this generalisation (ap Rees 1990). In barley leaves recovering from drought, consumption of the proline took approximately eight hours and could theoretically contribute to the TCA cycle at a rate sufficient to account for 20% of the total respiratory activity (Stewart & Voetberg 1985).

A number of observations in different biological systems support a role for proline as a primer for TCA cycle activity in plants recovering from osmotic stress. Proline is the primary mitochondrial fuel in energy-intensive processes such as insect flight (Gäde 1992) and may contribute to thermogenesis in the voodoo lily (Skubatz et al. 1989). High levels of proline oxidation in the bacteroids of nitrogen-fixing root nodules of ureide-producing legumes suggest that proline may be the primary energy source used in the energy-intensive process of nitrogen-fixation (Kohl et al. 1988). Additional evidence for proline priming oxidative respiration comes from studies involving animals deficient in the ability to oxidise proline. Both the PRO/Re mouse mutant (Blake 1972) and sluggish-A mutant of *Drosophila melanogaster* (Hayward et al. 1993) are incapable of proline oxidation and exhibit markedly reduced mobility, presumably as a result of an impaired respiratory rate. A large body of evidence obtained using honeybee drone retina supports an important role for proline catabolism to 2-oxoglutarate in meeting an essential anaplerotic function in TCA cycle activity of photoreceptor neurons (Tsacopoulos et al. 1997). Light stimulation of the retina caused a 200% increase of O_2 consumption, a 50% decrease of proline and of glutamate and a 60% increase in $^{14}CO_2$ production from ^{14}C-proline (Tsacopoulos et al. 1994).

Besides contributing carbon to the TCA cycle, the mitochondrial degradation of proline to 2-oxoglutarate may also provide reducing equivalents needed to support mitochondrial electron transport and the generation of ATP for recovery from stress and repair of stress-induced damage (Figure 2.14). Although proline degradation may serve as a means of repleting TCA cycle intermediates in insect flight muscles, experimental evidence suggests that the critical role played by proline in insects involves redox transfer (Balboni 1978). The possible involvement of proline metabolism in this aspect of intracellular redox regulation is discussed further in the section which follows.

2.5.2 Involvement of proline metabolism in the regulation of intracellular redox potential

From a metabolic perspective, the plant is a highly integrated system, which is dominated by the capture of energy by photosynthesis and its use in carbon and nitrogen assimilation. Under normal conditions, tight regulation of interacting metabolic pathways is ensured by their coupling though common intermediates including adenylates and pyridine nucleotides. Therefore, following the imposition of stress, even a small modification of one process may have considerably amplified effects on the rest of intermediary metabolism. Under such conditions, altered flux through certain pathways is likely to be an important mechanism used to stabilise metabolism and ensure survival when conditions are suboptimal.

Environmental perturbations, even if they do not directly affect the composition of the photosynthetic apparatus or its functions, almost invariably affect photosynthesis in the long term. The resultant imbalance between absorbed light energy and energy used through metabolism adversely affects productivity. Examination of adenylates and pyridine nucleotides in water stressed photosynthetic cells indicates that in the short term, water deprivation causes a dramatic increase in the ratio NAD(P)H/NAD(P)$^+$ and a decrease in ATP content (Turner & Wellburn 1985; Lawlor & Uprety 1993). Similar changes in cellular redox status and/or depletion of ATP are also reported for other stresses, including heavy metal toxicity (Alia & Pardha Saradhi 1991), nutrient deprivation (Dietz & Heilos 1990; Juszczuk & Rychter 1997), elicitor treatment of suspension-cultured cells (Robertson et al. 1995), cold shock (De Nisi & Zocchi 1996) and high irradiance (Savitch et al. 1996).

The argument that proline accumulated during stress may act as a non-toxic sink for excess reductant has been presented previously in relation to a possible role for proline accumulation in response to heavy metal toxicity (Alia & Pardha Saradhi 1991), salinity (Alia & Pardha Saradhi 1993) and water deficit (Bellinger & Larher 1987). In all instances, emphasis was placed on the effects of proline synthesis on mitochondrial respiration. The observation that inhibitors of mitochondrial electron transport stimulated proline accumulation in rice seedlings (Alia & Pardha Saradhi 1993) led these workers to conclude that a stress-associated decline in mitochondrial electron transport activity results in proline accumulation. However, the physiological significance of this finding requires further investigation since it may result exclusively from a block in proline degradation. The inhibition of proline and P5C dehydrogenation by rotenone, antimycin and KCN (Huang & Cavalieri 1979; Elthon & Stewart 1981, 1982) was not considered by these workers. Electrons from proline and P5C oxidation enter the respiratory chain directly prior to at least one of the rotenone sensitive iron-sulphur carriers (Elthon & Stewart 1981). Likewise, respiratory

deficient yeast strains contain inactive proline oxidase because of a non-functional electron transport chain, although *in vitro* activity can be detected if artificial electron acceptors are included in the assay (Wang & Brandriss 1987).

Here, the proposal that proline may act as a redox buffer under many adverse environmental conditions is extended in the light of more recent findings concerning stress-induced shifts in cellular redox potential and the cofactor preferences of both of the enzymes which catalyse proline biosynthesis from glutamate. These are considered in the context of several well-documented but poorly understood features of stress-related increases in proline synthesis. As detailed below, the preferential use of NADPH as the reductant used in proline synthesis suggests that during stress, proline accumulation is likely to impact more on relief from stress-related decreases in photosynthesis than from a depression in dark respiration. The involvement of NADPH as an ancillary electron carrier for a wide variety of enzymes involved in reductive biosynthesis suggests that the alteration in the level of reduction of the cellular NADP pool during stress is likely to have widespread influences on cellular metabolism, and that these negative effects may be counteracted in part by increased proline synthesis.

2.5.2.1 A role for enhanced proline biosynthetic rate in amelioration of stress-induced photoinhibition

The sensitivity of photosynthesis to all of the adverse environmental conditions capable of causing proline accumulation is well documented. The question of whether the primary limitation to net photosynthetic rate during water deprivation is stomatal or metabolic has been the subject of numerous discussions and remains controversial (Lawlor & Uprety 1993). Not surprisingly therefore, there is little consensus as to what may constitute the single most important factor in limiting net photosynthetic rates in response to other abiotic and biotic stresses.

All of the stresses capable of eliciting proline accumulation are also likely to increase levels of the generalised stress hormone ABA (Chandler & Robertson 1994), which is known to cause stomatal closure (Rob et al. 1995). As already discussed (Section 2.4.3), although a causal link between ABA and proline accumulation has been suggested for some species, in *Arabidopsis*, the effect of ABA is unlikely to account fully for the response. Furthermore, ABA may elicit its effect on proline accumulation at different levels, ranging from metabolic effects arising from stomatal closure to facilitating increases in the abundance of transcript encoding P5CS (Yoshiba et al. 1995; Savouré et al. 1997; Strizhov et al. 1997).

With regard to metabolic limitations to photosynthetic capacity under adverse conditions, photosystem II (PSII) appears to be the primary target for stress-induced photoinhibition (Baker 1991). It has been proposed that photoinhibition results from overreduction of PSII, which may damage the 32-kD PSII reaction centre D1 polypeptide (Aro et al. 1993a, 1993b), the product of the *psbA* gene (Section 2.2.2.3) . Although the exact significance of D1 protein turnover in contributing to photoinhibition remains controversial (Critchley & Russel 1994), the susceptibility of PSII to a range of stress factors including drought (He et al. 1995; Giardi et al. 1996), heat (Havaux 1992), UV-B radiation (Nogués & Baker 1995), air pollution (Lütz et al. 1992), low temperature (Fryer et al. 1995) and nutrient deficiency (Lütz et al. 1992; Vassiliev et al. 1995; Dannehl et al. 1996) has been described. In wheat leaves, water stress not only impaired the translatability of *psbA* mRNAs (He et al. 1995), but also caused a six-fold decrease in *psbA* transcript levels, presumably due to decreased mRNA transcriptional rates and/or altered transcript stability (He et al. 1998). The importance of D1 protein turnover as a general adaptive response to various types of environmental stresses has recently been reviewed (Giardi et al. 1997). Additionally, it is known that photophosphorylation is extremely sensitive to water stress. The chloroplast coupling factor (CF_0-CF_1), a protonmotive ATPase, is inhibited by the increased ionic concentration in stressed chloroplasts. The inhibition is associated with both altered conformation of the protein and decreased binding affinity for ADP (Boyer & Younis 1983). This is consistent with the experimentally observed decrease in ATP in water-stressed photosynthetic cells (Lawlor & Uprety 1993). Impaired ATP synthesis is likely to constitute a major metabolic limitation to photosynthesis as it will reduce the rate of RuBP regeneration. Furthermore, ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO; EC 4.1.1.39) requires activation by ATP-dependent RUBISCO activase before it exhibits catalytic activity (Portis 1992).

Similarly to water deprivation, the general picture from several studies is that in the short term, *low temperature-induced inhibition of photosynthesis results from feedback regulation and is the* result of thermodynamic constraints on the rate of sucrose synthesis (Savitch et al. 1996). The resultant Pi limitation in the chloroplast limits ATP biosynthesis, which in turn limits the regeneration of RuBP. Reduction in the maximum flux of electrons through the photosynthetic electron transport chain decreases the rate at which absorbed excitation energy can be dissipated by PSII photochemistry and increases the potential for photodamage to PSII reaction centres. Under normal conditions, oxidation of NADPH by the reductive pentose phosphate (Calvin) cycle continually restores the terminal electron acceptor of the photosynthetic electron transport chain, thereby permitting a steady rate of photochemical de-excitation of reaction centres and providing efficient protection against photoinhibition. Any condition that diminishes the use of photosynthetic energy in carbon metabolism will decrease the rate of linear electron flow from water to $NADP^+$ due to lack of regeneration of electron acceptors. The fact that the proline biosynthetic pathway

from glutamate involves a high rate of consumption of NADPH and ATP, coupled to the enhanced flux through the pathway during stress suggest that proline biosynthesis may be an important adaptive mechanism to ameliorate an imbalance between light energy absorbed through photochemistry and energy used in intersystem electron transport and carbon metabolism under conditions of stress. The biocompatible features of proline suggest that the accumulation of the end product of the biosynthetic pathway to high levels might be tolerated without deleterious effects on the stressed cell. Surprisingly, exogenous proline (10^{-5} M) decreased CO_2 fixation by isolated protoplasts from pea plants and the inhibition increased at higher concentrations (Fedina et al. 1994). However, pre-treatment of wheat leaves with 20 mM proline alleviated NaCl-mediated inhibition of net photosynthesis to some degree although apparently not to a significant extent (Rajasekaran et al. 1997).

This proposed link between enhanced proline synthesis and photoinhibition is consistent with the well-documented association of light with stress-induced proline accumulation. Proline accumulation in cold-stressed (Chu et al. 1978) as well as in several salt-stressed halophytes and glycophytes (Hanson & Tully 1979; Goas et al. 1982; Chiang & Dandekar 1995; Sanada et al. 1995) is promoted in the light and suppressed in the dark. A five-fold day/night fluctuation in free proline concentration in NaCl-stressed tobacco plants did not correlate with changes in cellular Na^+ concentration (Sheveleva et al. 1997; Bohnert & Sheveleva 1998). The fluctuation in proline levels during light-dark transitions is not a circadian rhythm (Goas et al. 1982; Sanada et al. 1995), thus confirming that stress-induced proline synthesis is light-dependent. It may also be of relevance to note that although roots are the first tissues to perceive salinity stress, several studies indicate that levels of free proline are lower in osmotically stressed roots than in photosynthetic shoot tissues (Verbruggen et al. 1993; Chiang & Dandekar 1995; Hare 1995; Sanada et al. 1995).

The importance of monitoring physiological, biochemical and molecular adaptations to stress as a function of irradiance and the disruption of redox balance as a feature common to most environmental stresses has been convincingly demonstrated by Huner and co-workers (Maxwell et al. 1995a, 1995b; Gray et al. 1996). These workers have suggested that changes in the redox poise of intersystem photosynthetic electron transport, estimated by the level of reduction of the first stable quinone acceptor of PSII reaction centres, may be part of a common signalling pathway that mediates photosynthetic adjustment to several environmental stimuli. Low temperature sensitivity has been correlated with a reduction of photosynthetic activity caused by a combination of light and low temperature (Huner et al. 1993), since chilling-sensitive plants are not adversely affected by low temperature in the dark (Hodgson & Raison 1989). While it is tempting to view a successful adaptive response to water deficit or temperature extremes in the

presence of light as one in which photon damage is avoided completely, the increasing realisation that both ROIs and antioxidants have potent signalling capacities (Section 2.2.2.3; Foyer et al. 1997; Hare et al. 1998) nonetheless suggests that *regulation* of perturbations in redox homeostasis associated with photoinhibitory conditions, and not their complete elimination, may be an important determinant of stress tolerance.

2.5.2.2 Involvement of proline metabolism in the regulation of stress-induced changes in carbohydrate metabolism

Besides contributing carbon to the TCA cycle (Section 2.5.1), the mitochondrial oxidation of proline to 2-oxoglutarate may also directly provide reducing equivalents needed to support mitochondrial electron transport and the generation of ATP for recovery from stress and for the repair of stress-induced damage (Figure 2.14).

Net photosynthesis (P_n) is the difference between gross photosynthesis (P_g) and the total respiratory loss of CO_2, *i.e.*, photorespiration (P_r) plus dark respiration (R_d) (Lawlor & Uprety 1993):

$$P_n = P_g - (P_r + R_d).$$

The observation that in both C_3 and C_4 plants subjected to water stress that P_g decreases relatively less than P_n suggests that either or both of the respiratory components increases proportionately during water deprivation (Lawlor & Uprety 1993). In contrast to the demonstration that environmental stress suppresses mitochondrial electron transport (Alia & Pardha Saradhi 1993), activation of respiratory electron transport systems by salt stress has also been reported (Fry et al. 1986; Jeanjean et al. 1993; Fedina & Tsonev 1997). Possibly, the production of ATP by unstressed chloroplasts in the light may suppress mitochondrial activity. This suppression, possibly at the level of ATP-mediated inhibition of phosphofructokinase, may be relieved during stress to ensure that levels of ATP and the energy charge of the cell are maintained (Lawlor & Uprety 1993). Accordingly, the expression of genes encoding certain glycolytic enzymes is upregulated in response to several environmental perturbations capable of eliciting proline accumulation (Yang et al. 1993; Forsthoefel et al. 1995; Laxalt et al. 1996). In addition to the requirement for enhanced osmolyte synthesis, increased maintenance respiration during stress is likely to be necessary to support ATPase-mediated sequestration of ions to the vacuole, the specific retention of K^+ within the cytoplasm and extrusion of Na^+ and H^+ (Niu et al. 1995; Zhu et al. 1998).

Despite some reservations (Biehler & Foch 1996), it is generally accepted that a reduction in internal CO_2 levels resulting from stress-induced stomatal closure results in elevated photorespiratory activity in C_3 species (Foyer 1997). An increase in the CO_2 compensation point as well as glycolate oxidase (EC 1.1.3.1) and phosphoglycolate phosphatase (EC 3.1.1.18) activities in salt-stressed pea plants (Fedina et al. 1994; Fedina & Tsonev 1997) indicates that NaCl treatment increases photorespiration. A salt stress-induced increase in stomatal resistance and approximate doubling of free proline in shoot tissue were also reported by Fedina and Tsonev (1997). Heat stress may also decrease the intracellular CO_2 content, owing to the increased solubility of O_2 and decreased solubility of CO_2 at elevated temperatures (Leegood et al. 1995). In leaves of C_3 plants, the photosynthetic carbon oxidation (PCO) cycle, which is restricted to the chloroplasts, peroxisomes and mitochondria, arises from the incomplete discrimination of CO_2 over O_2 by RUBISCO and involves a net consumption of reductant. A variety of photorespiratory mutants of *Arabidopsis* cannot grow in normal air, under conditions in which photorespiration may account for up to 40% of the net rate of CO_2 fixation (Leegood et al. 1995). Although these observations indicate that photorespiration is necessary for the survival of C_3 plants, the physiological function of photorespiration remains unclear. Long-standing suggestions as to the importance of photorespiration during stress are that the oxygenase activity of RUBISCO may provide a sink for photosynthetic electron transport and that the glycolate pathway may assist in mobilisation of carbon reserves into the reductive pentose phosphate pathway (Heber et al. 1996). By dissipating excess photochemically-generated energy, photosynthetic membranes are protected against light induced damage when CO_2 assimilation is limited. This proposal was corroborated by the demonstration that transgenic tobacco plants with twice the amount of plastidic glutamine synthetase (GS_2) had an increased tolerance to high intensity light, which correlated with an improved capacity for photorespiration (Kozaki & Takeba 1996). Transgenic plants with a reduced amount of GS_2 had a diminished capacity for photorespiration and were photoinhibited more severely by high-intensity light compared with control plants (Kozaki & Takeba 1996).

Although studies with *Arabidopsis* and barley mutants defective in essential enzymes of photorespiratory metabolism (Leegood et al. 1995) suggest that excessive loss of carbon from the PCO cycle under photorespiratory conditions may be lethal, amino acids can be imported to and removed from the pathway (Yamaya & Oaks 1987; Kumar et al. 1993). Photorespiration proceeds at rates greatly in excess of related processes which use common intermediates (e.g. 2-oxoglutarate, glutamate, glycine, serine, organic acids and N^5-methylenetetrahydrofolate). The process nonetheless shares these metabolites with other pathways, possibly by differential regulation of various reactions of the pathway dependent on temperature and the availability of light and CO_2 (Leegood et al. 1995). An important role for photorespiration in supporting not only

proline synthesis but also the synthesis of glycine betaine, has recently been suggested (Hare et al. 1998). Noctor et al. (1997a,b) have provided convincing evidence that light-dependent glycine formation through photorespiration is required to support maximum rates of GSH synthesis in poplar, particularly under conditions where the capacity for synthesis of its immediate precursor, γ-glutamylcysteine, is augmented. Thus, irrespective of whether or not photorespiration serves an intrinsically adaptive function, removal of a significant quantity of amino acids from the PCO cycle under stress appears to be not only energetically feasible, but may be beneficial. Presently, the synthesis of GSH is the only biosynthetic pathway that has been demonstrated to require photorespiratory intermediates. However, during stress, increased H_2O_2 production by elevated glycolate oxidase activity may further upset the balance between the production of ROIs and the quenching activity of antioxidants if lipid peroxidation disrupts peroxisomal membrane integrity, causing leakage of H_2O_2 into other compartments (Willekens et al. 1997). In C_3 plants, endogenous catalase activity in the light may be inadequate for destruction of H_2O_2 generated during photorespiration (Foyer et al. 1994). The photoinactivation of catalase represents an early widespread stress symptom in light, which usually accompanies photoinhibition of PSII and precedes the appearance of more general oxidative damage. Although under non-stressed conditions, the loss of catalase by photoinactivation can be compensated for by new synthesis, the capacity of regeneration of catalase activity is lost upon exposure to heat, cold, salt or toxic chemicals, which either enhance the rate of catalase inactivation or inhibit protein synthesis (Streb et al. 1997). Catalase activity decreased in drought-stressed wheat plants (Zhang & Kirkham 1994). Examination of transgenic tobacco plants with approximately 10% of WT catalase activity has indicated the importance of catalase in maintaining redox balance during salt stress, even under low-intensity light (Willekens et al. 1997). Light-induced damage in the catalase-deficient line was completely prevented under non-photorespiratory conditions (Willekens et al. 1997). This emphasises that eliminating photorespiratory H_2O_2 is an important component of tolerance to hyperosmotic stresses. Withdrawal of mitochondrial serine and N^5-methylenetetrahydrofolate to support increased glycine betaine synthesis, or the contribution of 2-oxoglutarate and/or glutamate to proline synthesis may be beneficial in mitigating excessive H_2O_2 production in stressed plants in the light. It is interesting to note that in tobacco plants expressing a P5CS transgene, proline accumulation occurred not only at the expense of glutamate, but also substantially reduced levels of glycine (Kavi Kishor et al. 1995).

Ideally, hypotheses that attribute an important link between osmolyte accumulation and photorespiration should account for the apparently poor distinction between patterns of osmolyte accumulation in C_3 plants and those displaying C_4 photosynthesis or Crassulacean acid metabolism (CAM). In the latter species, which constitute approximately 10% of higher plants, mechanisms that concentrate CO_2 at the site of carboxylation result in reduced photorespiratory

rates. Catalase deficiency in maize has no apparent detrimental effect (Willekens et al. 1997). Nonetheless, a capacity for proline and glycine betaine accumulation in certain C_4 species (Marcum & Murdoch 1994) and proline accumulation in *Mesembryanthemum crystallinum* even after the transition from C_3 photosynthetic metabolism to CAM (Sanada et al. 1995) does not negate an adaptive role for their synthesis in C_3 plants (Hare et al. 1998). Intriguingly, it is thought that the ratio of oxygenation:carboxylation of RuBP, which is very low in the leaves of C_4 plants under optimal conditions, may increase significantly under stress conditions which cause stomatal closure (Edwards & Walker 1983).

Although PDH activity apparently declines during stress, if a low level of cycling between proline and its precursors continues during stress, it may provide a mechanism for:

i) simultaneously moderating the level of reduction of the cytosolic and/or plastidic NADP pool as well as adenylate charge within the mitochondrion; and/or

ii) enabling interconversion of the phosphorylated and non-phosphorylated pools of pyridine nucleotide cofactors.

Unstressed leaves of barley and tobacco (Boggess et al. 1976a, Stewart et al. 1977; Iwai et al. 1979), as well as mitochondria isolated from unstressed shoot tissue of several species (Boggess et al. 1978; Huang & Cavalieri 1979; Elthon & Stewart 1981; Sells & Koeppe 1981) have a substantial capacity for *in vivo* oxidation of added proline. For barley and tobacco *in vivo* and for maize *in vitro*, the proline-oxidising capacity was lowered during water stress, although a contrasting situation applied in bean leaves (Stewart 1972). It is interesting that subsequent workers have also reported stimulation of PDH activity during cold treatment (Charest & Phan 1990) and water deprivation (Kohl et al. 1991). Xin and Browse (1998) recently reported a strong upregulation of both *AtP5CS1* and *AtPDH* expression in *Arabidopsis* plants exposed to 4°C. Using the viewpoint that the primary roles for shifts in proline biosynthesis during stress are biophysical, these workers could not account for the apparently anomalous induction of both proline biosynthetic and degradative capacities during cold stress. In seedlings of *Triticum durum*, PDH activity was insensitive to water stress, but declined significantly following NaCl treatment (Mattioni et al. 1997). Triadimefon, a triazole compound which mitigates the adverse effects of salinity stress in many plants, attenuated the loss of PDH activity in *Arachis hypogaea* seedlings in response to a mild salt stress (Muthukumarasamy & Panneerselvam 1997). The observation that the onset of severe dehydration in *Arabidopsis* is associated with accumulation of transcripts encoding both PDH and P5CS, with no net accumulation of free proline (Figure 2.10; Kiyosue et al. 1996) further corroborates the notion that cycling between proline and its precursors may be an important homeostatic mechanism to forestall redox imbalance associated with small water deficits of the order of those experienced on a daily basis. An important, although frequently

ignored consideration in interpreting plant responses to stress is the need to differentiate between short-term and long-term stress events as well as distinguishing between low stress effects, which can usually be at least partially compensated for by adaptation, acclimation and repair mechanisms, and chronic stress events which cause considerable damage and may eventually lead to cell and plant death. The events which occur when water deficits develop slowly but continuously over days or weeks may be very different from those where the same water deficit is imposed rapidly over a period of hours. The latter situation is almost always chosen in laboratory-based experiments (e.g. Kiyosue et al. 1996; Knight et al. 1997), owing to the rapidity, ease and reproducibility of this approach. Most field-grown plants are exposed to more marginal diurnal fluctuations in parameters such as photon flux density, temperature, air humidity and the availability of soil water. This would be consistent with shifts in proline metabolism acting a redox buffering. Thus, enhanced flux through this substrate cycle may confer a yield advantage at least under modest stress, although uncoupling of the process under severe stress might overwhelm the benefit. Elevated levels of proline accumulated after stress may simply be symptomatic of a metabolic adjustment of benefit only in the early stages of acclimation.

Interestingly, an isoform of P5CDH which is most abundant in exponentially growing *Nicotiana plumbaginifolia* cells, displayed significantly greater specific activity when the culture media was supplemented with NaCl, even though proline accumulation was noted (Forlani et al. 1997b). Whether or not this isoform oxidises P5C derived from proline or ornithine (Section 2.3.3.2) remains to be established. Under normal growth conditions, accumulation of transcript encoding PDH can be induced by high concentrations of proline (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996a), although this induction by proline is inhibited by treatment with 200 mM NaCl for 4 h (Peng et al. 1996). A proline shuttle is responsible for hydrogen transfer from cytosolically generated NADH to the mitochondrial respiratory chain in insect flight muscle (Balboni 1978). Although the malate-aspartate cycle is commonly considered to be responsible for transferring NADH reducing equivalents across the mitochondrial membrane, the interconversions of proline and glutamate may represent a means of transferring electrons from NADPH to NAD^+, thereby coupling the cytosolic oxidation of NADPH to mitochondrial electron transport and serving as a mechanism for generation of energy.

However, of perhaps more likely significance is the possible effect of enhanced proline synthesis on carbon flux through a redox sensitive pathway such as the OPPP, which is generally considered to account for at least 10-15% of the oxidation of carbohydrates in most plant tissues (ap Rees 1980). The two dehydrogenases responsible for transforming glucose-6-phosphate into ribose-5-phosphate are primarily regulated by the $NADP^+$/NADPH ratio, with both enzymes being strongly inhibited by NADPH. If left unchecked, the increased glycolytic flux under conditions of

stress may be at the expense of the OPPP. Excessive reduction of the NADP pool accompanying stress is also likely to inhibit the rate-limiting step catalysed by glucose-6-phosphate dehydrogenase (G6PDH). Dehydrogenase reactions that consume NADPH and produce $NADP^+$ would positively interfere with OPPP activity. As shown in Figure 2.14, the alternating oxidation of NADPH by proline synthesis and reduction of $NADP^+$ by the two oxidative steps of the OPPP potentially links these two pathways.

Although investigations of flux through the OPPP under conditions of stress are limited, a four-fold increase in activity of G6PDH was demonstrated in water-stressed barley (Argandona & Pahlich 1991) and transcripts encoding 6-phosphogluconate dehydrogenase and G6PDH accumulated in alfalfa following treatment with fungal elicitor (Fahrendorf et al. 1995). Application of the elicitor cryptogein to tobacco cells induces a large oxidation of NADPH that results in activation of the OPPP to regenerate the NADPH required for the generation of ROIs by the plasma membrane NADPH oxidase (Section 2.2.2.1; Pugin et al. 1997). Infection of cotyledons of *Curcurbita pepo* with cucumber mosaic virus had a large stimulatory effect on the capacity for the OPPP, glycolysis, the TCA cycle and oxidative electron transport (Técsi et al. 1994). Infection of tobacco leaves or leaf discs with tobacco mosaic virus increases the extractable G6PDH activity (Sindelarova et al. 1997). Rapid increases in G6PDH and 6PGDH activities in aluminium-resistant wheat cultivars, but not in Al-sensitive cultivars suggest a role for OPPP activity in overcoming Al toxicity (Ślaski et al. 1996). Oxidative stress is a central feature of the response of plants to inhibitory levels of Al (Richards et al. 1998). Glycolysis and the OPPP were activated in both a salt-tolerant and a salt-sensitive variety of wheat, although the increase in OPPP activity in the salt-susceptible variety occurred at the expense of the glycolytic pathway (Krishnaraj & Thorpe 1996). Drought stress increased the activity of the OPPP in soybean nodules from 55 d-old plants, but not those from younger plants (Kohl et al. 1991). Water stress did not seem to affect the ratio between fluxes through the OPPP and glycolysis in germinating seeds of *Citrullus lanatus* (Botha & Small 1985). The maximum catalytic activity of G6PDH increased during cold hardening of two cultivars of *Lolium perenne*, and was higher in the more cold-tolerant variety (Bredemeijer & Esselink 1995). Besides providing NADPH, increased OPPP activity during stress may be needed in order to support purine nucleotide biosynthesis (Figure 2.14). Although DNA biosynthesis is unlikely to be affected by water stress, rates of RNA synthesis are enhanced in water stressed millet seedlings (Kandpal & Rao 1985). Many of these transcripts are likely to encode products which are important in acclimation to stress. Alternatively, nucleotide biosynthesis might be essential to support re-initiation of growth following re-establishment of full turgor by osmotic adjustment or upon relief from stress.

The tight link between proline synthesis and OPPP activity has several precedents in both animal and plant systems. An elaborate series of studies conducted by Phang and co-workers (Phang 1985) provided evidence that in mammalian systems, proline and P5C constitute a redox couple and that their interconversion enables intercompartmental and intercellular transfer of reducing equivalents. In human erythrocytes, the increase in the ratio $NADP^+$/NADPH which accompanies proline synthesis activates the metabolism of glucose through the OPPP and thereby stimulates purine nucleotide synthesis (Yeh & Phang 1988). Levels of phosphoribosyl pyrophosphate increased when normal cells were treated with P5C but not in erythrocytes from patients with Mediterranean type G6PDH deficiency (Phang 1985). Based on these findings, Kohl et al. (1988) postulated that extremely high levels of proline synthesis in nitrogen-fixing nodules of soybean function primarily to regenerate $NADP^+$ needed to support high levels of OPPP activity required for purine synthesis. Purines are precursors to the ureides allantoin and allanoate, which are abundant nitrogen storage and transport molecules in tropical legumes. Using [1-^{14}C]glucose and [6-^{14}C]glucose, together with dehydroepiandrosterone, a potent inhibitor of G6PDH, Wu (1996) subsequently provided convincing evidence that glucose metabolism via the OPPP plays an important role in providing NADPH for proline synthesis from glutamate in porcine enterocytes.

Increased synthesis of phenylpropanoids is stimulated by various abiotic and biotic stresses including UV irradiation, high intensity light, pathogen attack, drought, low temperature and nutrient deficiency (Dixon & Paiva 1995; Solecka 1997). Like proline accumulation, transcripts encoding PAL and CHS accumulate in cold-stressed and pathogen-infected *Arabidopsis* in a light-dependent manner (Leyva et al. 1995). The OPPP and photosynthetic carbon reduction cycle provide carbon in the form of erythrose-4-phosphate, which together with phospho*enol*pyruvate acts as a precursor for phenylalanine biosynthesis via the shikimate pathway (Figure 2.14). As much as 60% of plant dry mass may be derived from compounds that have traversed this common trunk of phenylpropanoid biosynthesis (Jensen 1986) before it branches to give rise to a range of secondary metabolites (e.g. monolignols, flavonoids, alkaloids, coumarins, flavonols and flavanones). Although the reductant needed for secondary metabolite synthesis is likely to be in excess under extreme environmental conditions, reduced photosynthetic rates may necessitate higher rates of OPPP during stress to provide carbon for phenylpropanoid synthesis (Fahrendorf et al. 1995). Although ribulose-5-phosphate and erythrose-4-phosphate are also intermediates in the reductive pentose phosphate pathway in plants, the decline in photosynthetic rates accompanying stress is likely to necessitate increased rates of their production by the OPPP.

The physical properties of the cell wall change at low water potentials (Iraki et al. 1989; Nonami & Boyer 1990), with accelerated lignification reported to occur in the roots of salt stressed maize

(Azaizeh & Steudle 1991) and water stressed sorghum (Cruz et al. 1992). Lignin is a three-dimensional polymer of cross-linked phenylpropanoid alcohols (Section 2.2.2.2; Whetten & Sederoff 1995). Consistent with the involvement of peroxidases in lignin formation, Botella et al. (1994) suggested that a salt-inducible root-specific tomato peroxidase functioned primarily in lignin formation and suberin deposition under conditions of hyperosmotic stress. Besides imparting mechanical rigidity to plant tissues specialised in solute conductance or mechanical support, lignin formation is frequently observed at sites of wounding or pathogen attack, apparently in an effort to strengthen the cell wall at these sites of damage (Vance et al. 1980). [^{14}C]-glucose labelling experiments and measurement of G6PDH activity indicated elevated OPPP activity during lignification in stems of *Coleus blumei*, sunflower and pea (Pryke & ap Rees 1976, 1977). Much circumstantial evidence is consistent with a role for proline metabolism in mediating xylogenesis. Not only was a high level of transcript encoding P5CR found in vicinity of the protoxylem in the flowering stem of *Arabidopsis* (Hare & Cress 1996), but proline was also shown to stimulate auxin-induced xylogenesis in stem explants of *Coleus blumei* (Roberts & Baba 1968). A high level of proline biosynthesis in tobacco callus grown on media supplemented with 15% PEG correlated with stimulated xylogenesis compared with unstressed controls (Bornman & Huber 1979). The demonstration that proline induces ionically bound peroxidase activity in roots of rice seedlings (Chen & Kao 1995) is of particular interest in this regard since a clear correlation has been reported between peroxidase activity and the synthesis of lignin polymers (Lagrimini et al. 1987). Furthermore, Kwok and Shetty (1998) have recently tested the original proposal (Hare & Cress 1997) that proline synthesis linked to OPPP activity might have an role in regulation of the shikimate and phenylpropanoid pathways. While exogenous proline did not enhance total phenolic and rosmarinic acid contents in a high phenolic-producing shoot-based clonal line of thyme (*Thymus vulgaris*), increases in proline, total phenolics and rosmarinic acid synthesis were observed after supplementation of the growth medium with the proline analogues, hydroxyproline and azetidine-2-carboxylate (AZC). It was proposed that through their stimulatory effect on proline synthesis mediated by desensitisation of proline synthesis to feedback inhibition by proline, these analogues may have activated the OPPP, and thus phenylpropanoid synthesis by the coupling steps involved in $NADP^+$/NADPH interconversions (Kwok & Shetty 1998). The importance of proline-linked activation of phenylpropanoid synthesis via activation of the OPPP is currently been investigated in several species (K Shetty, University of Massachusetts, Amherst, MA; personal communication), since plant phenolics such as rosmarinic acid are useful food ingredients and often have valuable medicinal properties.

In plants, G6PDH exists in both the cytosol and plastidic stroma (von Schaewen et al. 1995). The cytosolic isoform is regulated by the $NADP^+$/NADPH ratio alone, whereas the chloroplastic counterpart is also post-translationally inactivated by covalent redox modification in the light in

order to avoid futile cycles with photosynthetic CO_2 fixation (Section 2.2.2.3; Buchanan 1991; Wenderoth et al. 1997). Since the OPPP in chloroplasts only operates in the dark, and stress-induced proline synthesis appears to be light-dependent, the system outlined above would most likely only implicate the involvement of proline synthesis in regulating cytosolic OPPP activity. Nonetheless, it is pertinent to note that an interesting variant on the view that the plastidic OPPP is inactive when reduction of $NADP^+$ can be accomplished by photosynthesis has been found in *Selenastrum minutum*. Although the plastidic G6PDH in this green alga is inhibited by reduction using electrons from the Fd/TRX system, if the demand for reducing power is increased in the light by feeding cells with nitrate, G6PDH activity increases to supplement photosynthetic reducing power (Huppe et al. 1992). This suggests some interdependence between the photosynthetic electron transport chain and the OPPP in supplying plastidic reducing power in the light and that TRX-mediated inactivation of G6PDH in the light may not be absolute.

A close link between proline biosynthesis and OPPP activity may account for the fact that glucose, but not glutamate or 2-oxoglutarate, can mimic the enhancing effect of light on ABA-induced proline accumulation in detached hydrated wheat leaves (Pesci 1993). This observation tends to discredit the likelihood that an abundance of metabolisable carbohydrate might be a requirement for supplying adequate carbon precursors for the process. At least two other roles for sugars in stimulating proline synthesis seem possible. Firstly, the known involvement of sugars in altering gene expression patterns (Section 2.4.5.2) cannot be dismissed. However, alternatively, it is worth noting that detached spinach leaves fed with glucose via the petiole had an elevated $NADPH/NADP^+$ ratio (Krapp et al. 1991). In darkness, cytosolic OPPP activity is likely to compete with OPPP activity in the plastids. In darkness, if reductant supply is more important than availability of carbon precursors to proline, enhanced carbohydrate availability may relieve this limitation. Whether this may account exclusively for the light-dependence of proline accumulation requires investigation.

It is generally believed that a restricted export of carbohydrates from source leaves causes oxidative stress because of an enhanced use of O_2 instead of $NADP^+$ as the electron acceptor in photosynthesis (Polle 1996). An increased carbohydrate content in source leaves of transgenic tobacco which express yeast invertase in the apoplastic space was associated with enhanced activities of catalase, APX and MDHAR as well as an increased ascorbate/dehydroascorbate ratio compared with source leaves of the WT (Polle 1996). The increase in APX activities observed is consistent with the demonstration that two cytosolic APX genes from *Arabidopsis* are regulated by a signal transduction pathway that is initiated by changes in the redox status of the plastoquinone pool (Karpinski et al. 1997). Polle (1996) proposed that carbohydrate-accumulating cells have increased availability of reductant, which can increase the degree of reduction of the

ascorbate system via GSH-related systems or via the activity of MDHAR. Intriguingly, potato plants expressing yeast invertase directed either to the apoplast, vacuole or cytosol accumulated not only sucrose and hexoses, but also proline to levels as high as 30-fold in excess of those found in the WT (Büssis et al. 1997). This is consistent with the extreme sensitivity of proline synthesis to the level of reduction of cellular $NADP^+$ and reinforces the proposal that proline synthesis may constitute an important means of regulating cellular redox potential. As was discussed in Section 2.4.5.2, Herbers et al. (1996) used the three classes of transgenic tobacco plants (expressing invertase in the cell wall, vacuole or cytosol) to justify their proposal that hexose sensing in the secretory pathway, but not elevated cytosolic hexokinase activity, mediates the activation of stress-related genes as well as the repression of photosynthetic genes. Induction of proline accumulation in all three classes of invertase-overexpressing potato plants (Büssis et al. 1997) suggests that if sugar abundance signals an increased proline biosynthetic capacity, then this is unlikely to be via the mechanism proposed by Herbers et al. (1996).

Although the likelihood that proline synthesis may participate indirectly in mounting anti-oxidative responses in photosynthetic tissue is diluted somewhat by the probability that the cellular pool of $NADP^+$ is likely to be primarily reduced under adverse conditions, it seems feasible to speculate that an additional benefit of proline-mediated replenishment of $NADP^+$ supply may be to support redox cycling, which was proposed to be important in antioxidant defence mechanisms (Babiychuk et al. 1995). In a screen for *Arabidopsis* cDNAs that confer resistance of the *yap1* mutant of *S. cerevisiae* to the thiol oxidising drug diamide, these workers recovered four NADPH-dependent oxidoreductases. Transcripts encoding three of the enzymes accumulated rapidly in *Arabidopsis* plants under various oxidative stress conditions (Babiychuk et al. 1995). It was proposed that the gene products play a role in plant antioxidant defence through the regulation of $NADP^+$/NADPH homeostasis, which is coupled to regulation of the OPPP (Babiychuk et al. 1995). Consistent with the need for NADPH to maintain GSH and ascorbate in the reduced state, it is known that the OPPP is an important component of antioxidative defence mechanisms in yeast (Juhnke et al. 1996) and animal cells (Pandolfi et al. 1995).

It seems pertinent to point out that although many workers have emphasised the significance of accumulated proline in the maintenance of turgor required for continued growth, an important consideration often neglected is that even if accumulation of this osmolyte does mediate osmotic adjustment, this alone is unlikely to be adequate to ensure continued survival under suboptimal conditions. Whereas cells with reduced turgor have a lower demand for assimilates, maintenance of turgor or its re-establishment after initial water loss is likely to maintain or even increase the demand for assimilates required for cell wall deposition or protein and nucleotide biosynthesis. Consideration of the implications of alterations in proline metabolism during and upon relief from

stress is not trivial as these changes suggest that the synthesis and degradation of a single metabolite may coordinate both the osmotic and metabolic requirements for continued growth under adverse conditions. The interconversion of proline and its precursors may thus be a metabolic rheostat that regulates several processes that do not involve carbon intermediates in proline biosynthesis or degradation. It is possible that the biocompatible features of the end product of the proline biosynthetic pathway enable proline to be accumulated to high levels until normal metabolic homeostasis is regained. In this scenario, any osmoprotective effects of proline accumulation on subcellular structure may be of secondary importance to the associated metabolic implications of proline synthesis and degradation.

2.5.2.3 The cofactor preference of plant P5CR - a valuable key to the importance of stress-induced proline synthesis?

Elucidation of whether accelerated rates of proline synthesis are likely to impact more on cellular energetics or the regulation of photosynthesis and reductive biosyntheses during stress requires critical analysis of the cofactor preferences of the two enzymes responsible for proline synthesis from glutamate. Validity of the scheme outlined above relies on preferential use of NADPH over its non-phosphorylated counterpart as the reductant used in proline biosynthesis. To date, the cofactor preferences of none of the known plant P5CS enzymes (Section 2.3.1.1) has been reported. Based on the NADPH-dependence of γ-glutamyl phosphate reductase from *Escherichia coli* (Hayzer & Leisinger 1980) as well as P5CS activity from mammals (Wakabayashi et al. 1991), it is generally accepted that NADPH is used. Zhang et al. (1995) demonstrated γ-glutamyl phosphate reductase activity in the recombinant *Vigna aconitifolia* P5CS by assaying the enzyme in the reverse direction using $NADP^+$. Examination of the available P5CS sequences prevents unambiguous assignment of a nucleotide-binding domain in plant enzymes (Savouré et al. 1995). Thus, validity of the scheme proposed above still relies in part on critical analysis of the K_m values of plant P5CS for NADH and NADPH.

Much more evidence is available concerning the cofactor preferences of P5CRs from several plants. Heretofore, many investigators have considered that NADH is the preferred cofactor for plant P5CR. Nevertheless, although most P5CRs characterised to date can use either NADH or NADPH to support the reduction of P5C (Table 2.12), NADPH appears to be the preferred reductant in the reduction of P5C in plants. Preference for NADPH is based not only on the markedly higher affinity for this cofactor, but also on elevated affinity for P5C with NADPH as cofactor (Table 2.12). However, it is worth considering that NADH is normally more ubiquitous in the cell, and may thus still be the primary reductant used *in vivo*, especially under conditions of NADPH limitation.

Table 2.12: Affinities of plant P5CRs for substrate and pyridine nucleotide cofactors. From Hare and Cress (1997).

Species	Ligand K_m			Reference
Nicotiana tabacum				
	P5C	0.15 - 0.18 mM	(0.41 mM NADPH)	
	P5C	0.60 mM	(0.51 mM NADH)	LaRosa et al. (1991)
	NADPH	0.03 mM	(0.7 mM P5C)	
	NADH	0.51 mM	(0.7 mM P5C)	
Glycine max				
	P5C	0.12 mM	(0.68 mM NADPH)	
(root nodules)	P5C	0.20 mM	(0.68 mM NADH)	Kohl et al. (1988)
	NADPH	0.06 mM	(0.5 mM P5C)	
	NADH	1.55 mM	(0.5 mM P5C)	
	P5C	0.212 mM	(0.24 mM NADPH)	
(leaves)	P5C	0.179 mM	(0.24 mM NADH)	Szoke et al. (1992)
	NADPH	0.031 mM	(0.48 mM P5C)	
	NADH	0.385 mM	(0.48 mM P5C)	
Pisum sativum				
(chloroplasts)	NADPH	0.12 mM	(2 mM P5C)	
	NADH	0.19 mM	(2 mM P5C)	Rayapati et al. (1989)
	NADPH	0.10 mM	(2 mM P5C)	
(etiolated seedlings)	NADH	0.43 mM	(2 mM P5C)	

This cofactor preference is not reflected in all P5CRs characterised thus far. For example, affinity of P5CR from the soil amoeba *Acanthamoeba castellanii* for NADPH is almost an order of magnitude lower than for NADH and maximum activity with NADH is eight times higher than with NADPH (Hellebust & Larochelle 1988). A highly purified form of *Clostridial* P5CR specifically used NADH (Costilow & Cooper 1978). Similarly, an unfactionated system from blowfly flight muscle (Balboni 1978) catalysed P5C reduction to proline only in the presence of NADH.

This enzymatic evidence is borne out by molecular data suggesting differences in affinities of both the phosphorylated and non-phosphorylated analogues of pyridine nucleotide cofactors. Examination of all sequences encoding P5CR currently available indicates that most possess an N-terminal domain with homology to known NAD(P)-binding sites (Figure 2.15). However, whereas sequences from plant and human P5CRs bear almost perfect homology to a key for NADPH-binding sites (Hanukoglu & Gutfinger 1989), the corresponding domains of P5CRs from *S. cerevisiae*, *M. smithii*, *T. thermophilus* and *M. leprae* display greater homology to an NADH-binding site consensus (Branden & Tooze 1991). A single mismatch occurs for the NADPH

A) NADPH-binding site consensus (Hanukoglu & Gutfinger 1989)

+XXXXGXGXXAXXXAXXXXXXGX+XXXX

A. thaliana	KVGFIGAGKMAESIARGVVASGVLPPNR	(12-39)
G. max	TLGFIGAGKMAESIARGAVRSGVLPPSR	(12-39)
P. sativum	TLGFIGAGKMAESIAKGASRSGVLPSSR	(12-39)
A. deliciosa	KLGFIGAGKMAESIARGVVKSGVLPASR	(15-42)
H. sapiens	SVGFIGAGQLAFALAKGFTAAGVLAAHK	(2-29)
P. aeruginosa	RIAFIGAGNMAASLIGGLRAQGVPAAQI	(5-32)
B. subtilis	KVAFIGAGSMAEGMISGIVRANKIPKQN	(18-45)
E. coli	KIGFIGCGNMGKAILGGLIASGQVLPGQ	(4-31)

B) NADH-binding site consensus (Branden & Tooze 1991)

XXXXXGXGXXGXXXXXXXXXXXXXXX-

S. cerevisiae	TLAILGCGVMGQALLSAIYNAPKAAD	(4-29)
M. smithii	NLGIIGYGNIGELLSQNI----ISHD	(2-23)
T. thermophilus	RLAFVGLGKMGRSILKGALERGFLRPE	(2-28)
M. leprae	RIAIIGGGSIGEALLSGLLRAGRQVKD	(12-33)

Figure 2.15: Amino acid sequences of N-terminal NAD(P)H-binding sites in P5CRs currently listed in international sequence databases. N-terminal sequences of P5CRs from human (Accession No. P32322), the plants *Arabidopsis thaliana* (M76538), soybean (X16352), pea (Q04708), kiwifruit (U92287), the bacteria *Pseudomonas aeruginosa* (JO0418), *Escherichia coli* (P00373), *Bacillus subtilis* (P14383), *Thermus thermophilus* (JC2078), *Mycobacterium leprae* (U00018) and *Methanobrevibacter smithii* (36534) as well as the yeast *Saccharomyces cerevisiae* (P32263) are aligned with NADPH- (Hanukoglu & Gutfinger 1989) and NADH- (Branden & Tooze 1991) binding site consensus sequences. Invariant residues in the consensus sequences are indicated in bold. In the consensus sequences, X represents any amino acid. Hydrophobic residues found at the same positions as hydrophobic residues in consensus sequences are underlined. Positively charged residues in consensus sequences are denoted + and negatively charged residues as -. Positive residues at the N-termini of P5CRs with NADPH binding sites are italicised. The amino acid residues in each sequence which contribute to the NAD(P)H binding site are indicated in parentheses. Modified from Hare and Cress (1997).

binding site consensus in the P5CR sequence from *P. aeruginosa* and two mismatches for the same site occur in the N-terminal stretches of P5CR sequences from *E. coli* and *B. subtilis*.

Given that NADPH is metabolically more expensive than NADH, the definite cofactor preference exhibited by P5CRs from multicellular eukaryotes is likely to be of some significance. It supports the hypothesis that P5CR activity is involved in the regulation of cellular redox potential by affecting the level of reduction of the NADPH and is therefore important in metabolic regulation. In keeping with this suggestion, soybean P5CR is inhibited by $NADP^+$, but not by proline, NAD^+ or ATP (Kohl et al. 1990; Szoke et al. 1992).

Several workers (Boggess et al. 1976b; Argandona & Pahlich 1991; LaRosa et al. 1991; Szoke et al. 1992; Yoshiba et al. 1995) have concluded that P5CR is unlikely to play a major role in the control of flux through the biosynthetic pathway of proline in plants. Although proline levels in salt-adapted tobacco cells were two orders of magnitude in excess of proline levels in nonadapted cells, P5CR activities in the two cell types were not significantly different (LaRosa et al. 1991). Furthermore, a 50-fold enhancement of P5CR activity in transgenic tobacco plants expressing soybean P5CR did not result in any significant increase in proline formation (Szoke et al. 1992). Thus, *in vivo* activity of P5CR in tobacco is apparently limited by the availability of the substrate P5C, and the enzyme functions at only a fraction of its V_{max}. In the light of these conclusions that P5CR is not important in controlling proline production, it seems feasible to postulate that an important role of the enzyme during acclimation to environmental stress might be stabilisation of the $NADP^+$/NADPH couple in stressed cells. As outlined above, absolute carbon flux through the biosynthetic pathway from glutamate may be quantitatively more important than assessment of the contribution of P5CR to proline accumulation *per se*.

It was estimated that the specific activity of salt-adapted tobacco cells is 86- to 200-fold greater than the net rate of proline synthesis. In unadapted cells, P5CR activity was estimated to be 1053- to 4227-fold greater than net proline synthesis (LaRosa et al. 1991). In contrast to the conclusion of these workers that P5CR is therefore of limited significance in acclimation to stress, it seems possible that these high levels of P5CR activity may in fact indicate the extreme importance of this enzyme in the regulation of intermediary metabolism. To discredit the value of further characterisation of P5CR in phenomena relating to stress-induced proline synthesis also conflicts with several reports that transcript encoding the enzyme accumulates following salinisation (Delauney & Verma 1990; Williamson & Slocum 1992; Verbruggen et al. 1993; Yoshiba et al. 1995) and dehydration (Hare & Cress 1996). Increases in total extractable P5CR activity following hyperosmotic stress have also been reported for several species (Table 2.1). Lack of functionality for this upregulation of gene transcription would imply that this is a metabolically wasteful process. In seedlings of *Triticum durum*, exposure to water or salt stress did not affect levels of transcript encoding P5CR, although both stresses increased the activity of the enzyme (Mattioni et al. 1997).

In view of the suggestion that redox modulation accompanying proline synthesis (proline is a redox shuttle molecule) may be a more important endpoint than proline production *per se*, it is unfortunate that transgenic approaches to resolving the issue of a functional significance of proline accumulation (Szoke et al. 1992; Kavi Kishor et al. 1995) have focused on assessment of absolute levels of proline rather than absolute flux through the proline biosynthetic pathway. If continuous cycling between cytoplasmic proline and its mitochondrial degradation products does occur, particularly under normal conditions, then high levels of P5CR activity may not necessarily result in the expected levels of proline accumulated if only a synthetic route is considered. The limitations of direct extrapolation of proline accumulation from assessment of levels of proline biosynthetic capacity are exemplified by a study on salt-tolerant *Brassica juncea* (Madan et al. 1995). A 34-fold increase in proline level was accompanied by only three- to four-fold increase in the activities of proline biosynthetic enzymes.

2.5.2.4 Physiological roles for proline-mediated alteration of redox potential in the absence of stress

If shifts in proline metabolism regulate cellular redox potential under conditions of stress, one might also anticipate its involvement in modulating other metabolic responses. A number of studies using both plant and animal systems have provided evidence that proline metabolism may be related to a metabolic endpoint other than the exchange of carbons. For example, the stimulatory effect of proline on cytokinin-induced shoot organogenesis in *Cucumis melo* was proposed to result from an increase in the supply of both $NADP^+$ for purine biosynthesis and reducing equivalents for ADP phosphorylation, which would be needed to maintain high rates of cell division (Shetty et al. 1992). The most salient observation arising from this study was that thioproline (L-thiazolidine-4-carboxylic acid), a proline analogue known to inhibit proline oxidation (Elthon & Stewart 1984), markedly reduced the extent of proline-mediated stimulation of shoot formation. Since the process of proline synthesis, rather than the presence of proline *per se*, is important in enhancing activity of the OPPP, these findings are in keeping with the proposal that proline metabolism is intimately linked with nucleotide biosynthesis and oxidative phosphorylation. One product of P5C synthesis and reduction, proline, is transported into the mitochondrion where it is dehydrogenated, while $NADP^+$, the other product from both biosynthetic steps from glutamate, stimulates OPPP activity.

A high level of OPPP activity is traditionally associated with tissues characterised by high rates of cell division and differentiation. Accordingly, changes in G6PDH activity were recently suggested to be a reliable marker for determining the regenerability and recalcitrance of plant

callus tissues (Gahan et al. 1997). Two isoforms of P5C dehydrogenase in *Nicotiana plumbaginifolia* suspension cultured cells are differentially regulated throughout the growth cycle (Forlani et al. 1997b). The isozyme associated with rapidly proliferating cells had much higher affinities for NAD^+ and P5C (Forlani et al. 1997b). Furthermore, consistent with the high levels of transcript encoding P5CR in the vascular cambium of the flowering stem of *Arabidopsis* (Hare & Cress 1996), Hua et al. (1997) found a high rate of transcriptional activity of the promoter of the *AtP5CR* gene in rapidly dividing cells of *Arabidopsis* seedlings. Another recent study provided evidence consistent with the notion of an important role for proline biosynthesis in cell division. The *AtP5CS2* gene from *Arabidopsis* contributes 20-40% of total P5CS mRNA in differentiated plant tissues, but is solely responsible for the synthesis of abundant P5CS mRNA in rapidly dividing cell cultures. In contrast, expression of the previously characterised *AtP5CS1* gene (Savouré et al. 1995; Yoshiba et al. 1995) occurs in differentiated tissues, but cannot be detected in dividing cell cultures in the absence of stress stimuli (Strizhov et al. 1997).

A role for proline metabolism in directing cell differentiation in certain mammalian cancer systems has been proposed (Phang 1985). Proline is capable of reversing the inhibition of differentiation by AZC (a proline analogue) of Leydig cells in the rat fetal testis (Jost et al. 1988). The proposal is also consistent with the observation that thioproline can reduce certain types of animal tumours. In this context, it is interesting to note that following injection of *Agrobacterium tumefaciens* Conn. into stems of tobacco and tomato, levels of proline in the resultant crown galls are 70 and 22 times higher than those found in normal stem tissues of the respective plants (Seitz & Hochster 1964). This may be associated with the rapid proliferation of cells within the crown gall tumours, which is likely to depend on augmentation of ribonucleotide production for DNA and RNA synthesis.

Exogenous application of proline markedly inhibits shoot elongation (Garcia et al. 1997) and root growth of rice seedlings (Chen & Kao 1995) as well as growth of *Brassica napus* callus (Chandler & Thorpe 1987). This introduces the intriguing possibility that high levels of proline accumulated under conditions of stress may account, at least in part, for the reduced growth rate associated with exposure to adverse conditions. When assayed *in vitro*, recombinant P5CS from *Vigna aconitifolia* is completely feedback inhibited by 10 mM proline (Zhang et al. 1995), although one of the P5CS isoforms from tomato is 70- to 250-times more sensitive to inhibition by proline (García-Ríos et al. 1997). If under normal conditions, continuous cycling occurs between proline and its ultimate precursor glutamate (Figure 2.14), and this is blocked by high levels of proline, exogenously applied proline may inhibit carbon flux through the OPPP and ADP phosphorylation, both of which are likely to be of importance in sustaining a rapid growth rate. In a study which demonstrated that 10 mM proline inhibited the growth of salt grass suspension cultures in the

presence of 260 mM NaCl (Rodriguez & Heyser 1988), it was reported that [^{13}C]-proline inhibited the normal biosynthesis of proline that would have occurred in suspensions grown at this salinity level. These findings bolster the argument that the synthesis of proline from glutamate and not merely its presence, is of importance in counteracting the effects of hyperosmotic stress.

Exogenous proline had no effect on either the mitotic index of meristems of excised pea roots or the proportion of nuclei arrested in the G2 phase of the growth cycle (Tramontano & Jouve 1997). Nonetheless, the highest concentration of proline tested by these workers was only 0.1 mM proline and a limitation in adequate proline uptake cannot be excluded. Interestingly, a temperature sensitive mutant of *S. cerevisiae*, named *ore2*, arrests in the G1 phase of the cell cycle at a non-permissive temperature, although growth can be restored under this condition on a synthetic medium supplemented with proline (Neuville & Aigle 1992). The disrupted gene was found to encode P5CR. In eukaryotic cells, the G1 period occurs between the completion of cytokinesis and the onset of DNA replication. Several factors, including plant hormones, mediate passage though the plant cell cycle, which is effectively a protein phosphorylation cascade (Hare & van Staden 1997). The accurate timing and coordination of events in cell cycle transitions is dependent on signalling pathways known as checkpoints, which ensure firstly that cells do not make a foolish commitment to divide and that once begun, particular programmes in any phase of the mitotic cycle have been successfully completed before progression to subsequent phases (Hare & van Staden 1997). The phenotype of the yeast *ore2* mutant suggests that the reduction of P5C may provide an important signal which coordinates exit from the G1 phase of the growth cycle, and might thus participate in important developmental decisions. Recent studies involving investigation of the involvement of OPPP activity in mediating meiotic induction in mouse oocytes (Downs et al. 1998) may be consistent with this idea. Artificial electron acceptors (methylene blue and phenazine ethosulphate) or P5C triggered a dose-dependent increase in meiotic maturation. The effects of these agents were attributed to activation of OPPP activity and thus purine synthesis via the oxidation of NADPH (Downs et al. 1998).

A high rate of carbon flux through the OPPP relative to glycolysis is considered to support a high rate of biosynthesis in an actively growing or differentiating tissue. Accordingly, stimulation of OPPP activity has been proposed to be necessary for the breaking of seed dormancy (Section 2.2.3). The reservoir of NADP in the dry seed is apparently in the reduced state and requires to be oxidised to initiate the OPPP (Botha et al. 1992). The observation that the proline content in wheat seeds increased more than seven-fold 48 h after imbibition, but thereafter declined and had stabilised after 120 h (Mumtaz et al. 1995) lends some credence to the idea that proline synthesis may activate OPPP activity during germination. Of all of the plant parts of *Arabidopsis* studied, seeds have the highest levels of P5CR transcript (Verbruggen et al. 1993). In

Arabidopsis, dry seed has considerably higher levels of transcript encoding PDH, than do the peduncle, leaf, root or silique, although levels in seed are lower than those found in the florets of mature plants (Verbruggen et al. 1996a). The potential capacity for high levels of both proline synthesis and degradation in dry seeds is consistent with an important role for cycling between proline and its precursors during the breaking of dormancy. Although such a cycle is futile in terms of carbon exchange, it could have enormous implications on redox potential and adenylate charge, both of which are likely to be of importance. Furthermore, the germinating seed represents a heterotrophic system, suggesting that in the absence of photosynthetic capacity, the OPPP may assume a larger role in redox regulation than in autotrophic mature plants.

Like seed germination, floral initiation involves differentiation of a primarily heterotrophic tissue. A role for proline in initiation of flower development has been proposed for kiwifruit (Walton et al. 1991, 1998) and other species (Bernier et al. 1981). This relationship may also involve stimulated carbon flux through the OPPP. Increased G6PDH activity per unit fresh weight was found in the shoot apical meristems of spinach within a few hours after the critical day length required for floral induction was met (Auderset et al. 1980). A transient stimulation of OPPP activity at the time of bud-break in poplar (Sagisaka 1974) coincided with a transient increase in free proline reported in a subsequent paper (Sagisaka & Araki 1983). Other workers have provided evidence of a role for proline oxidation in anthesis in several species of the Araceae (Skubatz et al. 1989) and increased proline synthesis in P5CS-overexpressing tobacco enhanced flower development under drought conditions (Kavi Kishor et al. 1995). In *Arabidopsis*, levels of both free proline and transcript encoding P5CS are much higher in flowers than in roots, leaves and siliques (Savouré et al. 1995) and the florets also contain high levels of P5CR transcript (Verbruggen et al. 1993). In *Arabidopsis*, free proline constitutes 26.08% and 16.69% of the total amino acids found in the florets and dry seeds respectively (Chiang & Dandekar 1995). These findings are consistent with a role for proline synthesis in mediating developmental processes that may be dependent on increased OPPP activity. They may, however, also relate to the abundance of proline- and hydroxyproline-rich glycoproteins[1] in both the male and female tissues of flowering plants (Sommer-Knudsen et al. 1997). The biological functions of these gene products in sexual tissues has not yet been established unequivocally.

Supplementation of embryo-production medium with 1 mM proline increased fatty acid accumulation in somatic embryos of celery (*Apium graveolens*). Although an approximately 17% increase in fatty acid levels obtained by incubation in the presence of 1 mM proline was less than that obtained in the presence of 1 µM ABA (approximately 38%), when both effectors were provided together, levels of free fatty acids were approximately 73% higher than in the absence of either (Kim & Janick 1991). These workers suggested that the improved desiccation tolerance

of celery somatic embryos observed in the presence of ABA and proline was the result of increased fatty acid levels.

The synthesis of one molecule of palmitic acid requires 14 molecules of NADPH and seven molecules of ATP. In leaves, the photosynthetic electron transport chain provides ATP and NADPH, while in heterotrophic tissues, such as developing seeds, energy must be derived from sucrose imported from the leaves (Schmid et al. 1997). Plastidic OPPP activity provides reductant, while ATP for acetyl-CoA carboxylase is derived from glycolysis. In non-photosynthetic tissues, pyruvate provided by the glycolytic pathway provides acetyl CoA as the building block for the assembly of fatty acids (Schmid et al. 1997). By studying the metabolism of glucose-6-phosphate and pyruvate by plastids isolated from developing embryos of oilseed rape (*Brassica napus*), Kang and Rawsthorne (1996) concluded that OPPP can contribute to the NADPH demand created during fatty acid synthesis in this system, although direct evidence for linkage of the two pathways has yet to be achieved. Recent evidence indicates that in pea chloroplasts, reduction of a disulphide bond(s) in acetyl-CoA carboxylase, which catalyses the first committed step in fatty acid synthesis, is necessary for its activation. This could be achieved *in vitro* using TRX reduced enzymatically with NADPH and NTR (Sasaki et al. 1997). Thus redox potential apparently plays an important regulatory role in fatty acid biosynthesis.

In view of the proposal that flux through the proline biosynthetic and degradative pathways regulates the level of reduction of NADP and mitochondrial adenylate charge (Figure 2.14), the simplest interpretation for the fatty acid accumulation observed in celery somatic embryos cultured in the presence of ABA and proline, either singly or in combination (Kim & Janick 1991), is that exogenous proline activates a cycle between itself and its precursors (Figure 2.14), while ABA induces proline biosynthetic capacity through transcriptional activation of P5CS (Section 2.4.3.1; Savouré et al. 1997; Strizhov et al. 1997). When both proline and ABA are provided together, the increased proline biosynthetic capacity together with greater availability of substrate to prime cycling, might account for the synergistic effect observed. Interestingly, free proline (Chiang & Dandekar 1995; Hare 1995) as well as transcriptional activity of the promoter of the *AtP5CR* gene (Hua et al. 1997), although not *AtP5CS1* transcripts (Savouré et al. 1995) are found at extremely high levels in ripening siliques of *Arabidopsis*, which has lipid-rich seeds. Free proline contributes as much as 6.6% of the dry weight of the silique of *Brassica napus* (Flasinski & Rogozinska 1985). It has been proposed that proline participates in dehydration in siliques (Chiang & Dandekar 1995). A link between proline metabolism and fatty acid biosynthesis is an alternative, although not mutually exclusive, explanation for an important role for proline metabolism in embryogenesis. In view of the definite, albeit somewhat confusing involvement of ABA in proline accumulation (Section 2.4.3.1), a high level of proline synthesis during embryogenesis may also

be consistent with a peak in ABA levels within developing seeds around the time of maximum fresh weight (Chandler & Robertson 1994).

2.5.3 Does proline accumulation elicit a stress-related signal?

If the interconversions of proline and P5C involve transfer of redox potential, then changes in proline metabolism may constitute a form of metabolic signalling. This extends into the intriguing possibility that proline may serve as an osmoticum and a regulatory signal at the same time. Considerable evidence indicates that many plant genes are regulated not only by hormones, but also by changes in the levels of intermediates or end products of central pathways in carbohydrate metabolism (Section 2.4.5.2). In the light of the increased levels of proline that accompany imposition of a diverse range of environmental stresses (Table 2.4), it seems possible that synthesis of this imino acid may be one of the earliest metabolic responses triggered in the signal transduction pathway that links the perception of many environmental stresses to the elicitation of physiological responses at the cellular level (Section 2.4.2). Proline itself or a signal derived from its synthesis would be a good candidate as a second messenger derived from the primary signal following perception of adverse environmental conditions (Hare & Cress 1997).

Several observations are consistent with this proposal. The *rss* (reduced salt sensitivity) mutants of *Arabidopsis* accumulate proline to a significantly lesser degree than WT when they are exposed to either salt or osmotic stress (Werner & Finkelstein 1995). Although vegetative tissues of these mutants do not exhibit enhanced tolerance of NaCl, the *rss* mutants demonstrate reduced sensitivity to salt and osmotic stress during germination. Furthermore, proline increases ionically bound peroxidase activity in roots of rice seedlings (Chen & Kao 1995) and ameliorates the effect of salt stress and partial dehydration on ethylene synthesis in *Allenrolfea occidentalis* (Chrominski et al. 1989). Of particular interest are the recent demonstrations that proline or intermediates in proline synthesis and catabolism such as glutamate and P5C selectively increase the expression of certain stress-regulated genes in rice (Garcia et al. 1997; Iyer & Caplan 1998) even in the absence of salinity stress.

At 50 mM, proline induced a two-fold higher level of expression of the rice *salT* gene than was observed following exposure to 170 mM NaCl alone (Garcia et al. 1997). The *salT* gene product is hydrophilic, rich in glycine and contains an abundance of negatively charged amino acid residues, but is distinct from any of the osmotically regulated gene families identified in other plant species. Although the function of the *salT* gene remains unclear, its mRNA accumulates upon different osmotic and ionic stresses (Claes et al. 1990; Moons et al. 1997). This induction appears

to be a primary response to hyperosmotic stress and is not symptomatic of prolonged damage. The observation that a combination of NaCl and proline gave a higher expression than the sum of either treatment alone was interpreted as being indicative of the two stimuli acting independently at some point in the signal transduction pathway which regulates *salT* (Garcia et al. 1997). Iyer and Caplan (1998) subsequently demonstrated that 1 mM P5C or its analogue 3,4-dehydroproline (DHP) produced a greater effect on *salT* transcript accumulation than 1 mM proline or 75 mM NaCl. Glutamate (1 mM) caused some enhancement of *salT* expression, but was less effective than DHP. Whereas CHX prevented NaCl-mediated induction of *salT* expression, inhibition of protein synthesis had no effect on induction of *salT* transcript accumulation by P5C (Iyer & Caplan 1998). Like 75 mM NaCl, 1 mM of either P5C or DHP (24 h treatment for all three inducers) selectively increased the expression of two other osmotically regulated genes in rice, *viz. DHN4* and *RAB16A*, but decreased levels of a transcript encoding *S*-adenosylmethionine synthetase (Iyer & Caplan 1998) and had no effect on levels of *Em*, another osmotically regulated mRNA. Although the data were not presented, Jang and Sheen (1994) observed no apparent repression of selected photosynthetic genes when an excessive amount of proline was delivered into maize mesophyll protoplasts by electroporation. Both P5C and DHP only marginally increased levels of a cold-regulated dehydrin, *DHN5* and DHP decreased levels of transcript encoding *HSP70*, a heat shock protein which can be induced by severe osmotic stress (Iyer & Caplan 1998). Unlike induction of *salT* by NaCl, accumulation of *salT* transcript by P5C is CHX-insensitive (Iyer & Caplan 1998). However, as was observed for *DHN4* mRNA abundance, CHX reduced the maximal level of *salT* transcript induced by P5C. Plants that were treated with P5C or DHP consumed less O_2, had reduced NADPH levels, had increased NADH levels, and accumulated many osmolytes associated with osmotically stressed rice (Iyer & Caplan 1998).

It was recently suggested (Hare et al. 1998) that changes in cellular redox potential, mediated by altered flux through the proline biosynthetic and catabolic pathways may signal the changes in gene expression observed by Iyer and Caplan (1998). Although redox control of plant gene expression is presently not as well characterised as in animal cells, evidence implicating the involvement of the redox status of chloroplastic TRX (Danon & Mayfield 1994) and plastoquinone (Escoubas et al. 1995) in the regulation of photosynthetic genes has been presented (Section 2.2.2.3). The DNA-binding capacities of the large family of plant MYB-domain transcription factors, at least one of which has been implicated in the response to dehydrative stress (Urao et al. 1993; Abe et al. 1997), also appear to be subject to regulation by the reduction or oxidation of conserved cysteine residues (Martin & Paz-Ares 1997; Williams & Grotewold 1997). Given the dependence of animal ADP-ribose cyclase on NAD^+ and $NADP^+$ availability (Graeff et al. 1998), and the levels of these oxidised pyridine nucleotides in plant tissues (Gibon & Larher 1997), it is

tempting to speculate that proline synthesis may provide substrate for $NAADP^+$ or cADPR synthesis and that these agents of Ca^{2+} release (Section 2.4.3.3; Wu et al. 1997) subsequently induce stress-regulated genes. A 43% decrease in the NADPH levels of sheath segments from rice plants grown in the presence of P5C (Iyer & Caplan 1998) supports this proposal. In view of the level of reduction of the NADP pool in photosynthetic tissues (Gibon & Larher 1997), this may account for an approximately 20% increase in $NADP^+$, assuming that P5C treatment does not affect the overall size of the NADP pool. A role for the availability of substrate in regulating ADP-ribose cyclase activity is consistent with the demonstration that NADase, an enzyme that degrades NAD^+, the precursor of cADPR, blocks induction of *RD29A* and *KIN2* by ABA (Wu et al. 1997). Unfortunately, the effects of reducing $NADP^+$ levels on stress-related gene expression were not reported. Nonetheless, microinjection studies have indicated that $NAADP^+$ apparently plays a similar role to cADPR in ABA signal transmission (Y Wu, Rockefeller University, NY; personal communication).

Besides a physiologically significant effect on transcriptional activation under adverse conditions, proline or metabolites thereof may also play an important role in regulation gene expression at the translational level. Several studies have indicated a decrease in the overall rate of total protein synthesis in leaves subjected to water deficit (Bewley et al. 1983; Dasgupta & Bewley 1984; Bray 1988). In greening barley seedlings subjected to -1.0 MPa PEG, protein synthesis in primary leaves was only 12% of the control (Dasgupta & Bewley 1984). Exogenous proline (100 mM) decreases protein synthesis in maize suspension-cultured cells (Xin & Li 1993). Mick et al. (1988) observed that P5C not only inhibited synthesis of globin in rabbit reticulocyte lysates in a dose-dependent manner, but also inhibited translation from brome mosaic and alfalfa mosaic virus mRNAs. The P5C-mediated inhibition of globin synthesis could be at least partially overcome by an *in vitro* NADPH generating system (Mick et al. 1988). On the basis that the inhibition of globin synthesis in the cell-free system by 1 mM P5C was greater than that observed with equimolar oxidised GSH or $NADP^+$, Mick et al. (1988) concluded that P5C was not acting solely by its ability to generate $NADP^+$ during its catabolism to produce proline. Nonetheless, consideration of the notion that proline and its precursors may participate in a substrate cycle (Hare & Cress 1997; Hare et al. 1998) may account for their observations without eliminating this possibility. Whereas the conversion of P5C to proline generates a single $NADP^+$, further metabolism of the proline to glutamate and its conversion back to proline generates an additional two $NADP^+$. The 43% decrease in the NADPH levels of sheath segments from rice plants grown for 24 h in the presence of P5C (Iyer & Caplan 1998) is consistent with this proposal. In tissue with a high rate of cycling between proline and its precursors, the apparent discrepancy between the effects of equimolar concentrations of P5C and $NADP^+$ observed by Mick et al. (1988) may be ascribed to non-stoichiometrical amounts of $NADP^+$ generated per mole of P5C reduced to proline. Studies

involving inhibitors of mitochondrial electron transport, which are known to block proline degradation, are consistent with a fairly substantial turnover of the free proline pool in unstressed rice seedlings (Alia & Pardha Saradhi 1993). Nonetheless, this interpretation is confounded somewhat by the demonstration that DHP is more effective than P5C in induction of *salT* transcript accumulation, although P5C was more effective in increasing *DHN4* and *RAB16A* levels. As pointed out by Iyer and Caplan (1998), if DHP is converted to proline and subsequently into P5C, all three inducers of *salT* expression should be equally effective. In contrast, P5C was more effective than proline (Iyer & Caplan 1998).

Treatment of rice plants with proline, P5C and DHP apparently did not affect ABA levels, although this was only monitored at a single time (24 h) after commencement of the treatment (Iyer & Caplan 1998). Xin and Li (1993) found no induction of ABA accumulation in maize cultured cells 6 h, 12 h or 24 h after treatment with proline. With the increasing recognition of the importance of metabolite regulation of plant gene expression (Section 2.4.5.2), the regulatory interactions of metabolite and hormonal signals will need to be defined. It also worth noting that alterations in proline metabolism (Figure 2.14) potentially affect the adenylate energy charge (Atkinson 1977), which is defined as:

$$\frac{[ATP] + 0.5[ADP]}{([ATP] + [ADP] + [AMP])}.$$

Energy charge can theoretically vary between 0 (i.e all the adenylate is present as AMP) and 1 (i.e. all the adenylate is present as ATP). Such extremes are not found in living cells, but it is generally recognised that the adenylate charge of normally metabolising cells is in excess of 0.8, with values of 0.5 or less being indicative of quiescent or senescent cells. It is plausible that adenine nucleotides are not only important allosteric regulators of a number of metabolic reactions, but may regulate signalling cascades that control plant gene expression. Several plant homologues of stress-activated yeast sucrose nonfermenting (SNF1)/mammalian AMP-activated protein kinases have recently been identified (Monger et al. 1997). This introduces the possibility that the equilibrium between adenine nucleotides may not only regulate metabolism at the level of their availability as substrates, but also in activating plant signalling pathways downstream of "pseudoreceptors" such as homologues of yeast SNF1. Although activation of plant SNF1-homologues by AMP does not appear to have been reported, these kinases potentially play an important role in the regulation of carbon partitioning and coordination of sucrose and amino acid synthesis with photosynthetic activity (Monger et al. 1997). Yeast SNF1 mediates the transcriptional response to glucose starvation (Smeekens & Rook 1997) and activation of the AMP-activated protein kinase in response to environmental and nutritional stresses in mammalian cells leads to inactivation of several biosynthetic enzymes (Monger et al. 1997).

2.5.3.1 Intercellular signalling

It has been proposed that in addition to intercompartmental transfer of reducing equivalents at the subcellular level, the interconversions of proline and P5C in animal cells might transfer redox potential across cell membranes between different cell types (Phang 1985). There is a well established asymmetry of P5C and proline metabolic enzymes in different mammalian cells (Phang 1985). Differential accumulation of proline (Chiang & Dandekar 1995) as well as mRNA transcripts encoding P5CR (Verbruggen et al. 1993), P5CS (Savouré et al. 1995) and PDH (Verbruggen et al. 1996a) have been demonstrated in organs of *Arabidopsis*. Chiang and Dandekar (1995) reported that proline formed 17-26% of the total free amino acid concentration in reproductive tissues (floret and seed), but only 1-3% of the total free amino acid concentration in vegetative tissues (rosette leaf and root). At the tissue-specific level, the highest levels of transcript encoding P5CR were found in phloem, meristematic tissues and photosynthetically active cells of the flowering stem of mature *Arabidopsis* plants (Hare & Cress 1996). Most of the cell types of a mature plant are represented in the stem.

In the scheme proposed in Figure 2.16, proline synthesised in an effector cell might exit from this cell and be transported to a target tissue where conversion back to P5C or glutamate might generate reducing equivalents to drive TCA cycle activity. The effector tissue would be characterised by a need to generate $NADP^+$ to drive ribonucleotide biosynthesis (e.g. meristematic tissue), whereas the target tissue may have high energy requirements. Apparently, intersystemic transport of P5C in plant systems has never been investigated. However, if P5C like glutamate and proline is capable of movement between tissue systems, it seems feasible to speculate that precursors of proline synthesis produced in the target tissue might be transported back to the effector tissue to constitute a cycle. In this way, intercellular transport of proline and its biosynthetic precursors might enable communication between different tissues and coordinate different metabolic requirements of different tissue systems. Depending on the rates of export from the effector cell-type or the rate of proline oxidation in cells of the target tissue, either or both cell-types could benefit from any associated osmotic adjustment effects conferred by proline. Transport of proline to meristematic tissues appears to be of particular importance during stress (Voetberg & Sharp 1991; Girousse et al. 1996), possibly because metabolically highly active, most rapidly growing regions may be more susceptible to harm by adverse conditions.

In keeping with this suggestion, Girousse et al. (1996) indicated that the most striking change in amino acid composition of phloem sieve tubes following the imposition of water deficit in alfalfa was an approximately sixty-fold increase in proline levels. Glutamate levels also increased, although the increase was not as dramatic as that observed for proline (Girousse et al. 1996).

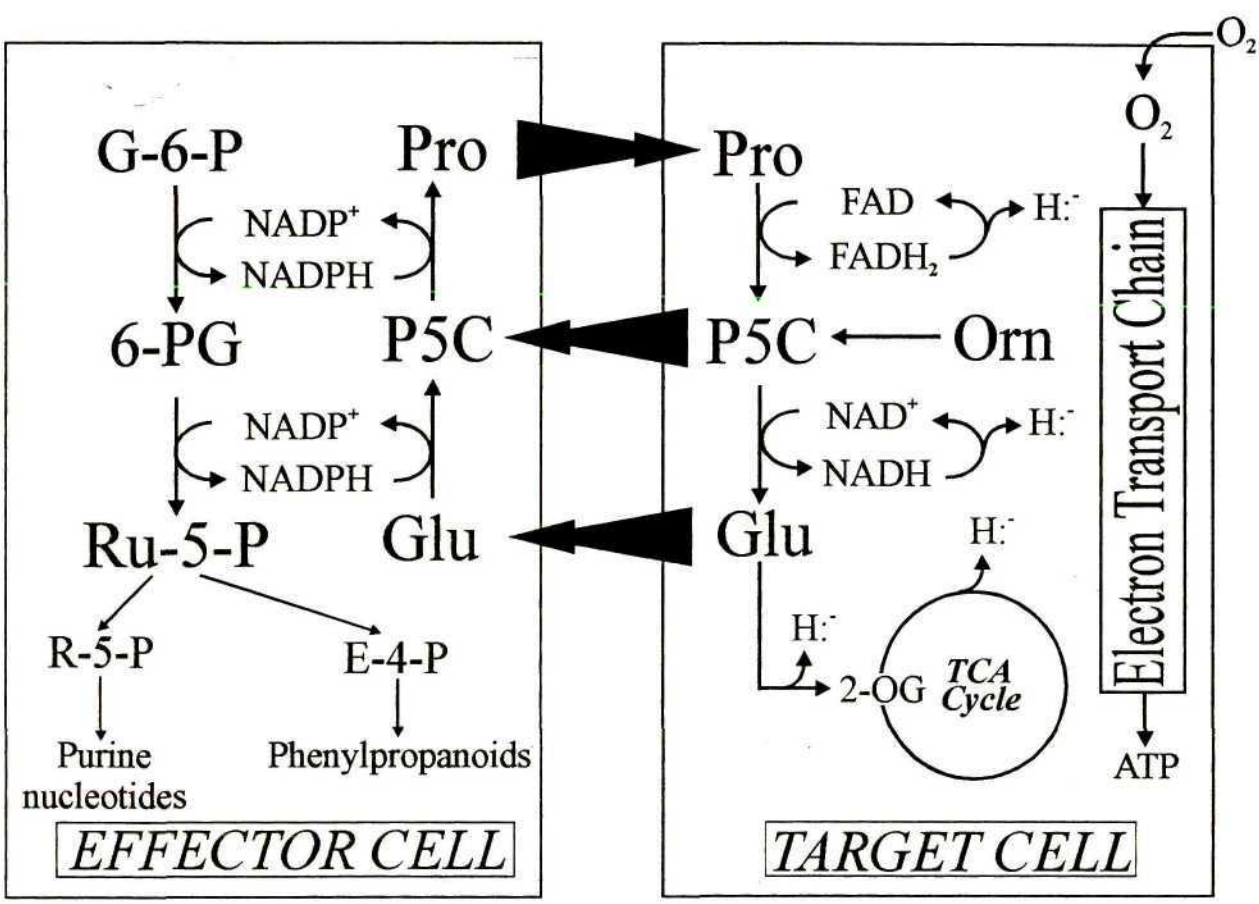

Figure 2.16: A proposed scheme whereby the interconversions of proline and P5C might act as an intercellular signalling system. Proline produced from P5C in an effector cell is transported via the phloem to a target tissue characterised by high energy requirements. Here, proline degradation generates reducing equivalents needed to drive TCA cycle activity. The P5C or Glu thus generated may be translocated back to the effector cell type where conversion back to proline generates $NADP^+$ needed to prime activity of the OPPP, which provides carbon for the synthesis of nucleotides or secondary metabolites. In this way, proline metabolism might transfer metabolic information between tissues with different metabolic requirements in the form of redox potential and activate metabolic pathways that do not involve any of the intermediates or products of proline metabolism. Abbreviations are the same as those used in Figure 2.14. From Hare and Cress (1997).

With regard to the hypothesis outlined in Figure 2.16, it would be interesting to know whether levels of P5C are altered in phloem tissue during stress, as well as the effects of stress on levels of proline, glutamate and P5C in xylem sap. Although the leaves of wheat plants supplied with 15.0 mM NO_3^- had higher levels of free proline than those supplied with 1.0 mM NO_3^-, the abundance of proline in phloem exudates relative to that of the other free amino acids was approximately three-fold higher in the nitrogen-limited plants than in those supplied with 15.0 mM NO_3^- (Caputo & Barneix 1997). Certain other amino acids (e.g. arginine and glutamine) were present at a lower relative abundance in phloem sap under nitrogen-limiting conditions. Furthermore, although a positive linear correlation exists between the concentrations of amino acids in phloem exudate and their levels in leaf tissue, glutamate was the only amino acid found

to be exported at much higher levels than predicted when nitrogen supply was low (Caputo & Barneix 1997). In contrast to the selective loading of amino acids into sieve tubes, the rate of sugar export to the phloem was unaffected by the nitrogen status of the plant (Caputo & Barneix 1997). Together, these findings corroborate the view that certain amino acids (e.g. proline and glutamate) are transported preferentially to the phloem under adverse conditions, while for others, transport into the sieve tubes is restricted (Section 2.3.4). They also underscore the likely importance of elucidating the molecular mechanisms responsible for the inter-organ transport of proline in evaluating whether shifts in proline metabolism may play an important regulatory role in both developmental transitions and responses to adverse conditions.

2.5.4 The situation for non-proline accumulators

It is appropriate to note that many plant species, including cucumber (Itai & Paleg 1982), pigeon pea (Joshi 1984), sugarcane (Naik & Joshi 1983), *Andropogon glomeratus* (Bowman 1988) and *Coleus blumei* (Gilbert et al. 1998) do not accumulate substantial amounts of proline following the imposition of hyperosmotic stress. As a generalisation, euhalophytes are typically not proline accumulators. Little or no accumulation of proline or polyols could be detected in the mangrove plant *Avicennia marina* (Ashihara et al. 1997), although proline accumulated in osmotically stressed *Triglochin maritima* may account for up to 10% of the dry weight (Stewart & Lee 1974). Although members of the Chenopodiaceae, which exhibit amongst the highest salt tolerances reported, accumulate glycine betaine in preference to proline, some proline synthesis is frequently observed as an initial response to osmotic stress imposed very rapidly or to levels much higher than those promoting normal growth (Storey & Wyn Jones 1977; Doddema et al. 1986). This may represent a rapid mechanism of maintaining metabolic homeostasis until more permanent and effective adaptive responses can be activated.

The pathways of proline biosynthesis and degradation are present in all plants. If a functional significance for stress-induced proline synthesis is to be assumed for many species, how then might one account for the failure of others to accumulate high levels of this imino acid during stress? In this regard, it may be relevant to note that, like proline, other common cellular osmolytes including betaines, sucrose, hexitols and cyclitols are also highly reduced (Hare & Cress 1997). Their accumulation might thus also contribute to biocompatible storage of excess reductant during stress. The low K_m values for $NADP^+$ observed for sorbitol dehydrogenase (also referred to as aldose reductase; EC 1.1.1.21) and G6PDH in silkworm suggested coupled activity of these enzymes in insects, where sorbitol accumulation has been associated with cold resistance (Konichev 1997). Although higher plants do not accumulate glycerol in response to

hyperosmotic stress, glycerol is the major compatible solute in microorganisms such as *S. cerevisiae* and its synthesis has been shown to be related to maintenance of cellular redox potential (Ansell et al. 1997).

One approach to elucidate the physiological significance of differences in the type(s) of osmotic solutes accumulated by various species is to consider the preferential accumulation of any osmolyte as a reflection of the availability of its precursors under adverse conditions (Hare et al. 1998). While much has been elucidated concerning the biochemistry and molecular genetics of proline synthesis and degradation to glutamate, less attention has been directed to the changes in metabolism which are required to support these processes. In trying to postulate an adaptive function for proline accumulation, most workers have considered the physical implications of various stresses in preference to assessment of the metabolic problems that the stressed cell must surmount. Intriguingly, there appears to be a reciprocal relationship between accumulation of proline and other osmolytes, not only in different species (Larher et al. 1993; Erskine et al. 1996), but even in near-isogenic lines (Yang et al. 1995). This suggests that higher plants may compensate for an inability to synthesise certain osmolytes by accumulating others. In *Aster tripolium*, the metabolic pathways involved in proline and glycine betaine synthesis are highly coordinated (Goas et al. 1982). The dependence of proline accumulation on the carbohydrate availability (Stewart 1978; Pesci 1993) suggests that differences between species or even varieties in their capacities for starch mobilisation may also account for differences in their ability to use activation of proline biosynthesis to ameliorate the consequences of environmental stress.

Another unifying feature that appears to be common to all metabolic processes that support the synthesis of compatible solutes is that these pathways apparently accommodate high fluxes in the absence of stress (Hare et al. 1997, 1998). It was proposed that apparently "wasteful" and "futile" metabolic pathways, including photorespiration, may provide the metabolic flexibility needed to facilitate acclimation to adverse environmental conditions, which are always associated with a discrepancy between energy absorption and its use by metabolism (Hare et al. 1998). Through their buffering capacity, substrate cycles may help to avert the need for a major reorientation of metabolism under stressful conditions. While energetically expensive, plants may permanently keep these adaptive mechanisms in place in anticipation of inevitable changes in environmental conditions. A mannitol cycle involving fructose, fructose-6-phosphate and mannitol-1-phosphate was recently identified in a eulittoral mangrove red alga and proposed to function in biochemical adaptation to fluctuating environmental extremes in this habitat (Karsten et al. 1997). Constitutive acclimatory mechanisms are likely to be of particular value in amelioration of stress damage in the short term, although amplification of their efficacy through subsequent modulation of gene expression may prevent damage arising from long term exposure to adverse

conditions. In addition, many osmolytes are products of "dead-end" pathways. Of the osmolytes which are present at detectable levels in the unstressed state, none participate in equilibrium reactions central to intermediary metabolism. In a teleological sense, the proposal that osmolytes play a role in scavenging free radicals is consistent with this generalisation. It also reinforces the argument that substrate availability may be an important criterion in evolutionary strategies that determine osmolyte preferences in different species.

Finally, it is appropriate to re-emphasise the suggestion of others (Delauney & Verma 1993) that the absence of a positive correlation between proline accumulation and osmotolerance in many species does not negate an adaptive role for the response *per se*. Rather, it may reflect the predominance in these species of alternative strategies towards adaptation to stress. Selection for such mechanisms may be influenced by carbon and nitrogen allocation patterns, plant size and total surface area, stomatal density, root characteristics or hormonal balance and its influence on stomatal physiology. The occurrence of such long-term adaptations may circumvent the need for a flexible homeostatic system capable of rapid responses to steadily occurring shifts in cellular metabolism in a constantly changing environment. As emphasised above, the dramatic proline accumulation observed in many plants in response to sudden and often dramatic stress events (the type most frequently used in the laboratory context) may simply be symptomatic of uncoupling between a proline metabolic cycle and the physiological processes which it serves to modulate. Thus, accumulation of free proline *per se* may simply be symptomatic of severe damage which occurs after the true stress coping mechanism has been overworked. Constitutive adaptations at the morphological (e.g. leaf waxiness or the development of a deeper rooting system), developmental (e.g. time of flowering) or physiological (e.g. active salt exclusion or vacuolar sequestration of ions) levels are likely to predominate in species indigenous to extremely hostile environments.

Therefore, in assessment of the functional significance of proline accumulation, the process should always be seen holistically in the context of other available mechanisms that facilitate adaptation to stress. Indeed, examination of the effects of transgene-mediated enhancement of proline synthesis in species which preferentially accumulate glycine betaine or sugars may provide considerable insight into the functional significance of proline accumulation in systems such as tobacco, barley, wheat and *Arabidopsis* in which the physiology of proline accumulation has been more commonly studied. Uptake of exogenous proline by cucumber, a non-proline accumulating and stress-sensitive species, failed to increase stress tolerance (Itai & Paleg 1982).

2.5.5 Conclusion

While a number of observations suggest a direct, adaptive role for proline in counteracting the effects of osmotic stress, free cytosolic proline often does not accumulate to levels sufficient to account exclusively for an osmoprotective role. It seems feasible to posit that the value of stress-induced proline accumulation may be mediated largely via the effects of its synthesis and degradation on cellular metabolism. Regulation of cellular redox potential by decreasing the elevated reductant charge resulting from an inhibition of photophosphorylation is likely to be important for cellular homeostasis and continued efficiency of metabolic reactions under conditions of stress. A small change in the intracellular $NADP^+$/NADPH ratio mediated by enhanced proline biosynthesis is likely to have a large effect on flux through a redox-sensitive pathway such as the OPPP, which is regulated by the level of reduction of the pool of cellular NADP.

It does not seem unreasonable to imagine that the effects of an accumulation of free proline on stress tolerance are multifaceted. Proline itself may act not only as an osmoticum but also as a substrate for the TCA cycle during recovery from stress, while the interconversions between proline and its precursors may be involved in the regulation of cellular pH and redox potential. Although turgor maintenance is the driving force for cell expansion and thus organ growth, it is important to emphasise that these processes are under metabolic control. Turgor provides only the physical force for expansion and is in itself not necessarily compatible with the continuation of metabolic processes. The rather energetically expensive process of proline accumulation might thus simultaneously maintain water balance while ensuring homeostasis necessary for the continuation of metabolic processes. Functions for this common physiological response to stress in addition to its likely role as an osmolyte during water deprivation may account for the induction of proline accumulation by imposition of several abiotic stresses which do not possess an overt osmotic component. Finally, the emerging view that the capacity for proline synthesis is not as sensitive to CHX as is the accumulation of free proline (Section 2.4.2) is consistent with the proposal that proline synthesis, and not free proline itself, may be of primary importance in adaptation to stresses which cause cellular dehydration (Hare & Cress 1997; Hare et al. 1998).

2.6 Perspective

This review of the literature, which provides a background for the presentation and interpretation of the results of this study, has covered a broad range of topics. At this point, it may be useful to the reader if the rationale which underlied the compilation of this literature review is concisely summarised in the context of the overall objectives of this study.

Despite considerable investigation, the extreme sensitivity of the proline metabolism to environmental changes remains largely enigmatic. The inadequacy of hypotheses which implicate the importance of free proline in mediating osmotic adjustment and the protection of subcellular structure has led to the suggestion that the metabolic effects associated with shifts in proline metabolism may be the primary benefit associated with stress-induced increases in proline biosynthesis. Converging experimental evidence, which has not been discussed here, suggests that similar arguments may apply for the accumulation of several other commonly-studied osmolytes (Hare et al. 1998). This body of largely circumstantial evidence does not invalidate any possible osmoprotectant effects associated with the accumulation of so-called compatible solutes, but it potentially sets the scene for more critical examination of this central aspect of the plant stress response.

The mechanisms which have been suggested to account for the functional significance of stress-induced changes in proline metabolism involve alterations in the flux through metabolic pathways that involve neither proline nor any of the precursors that provide carbon skeletons for proline synthesis. Thus, as is reflected in the title of this thesis, the proline metabolic system may have a truly regulatory role. A central feature of this role is the likely involvement of cellular redox potential in the regulation of a wide range of metabolic and signalling events that control various aspects of growth and development. The well-documented disruption of cellular redox potential under adverse conditions, as well as regulated changes in redox homeostasis throughout plant development, sets the stage for assessment of whether or not these processes can be correlated with changes in the capacity for proline synthesis and/or degradation.

Over the past decade, the application of various molecular biological approaches to the study of proline metabolism has provided convincing evidence of a functional role for proline. Following stress, the specific induction of genes involved in proline synthesis conforms with the view that increases in proline synthesis serve a functional role and tends to discredit the notion that proline accumulation may merely be a metabolic artifact which results from changes in the availability of the precursors that support proline synthesis. This direct link between the perception of stress and the regulation of genes involved in proline metabolism has yet to be further substantiated through the identification in proline metabolic genes of fairly ubiquitous promoter elements that have been

characterised in other stress-responsive plant genes. Ideally, the metabolic interconversions which regulate plant growth and development should always be viewed in the light of the signals that regulate the expression of the genes that participate in these processes. Besides the ability to begin to assign regulation of proline synthesis to different branches of the signalling network that controls genetic responses to stress, other DNA elements in the upstream regulatory regions of genes involved in proline synthesis from glutamate may also provide some useful indicators regarding the functional significance of proline metabolism. For instance, the recent demonstration (Strizhov et al. 1997) that levels of transcript encoding an *Arabidopsis* P5CS are sensitive to the availability of light may be consistent with the proposed role of proline synthesis in the modulation of light-dependent stress phenomena such as photoinhibition and high levels of photorespiration. Likewise, the induction of proline synthetic genes by auxin and cytokinin introduces the question of whether the apparent ability of these hormones to target the promoter regions of genes involved in proline synthesis may relate to the apparent coordination of a high rate of proline synthesis with rapid cell division. While the mechanisms by which plants perceive cellular redox status and translate this information into appropriate changes in gene expression are poorly understood, an evaluation of redox-regulated expression of proline biosynthetic capacity promises to substantiate the proposed importance of the proline biosynthetic pathway as a redox buffering mechanism.

Since biological studies at the molecular level should always be assessed at the level of the whole organism, the use of a genetic approach to inactivate proline biosynthetic genes potentially offers a powerful approach to test all of the hypotheses outlined in Section 2.5. Antisense suppression of specific genes involved in proline synthesis also enables assessment of functional redundancy in proline synthesis and potentially offers some insight into how the genes encoding proline biosynthetic enzymes are regulated.

Most of the studies aimed at assessing the role of proline accumulation have used an *in vitro* approach and have focussed on the biophysical properties of the end product of the biosynthetic pathway. The feedback inhibition of proline synthesis by proline offers a useful system to simultaneously test the viability of the compatible solute hypothesis *in vivo* as well as assessment of how obstruction of proline synthesis may affect different aspects of plant growth and development. Since PDH activity is induced by proline at the transcriptional level, exposure of plants to levels of proline that do not feedback inhibit P5CS activity should increase flux through the metabolic cycle involving both proline synthesis and degradation (Figure 2.14). In the light of increasing evidence that a signal derived from flux through the proline biosynthetic and catabolic pathways may control gene expression (Section 2.5.3; Iyer & Caplan 1998; Hare et al. 1998), the effects of exogenously applied proline on whole plant physiology should ideally be correlated with the changes in gene expression which accompany these changes in growth and development.

3. Materials and Methods

3.1 Reagents

All chemicals used were of the highest quality available. Generally, inorganic salts were purchased from BDH (Poole, England). Agar, tryptone and yeast extract were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England). Gelrite was from Labretoria (South Africa). Agarose was purchased from Promega (Madison, WI). Sequencing grade polyacrylamide was obtained from Stratagene Inc. (La Jolla, CA). D-[1-^{14}C]-glucose, D-[6-^{14}C]-glucose and [α-^{35}S]dATP were purchased from Amersham International (Buckinghamshire, England). L-ornithine, phenazine ethosulphate, D-proline, L-proline, and thioproline (L-thiazolidine-4-carboxylic acid) were obtained from Sigma Chemical Co. (St Louis, MO). Unless specified, proline refers to the naturally-occurring isomer (L-proline). Azetidine-2-carboxylic acid was from ICN Biochemicals (Aurora, OH). Unless otherwise specified, all enzymes, metabolites, deoxynucleotides and antibiotics were obtained from Boehringer Mannheim (Randburg, South Africa) and were used according to the instructions of the manufacturer.

3.2 Standard molecular biology techniques and microbial growth conditions

Standard procedures used in the purification of plasmid and plant genomic DNA, transformation of *Escherichia coli*, cloning of DNA fragments and Southern hybridisation are not outlined here in full. The same protocols as those previously outlined in detail (Ausubel et al. 1987; Hare 1995; Hare & Cress 1996) were used without modification. Any modifications to previously published protocols indicated in the text are outlined in detail.

Analysis of DNA fragments generated by restriction digestion was performed in TAE buffer (0.04 M Tris-acetate, 0.002 M Na_2EDTA, pH 8.0). Unless otherwise specified, growth of all bacterial strains was in LB medium (10.0 g l^{-1} tryptone, 10.0 g l^{-1} yeast extract, 10.0 g l^{-1} NaCl, pH 7.0 unless otherwise specified), which was solidified, where necessary, by the addition of agar at 15.0 g l^{-1}. All media used for bacterial and plant growth was adjusted to the specified pH, prior to autoclaving, by the addition of 1 M KOH. Where appropriate, ampicillin or kanamycin were included at final concentrations of 100 mg l^{-1}, unless specified otherwise. *Escherichia coli* strains were grown at 37 °C. *Agrobacterium tumefaciens* strains were grown at 28 °C. Stocks of all bacterial strains were maintained at -70 °C as a 1:1 mixture of a logarithmic-phase culture in LB

with the appropriate antibiotics, to a glycerol-based storage solution [65% (v/v) glycerol, 0.1 M $MgSO_4$, 0.025 M Tris-HCl, pH 8.0].

3.3 Characterisation of an incomplete cDNA encoding an *Arabidopsis* P5CS

A clone encoding an incomplete P5CS cDNA from *Arabidopsis thaliana* ecotype Columbia (EST No. 105E5T7, Genbank Accession No. T22627) was obtained from the *Arabidopsis* Biological Resource Centre (Ohio State University, Columbus, OH). The Lambda-PRL2 library from which the clone was isolated was derived from equal quantities of four pools of mRNA (Newman et al. 1994). The mRNA sources were: (i) seven d-old etiolated seedlings, (ii) tissue culture grown roots, (iii) rosettes from plants half of which were grown under continuous light and half grown under a 16 h light and 8 h dark regime and (iv) stems, flowers and siliques from the same plants described in (iii).

Plasmid DNA was sequenced by the dideoxy termination method (Sanger et al. 1977) , using the Sequenase Version 2.0 Sequencing Kit (US Biochemical Corp., Cleveland, OH) according to the specifications of the manufacturer. Subcloning of restriction fragments into the plasmid pBluescriptII SK$^-$ was performed using standard procedures (Ausubel et al. 1987; Hare 1995). Sequential deletions of the insert within 105E5T7 were generated using the Erase-A-Base system (Promega, Madison, WI) according to the manufacturer's instructions. The approach used to generate a nested set of deletions in the target DNA, thereby effectively moving the priming site closer to the sequence of interest is based on the procedure developed by Henikoff (1984), in which exonuclease III is used to specifically digest insert DNA from a 5' overhang or blunt end. The adjacent sequencing primer binding site is protected from digestion by a 4 bp 3' overhang restriction site. Clones used in the sequencing of 105E5T7 are described in Table 3.1. The strategy used to sequence the insert within 105E5T7 is represented diagrammatically in Figure 3.1.

DNA sequences were analysed using DNASIS (Hitachi Software Engineering, Inc., San Bruno, CA) or by GenBank database searches using the programs BLAST (Altschul et al. 1990) and Gapped-BLAST (Altschul et al. 1997) accessed via the Internet (http://www.ncbi.nlm.nih.gov).

Table 3.1 : Clones used in the sequencing of the insert within 105E5T7. The clone 105E5T7 was obtained from the *Arabidopsis* Biological Resource Centre (Ohio State University, Columbus, OH). All other clones were produced in this study. The plasmids pBluescriptII SK⁻ and pZL1 are products of Stratagene and Gibco BRL respectively.

Clone name	Description
105E5T7	1050 bp insert cloned into the *Sal*I and *Not*I sites of pZL1
105E5PB	315 bp fragment generated by double digestion of 105E5T7 with *Pst*I and *Bam*HI cloned into the *Pst*I and *Bam*HI sites of pBluescriptII SK⁻
105E5ND1	580 bp fragment generated by exonuclease III-mediated deletion from the *Eco*R1 site in 105E5T7[1]
105E5ND2	363 bp fragment generated by exonuclease III-mediated deletion from the *Eco*R1 site in 105E5T7[1]

[1] Plasmid 105E5T7 was double-digested with *Kpn*I and *Eco*RI before digestion with exonuclease III and selection of deletion subclones as described by Promega (Erase-a-Base Technical Manual).

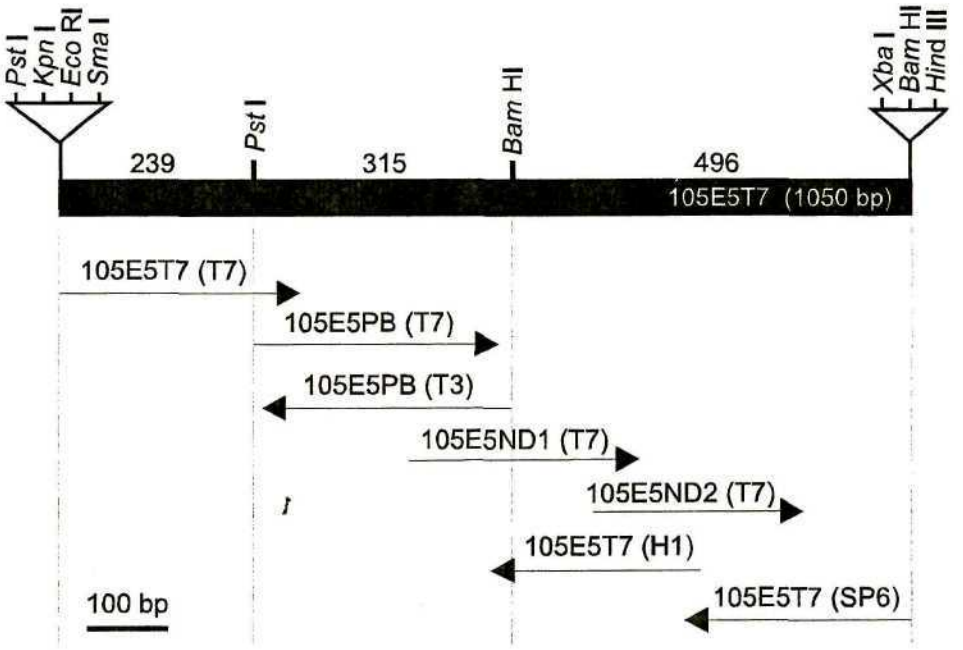

Figure 3.1: Strategy employed for the sequencing of 105E5T7. Arrows indicate the sequencing direction and the length of sequence obtained in individual sequencing experiments. Restriction sites that flank the termini of the insert are found within the polycloning site of the vector pZL1 (Gibco BRL). The clone used for each sequence determination (Table 3.1) is shown above the arrow. The sequencing primer used in each reaction is indicated in parentheses. The H1 primer (5'-CACTACATAAGCGAGGGTTT-3') was synthesised by Professor D. York (Faculty of Medicine, University of Natal, Durban). The T3-, T7- and SP6-promoter-specific sequencing primers were purchased from Boehringer Mannheim (South Africa). The scale bar represents 100 bp.

3.4 Construction of the binary transformation vectors pBI-P5CS1(AS) and pBI-P5CR(AS)

Both of the 105E5T7 and YAP057 cDNAs were directionally cloned into the *Bam*HI and *Sma*I sites of pBI121 (Jefferson et al. 1987; obtained from Clontech Laboratories Inc., Palo Alto, CA), thereby placing the 3' termini of the coding strands proximal to the CaMV35S promoter. Owing to the presence of an internal *Bam*HI site (position 554) in the insert of 105E5T7, in the generation of pBI-P5CS1(AS), the 1050 bp fragment was liberated by timed digestion of *Sma*I-digested 105E5T7 with *Bam*HI. Twenty µg of linearised, *Sma*I-digested 105E5T7 was incubated with 10 units of *Bam*HI (Boehringer Mannheim, South Africa) at 30 °C. Five aliquots, each containing 4.0 µg of digested plasmid DNA, were withdrawn at 2 min intervals and the reaction terminated by incubation of the reaction vessel in boiling water for 5 min. The reaction products were separated on a 1.1% (m/v) agarose gel and the 1050 bp fragment liberated after 2 min, 4 min and 6 min incubations was excised from the gel and purified using the Geneclean system (Bio101 Inc., La Jolla, CA) according to the manufacturer's instructions. To create pBI-P5CR(AS), the YAP057 plasmid was restricted with *Kpn*I, end-filled with the large fragment of DNA polymerase I, and then digested with *Bam*HI. Following electrophoretic separation, the 1.0 kb fragment was excised from a 1.1% (m/v) agarose gel and purified as described above. The purified fragments were inserted into pBI121 that had been linearised by digestion with *Bam*HI and *Sma*I, and pretreated with calf intestinal alkaline phosphatase. The cloning strategy used in the construction of pBI-P5CR(AS) resulted in the loss of the *Sma*I site in the polylinker of pBI121.

Escherichia coli strain JM109 (F' *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZΔM15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA*) was transformed with ligation mixtures as described previously (Inoue et al. 1990). Putative recombinants recovered from growth media supplemented with 100 mg l^{-1} kanamycin were screened by restriction analysis of plasmid DNA, which was purified as described previously (Feliciello & Chinali 1993). Positive isolates of pBI-P5CS1(AS) and pBI-P5CR(AS) were identified by digestion of the plasmid DNA with *Bam*HI and *Eco*RI respectively.

The structures of the pBI-P5CS1(AS) and pBI-P5CR(AS) plasmids were confirmed by more extensive restriction analysis and Southern hybridisation. Southern hybridisation (Southern 1975) was performed using Hybond N$^+$ nylon membranes, Hyperfilm-ECL and chemiluminescent detection reagents provided with the ECL™ Direct Nucleic acid Labelling and Detection System, exactly as recommended by the manufacturer (Amersham). The primary wash buffer contained a final concentration of 0.1 x SSC (0.0015 M Na_3-citrate, 0.015 M NaCl, pH 7.0) in order to ensure a high stringency of hybridisation (Amersham). The orientation of the inserts within both pBI-

P5CS1(AS) and pBI-P5CR(AS) relative to the CaMV35S promoter were further confirmed by sequencing into the 5' ends of the coding strands of both P5CS and P5CR cDNAs using a primer (5'-TCACGGGTTGGGGTTTCTAC-3'; Clontech) specific to the 5' end of the template strand of the *GUS* gene, which encodes β-glucuronidase. This 20-mer hybridises to the sequence between positions +32 and +13 nucleotides from the A of the *GUS* gene ATG initiation codon (Clontech).

Both pBI-P5CS1(AS) and pBI-P5CR(AS) plasmids were mobilised in *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) using a triparental mating procedure (Van Haute et al. 1983), which employs the helper plasmid pRK2013 (Ditta et al. 1980). Transconjugants were selected on AB minimal medium (3.0 g l^{-1} KH_2PO_4, 1.0 g l^{-1} $NaH_2PO_4.H_2O$, 1.0 g l^{-1} NH_4Cl, 0.3 g l^{-1} $MgSO_4.7H_2O$, 0.15 g l^{-1} KCl, 0.15 g l^{-1} $CaCl_2$, 2.5 mg l^{-1} $FeSO_4.7H_2O$, 2 g l^{-1} glucose, pH 7, solidified with 15.0 g l^{-1} agar) containing 50 mg l^{-1} kanamycin and 150 mg l^{-1} rifampicin and were subsequently maintained on LB medium supplemented with the same concentrations of both antibiotics.

3.5 Plant material

Unless specified otherwise, the *Arabidopsis thaliana* (L.) Heynh. ecotype used was Columbia (Col-0; purchased from Lehle Seeds, Round Rock, TX). The ecotype Landsberg *erecta* (Ler-0) and the hormonal response mutants *abi3-1* (Koornneef et al. 1984) and *gai* (Koornneef et al. 1985), both of which are also in the Ler-O background, were obtained from the *Arabidopsis* Biological Resource Centre (Ohio State University, Columbus, OH).

In vitro growth of whole plants was on MS-based (Murashige & Skoog 1962) media. *Arabidopsis* seeds were surface-sterilised by vigorous agitation in 70% (v/v) ethanol for 1 min, followed by a 15 min incubation with continuous stirring in a 35.0 g l^{-1} NaOCl solution containing one drop of Tween-20 per 100 ml. The NaOCl was removed by agitation of the seeds in five changes of the same volume of sterile distilled water (dH_2O). A small volume of dH_2O containing the surface-sterilised seeds (< 0.5 ml) was added to 4.0 ml of sterile, melted agar (6.0 g l^{-1} in dH_2O or MS-based medium, depending on the application) which had been cooled to 45 °C. The agar was then poured immediately onto the appropriate solidified medium in order to disperse the seeds evenly over the surface of the solidified agar in the petri dish (90 mm i.d.). Where necessary, surface sterilised seeds were transferred to the experimental media using a sterile toothpick. Depending on the experiment, seeds were either transferred directly to experimental medium (e.g. most germination experiments) or first incubated at 4 °C in the dark for 48 - 60 h (e.g. for root elongation assays and stress treatments). The latter vernalisation treatment is routinely used in

order to synchronise the germination of *Arabidopsis* seeds. Where experiments involved the growth of *Arabidopsis* seedlings in liquid culture, approximately 0.5 ml of dH_2O containing 30 - 50 surface-sterilised seeds was transferred under sterile conditions to 30 ml of MS-based medium in a 250 ml flask, using a wide-bore tip fitted to an automatic pipettor.

3.6 Plant growth conditions

Seed stocks of all *Arabidopsis* lines were obtained from plants grown in a mixture of peat-moss, vermiculite and perlite (1:1:1) and fertilised weekly with a complete nutrient solution (Somerville & Ogren 1982) in a thermostatically controlled greenhouse. Greenhouse temperatures varied between 18 °C and 26 °C. The transmittance of the greenhouse to solar radiation was approximately 35%.

All *in vitro* experiments involving plant material were conducted in a growth room maintained at 25 °C ± 1 °C and illuminated continuously with cool, fluorescent tubes to provide 175 - 225 µmol m^{-2} s^{-1} photosynthetically active radiation. Experiments involving growth in the dark were conducted in the same room, but with petri dishes wrapped in two layers of aluminium foil and transferred to a cupboard.

3.6.1 Seed germination studies

For germination assays, surface-sterilised seeds (Section 3.5) were transferred immediately (within 3 h of sterilisation) to media containing half-strength MS (Murashige & Skoog 1962) salts (MS/2 medium, pH 5.7) without sucrose. The media was solidified by the addition of 8.0 g l^{-1} of agar. Where required, supplements (proline, methylene blue, phenazine ethosulphate, NaCl, sorbitol or glycerol) were added as filter-sterilised stocks (pH 5.5 - 6.0) once the autoclaved media had cooled to at least 50 °C. For each treatment, four batches of 50 seeds each were transferred to the media in petri dishes (90 mm i.d.) using a sterile toothpick and a grid containing 50 evenly-spaced units, which was placed under the petri dish. Radicle emergence was used as the criterion for germination, and was assessed by visualisation of the plates above a light-bench.

3.6.2 Determination of seedling growth

For the determination of root and hypocotyl lengths, surface sterilised seeds (Section 3.5) which had been incubated at 4 °C in darkness for 48-60 h were transferred to MS/2 medium (pH 5.7

before autoclaving) containing 5.0 g l^{-1} sucrose and solidified with 15.0 g l^{-1} agar. Filter-sterilised stocks of supplements (NaCl, proline, glutamate; adjusted where necessary to pH 5.5 - 6.0 before filter-sterilisation) were added to the required concentration after the media has cooled to at least 50 °C. Generally, between 35 and 50 seeds were transferred to each petri dish (90 mm i.d.) using a sterile toothpick. The plates were sealed with two layers of Parafilm (American National Can) and transferred to either continuous light or incubated in darkness for the designated time. For dark-grown seedlings, the imbibed seeds were exposed to continuous light at 25 °C for 2 h prior to transfer to the dark, in order to promote their germination. Petri dishes were held vertically throughout the incubation period to enable the roots to grow along the surface of the agar and to ensure no mechanical constraints on hypocotyl elongation. After the time indicated, seedlings were removed from the dishes with forceps, taking care not to break any of the roots, which occasionally penetrated the agar. The seedlings were immersed in solution of 0.1% (w/v) toluidine blue dissolved in 0.1% (w/v) Na_3BO_3 for 2 - 5 min, and then rinsed with a large excess of dH_2O. Toluidine blue specifically stained the roots of young *Arabidopsis* seedlings, probably because of the inability of the stain to penetrate the wax layer covering the hypocotyls and cotyledons (Cao et al. 1993). Root and hypocotyl lengths were determined by laying the seedling on a steel ruler graduated in 0.5 mm increments.

The MS/2-N medium used to examine the effects of nitrogen deficiency on the growth of pBI-P5CS1(AS) plants was identical to MS/2 medium containing 5.0 g l^{-1} sucrose, but both KNO_3 and NH_4NO_3 were omitted.

3.6.3 Stress treatments

Using a sterile toothpick, surface-sterilised seeds were transferred to sterile circular nylon filter discs (Biotrace 0.45 µm blotting membrane, 90 mm diameter; generously donated by Gelman Sciences, Ann Abor, MI) placed on top of MS/2 medium containing 5.0 g l^{-1} sucrose and solidified with 8.0 g l^{-1} agar. After 14 d incubation under continuous light at 25 °C, the filters carrying 25 - 30 plantlets were carefully lifted off the agar surface and transferred to sterile glass petri dishes (90 mm i.d, 8 mm deep). For salinity and osmotic stresses, 10 ml of MS/2 medium containing 5.0 g l^{-1} sucrose and 0.250 M NaCl or 0.550 M sorbitol was transferred to the discs to ensure that the roots were covered with the test solution. Plants which were irrigated with 10 ml of liquid MS/2 medium containing 5.0 g l^{-1} sucrose served as controls. All manipulations were done under sterile conditions. The plates were transferred to a growth room and incubated at 25 °C under continuous light for 24 h. At the end of all stress treatments, excess liquid was removed by rapidly blotting the seedlings on tissue paper. The fresh weights of the samples were determined and the

samples were immediately frozen in liquid nitrogen. Where necessary, samples were stored at -70 °C before free proline was assayed. However, samples were always assayed within at least 48 h of freezing.

Cold stress was imposed by transfer of 14 d-old seedlings grown at 25 °C (MS/2 medium with 5.0 g l^{-1} sucrose, pH 5.7, solidified with 8.0 g l^{-1} agar) to a controlled environment chamber with continuous light (125-175 µmol m^{-2} s^{-1} photosynthetically active radiation), where they were incubated at 5 °C for the time specified. For cold stress experiments, plantlets were not grown on nylon filters. At the end of the cold treatment, the seedlings were carefully removed from the agar, taking care not to sever the roots.

3.7 *In vitro* shoot organogenesis and transformation of *Arabidopsis*

The procedures used for *in vitro* shoot organogenesis and the regeneration of transgenic *Arabidopsis* plants after co-cultivation with *A. tumefaciens* are based on methods described previously (Akama et al. 1992). Both of the hormonally-supplemented regeneration media used (callus-inducing medium, CIM and shoot-inducing medium, SIM) are based on B5 mineral salts (Gamborg et al. 1968) and contain 20.0 g l^{-1} glucose, 0.1 g l^{-1} *myo*-inositol, 30 mg l^{-1} thiamine-HCl, 2 mg l^{-1} nicotinic acid, 1 mg l^{-1} biotin and 1 mg l^{-1} pyridoxine.

Surface-sterilised seeds (Section 3.5) of the *Arabidopsis* ecotype Columbia (Col-0) were resuspended in 4.0 ml of germination medium (GM; full-strength MS salts, 10.0 g l^{-1} sucrose, pH 5.7) containing 6.0 g l^{-1} melted agar (cooled to 45 °C) and immediately poured onto the surface of 20-25 ml solidified GM (8.0 g l^{-1} agar) in a glass petri dish (90 mm i.d., 8 mm deep). After incubation in the dark at 4 °C for 48 h - 60 h, the plates were transferred to the growth room and exposed to continuous white light for at least 2 h in order to promote seed germination, before incubation in darkness at 25 °C for 10 d.

After 10 d, the hypocotyls of etiolated seedlings were excised and cut into segments 7.0 - 10.0 mm in length. The hypocotyl explants were transferred to callus-inducing medium (CIM; B5 salts with the supplements outlined above, 0.5 mg l^{-1} 2,4-diphenoxyacetic acid, 0.05 mg l^{-1} kinetin, pH 5.7, solidified with 2.5 g l^{-1} Gelrite) and incubated for 4 d in continuous light at 25 °C. A callus-induction step is necessary to ensure shoot regeneration throughout the explant (Valvekens et al. 1988). Omission of this step before transfer to SIM results in bud formation at the ends of the explant only.

Fourteen d after the commencement of germination, explants were transferred from CIM to a shoot inducing medium (SIM; B5 salts with the supplements outlined above, 0.4 mg l^{-1} N^6-benzyladenine, 0.2 mg l^{-1} 1-naphthalene acetic acid, pH 5.7, solidified with 2.5 g l^{-1} Gelrite) containing the supplements (proline, kanamycin, glutamate, ornithine or proline analogues) appropriate to the particular experiment. Where used in tissue culture experiments, kanamycin or any of the amino acids or amino acid analogues were never added to CIM. The SIM used differs from that used by Valvekens et al. (1988) and Akama et al. (1992) in that N^6-(Δ^2-isopentenyl)adenine and indole-3-acetic acid used by these workers was replaced by N^6-benzyladenine and 1-naphthalene acetic acid respectively. Preliminary studies indicated that these two synthetic hormones gave a slightly better shoot induction response than their naturally-occurring counterparts. For studies involving the effects of proline, proline analogues and amino acids on shoot organogenesis, callus formation and shoot regeneration were scored 21 - 24 d after transfer to SIM or SIM containing the appropriate supplements. Evaluation of callus formation, and shoot regeneration was based on number of explants displaying the response as a percentage of the total number of explants. For these studies, each petri dish contained between 35 and 50 explants.

For the regeneration of transgenic lines, *A. tumefaciens* LBA4404 strains carrying the appropriate binary vector [pBI121, pBI-P5CS1(AS) or pBI-P5CR(AS)] were grown to mid-logarithmic phase in LB broth (pH 5.6) containing 100 mg l^{-1} kanamycin, 150 mg l^{-1} rifampicin and of 20 µM acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone, Sigma). The cells in 40 ml of the bacterial culture were pelleted by centrifugation at 25 °C and resuspended in 15 ml of LB broth (pH 5.6) without antibiotics, but containing 20 µM acetosyringone. Explants (5.0-8.0 mm in length) from callus-induced hypocotyls were co-cultivated with the resuspended bacteria for 30 min in a glass petri dish with slight agitation on an orbital shaker. The co-cultivated explants were transferred directly to SIM supplemented with 20 µM acetosyringone and 1 mM proline and incubated under continuous light at 25 °C for 48 h. After this time, slight bacterial growth around the edges of the explants was evident. The explants were washed by agitation in liquid SIM supplemented with 20 µM acetosyringone and 1 mM proline to remove the bacteria, and then transferred to SIM containing 50 mg l^{-1} kanamycin, 1 mM proline and 0.5 g l^{-1} vancomycin hydrochloride [generously donated by Lederle Laboratories, South African Cyanamid (Pty) Ltd, Isando, South Africa]. The bactericidal action of vancomycin results primarily from inhibition of bacterial cell wall synthesis, although the antibiotic also affects bacterial cell membrane permeability and RNA synthesis (Lederle).

After 28 d, green (putatively transformed) calli that had not yet formed shoots were transferred individually to culture tubes (20 mm i.d.;100 mm deep) each containing 10 ml of SIM

supplemented with 50 mg l^{-1} kanamycin and 1 mM proline. The media was solidified by 2.5 g l^{-1} of Gelrite. Calli which regenerated shoots were subsequently transferred to boiler tubes (35 mm i.d., 200 mm deep) containing 30 ml of GM supplemented with 50 mg l^{-1} kanamycin and 1 mM proline and sealed with a cottonwool bung to ensure adequate gas exchange. Under these conditions of low humidity, several of the kanamycin-resistant (Kan^r) plants set seed in sterile culture. Root formation was not a requisite for flowering *in vitro*. Seeds from the dehiscent siliques of the primary transformants (T_0 generation) were transferred under sterile conditions to GM (solidified with 8.0 g l^{-1} agar) supplemented with 50 mg l^{-1} kanamycin and 1 mM proline. Viable seeds were recovered from seven independent pBI-P5CR(AS) transformants and 13 pBI-P5CS1(AS) transformants. The green Kan^r progeny were transferred to potting medium (Section 3.6), covered with a plastic sheet and transferred to a controlled environment chamber with continuous light (125-175 µmol m^{-2} s^{-1} photosynthetically active radiation, 25 °C, 95% relative humidity). After 5 d, the sheet of plastic was removed and the plants left under the same conditions for a further 10 d before transfer to greenhouse conditions. Using this procedure, the efficiency of successful hardening-off was usually greater than 80% and none of the transgenic lines were lost at this stage. In the greenhouse, plants of different lines were separated by a radius of at least 2 m in order to prevent cross-pollination. The seeds were collected and the process of germination in the presence of kanamycin, hardening-off and growth to maturity repeated in order to obtain seeds from the T_2 generation.

3.8 Characterisation of *Arabidopsis* transformants

3.8.1 PCR-mediated gene detection

Transformation of the T_1 generation of Kan^r lines transformed with pBI121, pBI-P5CS1(AS) and pBI-P5CR(AS) was confirmed by PCR-mediated amplification of the *GUS* and *NPTII* genes from plant genomic DNA using the primer sets 5'-GGTGGGAAAGCGCGTTACAAG-3'/5'-GTTTACGCGTTGCTTCCGCCA-3' and 5'-GAGGCTATTCGGCTATGACTG-3'/5'-ATCGGGAGCGGCGATACCGTA-3' respectively. These primers were generously donated by Dr D Berger (Agricultural Research Council, Roodeplaat, South Africa). *Arabidopsis* genomic DNA was isolated from *in vitro* grown plants as described previously (Hare & Cress 1996). Each amplification reaction (50 µl total volume) contained 100 ng of genomic template DNA, 0.5 µM of each primer, 10 mM of each of dATP, dATP, dTTP and dCTP, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 % (w/v) gelatin and I.25 units of *Taq* DNA polymerase (Boehringer Mannheim, South Africa). Samples were overlaid with 50 µl of autoclaved paraffin oil and

subjected to 36 cycles of amplification using a Hybaid thermal cycler. The first 35 cycles each comprised a 94 °C melting temperature (30 s), a 60 °C primer annealing step (30 s) and a 72 °C primer extension step (45 s). During the final cycle, an extension step of 5 min was used. Upon completion of amplification, all reaction mixes were stored at -20 °C until electrophoresis on 0.8% (w/v) agarose gels. Genomic DNA from pBI-P5CS1(AS) transformants was also used as the template for amplification of the 105E5T7::*GUS* fusion between the T-DNA borders of the pBI-P5CS1(AS) construct. The H1 primer (Figure 3.1) and the *GUS* right-hand side primer (5'-GTTTACGCGTTGCTTCCGCCA-3') were used for this purpose.

3.8.2 β-glucuronidase (GUS) assays

β-glucuronidase (GUS) activity in the T_2 progeny of selected lines was assayed essentially as described by Jefferson (1987). Seven d-old seedlings were germinated and grown in liquid MS supplemented with 5.0 g l^{-1} sucrose. Media used for the growth of transgenic lines also contained 50 mg l^{-1} kanamycin. After growth on an orbital shaker under continuous light at 25 °C, excess liquid was removed by blotting the seedlings on adsorbent tissue paper. The seedlings were incubated at 37 °C for 16 h in sealed microfuge tubes containing 1 ml of the assay solution [0.5 g l^{-1} 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc, Sigma) dissolved in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100].

The seedlings were incubated for 10 min in a fixative (35% ethanol, 5% acetic acid, 2% formaldehyde), then transferred to 50% ethanol for at least 5 min and then incubated in 95% ethanol for 3 h. Two additional incubations in 95% ethanol were required to remove all of the chlorophyll in the cotyledons. The seedlings were rehydrated by transfer through a graded ethanol series (90%, 80%, 70%, 60%, 50% ethanol) before visual assessment of GUS activity.

3.9 Metabolite assays

3.9.1 Amino acids

Free proline was extracted and quantified by the spectrophotometric method described by Bates et al. (1973). Between 0.2 and 0.5 g of plant tissue was used per assay. Instead of filtration (Bates et al. 1973), the homogenate of plant tissue in 30.0 g l^{-1} of sulphosalicylic acid was clarified by centrifugation (10,000 x *g*) for 20 min at 4 °C. The reaction mixture containing ninhydrin (Merck, Darmstadt, Germany) was extracted with toluene (Bates et al. 1973) and the absorbance of the

toluene phase containing the red chromophore was measured at 520 nm using a Beckman DU-65 spectrophotometer.

For the determination of changes in free amino acids during the course of *Arabidopsis* seed germination, surface-sterilised seeds (Section 3.5) were transferred immediately to 20 ml aliquots of liquid MS/2 media in a 50 ml flasks. The flasks were incubated under continuous light (175 - 225 µmol m^{-2} s^{-1} photosynthetically active radiation) on an orbital shaker (120 rpm) at 25 °C. Samples harvested at 12h intervals throughout the germination process were frozen in liquid nitrogen after removing as much of the surface liquid as possible and stored at -70 °C until the last sample had been taken. The material was homogenised in 30.0 g l^{-1} sulphosalicylic acid. After clarification of the extract by centrifugation (14, 000 x *g* for 10 min at 4 °C), the supernatant solution was stored at - 20 °C for 72 h before total amino acid analysis using a post-column ninhydrin derivitisation HPLC-based procedure (Beckman System 6300 High Performance Amino Acid Analyser).

3.9.2 Total protein

Protein concentration was determined according to the method of Bradford (1976) using a commercially-available reagent (Bio-Rad Laboratories, Hercules, CA) according to the instructions of the manufacturer. The absorbance at 595 nm was measured using a Beckman DU-65 spectrophotometer. Serial dilutions of bovine serum albumin (Boehringer Mannheim) were used to prepare standard curves.

Degradation of *Arabidopsis* seed storage proteins was monitored using SDS-PAGE (Laemmli 1970). Total seed and seedling proteins were extracted as described by Heath et al. (1986). Proteins were resolved on a 16 cm slab gel (16% polyacrylamide) using a vertical slab gel apparatus (Model SE 400, Hoeffer Instruments, San Francisco, CA). Protein gels were stained with Coomassie Brilliant Blue R250 (Sigma) in 7% (v/v) acetic acid and 40% (v/v) methanol.

3.9.3 Chlorophyll

Determination of chlorophyll content was performed essentially as described by Graan and Ort (1984). For determinations in *Arabidopsis* seedlings, 21 d-old plantlets grown on solidified MS/2 medium with 5.0 g l^{-1} sucrose either with or without supplemental proline were excised at the interface between the hypocotyl and the root, the shoot tissue (0.1 - 0.2 g fresh weight) was

homogenised in a pestle and mortar and ground further after addition of 5 ml of 80% (v/v) acetone. The homogenate was immediately transferred to a glass test tube, sealed with Parafilm to prevent volatilisation of the acetone, and stored at 4 °C prior to its clarification by centrifugation (14, 000 x *g* for 10 min at 4 °C). The absorbance of the supernatant solution at 647 nm and 664 nm was measured using a Beckman DU-65 spectrophotometer.

3.9.4 Histochemical determination of phenylpropanoids

Histochemical analysis of the phenolic content of the hypocotyls of *Arabidopsis* seedlings involved staining with phloroglucinol, which reacts with lignin and unpolymerised precursors of lignin. Seedlings were immersed in phloroglucinol solution [8.0 g l^{-1} phloroglucinol in 60% (v/v) ethanol, 2.0 M HCl] and incubated at room temperature for 5 - 10 min. The samples were washed three times with a large excess of water to remove superfluous stain and photographed within 1 h of staining.

3.10 Enzyme assays

All spectrophotometric assays were conducted using a Beckman DU-65 spectrophotometer with the cuvette holder connected to a circulating water bath maintained at 30 °C. Aliquots of enzyme extracts used for protein determinations were stored at -70 °C until the enzyme assays were completed.

3.10.1 Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

Plant material was prepared exactly as described in Section 3.9.1 for the study involving total amino acid analysis. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were determined based on the method described by Lozano et al. (1996). Approximately 0.2 g of seed or seedling tissue was homogenised in 2 ml of extraction buffer (0.05 M Tris-HCl, pH 7.9; 0.001 M Na_2EDTA) in a pre-cooled mortar. The crude homogenate was transferred to microcentrifuge tubes held at 4 °C and cleared of debris by centrifugation (14, 000 x *g*) for 10 min. The supernatant solutions were filtered through a 0.22 µm syringe filter (Millipore) to remove residual insoluble material and maintained at 4 °C. For both assays, 50 µl of the clarified enzyme extract was added to 950 µl of the assay buffer (0.05 M Tris-

HCl, pH 7.9; 0.005 M $MgCl_2$; 0.25 mM $NADP^+$) in a quartz cuvette. After measuring the low level of background reduction of $NADP^+$ by the extract over 3 min at 30 °C, 50 µl of 0.1 M glucose-6-phosphate or 50 µl of 6-phosphogluconate was added. The contents of the cuvette were mixed by quickly inverting the cuvette and returning it to the thermostatically regulated cuvette holder. Activity of both dehydrogenases was measured by monitoring the increase in the absorbance at 340 nm, which reflects the steady state production of NADPH. The activities were corrected for endogenous $NADP^+$-reducing activities, and expressed as the rate of increase in A_{340} per µg of protein.

3.10.2 Peroxidase

For *in vitro* determination of total peroxidase activity in *Arabidopsis* seedlings, surface sterilised seeds were germinated in liquid MS medium containing 10.0 g l^{-1} sucrose. The 250 ml flasks, each containing 50 to 100 seeds in 30 ml of medium were incubated under continuous light (175 - 225 µmol m^{-2} s^{-1} photosynthetically active radiation) on an orbital shaker (120 rpm) at 25 °C. Plantlets were collected at the designated times, the excess surface liquid removed by blotting on adsorbent tissue paper, weighed and immediately frozen in liquid nitrogen. After storage overnight at -70 °C, 1.0 - 2.0 g of seedling tissue was homogenised in extraction buffer (0.05 M potassium phosphate, pH 5.8; 0.8 M KCl; 2.0 % insoluble polyvinylpyrolidone) using a pestle and mortar. Two ml of the extraction buffer were used per g fresh weight of tissue. The extract was maintained at 4 °C for at least 10 min and then clarified by centrifugation (14, 000 x *g* for 10 min). Peroxidase activity in the supernatant solution was usually examined within 3 h of extraction, although it was found that little activity was lost after even 48 h storage at 4 °C.

To assay total peroxidase activity, 0.01 ml of the clarified enzyme extract was added to 2.99 ml of the assay mixture (0.05 M potassium phosphate, pH 5.8; 0.007 M guaiacol, 0.012 M H_2O_2) and the change in the absorbance at 470 nm monitored at 30 °C using a Beckman DU-65 spectrophotometer containing a cuvette-holder which was connected to a thermostatically regulated water bath. The total peroxidase activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol and expressed on a per mg protein basis. One unit of peroxidase activity was defined as the amount of enzyme that caused the formation of 1 µmol of tetraguaiacol per min (Chen & Kao 1995).

Separation of peroxidase isoforms by electrophoresis through polyacrylamide gels was performed as described by Herbers et al. (1996). One hundred and fifty µg of protein from the crude extract described above was loaded per well in a 12% polyacrylamide gel and separated by

electrophoresis at 60 mA for 5 h. Electrophoresis was conducted at 10 °C using a vertical slab gel apparatus (Model SE 400, Hoeffer Instruments, San Francisco, CA). Gels were immediately soaked in 0.05 M sodium acetate buffer (pH 5.5) for two 15 min incubations with gentle agitation. Staining for peroxidase activity was by a modification of the procedure described by Wendel and Weeden (1989). The gel (12 cm x 14 cm) was transferred to a flat dish containing 400 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.01M $CaCl_2$. Immediately prior to commencement of the detection step, 0.2 g of 3-amino-9-ethylcarbazole (Janssen Chimica, Belgium) was dissolved in 32 ml of dimethylformamide, added to the contents of the dish and rapidly mixed with the buffer by agitation. The reaction was initiated by addition of 2 ml of 3% H_2O_2. The gel was gently agitated at room temperature. Colour development was complete within 20 min. Gels were washed in 70% (v/v) ethanol for 20 min prior to photographing. A denaturing polyacrylamide gel was loaded with duplicate samples and stained for protein using Coomassie Brilliant Blue R250 (Sigma) to check for equal loading.

3.11 Metabolism of ^{14}C-labelled glucose during *Arabidopsis* seed germination

In order to determine changes in the relative contributions of [1-^{14}C]-glucose and [6-^{14}C]-glucose to respired CO_2 throughout the course of *Arabidopsis* seed germination, plant material was prepared exactly as described in Section 3.9.1 for the study involving changes in free amino acid levels throughout the germination process. At the end of each of the indicated incubation periods, 1.0 - 2.0 g (fresh weight) of material was collected, excess surface liquid was removed and the material was weighed. The material was carefully transferred to a plastic vial (13 mm i.d., 50 mm deep) routinely used for radioactive scintillation counting and 0.5 ml of MS/2 medium containing 0.1 µCi of either D-[1-^{14}C]-glucose or D-[6-^{14}C]-glucose (approximately 60 mCi mmol^{-1}) added to the medium. Using this volume of incubation solution, it is unlikely that the tissue experienced severe anaerobiosis and that ethanolic fermentation would have contributed substantially to the $^{14}CO_2$ evolved. After ensuring that radiolabelled solution was evenly distributed over the surface of the plant material, a PCR tube (0.5 ml volume) containing 50 µl of freshly-prepared 200 g l^{-1} KOH was placed within each vial, the vials were sealed tightly with their screw caps, and the material was incubated at 25 °C. After 30 min, 60 min, 90 min and 120 min, a 12 µl aliquot of the concentrated KOH solution, which absorbed respired $^{14}CO_2$, was transferred to a scintillation vial containing 4 ml of dioxan-based scintillation fluid (Beckman Ready Value, Ireland). After 120 min incubation, a 20 µl aliquot of the incubation medium was also added to a scintillation vial containing 4 ml of scintillation fluid for assessment of the total amount of radiolabelled glucose that was taken up by the tissue. Radioactivity was measured using a Beckman LS 6000LL scintillation counter. All incubations were performed in triplicate. The C_6/C_1 ratios at each time

point were determined by dividing the amount of $^{14}CO_2$ respired from samples supplied with [6-^{14}C]-glucose (C_6) by that from samples supplied with [1-^{14}C]-glucose (C_1). All C_6 and C_1 values were normalised by their expression on a per g fresh weight basis.

3.12 Electron microscopy

3.12.1 Transmission electron microscopy

Leaves from 21 d-old plants grown on MS/2 medium with 5.0 g l^{-1} sucrose with or without supplemental proline (Section 3.9.3) were excised. Leaf segments (approximately 3 mm^2) were fixed by incubation at 4 °C for 16 h in 3% (v/v) glutaraldehyde buffered to pH 7.2 with 0.05 M sodium cacodylate. Excess glutaraldehyde was removed by two washes (30 min each) in 0.05 M sodium cacodylate (pH 7.2) before postfixation for 2 h in 2% (w/v) osmium tetroxide (in 0.05 M sodium cacodylate, pH 7.2). After two additional rinses (30 min each) in the cacodylate buffer, the samples were dehydrated in an ethanol series and embedded in Epon-Araldite resin (Electron Microscopy Sciences, Fort Washington, PA). These steps were conducted at room temperature.

Sections (80 nm thickness) were cut with a diamond knife using an LKBIII ultramicrotome and mounted on uncoated 200-mesh copper grids. Following double staining with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963), the sections were visualised using a transmission electron microscope (Model 100CX, Jeol Ltd, Tokyo, Japan) operating at an accelerating voltage of 80 kV. Sections from three separate samples for each treatment were examined.

3.12.2 Scanning electron microscopy

Ten d-old *Arabidopsis* seedlings were mounted on a copper stub using Tissue-Tek adhesive embedding medium (Miles Scientific, Napierville, IL). The stub was immediately fitted to the end of a transfer rod and plunged into a bath of liquid nitrogen contained within an Emscope SP2000 "sputter-cryo" cryogen. The stub was transferred to the specimen chamber within the cryogen under vacuum and sputter coated with a palladium/gold mixture at -140 °C. The frozen specimen, still under vacuum, was then transferred to the stage of a scanning electron microscope (Model S570, Hitachi) for visualisation. The temperature of the stage was maintained at -175 °C. For each experimental treatment, hypocotyls of two representative seedlings were analysed.

3.13 Statistical analyses

All statistical analysis was performed using the software STATGRAPHICS Version 5.0 (STN Inc., St Louis, MO). The significance of differences was evaluated using either Student's *t*-test or Duncan's multiple range test. A P value < 0.05 was considered significant and $P < 0.01$ was considered highly significant.

4. RESULTS

4.1 Characterisation of an incomplete cDNA encoding an *Arabidopsis* P5CS

The *Arabidopsis* cDNA clone 105E5T7 was obtained from the *Arabidopsis* Biological Resource Centre (Ohio State University, Columbus, OH) on the basis of its high homology to a P5CS cDNA from *Vigna aconitifolia* (Hu et al. 1992). Sequencing of 105E5T7 revealed that it contained an insert of size 1050 bp. The sequence of the 5' end of the coding strand, previously partially deduced during the initial characterisation of the EST, was confirmed using the T7 promoter-specific primer (Figure 3.1). In addition, this resolved the ambiguity in identifying the 17 nucleotides not determined in the initial characterisation of the 426 bp of the clone closest to the T7 primer (Genbank Accession No. T22627). Sequence analysis of the other end of the insert within 105E5T7 (using the SP6 promoter-specific primer) indicated that the clone is complete at the 3' end, as evidenced by a 23 bp poly(A) tail (Figure 4.1). Examination of the 279 bp of sequence deduced by priming from the 3' end of the cDNA resulted in selection of a site suitable for priming further into the 5' end of the clone. Sequencing using a 20-mer (5'-CACTACATAAGCGAGGGTTT-3', named the H1 primer; Figure 3.1) yielded a further 267 bp of sequence of the 3' end of the cDNA, including a 21 bp overlap with the sequence previously determined using the SP6 primer. Sequencing of the clones 105E5ND1 and 105E5ND2, which were generated from 105E5T7 using the nested deletions method (Henikoff 1984; Table 3.1), provided confirmation of the sequences deduced by sequencing of the opposite strand. Likewise, the results of nucleotide sequencing of both strands for sections of the insert flanked by the *Pst*I and *Bam*HI sites were unequivocal and confirmatory of one another.

The nucleotide sequence of the insert within the 105E5T7 clone and the translation product of the largest open reading frame (ORF) is shown in Figure 4.1. This is not a complete ORF as it lacks a start codon. The deduced incomplete ORF of the insert is terminated by the ochre stop codon TAA (Figure 4.1). A further 541 bp of untranslated sequence including the putative polyadenylation signals AAAATA and ATACTA (Dean et al. 1986) ends with a poly(A) tract 23 nucleotides in length (Figure 4.1).

```
   1 GAGGAAGCAA CAAGCTTGTT ACTCAGATAA AAAATACTAC AAAAATCCCT
       G  S  N  K  L  V  T  Q  I  K  N  T  T  K  I  P
  51 GTGCTAGGTC ATGCTGATGG AATCTGTCAT GTATATGTCG ACAAGGCTTG
     V  L  G  H  A  D  G  I  C  H  V  Y  V  D  K  A  C
 101 TGATACGGAT ATGGCAAAGC GCATAGTTTC TGATGCAAAG TTGGACTATC
       D  T  D  M  A  K  R  I  V  S  D  A  K  L  D  Y  P
 151 CAGCAGCCTG TAATGCGATG GAAACCCTTC TTGTGCATAA GGATCTAGAG
       A  A  C  N  A  M  E  T  L  L  V  H  K  D  L  E
 201 CAGAATGCTG TGCTTAATGA GCTTATTTTT GCTCTGCAGA GCAATGGAGT
     Q  N  A  V  L  N  E  L  I  F  A  L  Q  S  N  G  V
 251 CACTTTGTAT GGTGGACCAA GGGCAAGTAA GATACTGAAC ATACCAGAAG
       T  L  Y  G  G  P  R  A  S  K  I  L  N  I  P  E  A
 301 CACGGTCATT CAACCATGAG TACTGTGCCA AGGCTTGCAC TGTTGAAGTT
       R  S  F  N  H  E  Y  C  A  K  A  C  T  V  E  V
 351 GTAGAAGACG TTTATGGTGC TATAGATCAC ATTCACCGAC ATGGGAGTGC
     V  E  D  V  Y  G  A  I  D  H  I  H  R  H  G  S  A
 401 ACACACAGAC TGCATTGTGA CAGAGGATCA CGAAGTTGCA GAGCTATTCC
       H  T  D  C  I  V  T  E  D  H  E  V  A  E  L  F  L
 451 TTCGCCAAGT GGACAGCGCT GCTGTGTTCC ACAACGCCAG CACAAGATTC
       R  Q  V  D  S  A  A  V  F  H  N  A  S  T  R  F
 501 TCAGATGGTT TCCGATTTGG ACTTGGTGCA GAGGTGGGGG TAAGCACGGG
     S  D  G  F  R  F  G  L  G  A  E  V  G  V  S  T  G
 551 CAGGATCCAT GCTCGTGGTC CAGTCGGGGT CGAAGGATTA CTTACAACGA
       R  I  H  A  R  G  P  V  G  V  E  G  L  L  T  T  R
 601 GATGGATAAT GAGAGGAAAA GGACAAGTTG TCGACGGAGA CAATGGAATT
       W  I  M  R  G  K  G  Q  V  V  D  G  D  N  G  I
 651 GTTTACACCC ATCAGGACAT TCCCATCCAA GCTTAAACAA GACTTCCGAG
     V  Y  T  H  Q  D  I  P  I  Q  A  *
 701 TGTGTGTTTG TGTATTTGGT TGAGACTTGA GGAGAGACAC AGAGGAGGAT
 751 GGGCTTTTTT GTTTCCTCTC TGCTTAGTAC TCATATCCTA TCATTATTAT
 801 TATTACTACT ACTTATTATT GAAACCCTCG CTTATGTAGT GGTTTTGATT
 851 TAGGGTTAGG ATTGCACCAA AAATAAGATC CACTTTACCA CTTAGTCTTG
 901 CTCATAAGTA CGATGAAGAA CATTTAATTA GCTTCTCTTC TTGTCATTGT
 951 AAGCTACCTA CACATTTCTG ATCTTTATCA AGATACTACT ACTTTTCATT
1001 TCGCTTATCT ATAAATATAT TTCGATTAAA AAAAAAAAAA AAAAAAAAAA
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Figure 4.1: Nucleotide sequence and translation product of the insert within 105E5T7. The deduced amino acid sequence indicated below the nucleotide sequence represents the translation product of the largest incomplete open reading frame within the insert of 105E5T7. A putative leucine zipper motif in the translation product is underlined. The translation stop codon is marked by an asterisk. The *Pst*I (cleavage site at nucleotide position 239) and *Bam*HI (cleavage site at nucleotide position 553) recognition sites which were used in the subcloning of the fragment found in 105E5PB (Table 3.1) are in bold. Putative polyadenylation signal sequences (Dean et al. 1986) are underlined twice.

Subsequent reports of the complete sequences of cDNAs encoding P5CS from *Arabidopsis* (Savouré et al.1995; Yoshiba et al. 1995; Strizhov et al. 1997) confirmed that the deduced nucleotide sequence of the insert within is identical to the corresponding region of the complete *AtP5CS1* cDNA sequence determined by these workers. A second isoform of P5CS in *Arabidopsis*, encoded by the *P5CS2* gene, has subsequently been characterised (Strizhov et al. 1997). An alignment of the sequence encoded by the insert within 105E5T7 with the published amino acid sequences of both *Arabidopsis* P5CS proteins and those characterised from six other plant species is shown in Figure 4.2. At the time of submission of this document, this appears to be the most comprehensive alignment of plant P5CS sequences.

4.2 Characterisation of the 5'-UTRs of *AtP5CS1*, *AtP5CS2* and *AtP5CR*

While it is well known that gene expression may regulated at several different levels (e.g. the regulation of mRNA transcript stability, translational initiation, protein turnover and post-translational modification of the activity of the gene product), the regulation of transcriptional initiation is accepted to be an important mechanism whereby all organisms selectively express genes under the appropriate developmental and environmental conditions. Responses of eukaryotic cells to both endogenous signals and external stimuli are at least in part modulated by changes in gene expression at the level of promoter activity. In general, a given promoter contains several discrete functional modules, each of which contains one or more recognition sites for proteins that are required for the initiation of transcription. Both the sum and the interplay between several constituent *cis*-acting elements explains the full regulative capacity of a promoter. Several of the proteins within the transcriptional complex are likely to affect the overall level of transcription of the gene, whereas others act as regulatory elements that determine tissue-specificity and the response to external cues. Since these cues regulate the expression of a range of genes, binding sites within a given promoter are likely to reside in the promoters of other genes that are subject to regulation by the same stimuli. Any assessment of the physiological function of a metabolic process should thus include examination of the 5' untranslated regions (5'-UTRs) of the genes which regulate that process.

Sequences of the 5'-UTRs of *AtP5CS2* and *AtP5CR* have been published. Surprisingly, Zhang et al. (1997) did not analyse the 5'-UTR of *AtP5CS2* for regions with homology to known plant promoter elements. Furthermore, the rapid advances in our understanding of plant promoter elements over the past five years now enable a far more extensive interpretation of the 5'-UTR of the *AtP5CR* gene (Verbruggen et al. 1993) than was possible when this sequence was published. These workers have subsequently used fusions of 5' deletions of the *AtP5CR* promoter

```
AtP5CS1   ME E - L DR S R AF A R DV K R I V V K V G T A V V T G K G G R L A L G R L G A L C E Q L A E L N S D G F E  54
AtP5CS2   MT E - I DR S R AF A K DV K R I V V K G V T A V V T G K G G R L A L G R L G A I C E Q L A E L N S D G F E  54
VaP5CS    ME S A V DP S R G F MK DV K R V I I K V G T A V V T R E E G R L A V G R L G A L C E Q I K Q L N S L G Y D  55
OsP5CS    MA S - V DP S R S F V R DV K R V I I K V G T A V V S R Q D G R L A L G R V G A L C E Q V K E L N S L G Y E  54
LeP5CS    ME - T V DS T R AF V K N V K R L I V K V G T A V V T R A D G R L A L G R L G A L C E Q L Q E L N S Q G Y E  54
MsP5CS    MA N A - DP C R E F V K DV K R I I I K V G T A V V T R Q D G R L A V G K L G A L C E Q I K E L N I L G Y E  54
AdP5CS    MD - A V DS T R AF V K G V K R V I I K V G T A V V T R A D G R L A L G R L G A L C E Q I H E L N S Q G F E  54
McP5CS    MD - A - - - T R AF V K DV K R V V V K V G T A V V T R S D G R L A L G R L G S L C E Q L K E L N S D G Y E  51

                    ATP-binding site
AtP5CS1   V I L V S S G A V G L G R Q R L R Y R Q L V N S S F A D L Q K P Q T E L D G K A C A G V G Q S S L M A Y Y E T 109
AtP5CS2   V I L V S S G A V G L G R Q R L R Y R Q L V N S S F A D L Q K P Q M E L D G K A C A G V G Q S S L M A Y Y E T 109
VaP5CS    I I L V S S G P V G I G R Q R L R F R K L I N S S F A D L Q K P Q L E L D G K A C A A V G Q N S L M A L Y D T 110
OsP5CS    V I L V T S G A V G V G R Q R L R Y R K L V N S S F A D L Q K P Q M E L D G K A C A A V G Q S G L M A L Y D M 109
LeP5CS    V I L V T S G A V G V G R Q R L R Y R K L L N S S F L D L Q K P Q T E L D G K A C A A V G Q N G L M A L Y S S 109
MsP5CS    V I L V S S G A V G L G R Q R L R Y R K L I Q S S F A D L Q K P Q V E L D G K A C A A V G Q S S L M A T Y D I 109
AdP5CS    V I L V T S G A V G V G R Q R L R Y R K L V N S S F A D L Q K P Q I E L D G K A C A A V G Q N G L L A L Y D T 109
McP5CS    V I L V T S G A V S A G R Q R L R F R K L V N S S F A D L Q K P Q V E L D G K A C A A V G Q N G L M A L Y D T 106

                                  *   *
AtP5CS1   M F D Q L D V T A A Q L L V N D S S F R D K D F R K Q L N E T V K S M L D L R V I P I F N E N D A I S T R R A 164
AtP5CS2   M F D Q L D V T V A Q M L V T D S S F R D K D F R K Q L S E T V K A M L R M R V I P V F N E N D A I S T R R A 164
VaP5CS    L F T Q L D V T S A Q L L V T D N D F R D K D F R K Q L T E T V K S L L A L K V I P V F N E N D A V S T R K A 165
OsP5CS    L F N Q L D V S S S Q L L V T D S D S E N P K F R E Q L T E T V E S L L D L K V I P I F N E N D A I S T R K A 164
LeP5CS    L F S Q L D V T S A Q L L V T D N D F R D P D F P R Q L N D T V N S L L S L K V I P I F N E N D A I S T R R A 164
MsP5CS    L F S Q L D V T S A Q L L V T D N D F R D Q D F R K Q L S E T V R S L L A L K V I P I F N E N D A V S T R K A 164
AdP5CS    L F S Q L D V T S A Q L L V T D N D F R D P E F R K Q L T E T V E S L L N L K V I P I F N E N D A V S T R K A 164
McP5CS    L F S Q L D L T A A Q L L V T D N D F R D P S F R T Q L T E T V Y Q L L D L K V V P V L N E N D A V S T R K A 161

                          conserved leucine zipper
AtP5CS1   P Y Q D S S G I F WD N D S L A A L L A L E L K A D L L I L L S D V E G L Y T G P P S D P N S K L I H T F V K 219
AtP5CS2   P Y K D S T G I F WD N D S L A A L L S L E L K A D L L I L L S D V E G L Y T G P P S D S T S K L I H T F I K 219
VaP5CS    P Y E D S S G I F WD N D S L S A L L A L E L K A D L L V L L S D V E G L Y S G P P S D P H S K L I Y T Y N K 220
OsP5CS    P Y E D S S G I F WD N D S L A G L L A L E L K A D L L I L L S D V D G L Y S D P P S E P S S K I I H T Y I K 219
LeP5CS    P Y E D S S G I F WD N D S L A A L L A L E L K A D L L V L L S D V D G L Y S G P P R D P D S K L I H T Y I K 219
MsP5CS    P Y E D S S G I F WD N D S L S A L L A L E L K A D L L I L L S D V D G L Y N G P P S D P L S K L I H T Y I K 219
AdP5CS    P Y E D A S G I F WD N D S L A A L L A L E L K A D L L V L L S D V E G L Y S G P P S D P Q S K L I H T Y I K 219
McP5CS    P Y E D S S G I F WD N D S L A A L L A L E L K A D L L I L L S D V D G L Y N G P P S D P R S K L I S T Y V K 216
```

Figure 4.2 (legend on p. 161)

putative γ-GK domain

```
AtP5CS1   EKHQDEITFGDKSRLGRGGMTAKVKAAVNAAYAGIPVIITSGYSAENIDKVLRGL 274
AtP5CS2   EKHQDEITFGEKSKLGRGGMTAKVKAAVNAAYGGVPVIITSGYAAENISKVLRGL 274
VaP5CS    EKHQNEITFGDKSRVGRGGMTAKVKAAVHAAEAGIPVVITSGFAPENIINVLQGQ 275
OsP5CS    EKHQQEITFGDKSRVGRGGMTAKVKAAVLASNSGTPVVITSGFENRSILKVLHGE 274
LeP5CS    EIHERVITFGDKSRVGRGGMTAKVKAAMYAAYAGIPVVITSGFATDNIIKVLHGE 274
MsP5CS    EKHQNEITFGDKSRVGRGGMTAKVKASVHAADAGIPVIITSGNAAENLTKILQGQ 274
AdP5CS    EMFEGLITFGDKSRVGRGGMTAKVKAAVYAAHAGIPVVITSGYATNNIIKVLQGE 274
McP5CS    EKHQGEITFGDKSRLGRGGMTAKVKAAVYAAYAGIPVIIASGKATDNIIKVIDGQ 271

AtP5CS1   RVGTLFIIQDARLWAPITDSNARDMAVAA---RESSRKLQ---------------- 310
AtP5CS2   RVGTLFIIQDAHLWAPVVDTTSRDMAVAA---RESSRKLQ---------------- 310
VaP5CS    RIGTLFIIKDAHEWAQVKEVDAREMAVAAGNVREGSRRY---------------- 312
OsP5CS    KIGTLFIIKNANLWESSKDVSTREMAVAA---RDCSRHLQ---------------- 310
LeP5CS    RIGTLFIICDANKWASIGETDAREMAVAA---RACSRRLQ---------------- 310
MsP5CS    RIGTLFIIKDAHKWVPSKEVDVREMAVAA---RDCSRRLQVSLISEQLENVSRIPG 325
AdP5CS    RIGTLFIIRDAQKWAPVGDVGARDMAVAA---RESSRRLQ---------------- 310
McP5CS    CVGTLFIIKDAHLWVQVKETGVRDMAVAA---RESSRRLQ---------------- 307

AtP5CS1   ----------------------ALSSEDRKKILLDIADALEANVTTIKAENELDVA 344
AtP5CS2   ----------------------ALSSEDRKQILHDIANALEVNEKTIKAENDLDVA 344
VaP5CS    ----------------------LQRKG-NKILLKIADALEANEKIIRIENEADVT 344
OsP5CS    ----------------------NLSSEERKKILLDVADALEANEDLIRSENEADVA 344
LeP5CS    ----------------------ALSSQERSKILQDIADALEANEKAILAENEADVV 344
MsP5CS    YYQASVYRSFQNMLISNLTLQAVSSEERKQILLNIADALQSREKEIRIENEADVV 381
AdP5CS    ----------------------AMSPQDRSKILLDVADALEANEKLIRIENEADLA 344
McP5CS    ----------------------AVSSEERKKILLDIADALEANEEKILAENEADVA 341

AtP5CS1   SAQEAGLEESMVARLVMTPGKISSLAASVRKLADMEDPIGRVLKKTEVADGLVLE 399
AtP5CS2   AAQEAGYEESLVARLVMKPGKISSLAASVRQLAEMEDPIGRVLKKTQVADDLILE 399
VaP5CS    AAQEAGYEKSLVARLALKPGKIASLANNMRIIANMEDPIGRVLKRTELSDGLILE 399
OsP5CS    AAQVAGYEKPLVARLTIKPGKIASLAKSIRTLANMEDPINQILKKTEVADDLVLE 399
LeP5CS    AAQQAGYEKSLISRLALNPGKISSLANSVRVLSNMDEPLGHTLKRTEIADGFILE 399
MsP5CS    AAQEAGYEKSLVARLVLKSEKIVGLANNIRIIANMEDPIGRVLKRTELAEGVILE 436
AdP5CS    AAQQAGYEKSLISRLALKSGKISSLAKSIRVLANMEEPIGHVLKRTEITDGLVLE 399
McP5CS    AAQYARYDRSLVARLAMNPDKISSLAKSIRVLADMEEPIGRILKRTEIADGLILE 396
```

Figure 4.2 (legend on p. 161)

NADPH binding

```
AtP5CS1   KTSSPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEARRSNAILHKVITD 454
AtP5CS2   KTSSPIGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEARRSNAILHKVITD 454
VaP5CS    KTSSPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEAKRSNAILHKVIIE 454
OsP5CS    KTSCPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEAIRSNTILHKVITD 454
LeP5CS    KSSSPLGVVLIIFESRPDALVQIASLAVRSGNGLMLKGGKEAKRSNAILHKVITS 454
MsP5CS    KTSSPLGVLLIIFESRPDALVQIASLAIRSGNGLLLKGGKEANRSNAILHKVITE 491
AdP5CS    KTSSPLGVLLIIFESRPDALVQIASLAIRSGNGLVLKGGKEAKRSNAILHKVITS 454
McP5CS    KTSCPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEAKRSNAILHKVITS 451
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```
105E5T7   GSNKLVTQIKNTTKIPVLG 19
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site

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AtP5CS1   AIPETVGGKLIGLVTSREEIPDLLKLDDVIDLVIPRGSNKLVTQIKNTTKIPVLG 509
AtP5CS2   AIPETVGGKLIGLVTSREEIPDLLKLDDVIDLVIPRGSNKLVSQIKNSTKIPVLG 509
VaP5CS    AIPDNVGGKLIGLVTSREEIPELLKLDDVIDLVIPRGSNKLVSQIKSSTKIPVLG 509
OsP5CS    AIPRNVGEKLIGLVTTRDEIADLLKLDDVIDLVTPRGSNKLVSQIKASTKIPVLG 509
LeP5CS    AIPVSVGERLIGLVTSREEIPELLKLDDVIDLVIPRGSNKLVSQIKASTKIPVLG 509
MsP5CS    AIPDTVGSKLIGLVTSRAEIPELLKLDDVIDLVIPRGSNKLVSDIKSSTKIPVLG 546
AdP5CS    AIPENVGPRLIGLVTSREEIPDLLKLDDVIDLVIPRGSNKLVSQIKESTKIPVLG 509
McP5CS    AIPDKVGEKLIGLVTSRDEIPDLLKLDDVIDLVIPRGSNKLVSQIKESTRIPVLG 506
```

putative

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105E5T7   HADGICHVYVDKACDTDMAKRIVSDAKLDYPAACNAMETLLVHKDLEQNAVLNEL 74
AtP5CS1   HADGICHVYVDKACDTDMAKRIVSDAKLDYPAACNAMETLLVHKDLEQNAVLNEL 564
AtP5CS2   HADGICHVYVDKSGKLDMAKRIVSDAKLDYPAACNAMETLLVHKDLEQNGFLDDL 564
VaP5CS    HADGICHVYVDKSANVEMAKRIVLDAKVDYPAACNAMETLLIHKDLIEKGWLKEI 564
OsP5CS    HADGICHVYIDKSADMDMAKLIVMDAKTDYPAACNAMETLLVHKDLMKSPGLDDI 564
LeP5CS    HADGICHVYVDKSADMDMAKRITVDAKIDYPAACNAMETLLVHKDLAQNGGLNDL 564
MsP5CS    HADGICHVYVDKSANLEMAKQIVLDAKTDCPSGCNAMETLLVHKDLVEKGWLNSI 601
AdP5CS    HADGICHVYVDKSANMDMAKKVVLDAKTDYPAACNAMETLLVHKDLVQNGCLDEL 564
McP5CS    HADGICHVYVDKSANMDMAKRIVLDAKTDYPAACNAMETLLVHKDLAENGGLNDL 561
```

leucine zipper domain

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105E5T7   IFALQSNGVTLYGGPRASKIL--NIPEARSFNHEYCAKACTVEVVEDVYGAIDHI 127
AtP5CS1   IFALQSNGVTLYGGPRASKIL--NIPEARSFNHEYCAKACTVEVVEDVYGAIDHI 617
AtP5CS2   IYVLQTKGVTLYGGPRASAKL--NIPETKSFHHEYSSKACTVEIVEDVYGAIDHI 617
VaP5CS    ILDLRTEGVILYGGPVASSLL--NIPQAHSFHHEYSSLACTAEIVDDVYAAIDHI 617
OsP5CS    LVALKTEGVNIYGGPIAHKAL--GFPKAVSFHHEYSSMACTVEFVDDVQSAIDHI 617
LeP5CS    IVELQTKGVSLYGGPKASSLL--MIPEARTFRHEYSSLACTVEVVEDVYAAIDHI 617
MsP5CS    SDDLRSEGVTLYGGPKASSLL--NVPLARSLHHEYCSLACTLEIVDDVYAAIHHI 654
AdP5CS    IVELQIKGVVIHGGPRASSLL--HIPEARSLHHEYSSLACTIEIVDDVYAAIDHI 617
McP5CS    IVDLRTEGVTMFGGPRIDALQEFNIQATQTFNREYSSPACTVEIVDDVYAAIEHI 616
```

putative GPR

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105E5T7   HRHGSAHTDCIVTEDHEVAELFLRQVDSAAVFHNASTRFSDGFRFGLGAEVGVST 182
AtP5CS1   HRHGSAHTDCIVTEDHEVAELFLRQVDSAAVFHNASTRFSDGFRFGLGAEVGVST 672
AtP5CS2   HQHGSAHTDCIVTEDSEVAEIFLRQVDSAAVFHNASTRFSDGFRFGLGAEVGIST 672
VaP5CS    NLYGSAHTDSIVAEDNEVANVFLRQVDSAAVFHNASTRFSDGARFGLGAEVGIST 672
OsP5CS    HRYGSAHTDCIVTTDDKVAETFLRRVDSAAVFHNASTRFSDGARFGLGAEVGIST 672
LeP5CS    HQHGSAHTDSIITEDQEVAEVFLRQVDSAAVFHNASTRFSDGFRFGLGAEVGIST 672
MsP5CS    NLYGSAHTDSIVTEDHEVADVFLRQVDSAAVFHNASTRFSDGARFGLGAEVGIST 709
AdP5CS    HRHGSAHTDSIITEDHEVAEIFLRQVDSSSVLHNASTRFSDGARFGLGAEVGIST 672
McP5CS    NHHGSAHTDCIIAEDHKVAETFLQLVDSAAVLHNASTRFCDGFRFGLGAEVGIST 671
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domain

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105E5T7   GRIHARGPVGVEGLLTTRWIMRGKGQVVDGDNGIVYTHQDIPIQA          227
AtP5CS1   GRIHARGPVGVEGLLTTRWIMRGKGQVVDGDNGIVYTHQDIPIQA          717
AtP5CS2   SRIHARGPVGVEGLLTTRWIMRGKGQVVDGDNGIVYTHKDLPVLQRTEAVENGI 726
VaP5CS    SRIHARGPVGVEGLLTTRWILKGRGQVVDGDRGVVYTHKDLAI            715
OsP5CS    GRIHARGPVGVEGLLTTRWILRGRGQVVNGDKDVVYTHKSLPLQ           716
LeP5CS    GRIHARGPVGVEGLLTTKWLARGSGQIVDGDKSIVYSHKDLTQQG          717
MsP5CS    SRIHARGPVGVDGLLTTRWLLKGSGQVVDGDKTVTYTHKDLTT            752
AdP5CS    SRIHARGPVGVEGLLTTRWIARGSGQVVDGDKGIVYTHKDLTSHA          717
McP5CS    SRIHARGPVGVEGLLTTRWVLKGSGQVVHGDKGVVYTHKDLPLVAQNS       719
```

Figure 4.2. Alignment of the deduced translation product of the insert within 1505E5T7 with complete plant P5CS sequences. The sequences used are from *Arabidopsis thaliana* (AtP5CS1, Genbank Accession No. X86777; AtP5CS2, Genbank Accession No. Y09355), *Vigna aconitifolia* (VaP5CS, Genbank Accession No. M92276), *Oryza sativa* (OsP5CS, Genbank Accession No. D49714), *Lycopersicon esculentum* (LeP5CS, Genbank Accession No. U60267), *Medicago sativa* (MsP5CS, Genbank Accession No. X98421), *Actinidia deliciosa* (AdP5CS, Genbank Accession No. U92286) and *Mesembryanthemum crystallinum* (McP5CS; Genbank Accession No. AF067967). The sequences were aligned visually. Amino acid residues which display absolute conservation at that position in all eight plant P5CS sequences are indicated in bold. Asterisks indicate the positions of two residues in VaP5CS (Asp_{126} and Phe_{129}) which are responsible for feedback inhibition of VaP5CS activity by proline (Zhang et al. 1995). Putative domains within plant P5CS proteins are assigned based on the alignment of AtP5CS1, AtP5CS2 and VaP5CS sequences with γ-GK and GPR sequences from *Escherichia coli* and *Serratia marcescens* (Strizhov et al. 1997).

to the β-glucuronidase (*GUS*) gene to study the tissue-specific and developmental regulation of the gene at the level of transcriptional initiation (Hua et al. 1997). Nonetheless, analysis of *AtP5CR* promoter activity during stress was not reported. Recently, the 5'-UTR of the *AtP5CS1* gene has become available following the submission to Genbank of the sequence of a 92,624 bp genomic clone (BAC T5I7; Genbank Accession No. AC003000) by workers at the Institute for Genomic Research (Rockville, MD). The complement of the region between nucleotides 39,436

and 45,572 in BAC T5I7 is identical to that of a genomic clone encoding *AtP5CS1* (Savouré et al. 1995; Genbank Accession No. X89414) which included only 100 bp of untranslated sequence upstream of the start codon. Consistent with the mapping data of Savouré et al. (1995) and Strizhov et al. (1997), the BACT7I7 sequence is derived from genomic DNA from chromosome 2 of *Arabidopsis* (Genbank Accession No. AC003000). A search for other ORFs upstream of the *AtP5CS1* sequence suggests that the nearest coding sequence occurs approximately 10 kb upstream from the start codon of *AtP5CS1* (Genbank Accession No. AC003000). The approximately 2.5 kb sequence used in the analysis of the 5' regulatory region of *AtP5CS1* (Appendix) is thus unlikely to contain any coding regions for other gene products.

In view of the comparative absence of any analysis of the 5'-UTRs of *AtP5CS1*, *AtP5CS2* and *AtP5CR* thus far, these sequences were examined for sequence homologies with known promoter elements in an attempt to elucidate some likely mechanisms by which transcription of these proline biosynthetic genes may be regulated.

4.2.1 MYB recognition sites

Most strikingly, the 5'-UTRs of all three genes contain several perfect matches with previously defined consensus recognition sites for MYB-related transcription factors (Table 4.1). The specific oxidation or reduction of nuclear proteins is an important regulatory mechanism that can affect their DNA binding activity and thus affect gene expression (Section 2.2.2.3). In higher plants, a large family of genes encode transcription factors structurally related to the c-MYB protooncogene family of mammals (Martin & Paz-Ares 1997; Romero et al. 1998). These play a wide role in the regulation of processes as diverse as phenylpropanoid metabolism (Moyano et al. 1996; Grotewold et al. 1998; Tamagnone et al. 1998), morphogenesis (Martin & Paz-Ares 1997; Kirik et al. 1998), phytochrome-mediated changes in gene expression (Wang et al. 1997), circadian timing (Schaffer et al. 1998; Wang & Tobin 1998) and the response to dehydration stress (Urao et al. 1993; Abe et al. 1997). Like c-MYB, the mammalian prototype of all eukaryotic MYB-domain proteins (Myrset et al. 1993), the binding of plant MYB-domain proteins to specific DNA sequences appears to be sensitive to the cellular redox status. At least three plant MYB-domain proteins have been shown to bind DNA only in the reduced state (Martin & Paz-Ares 1997; Williams & Grotewold 1997).

It was previously proposed that the ABA- and dehydration inducible *AtMYB2* gene product from *Arabidopsis* (Urao et al. 1993, 1996, Abe et al. 1997) may regulate expression of *AtP5CS1*, since both of these genes are overexpressed in the *sos1* mutant (Liu & Zhu 1997a). A subsequent

Table 4.1. Putative MYB recognition sequences within the 5'-UTRs of *Arabidopsis* genes involved in proline synthesis from glutamate. Complete sequences of the 5'-UTRs used in this analysis of the *AtP5CS1* (Genbank Accession No. AC003000), *AtP5CS2* (Zhang et al. 1997) and *AtP5CR* (Verbruggen et al. 1993) genes are provided in the Appendix. Putative TATA boxes are found at positions -153, -412 and -73 for *AtP5CS1*, *AtP5CS2* and *AtP5CR* respectively[1] (Appendix).

Consensus	*AtP5CS1*		*AtP5CS2*		*AtP5CR*		Reference
	Pos.[1]	Sequence	Pos.[1]	Sequence	Pos.[1]	Sequence	
CC(T/A)ACC	- 1206 - 609 -225	CCAACC[2] CCAACC CCTACC	- 790 - 722	CCAACC[2] CCTACC	- 865	CCAACC	Iturriaga et al. (1996)
(T/C)AAC(T/G)G	- 630 - 54	CAACGG[2] TAACTG[2]	-1289	CAACTG	- 881 - 218	CAACGG CAACTG	Iturriaga et al. (1996)
(T/C)AAC(T/C)(A/G)	- 2282 - 2255 - 2236 - 2048 -1368 - 599	TAACTA TAACCA[2] TAACTA[2] TAACTA TAACTG[2] TAACCA	- 791 - 777 - 658 - 335 - 311	CAACCA[2] TAACTA[2] TAACTA TAACTA[2] CAACCG	- 864 - 820 - 218	CAACCG CAACTA CAACTG	Abe et al. (1997) Shinozaki & Yamaguchi-Shinozaki (1997)
TTTGTTA	- 2473 - 249	TTTGTTA[2] TTTGTTA	-1053 - 632 - 361	TTTGTTA[2] TTTGTTA TTTGTTA[2]	-	-	Gubler et al. (1995)
GGATA	- 2278 - 1589	GGATA[2] GGATA[2]	- 501 - 47	GGATA GGATA[2]	-	-	Baranowskij et al. (1994)
TGGTAGGT	- 2377 - 1574	TGGTACGT[2] TGGTAGCT[2]	- 723	GGGTAGGA[2]	-	-	Li & Parish (1995)
(A/G/T)GGT(A/T)GGT	- 2163 - 1207 - 226	GGGTGGGC AGGTTGGC GGGTAGGT[2]	-	-	- 208 - 122	GGGTCGGA GGGTAGTT	Grotewold et al. (1994)
GTTAGGTT	- 891	ATTAGGTT	-	-	-	-	Sablowski et al (1994)

[1] For the *AtP5CS2* and *AtP5CR* 5'-UTRs, the position indicates the 5' end of the sequence upstream of the transcription initiation site. The second, downstream transcription initiation site (Zhang et al. 1997) was used in the case of *AtP5CS2*. This second transcription initiation site occurs 76 nucleotides downstream from the first transcription initiation site (Appendix, Zhang et al. 1997). Since the transcription initiation site(s) for *AtP5CS1* has not yet been defined, the position indicates the 5' end of the sequence upstream from the translation initiation site (start codon).

[2] The sequence indicated is found on the complement of the coding strand.

study found that NaCl-mediated induction of both *AtMYB2* and *P5CS1* was much stronger in *sos2* than in wild-type *Arabidopsis* seedlings (Zhu et al. 1998). Consistent with this proposal, the TAACTG recognition site used in the characterisation of AtMYB2 is found at two positions (-54 and -1368 upstream from the translation initiation site) in the *AtP5CS1* 5'-UTR (Table 4.1). Although this particular sequence is not found in either of the *AtP5CS2* or *AtP5CR* 5'-UTRs, it is worth noting that this target site may not be the optimal binding sequence for AtMYB2 (Urao et al. 1996). Where characterised, most plant MYB-domain proteins appear to have a relatively broad DNA binding specificity (Li & Parish 1995; Solano et al. 1995; Sainz et al. 1997; Uimari & Strommer 1997).

4.2.2 ABA-related and other stress-related promoter elements

The extensive amount of literature implicating the involvement of ABA in mediating stress-induced proline accumulation in several species (McDonnell et al. 1983; Stewart & Voetberg 1985; Chou et al. 1991; Ober & Sharp 1994; Igarashi et al. 1997) including *Arabidopsis* (Finkelstein & Somerville 1990; Yoshiba et al. 1995; Savouré et al. 1997; Strizhov et al. 1997) prompted investigation of whether any of the 5'-UTRs of *Arabidopsis* genes involved in proline synthesis from glutamate contain promoter elements which have previously been implicated in ABA-mediated changes in gene expression (Section 2.4.3.2). These results are presented in Table 4.2.

Interestingly, both of the *AtP5CS1* and *AtP5CS2* 5'-UTRs contain perfect matches with the consensus ABRE (Table 4.2). Two sequences which contain the CACC-core of the ABA coupling element CE1 (Shen & Ho 1997) are found immediately upstream of the putative ABRE in the *AtP5CS2* 5'-UTR (Table 4.2). Consistent with an inability of ABA to induce substantial *AtP5CR* transcript accumulation (Yoshiba et al. 1995; Savouré et al. 1997), this promoter does not contain any perfect matches with the ABRE consensus sequence, although a region with weak homology is found on the complement of the coding strand (Table 4.2). No CE1-like sequences are found in either of the *AtP5CS1* or *AtP5CR* 5'-UTRs.

Sequences similar to the recently identified GC-rich *RAB* activator (GRA) motif (CACTGGCCGCCC) were found in the 5'-UTRs of both the *AtP5CS2* and *AtP5CR* genes (Table 4.2). The GRA element of the maize *RAB17* gene is important for both basal and ABA-induced transcription in leaves, but is inactive in embryos (Busk et al. 1997). Both the *AtP5CS1* 5'-UTR and the *AtP5CR* 5'-UTRs contain putative Sph elements (Table 4.2) The palindromic Sph element apparently mediates both positive and negative regulation of ABA-responsive genes (Busk et al. 1997). Interestingly, although none of the putative Sph sites display complete identity with the

Table 4.2. Putative ABA-responsive promoter elements within the 5'-UTRs of *Arabidopsis* genes involved in proline synthesis from glutamate. Complete sequences of the 5'-UTRs used in this analysis of the *AtP5CS1* (Genbank Accession No. AC003000), *AtP5CS2* (Zhang et al. 1997) and *AtP5CR* (Verbruggen et al. 1993) genes are provided in the Appendix. Putative TATA boxes are found at positions -153, -412 and -73 for *AtP5CS1*, *AtP5CS2* and *AtP5CR* respectively[1] (Appendix).

Consensus	*AtP5CS1*		*AtP5CS2*		*AtP5CR*		Reference
	Pos.[1]	Sequence	Pos.[1]	Sequence	Pos.[1]	Sequence	
• ABA response element (ABRE)							
(C/T)ACGTGGC	- 2566 - 209	TACGTGGA[2] CACGTGGC	- 805	CACGTGGC	- 252	GACGTGGA[2]	Shinozaki & Yamaguchi-Shinozaki (1997)
• ABA coupling element (CE1)							
TGCCACCGG	-	-	- 874 - 821 - 58	CCCCACCAC CACCACCAC CCACACCGG	-	-	Shen & Ho (1997)
• GR-rich *RAB* activator (GRA)							
CACTGGCCGCCC	-	-	-1016 - 907 - 645	TACGCGCCGCCA[2] TCTCAGCCGCCA ATATGGCCGAAT[2]	- 457 - 41	AAAGGGTCGCCT[2] GGGAGGCCGCGA	Busk et al. (1997)
• Sph element							
CATGCATG	- 2302 - 1720	TATGCATA CATGCATT	-	-	- 238	GATGCATC	Busk et al. (1997)
• ABRE2 element							
CACACGTCC	-	-	- 815 - 35	CACACGTCA TCCACGTCC	- 252	TCCACGTCA	Busk et al. (1997)

[1] For the *AtP5CS2* and *AtP5CR* 5'-UTRs, the position indicates the 5' end of the sequence upstream of the transcription initiation site. The second, downstream transcription initiation site (Zhang et al. 1997) was used in the case of *AtP5CS2*. This second transcription initiation site occurs 76 nucleotides downstream from the first transcription initiation site (Appendix, Zhang et al. 1997). Since the transcription initiation site(s) for *AtP5CS1* has not yet been defined, the position indicates the 5' end of the sequence upstream from the translation initiation site (start codon).

[2] The sequence indicated is found on the complement of the coding strand.

consensus Sph motif, two of these elements are palindromes (Table 4.2). The possibility that the palindromic nature of the Sph site may impact on its functioning, taken together with the relatively close location of the putative Sph element in *AtP5CR* relative to the TATA box, suggest that this motif may participate in the regulation of *AtP5CR* transcriptional initiation.

As has already been discussed (Section 2.4.3), considerable evidence indicates that many stress-regulated genomic responses occur independently of ABA action. Some of the promoter elements that have been implicated in ABA-independent changes in stress-induced gene induction are included in Table 4.3.

The 5'-UTR's of *AtP5CS1, AtP5CS2* and *AtP5CR* all possess an identical 8 bp sequence which includes a hexamer that is recognised by a cold-regulated bZIP transcription factor from maize (Kusano et al. 1995). Upstream regions of both of the *AtP5CS* genes contain the CACATG motif which is recognised by a dehydration- and ABA-inducible MYC-type transcription factor RD22BP1, which cooperates with AtMYB2 in stress-mediated transcriptional activation of the *Arabidopsis RD22* gene (Abe et al. 1997). Sequences with weak homology to the DRE/C-repeat sequence are found in the 5'-UTRs of all three genes (Table 4.3), although the DRE-like element in the *AtP5CS2* 5'-UTR occurs downstream of the putative TATA box (Table 4.3). The DRE-like sequence in the *AtP5CR* 5'-UTR is identical to the DRE2 motif (ACCGACGC) found in the maize *RAB17* promoter, which was suggested to function as a coupling element involved in ABA-mediated expression (Busk et al. 1997). Interestingly, the bacterial OxyR response element, which coordinates changes in bacterial gene expression in response to oxidative stress (Storz et al. 1990; Zheng et al. 1998) and appears to play a similar role in eukaryotes (Duh et al. 1995; Guan & Scandalios 1998) occurs four times far upstream of the *AtP5CS1* start codon and twice in close proximity to the *AtP5CR* transcription initiation site (Table 4.3).

4.2.3 TEF1-like sequences

The potential involvement of high rates of transcriptional activation of the *AtP5CR* gene in rapidly dividing cells was first suggested by Hare and Cress (1996) on the basis of a high level of *AtP5CR* transcript in meristematic cells within the flowering stem of mature *Arabidopsis* plants. This suggestion, and the rationale which supported it, was subsequently used by Hua et al. (1997) to justify their demonstration that a 69 bp region within the 5'-UTR of *AtP5CR* directed strong expression of a marker gene in the rapidly dividing cells of the root and shoot apical meristems of *Arabidopsis* seedlings. These findings, together with the abundance of *AtP5CS2* transcript in rapidly dividing suspension cultured *Arabidopsis* cells (Strizhov et al. 1997), prompted

Table 4.3. Additional promoter elements within the 5'-UTRs of *Arabidopsis* genes involved in proline synthesis from glutamate. Complete sequences of the 5'-UTRs used in this analysis of the *AtP5CS1* (Genbank Accession No. AC003000), *AtP5CS2* (Zhang et al. 1997) and *AtP5CR* (Verbruggen et al. 1993) genes are provided in the Appendix. Putative TATA boxes are found at positions -153, -412 and -73 for *AtP5CS1*, *AtP5CS2* and *AtP5CR* respectively[1] (Appendix).

Consensus	*AtP5CS1*		*AtP5CS2*		*AtP5CR*		Reference
	Pos.[1]	Sequence	Pos.[1]	Sequence	Pos.[1]	Sequence	
• MYC-related bHLH-ZIP binding domain							
CACATG	- 2323	CACATG[2]	-795	CACATG[2]	-	-	Abe et al. (1997)
	- 2133	CACATG					
• mLip15 (bZIP transcription factor) recognition sequence							
ACGTCA	- 1265	ACATCATC[2]	- 812	ACGTCATC	- 249	ACGTCATC	Kusano et al. (1995)
	- 444	ACGTCATC					
• Drought response element (DRE)							
TACCGACAT	- 1916	TACCGACTT[2]	- 320	AACCGACAA	- 863	AACCGACGC	Shinozaki & Yamaguchi-Shinozaki (1997)
• Y-box motif / OxyR response element (ORE)							
GATTGG	-2372	GATTGG[2]					
	-2172	GATTGG	-	-	-228	GATTGG	Duh et al. (1995)
	-1248	GATTGG			-202	GATTGG	Guan & Scandalios (1998)
	-1167	GATTGG					
• Putative promoter element conferring meristematic tissue-specific expression							
TGG(G/T)CCC(A/G)(T/C)	-2161	TAGGCCCAC[2]	-1338	TGGACCCTT			
	-2160	TGGGCCTAA	-851	TGGCGCAGT[2]	-200	TAGGCCCAA[2]	Kosugi et al. (1995)
	-1282	TGGCTCTGT[2]	-802	TGGCGCCAC[2]	-199	TGGGCCTAC	
	-756	TGGTCCGCA					
• *as-1* box							
TGACG	- 626	TGACG	-811	TGACG[2]	-113	TGACG[2]	Lam et al. (1989)
	- 443	TGACG[2]			-248	TGACG[2]	

[1] For the *AtP5CS2* and *AtP5CR* 5'-UTRs, the position indicates the 5' end of the sequence upstream of the transcription initiation site. The second, downstream transcription initiation site (Zhang et al. 1997) was used in the case of *AtP5CS2*. This second transcription initiation site occurs 76 nucleotides downstream from the first transcription initiation site (Appendix, Zhang et al. 1997). Since the transcription initiation site(s) for *AtP5CS1* has not yet been defined, the position indicates the 5' end of the sequence upstream from the translation initiation site (start codon).

[2] The sequence indicated is found on the complement of the coding strand.

examination of the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes for promoter elements that might be associated with high rates of plant cell division.

As already mentioned (Section 2.2.2.3), the TEF1 (Translation Elongation Factor 1) box is a ubiquitous *cis*-acting motif which has been implicated in the activation of transcription in rapidly dividing cells (Regad et al. 1995). This domain is the target for *trans*-acting factors identified in nuclear extracts prepared from *Arabidopsis* (Curie et al. 1991). Since its identification in the promoters of four genes which encode isoforms of the translation elongation factor EF-1α of *Arabidopsis* (Curie et al. 1991), TEF1 sequences have been found in the promoters of a tomato gene encoding EF-1α, ribosomal genes from *Arabidopsis* and soybean, genes encoding TRX *h* in both tobacco and rice, as well as a tobacco gene (*ParA*) which has extensive homology with glutathione *S*-transferases (Regad et al. 1995). In eukaryotes, the α-subunit (EF-1α) of translation elongation factor 1 is functionally homologous to the bacterial factor EF-TU and catalyses the binding of aminoacyl tRNA to ribosomal acceptor sites which is driven by the hydrolysis of GTP. All four members (A1-A4) of the *Arabidopsis* EF-1α gene family are actively transcribed in young tissues, in freshly isolated protoplasts and in cell suspensions in the growth phase. The tobacco *ParA* gene is highly expressed during the transition from the G_0 to the S phase of the cell cycle in tobacco mesophyll protoplasts cultured *in vitro* (Takahashi et al. 1990). The likelihood of an important role for TRX *h* in mediating redox-dependent events required for plant cell division has already been mentioned (Section 2.2.2.3).

Thus, the TEF1 regulatory element appears to be of importance in the expression of genes which are highly expressed in rapidly cycling cells and are involved in processes of translation or the regulation of cellular redox potential (Regad et al. 1995). Indeed, TEF1-dependent transcriptional activation appears to be a general feature associated with entry into the plant cell cycle (Regad et al. 1995). Gel retardation experiments suggest that TEF1-like sequences in the *Arabidopsis SRP18* gene (encoding a small ribosomal protein), a tobacco gene encoding a TRX *h*, and different *Arabidopsis* and tomato genes encoding EF-1α gene products are all targets for the same proteins as those which interact with the TEF1 box of the *Arabidopsis* EF-1α *A1* gene (Regad et al. 1995). This substantiates the notion that genes with TEF1 boxes may be upregulated simultaneously by a common mechanism that enables the coordination of several events required for passage through the plant cell cycle.

As shown in Figure 4.3, the 5'-UTRs of both the *AtP5CS1* and *AtP5CR* genes contain sequences with high homology to sequences used by Regad et al. (1995) to establish a consensus TEF1 sequence. Two TEF1-like sequences occur in the *AtP5CS2* 5'-UTR, although their alignment with the other sequences requires either the insertion or the deletion of a single nucleotide prior to the

CONSENSUS		aRGGRYaHNHHN-GTMaH	
AtP5CS1[1]	-609	GGGC**GG**ACA**A**AACC-**GTCA**ACGGAT	-632
AtP5CS2[1]	-909	GGTG**GG**GTA**A**TTTTGG**GTAA**TTTAAA	-933
AtP5CS2[1]	-139	CGTA**GG**ACA**A**CTT--**GTCA**AAATAG	-161
AtP5CR	-305	CG**AAGG**GTA**A**GTTTC-**GTCA**AATCCC	-282
AtEFA1	-784	AC**AGGG**GCA**A**TAATG-**GTAA**TTTAAA	-761
AtEFA2	-839	AT**AAGG**GTA**A**AAATT-**GTCA**TTACGC	-816
AtEFA3	-782	AT**AGGG**GTA**A**CGTTT-**GTAA**TTTGGC	-759
AtEFA4	-659	GT**AAGG**GCA**A**ATTA-**GTAA**AAGTAG	-636
LeEF1	-944	TA**AGGG**GCA**A**TTTAC-**GTAA**ATAGAT	-921
NtTRXh2	-158	GT**AGGG**ACA**A**AATCT-**GTAA**ATGTGT	-135
OsTRX	-167	GC**AAGG**GCA**A**CTCCG-**GTAA**TTTCTC	-144
NtParA	-625	AG**AAGG**ATA**A**TTTTA-**GTAA**TTCAAC	-602
NtParA	-736	AG**AAGG**ATA**A**TTTTA-**GTAA**TTCAAC	-713
AtSRP15	-146	ACTA**GG**GTTCCCAA-**GTA**TCCCACT	-123
AtSRP18	-159	TA**AAGG**GTA**A**ATTA-**GTAA**TTAACT	-136
GmSRP11[1]	-179	CT**AGGG**ATA**A**GTAA-**GTAA**TGTCGC	-202
SoRPS22[1]	-1160	AG**AAGG**ATA**A**TTTTA-**GTC**CACTTAT	-1183
StCI7	-1901	AT**AAGG**GTA**A**AAATT-**GTAA**CTTCAC	-1878

Figure 4.3. Alignment of putative TEF1 boxes in the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes with TEF1-like sequences in other plant genes. A single gap (-) was introduced into most of the sequences in order to facilitate alignment with a sequence in the 5'-UTR of the *AtP5CS2* gene. Invariant residues found in all of the sequences are indicated in bold and are underlined. Positions where only one of two possible nucleotides occur (R = A or G, Y = T or C, M = A or C) are underlined only. The consensus sequence is based on gene sequences not associated with proline synthesis. In the consensus sequence, three adenine residues found very frequently, but which are not invariant, are written in lower case in bold and are underlined. H = A or C or T, N = any nucleotide.

Sequences used in the alignment with regions within the 5'-UTRs of the *Arabidopsis thaliana* genes *AtP5CS1* (Genbank Accession No. AC003000), *AtP5CS2* (Zhang et al. 1997) and *AtP5CR* (Genbank Accession No. M76538) are from the 5'-UTRs of *Arabidopsis* genes encoding the four isoforms of translation elongation factor EF-1α (*AtEFA1* - *AtEFA4*, Genbank Accession No.s X16430, X16431, X16432, AB011483), a gene encoding elongation factor EF-1α from tomato (*LeEF1*, Genbank Accession No. X53043), thioredoxin *h* genes from tobacco (*NtTRXh2*, Genbank Accession No. Z11803) and rice (*OsTRX*, Genbank Accession No. D26547), the tobacco gene encoding the parA protein (*NtParA*, Genbank Accession No. D42119), genes encoding ribosomal proteins from *Arabidopsis* (*AtSRP15,* Genbank Accession No. Z23162; *AtSRP18*, Genbank Accession No. Z23165), soybean (*GmSRP11*, Genbank Accession No. L28831) and spinach (*SoRPS22*, Genbank Accession No. X80044) and the potato gene encoding a cold stress inducible protein named CI7 (*StCI7*, Genbank Accession No. U69633). Since transcription start sites have not been identified in all of the genes used in the alignment, the numbers indicate the nucleotide position upstream from the start codon of each gene.

[1] The sequence indicated is found on the complement of the coding strand.

GTMa motif (Figure 4.3). Included in Figure 4.3 are two sequences with TEF1-like elements which were not included in the original alignment presented by Regad et al. (1995). These sequences, from the 5'-UTRs of genes encoding a plastid-specific ribosomal protein from spinach (Li et al. 1995a) and a potato cold-stress inducible protein named CI7 (Kirch et al. 1997), were fortuitously discovered during this study through a search of the Genbank sequence database. The more extensive alignment presented in Figure 4.3 enables a slight refinement of the original TEF1 core consensus sequence (arGGRYAnnnnnGTaa) suggested by Regad et al. (1995). The sequences used to update the consensus TEF1 box did not include the sequences from the *AtP5CS1*, *AtP5CS2* or *AtP5CR* 5'-UTRs.

Interestingly, all four *Arabidopsis* EF-1α promoters, at least two *Arabidopsis* ribosomal genes and a ribosomal gene from soybean contain the conserved sequence AAACCCTA (or its complement TAGGGTTT) in association with a TEF1 box (Regad et al. 1995). This element has been named the TELO box because of its homology to the repeat motif AAACCCT found in plant telomeres (Richards & Ausubel 1988). The promoters of a tomato EF-1α and a tobacco TRX *h* gene contain elements homology (88% and 75% identity respectively) to the consensus TELO box, although the promoter of a rice gene encoding TRX *h* does not contain a TELO box-like element in the vicinity of the putative TEF1 sequence (Regad et al. 1995). Where it does occur, the TELO box or a TELO box-like element is found within a region between 12 and approximately 100 nucleotides downstream of the TEF1 motif (Regad et al. 1995). Although the TELO box is specifically bound by a nuclear protein (Regad et al. 1994), the significance of its occurrence near TEF1 sequences remains enigmatic. Apparently, TELO sequences do not participate in the transcriptional activation of genes containing TEF1 boxes (Curie et al. 1993; Regad et al. 1995). The promoter of a rice gene encoding TRX *h* does not contain a TELO box (Regad et al. 1995). Since discrete motifs or internal tracts of telomeric DNA act as silencers in *S. cerevisiae*, it was suggested that the TELO box may act as an antagonist of the TEF1 box and thereby participate in the repression of gene expression in quiescent or senescent cells (Regad et al. 1995).

Regions with complete identity to the TELO box do not occur in the immediate vicinity of any of the putative TEF1 sequences found in *Arabidopsis* genes involved in the synthesis of proline from glutamate (Figure 4.3). However, as indicated in Figure 4.4, several sequences with 75-88% homology to the consensus TELO box are found close to the putative TEF1 boxes in the 5'-UTRs of these genes. Assuming some significance of the frequent occurrence of a TELO box in the vicinity of functional TEF1 motifs in other plant genes (Regad et al. 1995), the occurrence of these regions within less than 50 bp of the putative TEF1 elements seems to support the likelihood that a general TEF1-mediated regulatory mechanism may participate in the transcriptional regulation of proline biosynthesis.

CONSENSUS		**AAACCCTA**	
AtP5CS1	-590	**AAACCC**CT -583	(18 bp)
AtP5CS2	-980	**AAACCT**TT -973	(39 bp)
AtP5CS2	-131	**AAACCC**GT -124	(7 bp)
AtP5CR	-355	**AAACCCAA** -348	(42 bp)

Figure 4.4. TELO box-like motifs in the vicinity of putative TEF1 boxes in the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes. The minimal consensus TELO box, or the complement thereof, is found within 110 bp downstream of the TEF1 boxes in four *Arabidopsis* EF-1α promoters, at least two *Arabidopsis* ribosomal genes, a ribosomal gene from soybean and one of the two TEF1 boxes in the tobacco *ParA* gene (Regad et al. 1995). The numbers indicate the positions of the sequences upstream of the start codons in the three proline biosynthetic genes (Appendix). The distance of each TELO-like element from the putative TEF1-box is indicated in parentheses.

4.2.4 Putative phytochrome-regulated promoter elements

A well-documented requirement for light in the induction of proline accumulation under conditions of osmotic stress has already been mentioned (Section 2.5.2.1). As was emphasised in Section 2.4.5.1, light is essential for normal plant growth and development not only as a source of energy but also as a stimulus that regulates many metabolic processes. Studies of many light-regulated genes from different species have indicated that DNA elements responsible for phytochrome-responsive gene expression are located within 5' upstream sequences (Gilmartin et al. 1990). Nonetheless, discrimination between whether the effect of light on any metabolic process is a direct effect related to its perception by a photoreceptor or whether the effect is mediated more indirectly (e.g. by products of photosynthesis or photorespiration) has proven to be difficult when using classical physiological approaches. Proposals that the light requirement for proline accumulation may reflect a demand for carbohydrate precursors (Pesci 1993; Hare & Cress 1997), a means of defusing excess reducing power under photoinhibitory conditions (Section 2.5.2.1; Hare & Cress 1997), or a beneficial effect related to prevention of a damaging accumulation of H_2O_2 during photorespiration (Section 2.5.2.2; Hare et al. 1998) do not preclude direct regulation of the proline biosynthetic genes by a photoreceptor. These proposals suggest possible functions for the light-dependence of proline synthesis during stressful conditions, but do not propose a mechanism by which the phenomenon may be regulated. Direct regulation of proline biosynthetic capacity by phytochrome is an appealing mechanism by which plants may integrate certain metabolic adjustments with the availability of light.

The extensive characterisation of the effects of light on the expression of genes which encode enzymes involved in amide amino acid biosynthesis provides a precedent for the notion that phytochrome may regulate the expression of proline biosynthetic genes. Light and metabolic status are the two primary signals which govern the regulation of amide amino acid metabolism (Lam et al. 1995). In keeping with the essential role played by glutamine synthetase (GS; EC 6.3.1.2) in the reassimilation of photorespiratory ammonia, accumulation of an mRNA encoding a plastidic isoform of GS in pea (GS2) is induced by white light (Edwards & Coruzzi 1989). Although a 3 min pulse of red light (R) also increases the level of *GS2* transcript and the role of phytochrome in mediating this effect is demonstrated by the far-red (FR) reversibility of the process, the maximum induction by R is approximately one-fifth of that observed after exposure to white light treatment (Edwards & Coruzzi 1989). This observation, together with slower kinetics of *GS2* mRNA accumulation in response to white-light in etiolated or dark-adapted green plants than in those with mature, functional chloroplasts, as well as selective induction of *GS2* transcript accumulation following transfer from conditions of elevated CO_2 to conditions that induce photorespiration, led Edwards and Coruzzi (1989) to conclude that the light-induced accumulation of *GS2* mRNA in pea leaves results from the action of phytochrome as well as light-induced changes in chloroplast metabolism. The specific metabolite signals which act in concert with the phytochrome-derived signal do not appear to have been characterised.

In contrast to the induction of GS expression by light, the expression of genes encoding isoforms of asparagine synthetase (AS; EC 6.3.5.4) are down-regulated by light via a phytochrome-dependent mechanism in several species (Tsai & Coruzzi 1990, 1991; Lam et al. 1994; Ngai et al. 1997; Neuhaus et al. 1997). This effect occurs at the transcriptional level (Tsai & Coruzzi 1991; Ngai et al. 1997; Neuhaus et al. 1997). Nonetheless, a light-induced change in expression is not a general feature of all gene families which encode amino acid biosynthetic enzymes. For instance, none of the *Arabidopsis* genes which encode the four isoforms of aspartate aminotransferase (EC 2.6.1.1) in this species are subject to regulation by light (Schultz & Coruzzi 1995). Similarly, levels of transcript encoding pea cytosolic GS are not influenced by light conditions (Tsai & Coruzzi 1991). Thus, the involvement of phytochrome is not a universal feature of the mechanisms that coordinate the availability of nitrogen and carbon skeletons from the TCA cycle with amino acid synthesis.

Two fairly recent reports substantiate the potential involvement of a phytochrome-derived signal in the regulation of proline biosynthetic capacity. Pesci (1996) demonstrated that ABA- and KCl-induced stimulation of proline accumulation in barley leaves by white light could be mimicked by R at a photon fluence rate of approximately one-twelfth of that required for maximal stimulation by white light. Unfortunately, investigation of whether stimulation by R could be reversed by a

flash of FR, the hallmark of phytochrome-mediated induction, was not reported. Secondly, Strizhov et al. (1997) demonstrated a 40-50% decrease in the levels of *AtP5CS1* transcript in *Arabidopsis* plants following their transfer from illuminated conditions to darkness for 3 d, thus indicating that the stimulative effects of white light on proline synthesis may be mediated, at least in part, through changes in the expression of a proline biosynthetic gene. Transfer to darkness did not substantially affect *AtP5CS2* transcript abundance, although a slight increase in *AtP5CS2* mRNA levels is evident from the results presented by Strizhov et al. (1997).

As already outlined (Section 2.4.5.1), an extensive series of microinjection studies have resulted in the proposal of a biochemical framework for phytochrome signalling (Bowler et al. 1994; Barnes et al. 1997; Mustilli & Bowler 1997). In this model, stimulation of PHYA by light activates at least one heterotrimeric G-protein, which in turn stimulates three biochemically-distinct signalling cascades. These involve Ca^{2+}, cGMP or a combination of both second messengers (Figure 2.12). Previous demonstrations that specific elements from light-responsive promoters are required for phytochrome regulation of transcription enabled Wu et al. (1996) to define at least two of the terminal target sequence elements of the Ca^{2+}- and cGMP-dependent pathways. The Box II element of the *RBCS-3A* promoter (Green et al. 1987, 1988; Lam & Chua 1990) and the Unit I element of the parsley *CHS* promoter (Weisshaar et al. 1991) are known to bind specific transcription factors. Consistent with previous studies using full promoter constructs from *RBCS* and *CHS* genes (Bowler et al. 1994), it was found that fusion of multiple copies of either the 15 bp phytochrome-responsive Box II element or the 52 bp Unit I element to a minimal promoter with no intrinsic light responsiveness enabled induction of reporter gene expression following stimulation by Ca^{2+} (for Box II constructs) or cGMP (for Unit I constructs). Although microinjection of PHYA and an activator of heterotrimeric G-proteins into hypocotyl cells of a phytochrome-deficient mutant was able to activate expression of both constructs, cGMP was not capable of stimulating Box II activation and microinjection of Ca^{2+} was incapable of activating expression of a Unit I-containing construct (Wu et al. 1996). Thus, Box II and Unit I are targets of the Ca^{2+} and cGMP pathways, respectively.

Examination of the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes indicated the presence of regions upstream of the *AtP5CS1* and *AtP5CS2* ORFs which have fairly substantial homology with Box II from pea *RBCS-3A* (Figure 4.5A). No regions of homology to Box II were found in the 5'-UTR of *AtP5CR*. To screen for the presence of the cGMP-responsive Unit I element in the 5'-UTRs of the three proline biosynthetic genes, it was broken into 15 overlapping segments, each 7 bp in length. None of the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* nor *AtP5CR* genes contained regions with substantial homology to any of these segments from the Unit I phytochrome-responsive sequence.

(A)

		▼▼	
Box II		TGTGTGGTTAATATG	
AtP5CS1[1]	-590	TGAATGGTTATGGCG	-604
AtP5CS2[1]	-328	TGTGTGATTATTTTG	-342

(B)

RE3		GATCTGGTGGGAGCTAG	
AtP5CS2	-1313	ATTACCGTGGGATCTAC	-1297
AtP5CS2	-605	AACACTGTGGGGCACAA	-589

Figure 4.5: Putative phytochrome-responsive elements in the 5'-UTRs of *AtP5CS1* and *AtP5CS2*. (A) Sequences from the 5'-UTRs of *AtP5CS1* and *AtP5CS2* with homology to the Box II element shown to lie at the terminus of a Ca^{2+}/CaM branch of the phytochrome signal transduction network (Wu et al. 1996). The arrows indicate two adjacent G residues which are critical for binding of the nuclear protein factor GT-1 to Box II (Green et al. 1987, 1988). **(B)** Sequences from the 5'-UTR of *AtP5CS2* are aligned with the RE3 element from the promoter of a pea gene (*AS1*) encoding asparagine synthetase. The RE3 element confers phytochrome-mediated repression of gene expression by a branch of the phytochrome signal transduction network which involves both Ca^{2+} and cGMP (Neuhaus et al. 1997). The 8 bp consensus core sequence recognised by a putative phytochrome-induced repressor (Neuhaus et al. 1997) is underlined. All numbers indicate the distance upstream from the translation initiation site of that gene.

[1] The sequence indicated is found on the complement of the coding strand.

Phytochromes are responsible not only for the selective induction, but also the repression of certain plant genes. Whereas steady state levels of *AtP5CS1* transcript are substantially reduced after transfer of light-grown plants to darkness for 3 d, *AtP5CS2* levels apparently increased slightly after extended incubation in darkness (Strizhov et al. 1997). Neuhaus et al. (1997) used a microinjection strategy to demonstrate that phytochrome-mediated repression of an isoform of asparagine synthetase from pea (encoded by the *AS1* gene) is mediated by the branch of phytochrome signalling which involves both Ca^{2+} and cGMP. Based on an alignment of a 17 bp *cis*-acting element (named RE3) within the *AS1* promoter, which is necessary and sufficient for phytochrome-mediated repression of this gene, with six other RE3-like elements found in other phytochrome-repressed plant genes (Neuhaus et al. 1997), a consensus core sequence:

N(A/G)TGGG(A/C/G)N(A/C/G)NN

can be deduced. Examination of the 5'-UTRs of the *AtP5CS1* and *AtP5CR* genes did not reveal any regions that matched this consensus perfectly. However, two regions in the 5'-UTR of *AtP5CS2* closely match the consensus and are aligned with the RE3 element in Figure 4.5B.

4.2.5 Auxin-related motifs

The possible involvement of auxin in the regulation of *AtP5CS1* and *AtP5CS2* expression was mentioned in Section 2.4.3.4. The synthetic auxin 2,4-D induces both of these genes in 28 d-old *Arabidopsis* plants, albeit with different kinetics. An involvement of auxin in the regulation of *AtP5CR* expression does not appear to have been investigated. An approximately three-fold increase in *AtP5CS1* transcript levels was evident in both root and shoot tissue within 24 h after exposure to 2,4-D at a concentration of 1 mg l^{-1} (approximately 4.5 µM), whereas an elevation in *AtP5CS2* transcript levels in leaves occurred within 6 h of auxin treatment (Strizhov et al. 1997). Auxin did not appear to affect *AtP5CS2* expression in roots. The induction of both of the *AtP5CS* genes by salt stress was unaffected by mutation at the *AUX1* locus, although stress-induced expression of both genes was abrogated in an *axr2* mutant (Strizhov et al. 1997). Unambiguous interpretation of the latter result is complicated by the pleiotropic effects of *axr2*, which confers resistance not only to auxin, but also to ABA and ethylene (Estelle & Klee 1994).

While the physiological roles of the classical plant hormones are well documented, the mechanisms by which their actions are transduced remain unclear (Hare & van Staden 1997). However, fairly recent advances in the identification of several auxin-responsive elements (AuxREs) that confer auxin-inducibility of gene expression enable assessment of whether these may account for the responsiveness of the two *AtP5CS* genes to auxin (Strizhov et al. 1997). A central problem in hormone physiology is discrimination between whether or not experimentally-observed effects of an exogenously applied growth regulator(s) reflect a normal role for that hormone(s) in the physiological process being investigated (Hare et al. 1997). While the fairly recent isolation of mutants which are disrupted in hormone responses offers a powerful approach to address this problem, the pleiotropic effects of many signalling mutants (Estelle & Klee 1994; Mayer et al. 1996; Section 2.4.5.4) often complicates interpretation of studies such as those of Strizhov et al. (1997). Identification of AuxRE-like motifs in the *AtP5CS* 5'-UTRs offers one approach to further assess the likely involvement of auxins in the regulation of proline biosynthetic capacity.

Auxins regulate various aspects of plant growth and development. Exogenous application of auxin to plant tissues has been shown to stimulate cell elongation, cell division, vascular tissue

differentiation and root initiation and to maintain apical dominance. It is widely accepted that changes in gene expression must underlie these physiological responses. Although several recent studies have investigated the roles of auxins in mediating plant adaptations to adverse environmental conditions (Dunlap & Binzel 1996; Leymarie et al. 1996; Lopez-Carbonell et al. 1996), their involvement in the plant stress response is not well understood. Consistent with the diversity of auxin action, the current view of auxin-mediated changes in gene expression is that most AuxREs consist of more than a single consensus element and that several types of AuxREs act both independently and together to confer auxin responsiveness to diverse sets of genes (Xu et al. 1997). Therefore, the "coupling model" of ABA-dependent gene expression (Section 2.4.3.2; Shen & Ho 1997) seems equally applicable to auxin-dependent gene expression, where a highly conserved AuxRE appears to require interaction with a more unique coupling element that confers spatio-temporal specificity of expression. Together, the AuxRE and coupling elements(s) forms an auxin responsive domain. The complexity that arises through the combinatorial use of both conserved and variable elements in triggering auxin-mediated transcriptional activation introduces considerable difficulty in inferring that any region within the 5'-UTRs of the *AtP5CS* genes identified exclusively by sequence comparison may be responsible for mediating the induction of their expression by auxin. Auxin effects may also be mediated by post-transcriptional processes (Napier & Venis 1995).

At least two putative AuxREs have been identified in promoters that are induced only by biologically active auxins. These are the (T/G)GTCCCAT element (Domain A) originally characterised in the pea *PS-IAA4/5* promoter (Oeller et al. 1993; Ballas et al. 1993, 1995), and the TGTCTC element in composite auxin responsive domains of the soybean *GH3* promoter (Ulmasov et al. 1995, 1997a, 1997b). Composite auxin-responsive domains comprise a constitutive element (which confers constitutive expression when fused to a minimal promoter) adjacent to or overlapping with a TGTCTC element that confers repression and activation of the constitutive element when auxin levels are low and high, respectively (Ulmasov et al. 1995). Although the TGTCTC motif is necessary for induction of *GH3* expression by auxin, without constitutive or coupling elements, a single copy of TGTCTC does not function as an AuxRE (Ulmasov et al. 1995). Nonetheless, the importance of the TGTCTC motif has been demonstrated through the cloning and characterisation of ARF1, a transcription factor which binds to this sequence *in vitro* (Ulmasov et al. 1997a). Mutational analysis indicated that the sequence requirements for ARF1 binding *in vitro* are identical to those that confer auxin responsiveness *in vivo*. Mutation of any of the first four nucleotides (TGTC) abolished both auxin responsiveness and ARF1 binding, while mutation of the T and C at positions 5 and 6 in the TGTCTC motif, either separately or together, indicated that these two residues make a smaller contribution to ARF1 binding and auxin inducibility (Ulmasov et al. 1997a). Since many transcription factors are known

to bind dyad symmetric sequences, Ulmasov et al. (1997a) further demonstrated that palindromic repeats of TGTCTC [GAGACA(N)$_n$TGTCTC or TGTCTC(N)$_n$GAGACA] function most effectively as AuxREs. The optimal spacing between the two half-sites both for *in vivo* responsiveness to auxin and for ARF1 binding is seven or eight nucleotides (Ulmasov et al. 1997a). A subsequent report by these workers indicated that mutation of the naturally occurring AuxRE in *GH3* (CCTCGTGTCTC) to CCTTTTGTCTC dramatically increased auxin responsiveness in a carrot protoplast system and that this synthetic element can function as a strong AuxRE when multimerised and properly spaced as palindromic repeats or direct repeats in either orientation (Ulmasov et al. 1997b).

It is worth noting that because the (G/T)GTCCCAT element (Oeller et al. 1993; Ballas et al. 1993, 1995) in the form TGTCCCat is related to the TGTCTC element, and TCTCCC can function as an AuxRE when substituted for TGTCTC in composite AuxREs (Ulmasov et al. 1997a), both of these elements may in fact respond to a common auxin-induction mechanism. Nonetheless, the existence of other functional AuxREs is reflected for instance by the demonstration that although the auxin-inducible soybean *SAUR15A* promoter contains both a TGTCTC element and a GGTCCCAT element within an auxin-responsive promoter fragment (Li et al. 1994), another AuxRE outside of this fragment functions also confers auxin-inducibility on *SAUR15A* expression, but contains no (G/T)GTCCCAT or TGTCTC element (Xu et al. 1997). Mutation of the TGTCTC and GGTCCCAT elements within the *SAUR15A* promoter resulted in only a slight loss of auxin inducibility, thus indicating that they were not exclusively responsible for the auxin responsiveness of this 5'-UTR (Xu et al. 1997). The second small fragment in the *SAUR15A* promoter that functions as an AuxRE contains two copies of the 7 bp sequence AAAACAA, a G-box-like sequence CACGTC and a palindromic CATATG sequence found in many auxin-responsive promoter regions (Xu et al. 1997). Individual mutation of all three elements reduced the auxin inducibility of the promoter to about half of the normal level (Xu et al. 1997). Interestingly, all three of these elements occur several times in the 5'-UTRs of the *AtP5CS1* and *AtP5CS2* genes (Figure 4.6A).

In contrast, the TGTCTC motif does not occur in either of the *AtP5CS1* or *AtP5CS2* 5'-UTRs. However, in view of the preference of ARF1 for binding to a palindromic sequence comprising an inverted repeat of TGTCTC with the two half elements separated by seven or eight bp (Ulmasov et al. 1997a), the presence of the sequence :

-821 GGACAAAGCGGTCTAATTTGTCT -799

in the *AtP5CS1* 5'-UTR may be of some significance. The common involvement of palindromic

(A)

AtP5CS1	-2202	**AAAACAA**	-2196			
AtP5CS1[1]	-2025	**AAAACAA**	-2031	-1815	**AAAACAA**	-1821
AtP5CS1[1]	-1809	**AAAACAA**	-1815	-1797	**AAAACAA**	-1803
AtP5CS1[1]	-895	**AAAACAA**	-901			
AtP5CS2	-1257	**AAAACAA**	-1251			
AtP5CS2	-854	**CACGTC**	-849			
AtP5CS2	-74	**CACGTC**	-69			
AtP5CS1	-1745	**CATATG**	-1740			
AtP5CS2	-1117	**CATATG**	-1112			

(B)

Domain B		MACATGGNMRTGTYYM	
AtP5CS1	-2316	AGCTGCT**GCGTGTCTA**	-2301
AtP5CS1[1]	-1183	**AA**TATG**A**TAATGTTTG	-1198
AtP5CS1[1]	-516	TATATGATTGTGTGAC	-531
AtP5CS2	-383	TACATGGTAGTTAGCA	-368
AtP5CS2[1]	-1178	TACAAGGAAATGTTTA	-1193
AtP5CS2[1]	-448	TATATAGAAATGTATC	-463
AtP5CS2[1]	-432	TATATGGTATTGTGCC	-447

(C)

AS-1 Type		TKASST--AAGNNCTKACGTMN	
AtP5CS2	-1326	**TGACCTTTTAGTGATTACCGTG**	-1305
Hex-1 Type		TGACGTGG	
AtP5CS1[1]	-439	**TGACGTCG**	-446
AtP5CS2[1]	-848	**TGACGTGT**	-855
AtP5CR[1]	-423	**TGACGTGG**	-430

Figure 4.6 : Putative auxin-responsive motifs in the 5'-UTRs of *Arabidopsis* genes involved in proline synthesis from glutamate. (A) Sequences from the 5'-UTRs of *AtP5CS1* and *AtP5CS2* which are identical to elements that contribute to the auxin-inducibility of *SAUR15A* gene transcription (Xu et al. 1997). **(B)** Sequences from the 5'-UTRs of *AtP5CS1* and *AtP5CS2* are aligned with the consensus Domain B sequence (Ballas et al. 1993; Oeller et al. 1993). **(C)** Sequences from the 5'-UTRs of the three proline biosynthetic genes are aligned with the consensus ASF-1 binding sites (Liu & Lam 1994). All numbers indicate the distance upstream from the translation initiation site of that gene. K = G or T, M = A or C, N = any nucleotide, R = A or G, S = C or G, Y = C or T.

[1] The sequence indicated is found on the complement of the coding strand.

sequences as functional *cis*-acting elements has frequently been interpreted as a reflection of the observation that proteins involved in sequence-specific protein-DNA interactions are often active as dimers or tetramers, binding with a twofold symmetry that matches the symmetry of the palindrome. It should be noted, however, that the involvement of the TGTCTC motif in a palindromic arrangement does not appear to have been noted in naturally occurring AuxREs.

Examination of the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes did not reveal any regions with homology to the (G/T)GTCCCAT motif (Domain A; Ballas et al. 1993). Nonetheless, as shown in Figure 4.6B, several regions within the 5'-UTRs of both the *AtP5CS1* and *AtP5CS2* genes show homology to the Domain B consensus sequence (Ballas et al. 1993). DNase I footprinting analysis has revealed the binding of nuclear proteins to the highly conserved B domain which is found in the promoters of at least six auxin-responsive genes (Oeller et al. 1993). Whereas Domain A acts as an auxin switch, domain B has an enhancer-like activity (Ballas et al. 1993).

The identification of several activation sequence (*as-1*) boxes in the 5'-UTRs of genes encoding enzymes involved in proline biosynthesis from glutamate (Table 4.3) may also have some bearing on the induction of *AtP5CS1* and *AtP5CS2* transcript accumulation by auxin (Strizhov et al. 1997). Tobacco ASF-1 was originally identified as a sequence-specific DNA binding activity that interacts with the AS-1 element in the CaMV35S promoter (Lam et al. 1989). In this viral promoter, AS-1 comprises a pair of tandem motifs each containing the half site TGACG (the *as-1* box). The two 5 bp repeats, spaced 7 bp apart [TGACG$(N)_7$TGACG] form two binding sites for ASF-1 and mutation of these *as-1* boxes inhibits the binding of ASF-1 to the CaMV35S promoter *in vitro* (Lam et al. 1989). Liu and Lam (1994) demonstrated that the dimeric (AS-1 type) of ASF-1 recognition site is responsive to 2,4-D when fused as a tetramer to a truncated pea *RBCS-3A* promoter or as a monomer within the CaMV35S promoter truncated to a position -90 bp upstream of the transcription initiation site. The observation that mutation of the conserved TGACG motifs within the AS-1 type element eliminated responsivity to auxin suggested a role for ASF-1 in mediating at least a subset of auxin responses *in vivo* (Liu & Lam 1994). As shown in Figure 4.6C, a region within the 5'-UTR of *AtP5CS2* has weak homology to the consensus AS-1 type sequence, which comprises perfect or imperfect tandem repeats of TGACG-like sequences in a perfect or imperfect palindromic structure (Liu & Lam 1994). Alignment of these sequence with the consensus AS-1 type domain required the insertion of two gaps in the consensus sequence (Figure 4.6C). The AS-1 type recognition site for ASF-1 has been most extensively studied in several promoters of viral and agrobacterial genes that are expressed in plants (Lam et al. 1989; Fromm et al. 1989). However, promoters of plant genes with a double TGACG-related element spaced approximately 7 bp apart have been identified in auxin-responsive genes of higher plants (Lam & Liu 1994;

Droog et al. 1995; Sakai et al. 1996). In at least two instances, it has been shown that mutation of an AS-1 type element in the promoters of auxin-inducible genes from tobacco completely eliminated auxin responsiveness (Droog et al. 1995; Sakai et al. 1996). Droog et al (1995) presented evidence that ASF-1 or an ASF-1-like factor is likely to mediate the induction of two glutathione *S*-transferase genes by auxin. However, Sakai et al. (1996) demonstrated that the presence of an AS-1 type element from the *parC* gene alone was incapable of conferring induction of gene expression by auxin. This result suggests that for this gene, an AS-1 type sequence is indispensable for auxin responsiveness, but that it requires the presence of a promoter auxiliary segment(s). For *parC*, this is unlikely to be a GGTCCCAT or TGTCTC element (Sakai et al. 1996).

In addition to the AS-1 (dimeric) type of ASF-1 recognition site, ASF-1 also binds to single copies of the TGACG motif (Lam et al. 1989). The Hex-1 (monomeric) type recognition site, which is named because of its identity to the HEX-1 element of the wheat histone *H3* gene, contains a single 5 bp *as-1* element within the consensus sequence TGACGTGG (Liu & Lam 1994). Of the five *as-1* motifs that occur in the 5'-UTRs of the three genes involved in proline synthesis from glutamate (Table 4.3), only three have appreciable homology to the Hex-1 consensus sequence (Figure 4.6C). Hex-1 type ASF-1 recognition sites are found in several auxin inducible genes (Liu & Lam 1994). In the auxin-inducible *GH3* promoter, two Hex-1-like elements (TGACGTAA and TGACGTGGC) were found to be strong binding sites for nuclear proteins from four different species (Liu et al. 1994). Mutation of these sites suggested that both contributed to the absolute level of auxin-inducibility of the gene, but that they functioned within the context of additional AuxREs within the *GH3* promoter. As will be discussed further in Section 5.1.3, some controversy surrounds the physiological significance of the auxin-inducibility conferred by either of the two types of ASF-1 binding sites. Owing to an association of ASF-1 recognition sites with root-specific expression and high levels of expression in auxin-inducible genes involved in cell division, as opposed to those that facilitate cell elongation, the occurrence of likely ASF-1 binding sites in the 5'-UTRs of proline biosynthetic genes also extends into their tissue-specific patterns of expression. These issues will be addressed in the Discussion.

4.2.6 Putative organ-specific and developmentally-regulated promoter elements

A search for homology of any stretches within the *AtP5CS1* or *AtP5CS2* 5'-UTRs with sequences deposited in the Eukaryotic Promoter Database (EPD) [http://www.ncbi.nlm.hih.gov] revealed that two regions within these 5'-UTRs have extensive homology to two separate stretches within the promoter of the pea *legA* gene (*PsLEGA*), which encodes a legumin (Lycett et al. 1985; Rerie et

al. 1991). The 11S and 12S globulin storage proteins are found in the seeds of most dicotyledonous plants. These proteins consist of two subunits, designated α and β, linked by a single disulphide bond. Each subunit pair is synthesised as a single precursor protein that is proteolytically cleaved after disulphide bond formation (Shewry et al. 1995). In members of the Fabaceae, the 11S storage proteins (legumins) form the largest class of seed storage proteins. A set of 12S A and B subunits resembling the globulin proteins represents 50-55% of the proteins in mature *Arabidopsis* seeds (Finkelstein & Somerville 1990). The percentage amino acid homologies of the *AtCRA1* and *AtCRB* gene products to the *PsLEGA* gene product are 43% and 41% respectively (Pang et al. 1988). The other main storage proteins in the Brassicaceae are the 2S proteins, resembling albumin or napin. 2S albumins are small water-soluble proteins of 9-kD and 4-kD chains linked by disulphide bridges (Shewry et al. 1995).

As is evident from the alignments shown in Figure 4.7A, in contrast to the 5'-UTR of the legumin gene, both of the P5CS-related promoter elements are found on the anticoding strand. The percentage homologies of the two segments from the *PsLEGA* promoter to the elements found in the 5'-UTRs of the *AtP5CS1* and *AtP5CS2* genes are 94% and 89% respectively.

(A)

PsLEGA	-509	**ATAAATATATTTGAAAAGA**	-491
AtP5CS1[1]	-2335	**ATAA**TATATTTGAAAAGA	-2353
PsLEGA	-343	**AAAGTTTATTCTTTATAAATCTTTGTAA**	-316
AtP5CS2[1]	-1337	**AAAGTTT**TTTA**T**TTATAAATCTTTTTAA	-1364

(B)

ZmZA1	-147	**GTTTGGAAAAAATACAAAAT**	-128
AtP5CS2	-732	**GTT**CA**GAAAAAATACAAAAT**	-713

Figure 4.7: Putative regulatory regions within the 5'-UTRs of *AtP5CS1* and *AtP5CS2* which have high homology to regions within the 5'-UTRs of genes encoding seed storage proteins in other species. Homology of the regions within the *AtP5CS* sequences (Appendix) with those from **(A)** the pea *legA* gene (*PsLEGA*; Lycett et al. 1985; Rerie et al. 1991; Genbank Accession No.'s X02982, X57666) and **(B)** a maize zein gene (*ZmZA1*; Spena et al. 1982; Genbank Accession No. V01474) was established through a search of the Eukaryotic Promoter Database (http://www.ncbi.nlm.hih.gov) using the BLAST program (Altschul et al. 1990). For each gene, the numbers indicate the distance upstream of the translation start site.

[1] The sequence indicated is found on the complement of the coding strand.

Two additional pieces of evidence support the proposal that transcriptional activation of P5CS expression may coincide with the synthesis of seed storage proteins at a specific stage in *Arabidopsis* seed development. Firstly, as shown in Figure 4.7B, another region within the *AtP5CS2* 5'-UTR (on the coding strand) has 90% homology to a 20 bp sequence found in the promoter of a maize zein gene (Spena et al. 1982). The α-zeins account for up to 80% of the prolamins, the largest class of seed storage proteins found in cereals (Shewry et al. 1995). The 2S albumins found in *Arabidopsis* seeds are believed to be related to the prolamins (Shewry et al. 1995). Secondly, the sequence AACCCA, which was identified to be responsible for seed-specific expression of the soybean β-conglycinin promoter during mid- to-late stages of embryogeny (Chen et al. 1988) is found in the 5'-UTRs of all three of the proline biosynthetic genes investigated here. This motif occurs at positions - 2486 (for *AtP5CS1*), - 247 (for *AtP5CS2*) and - 354 (for *AtP5CR*) [Appendix]. These positions refer to the distance upstream from the translation initiation codon of each of the genes. The AACCCA motif was recently found in the 5'-UTR of the drought- and ABA-responsive CDeT11-24 gene from the resurrection plant *Craterostigma plantagineum* (Velasco et al. 1998). Analysis of the expression of a reporter gene driven by the CDeT11-24 promoter indicated that it had high activity in mature seeds of both transgenic *Arabidopsis* and tobacco (Velasco et al. 1998).

If any of these sequences play an important role in seed-specific expression in *Arabidopsis*, one might anticipate their involvement in the regulation of storage protein synthesis in this species. Examination of the 5'-UTRs of two genes encoding *Arabidopsis* 12S storage proteins (*AtCRA1* and *AtCRB*; Pang et al. 1988; Genbank Accession No.'s X14312, X14313) and four genes encoding *Arabidopsis* 2S albumins (*At2S1*, *At2S2*, *At2S3*, *At2S4*; Krebbers et al. 1988; Genbank Accession No.'s Z24744; Z24745) revealed that none of them contained perfect matches to any of the three putative elements defined in Figure 4.7. Together, the *At2S1* and *At2S2* 5'-UTRs contain four 19 bp regions with 63% homology to one of the putative *PsLEGA* regulatory regions (positions -509 to -491 in Figure 4.7A). A 20 bp stretch within each of the *At2S1* and *AtCRB* genes contains 70% homology to the putative *ZmZA1* regulatory region shown in Figure 4.7B (data not shown). Despite the demonstration that the 5'-UTR of *AtP5CR* directs strong transcriptional activity in developing seeds (Hua et al. 1997), no regions with homology to any of the putative elements identified in Figure 4.7 were found in the *AtP5CR* 5'-UTR.

Besides the homology to regions within the promoters of the legumin and zein genes, the only other plant derived promoter sequence obtained through searching the EPD database (http://www.ncbi.nlm.nih.gov) was that from a tomato anther-specific gene *LAT52*, which has 87% homology with a 24 bp region on the noncoding strand of the 5'-UTR of *AtP5CS2* (Figure 4.8A).

An AAATGA motif occurs several times within the 5'-UTRs of both of the *AtP5CS1* and *AtP5CS2* genes, but is not found on either strand upstream of *AtP5CR* (Figure 4.8B). The AAATGA motif occurs twice within a 100 bp stretch upstream of the transcription start site of the pollen-specific *NTP303* gene from tobacco and is completely conserved in the similarly regulated promoter of the *Bp10* gene from *Brassica napus*, which encodes a homologue of *NTP303* (Weterings et al. 1995). Two elements from the *NTP303* promoter (17 bp and 27 bp in length) which contain this motif were capable of driving pollen-specific expression from a heterologous promoter. The effect of these regions on pollen-specific expression was additive and independent of their orientation (Weterings et al. 1995). Site-directed mutagenesis of the AAATGA motif in the more upstream element in the *NTP303* promoter to AAAGCC revealed that the TGA triplet was essential for a high level of pollen-specific expression (Weterings et al. 1995).

(A)

LeLAT52	-237	**ATCACTTCATTATTAATTTTAATT**	-214
AtP5CS2	-1259	**ATCA**GTTTATTC**TTAATTTTAATT**	-1282

(B)

AtP5CS1	-494	**AAATGA**	-489			
AtP5CS1[1]	-247	**AAATGA**	-252	-1283	**AAATGA**	-1288
AtP5CS1[1]	-778	**AAATGA**	-783	-1640	**AAATGA**	-1645
AtP5CS1[1]	-998	**AAATGA**	-1003	-1980	**AAATGA**	-1985
AtP5CS2	-628	**AAATGA**	-623	-716	**AAATGA**	-711
AtP5CS2[1]	-361	**AAATGA**	-366			

Figure 4.8: Putative regulatory regions within the 5'-UTRs of *AtP5CS1* and *AtP5CS2* which have high homology to regions within the 5'-UTRs of genes with anther- or pollen-specific expression in other species. (A) Homology of a region within the *AtP5CS2* 5'-UTR with a region upstream of the transcription initiation site of the tomato *LAT52* gene (*LeLAT52*; Twell et al. 1989; Genbank Accession No. X15855) was established through a search of the Eukaryotic Promoter Database (http://www.ncbi.nlm.hih.gov) using the BLAST program (Altschul et al. 1990). **(B)** Occurrence of the AAATGA motif within the 5'-UTRs of the *AtP5CS1* and *AtP5CS2* genes. The AAATGA motif is a *cis*-acting element responsible for the pollen-specific expression of the *NTP303* gene from tobacco and its homologue in *Brassica napus* named *Bp10* (Weterings et al. 1995). For each gene, the nucleotide positions denote the distance upstream of the translation start site.

[1] The sequence indicated is found on the complement of the coding strand.

No plant-derived sequences within the EPD displayed extensive homology to the 5'-UTR of *AtP5CR*. Besides the four elements listed in Figures 4.7 and 4.8, no other plant-derived promoter sequences were identified when the EPD was screened using the two *AtP5CS* 5'-UTR sequences.

4.2.7 Overall similarity of the 5'-UTRs of *AtP5CS1* and *AtP5CS2*

An obvious consideration in elucidating the functional significance of the redundancy in P5C synthesis from glutamate in *Arabidopsis* is whether or not this is related to divergence of the roles of both of the P5CS isoforms *in vivo*. Clearly, the 5'-UTRs of *AtP5CS1* and *AtP5CS2* contain many similar types of promoter elements, although the distribution of these putative regulatory sites differs between the two isogenes. The results presented thus far do not indicate whether or not there is substantial identity between the two 5'-UTRs at the nucleotide level.

Construction of a dot matrix plot (Boswell & Lesk 1988) provides an easy way to assess the extent and degree of homology of two nucleotide sequences and to represent this graphically. Using this method, complete identity between two sequences is indicated by a diagonal line across the matrix. Gaps in the diagonal indicate regions where homology over the defined window is below the assigned threshold value. As is evident from Figure 4.9B, the translated regions of the *AtP5CS1* and *AtP5CS2* genes have considerable homology. This high degree of similarity between the coding regions is also reflected at the amino acid level (Figure 4.2). Consistent with the use of the 5'- and 3'-ends of these cDNAs as gene-specific probes to distinguish between the two isoforms (Strizhov et al. 1997), there is limited homology at the two extremes of the *AtP5CS1* and *AtP5CS2* mRNAs (Figure 4.9B). In contrast, the 5'-UTRs of both genes indicate that considerable divergence has occurred in the upstream regions responsible for the transcriptional regulation of the two genes (Figure 4.9A).

Examination of the nucleotide similarity of the two 5-UTRs using BLAST (http://www.ncbi.nlm.nih) indicated that the only regions with extensive homology between both genes occur immediately upstream from the start codons of both *AtP5CS1* and *AtP5CS2*. These two regions are indicated below. The numbers indicating the distance upstream from the start codon of each gene.

AtP5CS1	-67	ACTGAGTCCGACTCAGTTAACTCGTTC	-41
AtP5CS2	-53	ACTGAGTCCGACTAAGTTGACTCGTTC	-27

While conservation of these elements owing to functional constraints cannot be formally

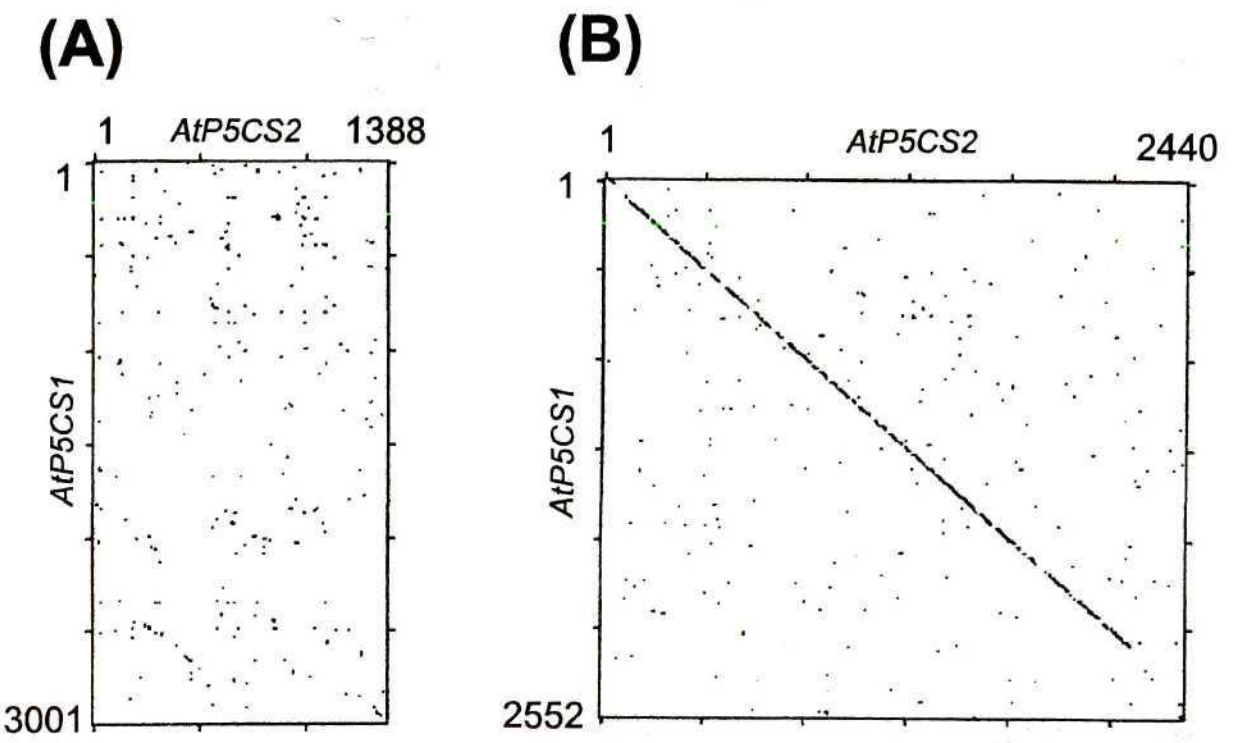


Figure 4.9. Dot matrix plots to examine the nucleotide similarity between the *AtP5CS1* and *AtP5CS2* genes in their 5'-UTRs (A) and translated regions (B). The similarities between the nucleotide sequences of *AtP5CS1* (y-axes) and *AtP5CS2* (x-axes) were assesses using the computer program DNASIS (Hibio). For both determinations, a window size of ten was used, with a stringency of nine [i.e. each dot in the matrix represents a match of at least nine bases in a sequence scan (window) of ten bases]. For **(A)**, the sequences used are a 3001 bp stretch upstream of the start codon of *AtP5CS1* (Genbank Accession No. AC003000) and the 1388 bp upstream of the start codon of *AtP5CS2* (Zhang et al. 1997). For **(B)**, the nucleotide sequences used are the sequences of cDNAs which encode AtP5CS1 (Strizhov et al. 1997; Genbank Accession No. X86777) and AtP5CS2 (Strizhov et al. 1997; Genbank Accession No. Y09355).

dismissed, their location downstream of the putative TATA boxes of both genes (Appendix) argues against any functional significance of the sequences in controlling transcriptional initiation.

4.3 Confirmation of the structures of the pBI-P5CS1(AS) and pBI-P5CR(AS) binary vectors

Over the past decade, the use of antisense RNA gene transcripts has become a well-established approach to suppress the expression of a specific protein *in vivo* (Bourque 1995). The technique involves the insertion, in an inverted or "antisense" orientation, of a cDNA or genomic sequence, or part thereof, between a promoter and terminator sequence that will be recognised and active within the plant genome. When transcribed *in planta*, this artificial gene produces a transcript that is complementary to the sense (coding) strand of the gene under investigation. The synthesis of

the antisense RNA frequently results in a substantial reduction in accumulation of the endogenous mRNA transcript and a reduced level of the encoded product, thus effectively reducing or blocking the biochemical function of the gene. An important advantage of this technique is the precision with which a single enzymatic activity can be decreased *in vivo* without appreciably altering the levels of other proteins. Furthermore, the observation that an interaction between two heterologous mRNA transcripts is capable of silencing the expression of an endogenous gene suggests that the antisense strategy can be effective in simultaneously downregulating the expression of isogenes within a multigene family, provided that the nucleotide sequences of their transcripts have not undergone extensive sequence divergence (Bourque 1995).

Since the use of the Ti (tumour-inducing) plasmid of *Agrobacterium tumefaciens* as a "gene ferry" for the transformation of plants is now well-documented, a detailed explanation of this approach to the generation of transgenic plants is beyond the scope of this document. Although many details of the transfer of *Agrobacterium* T-DNA to the plant genome are still unresolved, the major facets of the process which have been identified can be summarised as follows.

i) The T-DNA (transfer DNA) is flanked by two 25 bp imperfect repeats and is incorporated into the plant genome by a mechanism resembling bacterial conjugation.

ii) The proteins required by *Agrobacterium* both for sensing the wounded plant cell and for transfer of the T-DNA are encoded by the *vir* (virulence) region of the Ti plasmid.

iii) The T-DNA is transferred to the plant cell as a single-stranded intermediate and inserts stably into the nuclear genome, preferably into transcribed sequences, by a mechanism resembling illegitimate recombination.

For further details concerning our current knowledge of the mechanisms of T-DNA transfer, the reader is referred to the review articles of Zambryski (1992) and Zupan & Zambryski (1995). The development of binary vectors, such as pBI121 (Jefferson et al. 1987), was based on the observation that the 25 bp borders which flank the T-DNA are all that is required for the transfer of any segment of DNA which they enclose, provided that the transfer capability encoded by the *vir* region is supplied in *trans* (Hoekema et al. 1983). The obvious advantage of the use of binary transformation vectors is that they facilitate the cloning of any gene between the T-DNA borders. The DNA of interest replaces the normal *Agrobacterium* sequences, which are undesirable since they modify endogenous plant hormone levels and thus cause tumorigenesis.

As shown in Figure 4.10, the pBI-P5CS1(AS) and pBI-P5CR(AS) vectors contain inverted copies of the 105E5T7 and YAP057 cDNAs respectively. These are included within a region of the vectors that is flanked by the T-DNA borders which are recognised by *Agrobacterium tumefaciens* *vir* gene products. In the disarmed *A. tumefaciens* strain LBA4404, the virulence gene activities

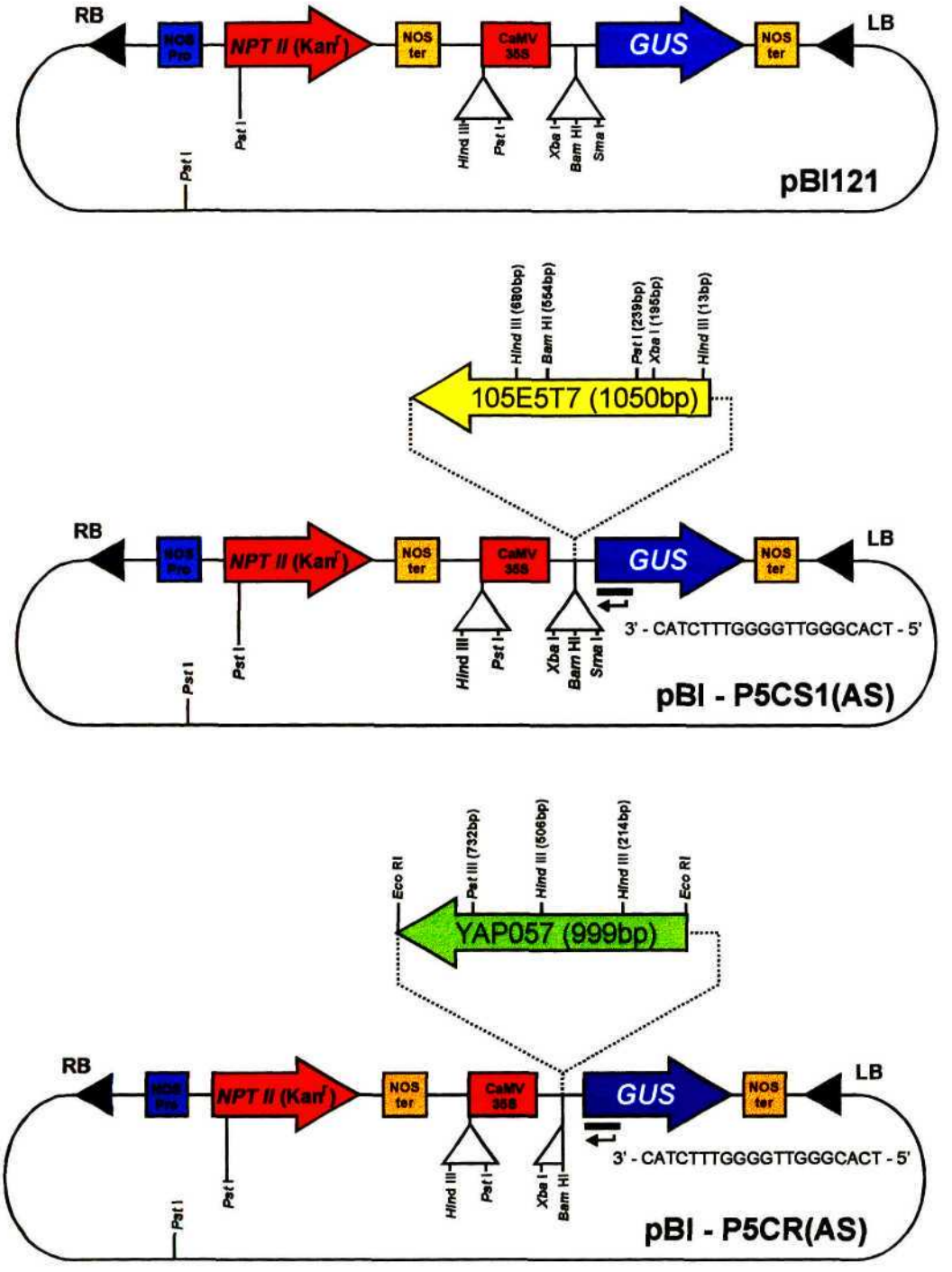


Figure 4.10: Structures of the pBI-P5CS1(AS) and pBI-P5CR(AS) constructs. The 1050 bp and 999 bp fragments of partial cDNAs encoding *Arabidopsis* P5CS and P5CR respectively, were inserted into pBI121 (Jefferson et al. 1987) in the antisense orientation between the cauliflower mosaic virus 35S rRNA promoter (CaMV 35S) and the coding sequence of the β-glucuronidase (*GUS*) gene. LB, T-DNA left border; NOS Pro, nopaline synthase gene promoter; NOS ter, nopaline synthase gene terminator; *NPTII*, neomycin phosphotransferase II gene; RB, T-DNA right border. Only the 105E5T7 and YAP057 inserts are drawn to scale. The numbers indicate the sites of cleavage relative to the 5' end of the cDNA inserts, as determined by sequence analysis (Figure 4.1; Hare 1995). The directionality of arrows within open reading frames denotes the 5'→3' orientation of the coding strand. The site of hybridisation of a sequencing primer used in the confirmation of the orientation of the 105E5T7 and YAP057 inserts is also indicated.

are provided in *trans*. The success of the cloning steps used in the construction of both of these pBI121-derived vectors was confirmed by restriction analysis and Southern hybridisation (Figure 4.11). Some non-specific cross-hybridisation was observed with pBI121-derived sequences when using both of the 105E5T7 (Figure 4.11B) and YAP057 (Figure 4.11B) probes. However, when using 105E5T7 as the probe, this was not evident for pBI121-derived fragments which were smaller than 20 kb (Figure 4.11B). This also applied when using YAP057 as the probe, with the exception of a 2.3 kb pBI121-derived fragment that resulted from digestion of pBI-P5CR(AS) with *Eco*RI (Figure 4.11C; lane h). Overall, the pattern of hybridisation obtained using both of the AtP5CS1 and AtP5CR cDNA probes is consistent with simplified restriction maps of these vectors provided in Figure 4.10.

Further confirmation of the antisense orientation of these cDNAs relative to the CaMV35S promoter was obtained through sequencing into the 5' ends of both of the 105E5T7 and YAP057 cDNAs using a primer (5'-TCACGGGTTGGGGTTTCTAC-3') which is complementary to a TATA-proximal region of the *GUS* (also sometimes referred to as *uidA*) coding strand (Clontech, Palo Alto, CA). Using this primer, 195 bp of sequence obtained using pBI-P5CS1(AS) template DNA and the 178 bp of sequence using pBI-P5CR(AS) template DNA were identical to the sequences of the 5' ends of the coding strands of 105E5T7 and YAP057 cDNAs respectively (data not shown).

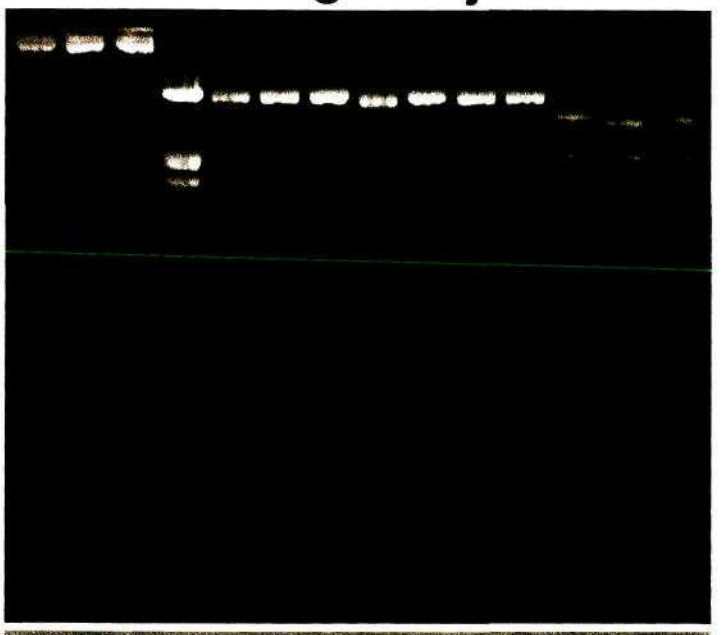
4.4 Transformation of *Arabidopsis* with pBI121, pBI-P5CS1(AS) and pBI-P5CR(AS)

In all experiments involving the regeneration of pBI-P5CS1(AS) and pBI-P5CR(AS) transformants, 1 mM proline was included in the media used as a measure to prevent selection against the regeneration of transformed cells which exhibit a strong antisense-mediated suppression of proline biosynthesis. The effects of exogenous proline on shoot organogenesis in wild-type (WT) *Arabidopsis* hypocotyl explants will be described in Section 4.10. Proline (1 mM) and kanamycin (50 mg l^{-1}) was also included in the germination medium (GM; Section 3.7) used for growth of the primary transformants (T_0 generation) to maturity as well as in all media used to germinate seed of the T_1 generation of individual lines.

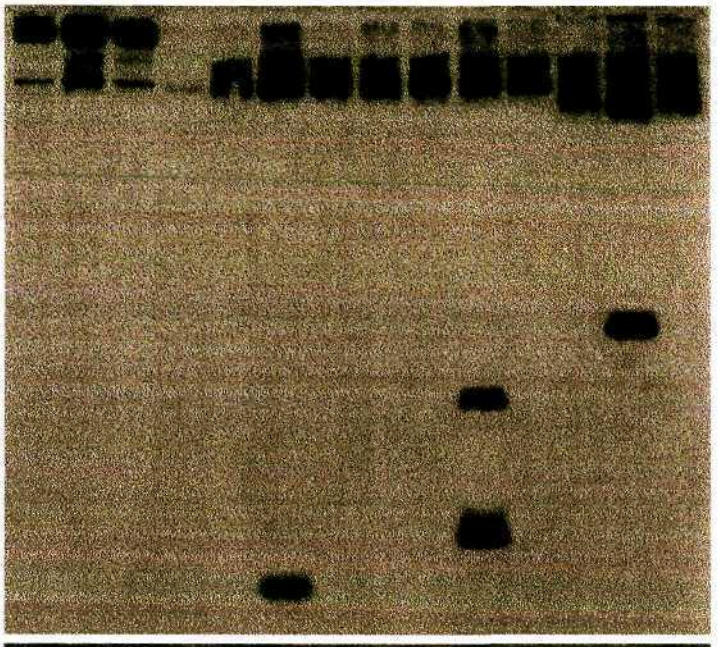
Despite the suggestion (Schmidt & Willmitzer 1988; Márton & Browse 1991) that acetosyringone is not necessary for the efficient transformation of explants from *Arabidopsis* and the omission of acetosyringone from standard protocols used in *Agrobacterium*-mediated transformation of this species (Valvekens et al. 1988; Akama et al. 1992), it was found that inclusion of 20 µM acetosyringone (Sheikholeslam & Weeks 1987) both in the media used to grow the

a b c d e f g h i j k l m n

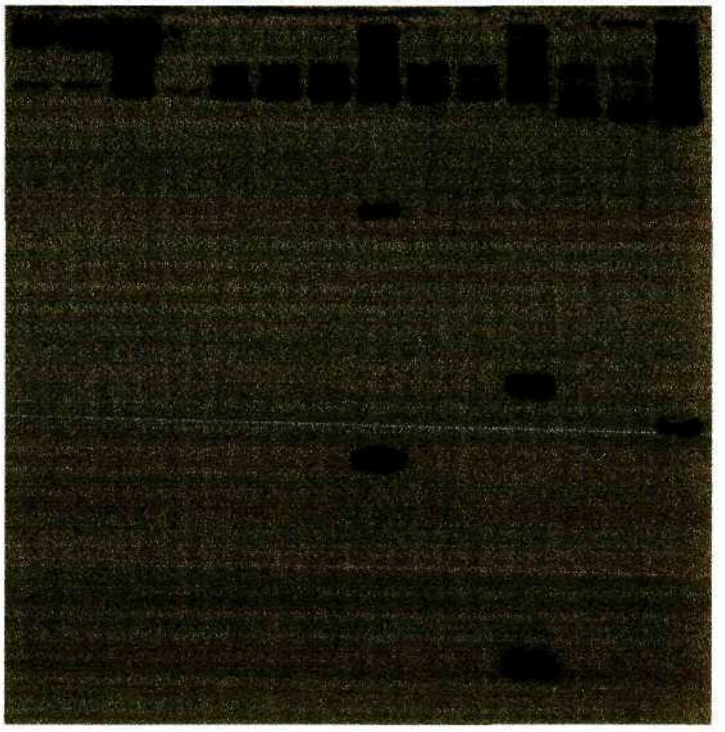
(A)



(B)



(C)



Agrobacterium strains and during co-cultivation on SIM was a critical requirement for acceptable transformation frequencies. Acetosyringone is essential for the induction of *vir* genes, some of which are responsible for pilus assembly. *Agrobacterium* pili are required for the transfer of DNA to plant cells in a process that resembles bacterial conjugation (Fullner et al. 1996).

Three to four weeks after infection of *Arabidopsis* hypocotyl explants with strains of *Agrobacterium tumefaciens* LBA4404 containing the plasmids pBI121, pBI-P5CS1(AS) and pBI-P5CR(AS), green kanamycin-resistant (Kan^r) *calli* were visible against a background of yellowish, untransformed kanamycin-sensitive (Kan^s) callus or hypocotyl explant. At least 120 putative Kan^r callus lines were obtained for each of the two antisense transformation vectors. Less effort was devoted to the generation of control lines transformed with pBI121. Approximately 30 putative pBI121-transformed calli were generated. Following transfer of the individual Kan^r calli to separate culture tubes containing fresh SIM supplemented with 50 mg l^{-1} kanamycin and 1 mM proline, less than 25% of these had undergone shoot regeneration within 2 months. Very few of the callus lines (less than 5%) were lost due to bacterial contamination, thus indicating that exposure to vancomycin during the initiation regeneration stage had effectively killed most of the *Agrobacteria*. After transfer of the regenerated shoots to boiler tubes containing GM supplemented with 1 mM proline and 50 mg l^{-1} kanamycin, most of the putative transformants rooted, although rooting was not a requisite for flowering and seed set *in vitro*. Since adequate gas exchange is an important consideration for the *in vitro* growth of *Arabidopsis* plants to maturity (Morris & Altmann 1994), loose cotton wool bungs were placed in the necks of the boiler tubes. Despite this measure, a low efficiency of seed set was noted for most of the lines (Table 4.4). Eleven pBI-P5CS1(AS) and nine pBI-P5CR(AS) lines were lost owing to their failure to set seed *in vitro*. This did not appear to be related to the phenotype of the antisense plants, since similar variability in the capacity for set seed was noted in WT plants grown *in vitro* on GM (data not shown) and pBI121 transformants grown on GM supplemented with 50 mg l^{-1} kanamycin (Table 4.4).

4.5 Characterisation of kanamycin resistance and β-glucuronidase (GUS) activity in transgenic *Arabidopsis* lines

Germination of seed collected from the T_0 generation of transgenic lines on GM supplemented with 50 mg l^{-1} kanamycin enabled confirmation of their transformation. Segregation of the T_1 progeny for T-DNA insertion was tested on the basis of kanamycin resistance. Inclusion of 50 mg l^{-1} kanamycin in GM reduced the rate of germination, but did not prevent radicle emergence and cotyledon expansion for the Kan^s WT. However, 14 d after germination, the cotyledons of WT seedlings had failed to accumulate chlorophyll, the seedlings never developed true leaves and

Table 4.4: Analysis of *Arabidopsis* pBI121, pBI-P5CS1(AS) and pBI-P5CR(AS) transformants for kanamycin resistance and β-glucuronidase (GUS) activity.

Line[a]		Segregation of kanamycin resistance [b]			GUS activity[c]
		No. of T_1 seed	Kan^r progeny	Kan^s progeny	
WT (Col-0)		NA	0	113	-
pBI121	A	33	31	2	+++
	B	12	11	0	+++
	C	21	20	1	+++
pBI-P5CS1(AS)	A2	13	12	1	+
	A3	18	17	0	+
	A5	24	21	3	-
	A7	13	12	0	ND
	A8	86	76	6	ND
	A9	25	23	2	++
	B5	6	6	0	+
	B6	67	48	14	+
	B7	71	61	5	ND
	B12	25	22	1	-
	C4	7	6	0	+
	C6	59	42	16	ND
	C10	20	18	1	ND
pBI-P5CR(AS)	A3	9	8	0	++
	A9	98	66	23	+
	B2	15	14	0	+
	D1	57	54	1	-
	D2	65	51	13	-
	E1	55	49	4	++
	F1	33	30	2	++

[a] All transgenic lines displayed a Kan^r phenotype during regeneration of the T_0 generation on SIM supplemented with 50 mg l^{-1} kanamycin. Transformation of all transgenic lines was confirmed by PCR-mediated amplification of fragments found within the *NPTII* and *GUS* genes (Figure 4.12) using genomic DNA isolated from Kan^r T_1 plants.

[b] For each transgenic line, all of the seed recovered from the primary transformant (T_0 generation) was tested for Kan^r growth on GM supplemented with 50 mg l^{-1} kanamycin and 1 mM proline. Seeds were harvested from *in vitro* grown plants, stored under sterile conditions for at least 21 days, and transferred to GM under sterile conditions. Wild-type (WT) plants displayed complete sensitivity to kanamycin. For all lines, a germination rate of at least 90% was noted.

[c] GUS activity in at least 50 seedlings from each transgenic line (T_2 generation) was assessed visually following histochemical staining (Section 3.8.2). Ratings of GUS activity as +++, ++, + and - denote strong, intermediate, weak and no detectable GUS activity, respectively.

Kan^r, kanamycin resistant; Kan^s, kanamycin sensitive; NA, not applicable; ND, not determined

root growth was strongly inhibited relative to WT seedlings grown in the absence of exogenous kanamycin. All of the putative transformants yielded Kan^r T_1 progeny. The ratios of Kan^r : Kan^s progeny of each transgenic line were determined 14 d after germination by counting the number of seedlings which had green cotyledons and at least two true leaves versus those that had bleached cotyledons and did not develop true leaves. Root growth in the Kan^r progeny was considerably better than that observed for the bleached Kan^s seedlings. Where an adequate number of T_1 seed were recovered, the progeny segregated for kanamycin resistance. Segregation of the kanamycin resistance character in self-pollinated T1 seed from the transformants revealed that most of the plants were likely to have more than one active T-DNA insert (Table 4.4). A segregation pattern of roughly 3:1 (Kan^r : Kan^s) is expected for expression from a single chromosomal locus. Following hardening-off, the Kan^r plants (T_1 generation) were grown to maturity under greenhouse conditions. None of the transgenic lines were lost at this stage.

Transformation of the pBI121 (positive control), pBI-P5CS1(AS) and pBI-P5CR(AS) lines was confirmed by PCR-mediated amplification of approximately 0.6 kb and 1.2 kb fragments found within the ORFs of the *NPTII* and *GUS* genes respectively (Figure 4.12). Genomic DNA isolated from shoot tissue from the T_1 generation of greenhouse-grown plants was used as the template for PCR-mediated gene amplification. No amplification occurred with the same primer sets using WT genomic DNA as template (Figure 4.12). Genomic DNA from each of the pBI-P5CS1(AS) transformants was also used as the template for PCR-mediated amplification of a 2.1 kb fragment flanked by the sequences recognised by the H1 primer (Figure 3.1) and a primer specific to a sequence found on the 3' side of the *GUS* gene (Figure 4.12). This confirmed that the fusion of the antisense 105E5T7 insert to *GUS* had been stably incorporated into these lines. No amplification product was obtained when genomic DNA from any of the pBI121 control lines was used as a template for amplification using the H1 primer and the *GUS*-specific right-hand side primer (Figure 4.12).

Assessment of GUS activity in the Kan^r progeny of the T_2 generation of each transgenic line indicated variable activity of the enzyme in different lines. As shown in Table 4.4 and illustrated for representative lines in Figure 4.13, GUS activity in many of the transgenic lines was relatively insensitive to fusion of either of the 105E5T7 or YAP057 cDNA fragments to the 5' end of the *GUS* gene. Visual assessment of the intensity of histochemical staining for GUS activity indicated a reduction in the intensity of staining of all of the pBI-P5CS1(AS) and pBI-P5CR(AS) lines relative to the three pBI121 control lines. Of the transgenic lines tested, the greatest reduction in GUS activity relative to pBI121 controls was in the pBI-P5CS1(AS) lines A5 and B12 and in the pBI-P5CR(AS) transformants D1 and D2 (Table 4.4). In these lines, the absence of GUS activity

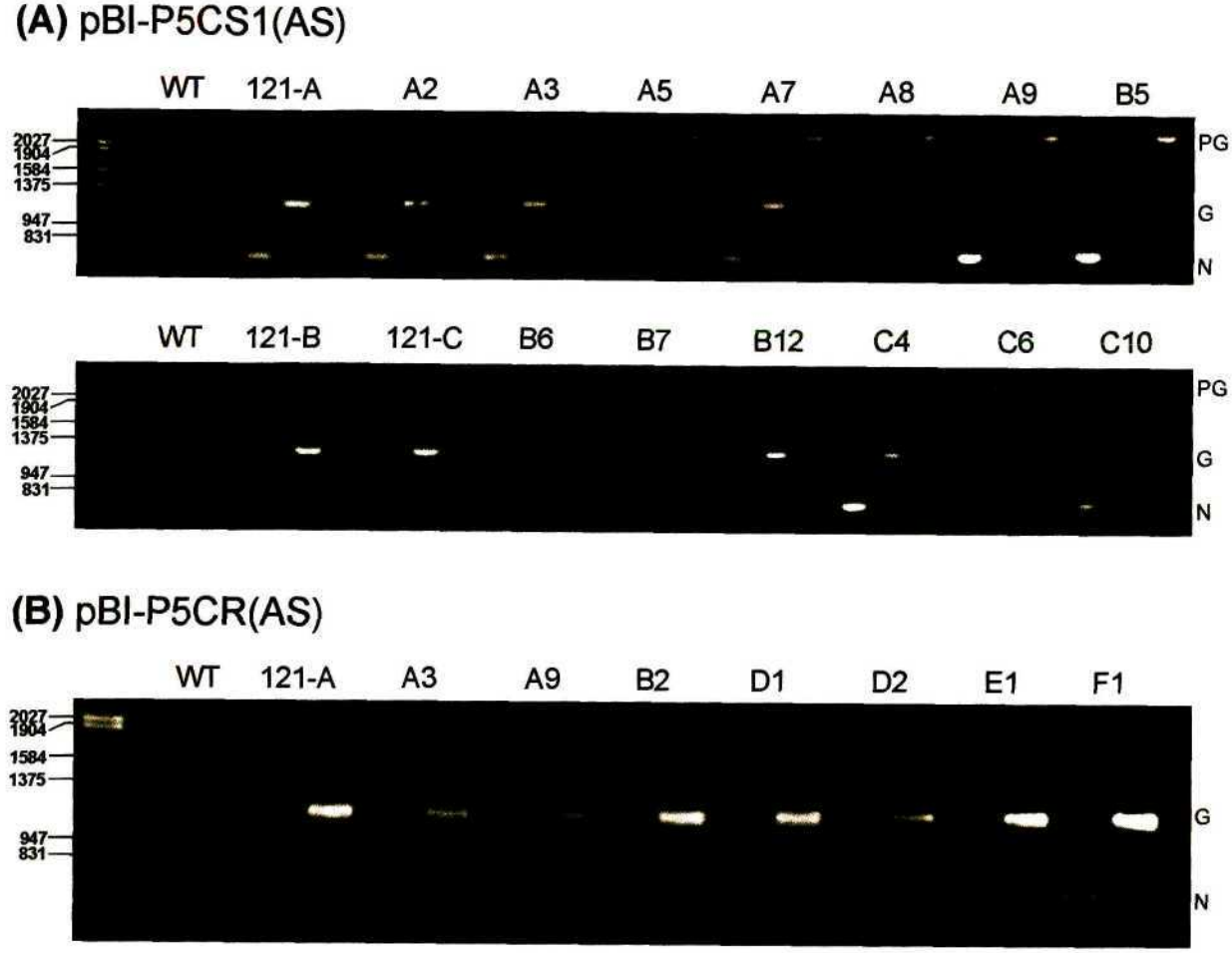


Figure 4.12: PCR-mediated amplification of DNA fragments within the *NPTII* and *GUS* coding regions using genomic DNA from *Arabidopsis* pBI121, pBI-P5CS1(AS) and pBI-P5CR(AS) transformants. (A) Amplification products obtained using genomic DNA from pBI-P5CS1(AS) lines, wild-type plants (negative control) and pBI121 transformants (positive controls). **(B)** Amplification products obtained using genomic DNA from pBI-P5CR(AS) lines, wild-type plants (negative control) and pBI121 transformants (positive control). Lanes containing the amplification products from two separate amplification reactions for each primer set using wild-type genomic DNA as template are denoted as WT. Lanes denoted 121-A, 121-B and 121-C contain the amplification products obtained using three independent lines transformed with pBI121. For all lines, the order of samples loaded is for amplification of a 0.6 kb fragment within *NPTII* (N) followed by the amplification of a 1.2 fragment within the *GUS* gene (G). Details of the PCR conditions are provided in Section 3.8.1. Confirmation of the inclusion of the 105E5T7 insert within pBI-P5CS1(AS) transformants was obtained through amplification of a 2.1 kb fragment (PG) following PCR-mediated amplification using the H1 primer (Figure 3.1) and the *GUS*-specific right-hand side primer (Section 3.8.1). Each gel slot was loaded with 20 µl of the 50 µl of amplification product following PCR (Section 3.8.1). All genomic template DNA was isolated from plants of the T_1 generation of each line.

resembled that observed for WT seedlings (Figure 4.13). The use of the Kan^r line CS3399 (obtained from the *Arabidopsis* Biological Resource Centre, Ohio State University, OH) which does not carry the *GUS* gene eliminates the possibility that growth of the pBI-P5CS1(AS) and pBI-P5CR(AS) seedlings in the presence of kanamycin may have influenced the result of the histochemical staining assay for GUS activity.

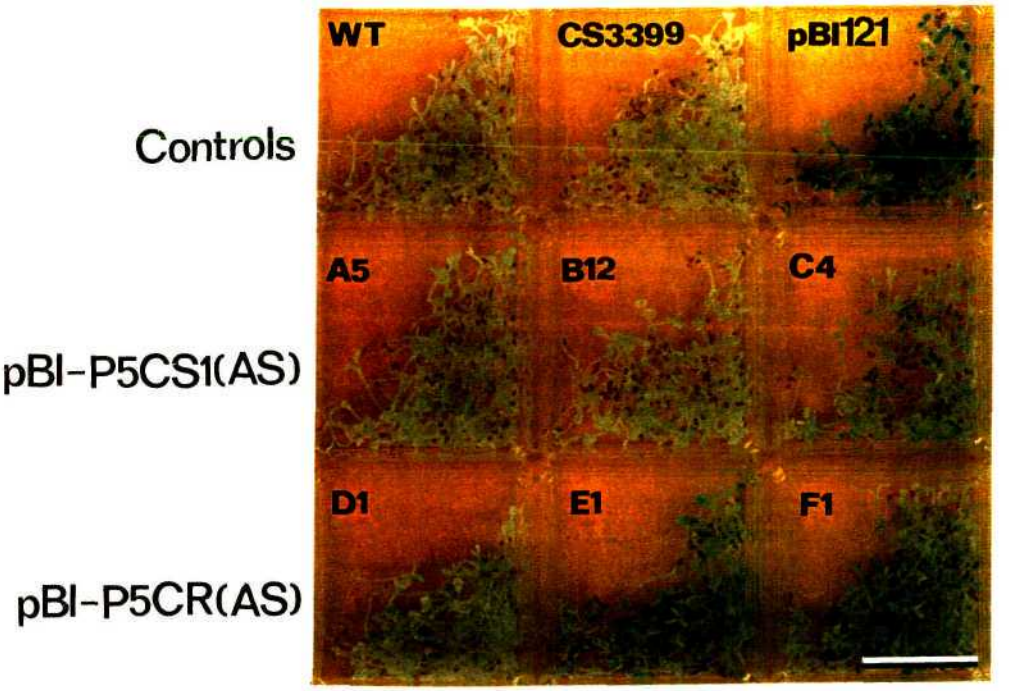


Figure 4.13 : Histochemical analysis of β-glucuronidase (GUS) activity in wild-type *Arabidopsis* and representative lines transformed with pBI121, pBI-P5CS1(AS) and pBI-P5CR(AS). Surface sterilised seed of the T_2 generation of all lines generated in this study was germinated in liquid MS medium (Murashige & Skoog 1962) containing 50 mg l^{-1} kanamycin and 30 g l^{-1} sucrose. β-glucuronidase activity was assayed seven days after germination as described in Section 3.8.2. No GUS activity was detected in wild-type (WT) plants or in a kanamycin-resistant line CS3399 (obtained from the *Arabidopsis* Biological Resource Centre, Ohio State University, OH) which does not carry the *GUS* gene. Some GUS activity in pBI-P5CS1(AS) line C4 and in pBI-P5CR(AS) transformants E1 and F1 indicates that expression of the *GUS* gene was not completely disrupted by fusion of the antisense copies of either 105E5T7 or YAP057 to the 5'-end of the *GUS* open reading frame. Nonetheless, where GUS activity was observed in antisense lines, it was always lower than that found in any of the three pBI121-transformed lines which were tested (Table 4.4). The scale bar represents 10 mm.

4.6 Characterisation of pBI-P5CS1(AS) and pBI-P5CR(AS) seedlings with respect to growth rates and proline accumulation in the presence and absence of stress

Shortly after transfer of the Kan^r plants of the T_1 generation from media containing proline to potting medium, it became apparent that none of the transgenic lines were proline auxotrophs. When grown under greenhouse conditions, the rosette and inflorescence morphologies of the various antisense lines were indistinguishable from those of the WT. Owing to the ease with which tobacco can be transformed, a series of parallel experiments involved transformation of *Nicotiana tabacum* cv. White Burley with both of the pBI-P5CS1(AS) and pBI-P5CR(AS) constructs. A total of 41 pBI-P5CS1(AS) and 29 pBI-P5CR(AS) tobacco transformants were

regenerated on hormonally-supplemented media containing 1 mM proline and 200 mg l^{-1} kanamycin. Like the *Arabidopsis* transformants, none of these lines showed a visible reduction in growth following hardening off of the T_0 plants and their growth to maturity under greenhouse conditions. At least four of the tobacco pBI-P5CS1(AS) transformants and three of the tobacco pBI-P5CR(AS) lines showed some evidence of abnormal morphology of certain of the flowers. The most common defect observed was an increase in the length of the style relative to that of the filaments. This caused the stigma to extend beyond the position of the anthers. Reduced seed yield in these lines most probably arose from a reduction in the capacity for self-pollination. More rarely, a few flowers in some of the affected plants were found to have petaloid stamens. Multiple corollas and a split floral tube were amongst other abnormalities observed in the affected pBI-P5CS1(AS) and pBI-P5CR(AS) tobacco transformants, although floral defects were never found in all of the flowers of a single plant. In two of the tobacco pBI-P5CS1(AS) lines, some of the leaves were malformed, with evidence of aberrant leaf shape and a crinkled appearance of the leaf margin. Abnormalities in either the leaf shape and floral morphology were not observed in any of six tobacco pBI121 (control) transformants. None of the pBI-P5CS1(AS) tobacco lines displayed an aberrant morphology in both their leaves and their flowers. Preliminary analysis of free proline levels in 14 d-old seedlings of the T_1 generation of four of the tobacco pBI-P5CS1(AS) and three of the tobacco pBI-P5CR(AS) lines which displayed the most severe morphological defects did not indicate any significant reduction in their proline levels (data not shown). Since the morphological aberrations in these tobacco lines may merely have originated from effects associated with somaclonal variation or insertional mutagenesis, the tobacco antisense lines were not investigated any further. Heterologous antisense sequences have often been found to inhibit gene expression quite efficiently and the effectiveness of antisense transcripts in silencing expression of the homologous gene in a different species is well documented (van der Krol et al. 1988; Temple et al. 1993; Kozaki & Takeba 1996). This indicates that a partial complementarity between the sense and antisense RNAs is frequently adequate to inhibit gene expression. While the focus of this study concerned examination of proline synthesis in *Arabidopsis*, the failure to observe any symptoms of proline auxotrophy in any of the antisense tobacco plants suggested that continued screening for *Arabidopsis* pBI-P5CS1(AS) and pBI-P5CR(AS) transformants with dramatic symptoms of proline auxotrophy was not warranted.

One advantage of the use of *Arabidopsis* as a model system, besides its streamlined genome and well-characterised genetics, is that its small size permits the growth of large numbers of seedlings on nutrient media plates. When the plates are oriented vertically, the roots grow along the surface of the agar media. Since the primary *Arabidopsis* root grows at an essentially constant radius, root elongation rates provide a facile means of assessing the overall growth rates of a large number of different genetic lines. Preliminary investigations of the inhibition of root elongation by NaCl in

the T_2 generation indicated that the most severely affected pBI-P5CS1(AS) lines were A5, B12 and C4, and the most severely affected pBI-P5CR(AS) lines were A3, D1 and D2. When at least fifty 10 d-old seedlings of each of the other antisense *Arabidopsis* lines were tested, none of them displayed a significant ($P < 0.05$; Student's *t*-test) reduction in root growth relative to the WT plants when grown in MS/2 medium (half-strength MS salts with 5.0 g l^{-1} sucrose) either with or without 100 mM NaCl (data not shown). Analysis of root elongation rates of three pBI121 transformants indicated that these were not significantly different to WT plants in the absence of stress or when germinated and grown in the presence of 100 mM NaCl (data not shown). More extensive analysis of the growth of pBI-P5CR(AS) lines A3, D1 and D2 on MS/2 supplemented with 5.0 g l^{-1} sucrose and a range of different salt concentrations (0 mM, 80 mM, 120 mM and 150 mM NaCl) indicated that none of the lines showed a significantly decreased growth rate relative to 10 d-old WT plants when grown in the absence of NaCl or when germinated and grown in media supplemented with 80 mM NaCl. At 120 mM NaCl, growth of all three of the pBI-P5CR(AS) lines was significantly less than that of WT seedlings. At 150 mM NaCl, growth of pBI-P5CR(AS) transformant D2 was not significantly different to that observed for the WT. This data is presented in Table 4.5.

Table 4.5: The effects of exogenous NaCl on root elongation of wild-type *Arabidopsis* seedlings and pBI-P5CR(AS) transformants A3, D1 and D2. At least fifty seedlings of each of the wild-type (WT) and pBI-P5CR(AS) transformants A3, D1 and D2 seedlings (T_2 generation) were grown on MS/2 medium (half-strength MS salts; Murashige & Skoog 1962) containing 5.0 g l^{-1} sucrose and the concentration of NaCl indicated. The length of the primary roots was measured 10 days after germination. Values in parenthesis indicate standard errors. Root lengths of pBI-P5CR(AS) transformants that are significantly different from the wild-type (WT) for that treatment ($P < 0.05$; Student's *t*-test) are denoted by an asterisk.

Treatment	Mean root length (mm)			
	WT	A3	D1	D2
0 mM NaCl	**26.84** (1.07)	**25.02** (0.90)	**25.88** (0.95)	**23.98** (1.16)
80 mM NaCl	**18.46** (0.91)	**16.51** (0.81)	**18.19** (0.71)	**18.02** (0.96)
120 mM NaCl	**13.04** (0.52)	**9.10 *** (0.57)	**7.13 *** (0.53)	**9.52 *** (0.89)
150 mM NaCl	**4.64** (0.46)	**2.36 *** (0.39)	**3.40 *** (0.34)	**3.35** (0.44)

Far more dramatic effects in the constitutive growth rate were observed for the pBI-P5CS1(AS) transformants A5 and B12 when grown on MS/2 medium containing 5.0 g l^{-1} sucrose (Figure

4.14A). Since these were the two most severely affected pBI-P5CS1(AS) lines, they were selected for use in all subsequent experiments. A low level of GUS activity in these two lines (Table 4.13; Figure 4.4) seemed consistent with the view that these two lines might display an effective antisense effect. Since the 105E5T7 cDNA in the antisense orientation was fused to *GUS* to produce a chimeric transcript, a lower level of GUS activity in these two lines relative to that found in other pBI-P5CS1(AS) transformants may reflect attenuation of the expression of 105E5T7::*GUS* fusion. Assuming that uncontrolled determinants of transgene expression, such as the site of transgene incorporation within a chromosome, do not have a dramatic effect on the variability in GUS expression between different lines, an efficient antisense effect with endogenous *P5CS* transcripts might be expected to destabilise expression of the attached *GUS* gene. As shown in Figure 4.14A, root growth of 10 d-old seedlings of both of the pBI-P5CS1(AS) transformants A5 and B12 was less than that of comparable WT seedlings at all of the concentrations of NaCl tested. The determination of an I_{50} value (concentration of NaCl which causes 50% inhibition in root growth relative to growth in medium without NaCl) is a useful indicator of the sensitivity of a genetic line to salinity stress. Surprisingly, despite the constitutively reduced growth rate of the pBI-P5CS1(AS) lines A5 and B12, an I_{50} value of between 90mM NaCl and 95 mM NaCl was found for both these lines as well as for WT seedlings (Figure 4. 15A). This suggests that neither of the lines is much more sensitive to NaCl than is the WT, at least at the seedling stage.

An important aspect of the use of transgenic antisense lines to study the effects of disruption of a metabolic reaction is the design of experiments which might reveal a physiological effect of the genetic modification which may not be evident under optimal growth conditions. Plant metabolism displays considerable flexibility. This plasticity might permit modifications in the rates of metabolic interconversions that can overcome a disruption in a single reaction and thereby mask the phenotypic changes that might be anticipated following the downregulation of expression of a single gene or members of a gene family. For instance, a block in P5C synthesis from glutamate could conceivably be overcome by OAT-catalysed P5C synthesis from ornithine (Figure 2.7). Delauney et al. (1993) reported an inverse relationship between the expression of genes encoding P5CS and OAT in *Vigna aconitifolia* that is related to the plant nitrogen status (Section 2.4.5.3). Although Roosens et al. (1998) did not assess the effects of plant nitrogen status on the relative contributions of P5CS and OAT activities to the P5C pool in *Arabidopsis*, it might be anticipated that the differences in growth rates between WT plants and the pBI-P5CS1(AS) transformants A5 and B12 would be more evident after growth under nitrogen-limiting conditions. For this reason, the growth of WT seedlings, and T_2 seedlings of the pBI-P5CS1(AS) lines A5 and B12 was tested on MS/2 media in which the nitrogen sources had been omitted (MS/2-N medium; Section 3.6.2). Surprisingly, omission of nitrogen (provided as 10 mM NH_4NO_3 and 10 mM KNO_3

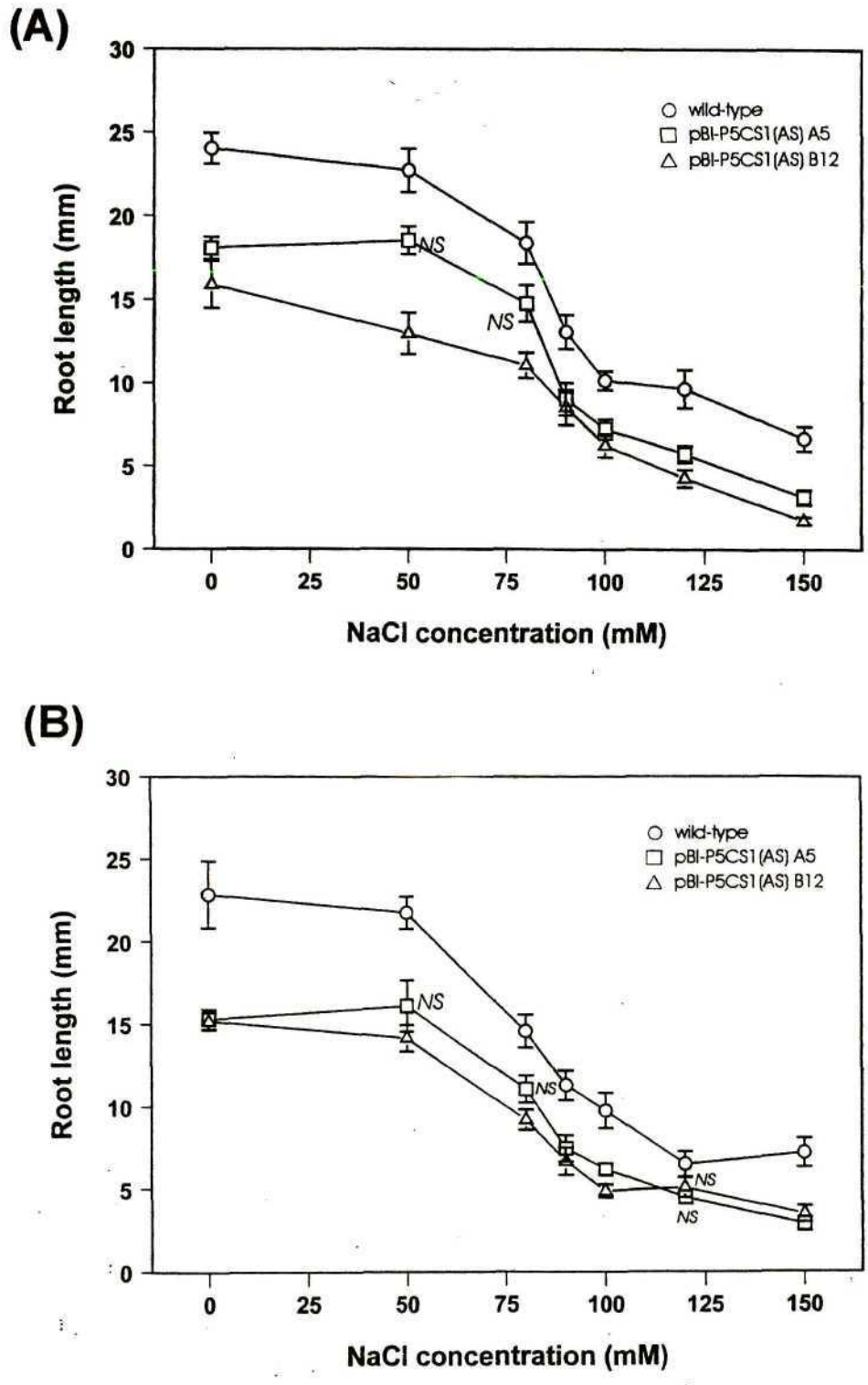


Figure 4.14: The effects of exogenous NaCl on root elongation of wild-type *Arabidopsis* seedlings and the pBI-P5CS1(AS) transformants A5 and B12. For each treatment, root lengths of at least 50 of each of wild-type (○), A5 (□) and B12 (△) seedlings were measured 10 d after germination. For the two pBI-P5CS1(AS) lines, seedlings of the T_2 generation were used. **(A)** Growth on MS/2 medium containing the concentration of NaCl indicated. Estimated I_{50} values are 95 mM NaCl (WT), 91 mM NaCl (A5) and 92 mM NaCl (B12). **(B)** Growth on MS/2-N medium containing the concentration of NaCl indicated. Estimated I_{50} values (concentrations of NaCl which cause 50% inhibition of root growth relative to growth in medium without NaCl) are 90 mM NaCl (WT), 90 mM NaCl (A5) and 87 mM NaCl (B12). Both basal media (Section 3.6.2) contained 5.0 g l^{-1} sucrose. Error bars indicate standard errors. Unless indicated, root lengths of each of the antisense lines at a given NaCl concentration were significantly different ($P < 0.05$; Student's *t*-test) than that of the wild-type. *NS* = $P > 0.05$ (Student's *t*-test).

in MS/2 medium) from the media did not have a dramatic effect on seedling growth (Figure 4.14B). In the absence of any NaCl stress, a slight decrease in root growth in the WT and both of the pBI-P5CS1(AS) lines A5 and B12 following omission of a nitrogen source from the media was not statistically significant ($P > 0.05$; Student's *t*-test). Comparison of the I_{50} values when plants were grown on a nitrogen-replete medium (Figure 4.14A) and in the absence of an exogenous nitrogen source (Figure 4.14B) suggested that nitrogen deficiency increased the sensitivity of WT and B12 seedlings to NaCl, but did not have a dramatic effect on the inhibition of root growth by NaCl in A5 (Figure 4.14).

The observations that root growth in the two pBI-P5CS1(AS) lines appears to be constitutively lower than that of the WT irrespective of the imposition of salinity stress and that the overall sensitivity of lines A5 and B12 to inhibitory concentrations of NaCl does not appear to be much higher than that observed for the WT complicates interpretation of the proposed importance of proline synthesis in mediating adaptation to stress. The apparent failure of incorporation of antisense *AtP5CS1* transgenes to affect the sensitivity to NaCl suggests that the reduction in growth rate observed for A5 and B12 seedlings may merely arise from a slight deficiency in proline which affects the synthesis of proline- and hydroxyproline-containing proteins. This interpretation is supported by the observation that the root growth rates of both of the pBI-P5CS1(AS) transformants A5 and B12 can be restored to WT levels by inclusion of 1 mM proline in GM-based growth medium, but not by 1 mM glutamate (Table 4.6). Glutamate appeared to stimulate growth of the WT seedlings, even in the presence of 80 mM NaCl, but the growth was always greater than for the two antisense lines, either in the presence or absence of a salinity stress. Exogenous proline increased root growth rates of the WT and both A5 and B12, although it was incapable of restoring growth of B12 seedlings to WT levels in the presence of 80 mM NaCl.

Owing to the small size of *Arabidopsis*, it is more difficult to quantify shoot growth in transgenic lines than it is to assess changes in the root lengths. No differences between the rates of leaf initiation in the WT and T_2 generation plants of either of the pBI-P5CS1(AS) transformants A5 or B12 were noted when plants were grown in the absence of kanamycin. Leaf initiation rates were determined by monitoring the increase in the number of true leaves for 14 d after germination. When grown on GM without kanamycin, the first pair of true leaves for both WT and both antisense lines appeared between 48 h and 72 h after germination. Within 5 d after germination, most of the seedlings had four true leaves and within 10 d, an additional two leaves were evident.

The significant decrease in root growth rate in both pBI-P5CS1(AS) lines relative to the WT, together with the ability of exogenous proline to restore their overall growth rate to WT levels

Table 4.6 : The effects of exogenous proline and glutamate on root elongation of wild-type *Arabidopsis* seedlings and the pBI-P5CS1(AS) transformants A5 and B12 in the presence and absence of salinity stress. Seedlings of the wild-type (WT) and pBI-P5CS1(AS) transformants A5 and B12 (T_2 generation) were grown on GM (Section 3.7) and the supplements indicated. The lengths of the primary roots were measured 10 d after germination. Each value is the mean of at least 50 independent measurements. Values in parenthesis indicate standard errors. Root lengths of pBI-P5CS1(AS) transformants that are significantly different from the WT for that treatment are denoted by asterisks (* = $P < 0.05$; ** = $P < 0.01$; Student's *t*-test).

Treatment	Mean root length (mm)		
	WT	A5	B12
0 mM NaCl	25.8 (0.89)	18.45 ** (0.99)	17.06 ** (1.81)
80 mM NaCl	21.63 (0.96)	14.94 * (1.27)	13.07 ** (1.13)
1 mM proline	23.01 (0.99)	23.99 (0.947)	22.45 (1.69)
80 mM NaCl + 1 mM proline	24.99 (1.61)	22.68 (1.63)	19.27 * (1.69)
1 mM glutamate	29.67 (1.10)	20.72 ** (1.08)	19.88 ** (1.12)
80 mM NaCl + 1 mM glutamate	27.71 (0.90)	19.56 ** (1.28)	17.82 ** (0.95)

(Table 4.6) prompted investigation of whether free proline levels in A5 and B12 are lower than those found in comparable WT plants. At this stage of the study, the report by Savouré et al. (1997) that ABI1 may exert a post-transcriptional effect on *AtP5CS1* mRNA accumulation after NaCl-stress, but not after exposure to an isosmotic concentration of sorbitol, suggested that it might be worthwhile to investigate whether or not an ABI1-regulated post-transcriptional event might be involved in at least partially overcoming the effects of any depression in total *AtP5CS* transcript levels in the transgenic lines. With this in mind, it was decided to use the same experimental conditions as those described by these workers (Savouré et al. 1997) to compare the effects of ionic and non-ionic osmotic stresses on proline accumulation in the WT with that observed in the pBI-P5CS1(AS) lines A5 and B12. In the absence of any stress, a slight but statistically significant decrease in free proline concentrations was observed in both of the antisense lines relative to WT controls (Figure 4.15). Both lines accumulated proline following the imposition of either NaCl- or sorbitol-mediated stress or prolonged exposure to low temperature. However, after all stress treatments, the magnitude of the increase in free proline in the antisense lines was less than that observed for the WT plants. For both A5 and B12, the percentage reduction in free proline levels relative to those found in the WT was greater following sorbitol-

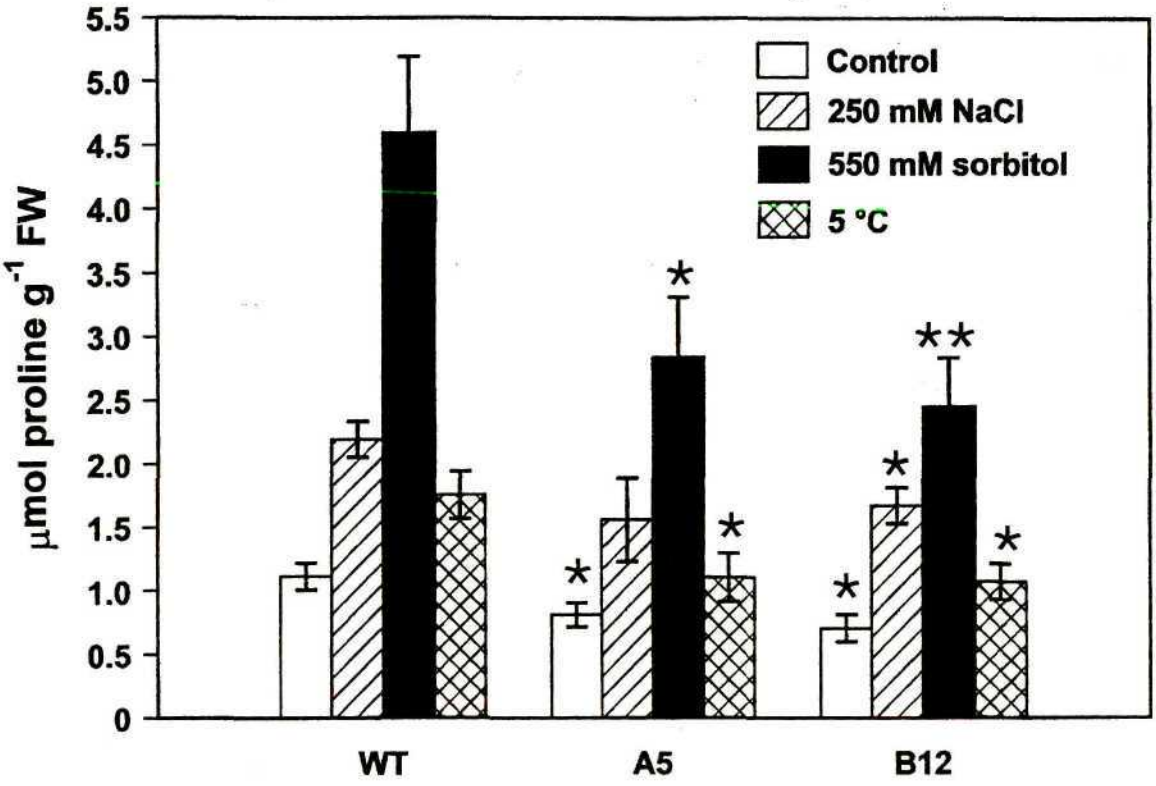


Figure 4.15: Levels of free proline in wild-type *Arabidopsis* seedlings and comparable seedlings of the T_2 generation of pBI-P5CS1(AS) transformants A5 and B12 before and after the imposition of NaCl-, sorbitol- or cold stress. Unstressed (open bars), NaCl-stressed (diagonally shaded bars), sorbitol-stressed (closed bars) or cold-stressed (cross-hatched bars) 14 d-old seedlings were prepared as described in Section 3.6.3. Proline concentrations in unstressed plants and following both NaCl- and sorbitol stress are the means of nine determinations involving three separate experiments. Determinations following cold treatment are the means of six separate determinations involving two separate experiments. Error bars indicate the standard deviation. For the results obtained using pBI-P5CS1(AS)-transformed lines A5 and B12, * denotes a significant difference ($P < 0.05$; Student's *t*-test) and ** denotes a highly significant difference ($P < 0.01$; Student's *t*-test) from the value obtained for that treatment using WT seedlings.

mediated osmotic stress than after incubation for the same time period in an isosmotic concentration of NaCl (Figure 4.15). Exposure of plants to cold stress (5 °C) for 24 h (Section 3.6.3) caused a slight increase in free proline levels in the WT and both pBI-P5CS1(AS)) transformants A5 and B12. However, cold-induced proline accumulation in both pBI-P5CS1(AS) lines A5 and B12 was significantly less than that observed for WT plants (Figure 4.15).

4.7 Physiological investigations concerning a regulatory role for proline metabolism in the regulation of *Arabidopsis* seed germination

If regulated changes in the rates of proline synthesis and degradation play a significant role in the modulation of cellular redox status during stress, then it might be anticipated that multiple functions for this quasi-constitutive metabolic system involved in regulating the levels of a proteinogenic imino acid may have been recruited to ensure metabolic homeostasis during the normal growth and development of plants. The effects of exogenous proline on *Arabidopsis* seedling growth and development will be described in Section 4.8. Exogenous proline had a dose-dependent inhibitory effect on the rate of radicle emergence in WT *Arabidopsis* seeds (Table 4.7). By 72 h after seed imbibition, radicle emergence in the presence of even the highest concentration of proline tested was not significantly reduced. Thus, exogenous proline at concentrations of 20 mM or less did not prevent seed germination.

Table 4.7: The effect of exogenous proline on radicle emergence in *Arabidopsis*. Seeds of wild-type *Arabidopsis* (ecotype Columbia) were transferred directly to MS/2 media supplemented with the concentration of proline indicated within 3 h after surface sterilisation and were incubated under continuous light at 25 °C (Section 3.6.1). The values indicate the average number of seeds that had germinated at that time, per replicate comprising 50 seeds. For each treatment, germination of 300 seeds was monitored (n = 6). Asterisks indicate differences that are significantly different from the control treatment (0 mM proline) at that time after imbibition (* = $P < 0.05$; ** = $P < 0.01$; Student's t-test). Similar results were obtained when the entire experiment was repeated, using 200 seeds per treatment.

	Mean number of seeds germinated per 50 seeds (hours post-imbibition)				
	36 h	**42 h**	**48 h**	**60 h**	**72 h**
0 mM proline	26.7	35.3	41.7	43.8	44.3
2.5 mM proline	20.2 *	34.0	40.8	43.5	45.0
5.0 mM proline	12.8 **	30.3 **	39.7	45.2	45.3
10.0 mM proline	4.2 **	19.3 **	34.8 **	41.7	44.2
20.0 mM proline	1.7 **	11.0 **	31.7 **	35.2 **	42.7

Chilling of imbibed *Arabidopsis* seeds at 4 °C for 48 h in the dark synchronises their germination and increases the speed or germination upon transfer to higher temperatures (Koornneef & Karssen 1994). As shown in Table 4.8, when WT seeds that are imbibed in the presence of

proline and stratified at 4 °C in darkness for 48 h before incubation at 25 °C under continuous light, the inhibitory effect of proline is not as evident as when seeds are not given a chilling pre-treatment (Table 4.7).

Table 4.8: The effect of exogenous proline on radicle emergence in *Arabidopsis* seeds following pre-incubation in the cold. Surface-sterilised seeds of wild-type *Arabidopsis* (ecotype Columbia) were transferred to MS/2 medium containing the concentration of proline indicated and incubated at 4 °C in darkness for 48 h prior to transfer to conditions of continuous light at 25 °C (Section 3.6.1). The time indicated denotes the time after transfer to 25 °C. The values indicate the average number of seeds that had germinated at that time, per replicate comprising 50 seeds. For each treatment, germination of 200 seeds was monitored (n = 4). The seeds used were from the same batch used to obtain the results presented in Table 4.7 and their germination was tested at the same time after the date of harvesting. Asterisks indicate differences that are significantly different from the control (0 mM proline) at that time after imbibition (* = $P < 0.05$; ** = $P < 0.01$; Student's t-test).

	Mean number of seeds germinated per 50 seeds (hours after transfer to 25 °C)		
	36 h	**48 h**	**60 h**
0 mM proline	40.5	43.5	45.0
2.5 mM proline	41.8	43.0	46.5
5.0 mM proline	35.0	41.0	44.3
10.0 mM proline	32.5 *	39.5	46.0
20.0 mM proline	26.5 **	36.5 *	43.8

As was mentioned in Section 2.2.3, changes in cellular redox potential have been suggested to be important in mediating the chain of events that ultimately results in the ability of embryo growth to overcome the mechanical constraint imposed by the testa. The ability of electron acceptors to stimulate germination in many species is consistent with the notion that activation of the OPPP is an important trigger of radicle emergence. Since the activities of the two dehydrogenases which catalyse the rate-limiting steps in the OPPP are inhibited by the NADPH which they generate, electron acceptors relieve feedback inhibition of OPPP activity through the maintenance of the NADP pool in a primarily oxidised state (Roberts 1973). Inclusion of either 5.0 µM methylene blue (MB) or 2.5 µM phenazine ethosulphate (PES) in MS/2 medium did not increase the rate of *Arabidopsis* seed germination when seeds were imbibed in the absence of exogenous proline (Table 4.9). However, when included in media containing either 10 mM or 20 mM proline, both 5.0 µM MB and 2.5 µM PES significantly increased rates of germination relative to the appropriate

controls when radicle emergence was tested 36 h and 42 h after imbibition (Table 4.9). Neither of these oxidants could restore germination frequencies to those observed in the absence of exogenous proline when germination was scored before 48 h after imbibition. By 48 h after imbibition, both MB and PES could restore germination rates in the presence of 10 mM proline to a level that was not significantly different from the rate of germination in the absence of exogenous proline (Table 4.9). The ameliorative effect of MB on proline-mediated inhibition of radicle emergence was greater at 5.0 µM than at concentrations of either 2.5 µM or 10 µM MB. Likewise, PES was more effective in overcoming the inhibition of germination by exogenous proline when used at a concentration of 2.5 µM than at either 1.0 µM or 5.0 µM (data not shown). Both MB and PES had an inhibitory effect on subsequent seedling growth. The seedlings displayed a dramatic reduction in root growth and the cotyledons appeared bleached after 10 d of growth in the presence of either of the oxidants (data not shown).

Table 4.9: The ameliorative effect of artificial electron acceptors on proline-mediated inhibition of radicle emergence in *Arabidopsis*. Surface-sterilised seeds of wild-type *Arabidopsis* (ecotype Columbia) were transferred directly to MS/2-based experimental media within 3 h after sterilisation and incubated under conditions of subdued light (50 - 70 µmol m^{-2} s^{-1}) at 25°C. The values indicate the average number of seeds that had germinated at that time per replicate comprising 50 seeds. For each treatment, germination of 300 seeds was monitored (n = 6). Means within one column which are followed by the same letter are not significantly different from each other ($P > 0.05$; Duncan's multiple range test). Similar results were obtained when the entire experiment was repeated, using 200 seeds per treatment. MB, methylene blue; PES, phenazine ethosulphate.

	Mean number of seeds germinated per 50 seeds (hours post-imbibition)				
	36 h	**42 h**	**48 h**	**60 h**	**72 h**
0 mM proline	**25.8** a	**37.3** a	**40.3** a	**42.0** a	**45.5** abc
0 mM proline + 5.0 µM MB	**27.3** a	**36.0** a	**39.7** a	**42.3** a	**44.2** abc
0 mM proline + 2.5 µM PES	**25.2** a	**35.8** a	**41.2** a	**43.0** a	**46.2** ab
10.0 mM proline	**3.0** b	**21.3** b	**34.2** bc	**40.8** ab	**44.2** abc
10.0 mM proline + 5.0 µM MB	**11.0** c	**29.8** c	**40.8** a	**47.0** c	**47.2** a
10.0 mM proline + 2.5 µM PES	**11.8** c	**30.7** c	**38.3** ab	**42.8** a	**46.8** a
20.0 mM proline	**1.7** b	**11.0** d	**31.3** c	**37.7** b	**42.0** c
20.0 mM proline + 5.0 µM MB	**5.7** d	**21.0** b	**31.0** c	**40.5** ab	**42.7** bc
20.0 mM proline + 2.5 µM PES	**6.0** d	**24.2** b	**32.5** c	**41.7** a	**44.7** abc

Owing to the critical importance of successful seed germination and seedling establishment in ensuring species survival, the transition from dormancy to germination is likely to be extremely sensitive to the presence of environmental stresses. Conflicting evidence regarding the ability of exogenous proline to alleviate the effects of salinity stress in different species has been presented (Bar-Nun & Poljakoff-Mayber 1977; Poljakoff-Mayber et al. 1994; Khan & Ungar 1997; Khan et al. 1998). The ability of 0.1 mM proline to alleviate the inhibitory effect of 120 mM NaCl on the germination of pea seeds (Bar-Nun & Poljakoff-Mayber 1977) and the alleviation of the innate dormancy of seeds of *Zygophyllum simplex* by both 0.1 mM and 1.0 mM proline (Khan & Ungar 1997) prompted investigation of whether exogenous proline at these concentrations might abrogate the inhibition of *Arabidopsis* seed germination by hyperosmotic stress. Neither 0.1 mM nor 1.0 mM proline caused a significant increase in germination rates in the absence of exogenous osmotica (Table 4.10). This was also noted 36 h after imbibition (data not shown). At the concentrations used, NaCl, sorbitol and glycerol all caused a significant reduction in the rates of germination. Neither of the two concentrations of proline tested were capable of significantly increasing germination rates in the presence of any of these three osmotica (Table 4.10). Glycerol, an inhibitor of OPPP activity in heterotrophic cultured cells (Aubert et al. 1994; Pugin et al. 1997), did not cause a significantly greater inhibition of radicle emergence than did an isosmolar concentration of sorbitol (Table 2.10). At 100 mM, NaCl caused a greater inhibition of germination than did either of the two non-ionic osmotica.

It was investigated whether hormonal mutants altered in their germination properties also displayed delayed germination when exposed to inhibitory concentrations of exogenous proline. Of the classical growth regulators, ABA and the gibberellins are the hormones most frequently suggested to control seed dormancy and germination (Hilhorst & Karssen 1992). Gibberellins are known to promote germination, obviate the requirement of seeds for various germination-promoting environmental cues and to counteract the inhibitory effects of ABA on radicle emergence (Bewley 1997). Although *abi1* and *abi2* mutants, which are primarily disrupted in responses of vegetative tissues to ABA (Table 2.10) display reduced seed dormancy, a second ABA-induced signalling pathway involving the *ABI3*, *ABI4* and *ABI5* gene products primarily affects the regulation of seed specific processes (Finkelstein & Somerville 1990; Finkelstein 1994). In the absence of exogenous proline, seeds of the *abi3-1* mutant (Koornneef et al. 1984) germinated at a significantly faster rate than did those of the WT, whereas seeds of the gibberellin-insensitive *gai* mutant (Koornneef et al. 1985) displayed a significant reduction in the germination rate (Table 4.11). Since both of these mutants have a Landsberg *erecta* background, the Ler-O line was used as the WT control in this experiment. Exogenous proline caused a significant reduction in the number of *abi3-1* seeds that had germinated 36 h and 48 h after imbibition (Table 4.11). For *gai*, a significant proline-mediated inhibition of radicle emergence was

Table 4.10 The effect of exogenous proline on *Arabidopsis* seed germination in the presence of hyperosmotic stress. Surface-sterilised seeds of wild-type *Arabidopsis* (ecotype Columbia) were transferred directly to MS/2-based experimental media within 3 h after sterilisation and incubated under continuous light at 25 °C (Section 3.6.1). The values indicate the average number of seeds that had germinated at that time per replicate comprising 50 seeds. For each treatment, germination of 300 seeds was monitored ($n = 6$). Means within one column which are followed by the same letter are not significantly different from each other ($P > 0.05$; Duncan's multiple range test).

	Mean number of seeds germinated per 50 seeds (hours post-imbibition)		
	48 h	**60 h**	**72 h**
0 mM proline	**41.2** a	**43.2** a	**46.2** a
0.1 mM proline	**42.2** a	**44.3** a	**44.7** a
1.0 mM proline	**40.5** a	**44.8** a	**45.3** a
100 mM NaCl	**13.5** b	**21.7** b	**27.3** b
100 mM NaCl + 0.1 mM proline	**16.5** bcd	**22.3** b	**24.0** b
100 mM NaCl + 1.0 mM proline	**14.5** bc	**20.0** b	**25.3** b
200 mM sorbitol	**20.0** de	**32.5** cde	**37.3** c
200 mM sorbitol + 0.1 mM proline	**18.7** de	**34.5** c	**42.3** ad
200 mM sorbitol + 1.0 mM proline	**21.3** e	**33.7** cd	**39.0** cd
200 mM glycerol	**17.5** cde	**28.7** e	**34.5** c
200 mM glycerol + 0.1 mM proline	**18.2** cde	**31.5** cde	**34.7** c
200 mM glycerol + 1.0 mM proline	**17.0** bcd	**29.2** de	**35.7** c

noted 60 h after imbibition. Thus, the effect of exogenous proline is apparently a metabolic perturbation that affects seed germination at a stage in the germination process that probably operates after changes in hormonal balance which are mediated by the *ABI3* and *GAI* gene products.

Table 4.11: The effect of exogenous proline on radicle emergence in the *Arabidopsis* ecotype Landsberg *erecta* and *abi3-1* and *gai* mutants. For each plant line, surface-sterilised seeds were transferred directly to MS/2 media supplemented with the concentration of proline indicated and incubated under continuous light at 25 °C (Section 3.6.1). The values indicate the average number of seeds that had germinated at that time, per replicate comprising 50 seeds. For each treatment, germination of 300 seeds of each line was monitored (n = 6). Means within one column which are followed by the same letter are not significantly different from each other (P > 0.05; Duncan's multiple range test).

	Mean number of seeds germinated per 50 seeds (hours post-imbibition)			
	36 h	**48 h**	**60 h**	**72 h**
Ler -O				
0 mM proline	5.0 a	31.2 a	40.0 ab	43.8 ab
10.0 mM proline	1.2 b	22.8 b	36.0 ac	43.5 ab
20.0 mM proline	0.2 b	19.5 b	32.3 c	40.8 a
abi3-1				
0 mM proline	24.8 c	44.3 c	46.2 d	46.7 b
10.0 mM proline	10.2 d	35.8 a	42.0 bd	45.7 b
20.0 mM proline	5.7 a	29.8 a	34.3 c	44.0 ab
gai				
0 mM proline	0.0 b	8.3 d	25.8 e	29.8 c
10.0 mM proline	0.0 b	5.0 d	20.7 f	29.2 c
20.0 mM proline	0.0 b	7.5 d	17.2 f	35.7 d

The amelioration by artificial electron acceptors of the inhibitory effect of exogenous proline on radicle emergence in *Arabidopsis* (Table 4.9) is consistent with the view that an elevation in endogenous proline content may increase the level of reduction of the NADP pool in the imbibed seed if feedback inhibition of P5CS activity prevents the oxidation of NADPH by the metabolic cycle outlined in Figure 2.14. Subsequent studies examined a possible role for OPPP activity in facilitating seed germination in *Arabidopsis*. *In vitro* assays of the specific activities of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) indicated that their activities increased following seed imbibition (Figure 4.16). Following seed imbibition,

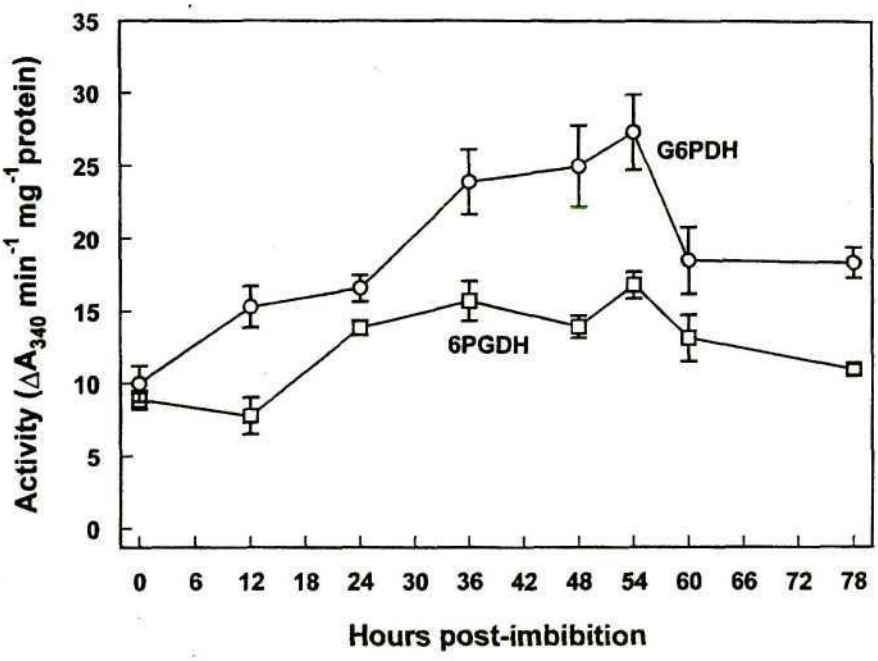


Figure 4.16: Changes in the total extractable activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase during the course of *Arabidopsis* seed germination. Plant material was prepared and the enzymes assayed as described in Section 3.10.1. The data for glucose-6-phosphate dehydrogenase (o; G6PDH) and 6-phosphogluconate dehydrogenase (□; 6PDGH) activities represent the averages of two experiments, each involving three independent assays for each of the enzymes at each time point. Assays were performed in duplicate. Error bars indicate the standard error. Radicle emergence had occurred in at least 70% of the seeds within 36 h after imbibition.

the mean specific activity of G6PDH was always greater than that of 6PGDH. Maximal G6PDH activity was observed from the time of radicle emergence of most of the seed grown in liquid culture (36 h) until approximately 18 h after most of the seeds had germinated (Figure 4.16).

Since the activities of extracted enzymes assayed *in vitro* are not necessarily an accurate measure of their *in vivo* activity, further experiments employed the use of radiolabelled glucose to assess the activation of the OPPP during *Arabidopsis* seed germination. By itself, analysis of the ratio of $^{14}CO_2$ released from [6-^{14}C]glucose relative to [1-^{14}C]glucose (C_6/C_1 ratio) cannot be used to quantify flux through the OPPP. Nevertheless, it serves as a quantitative indicator of changes in relative flux through the major pathways of carbohydrate oxidation (Section 2.2.3; ap Rees 1980). The observed decrease in the C_6/C_1 ratio following imbibition of WT *Arabidopsis* seeds indicates that during germination, there is a stimulation of OPPP activity relative to carbon flux through glycolysis. The greatest decrease in the C_6/C_1 ratio was observed 18 h after radicle emergence in most of the seeds (Table 4.12). The C_6/C_1 ratio at the time when germination had occurred in at least 70% of the seeds was significantly different to the C_6/C_1 ratio measured 12 h after imbibition (Table 4.12).

Table 4.12: Changes in the relative contributions of $^{14}C_6$- and $^{14}C_1$-labelled glucose to respired CO_2 during the course of *Arabidopsis* seed germination. Seeds or seedlings were incubated with [1-^{14}C]glucose and [6-^{14}C]glucose as described in Section 3.11. $^{14}CO_2$ production per gram of fresh wight was measured after 120 min of incubation and the data used to determine the C_6/C_1 ratio. Each ratio is the mean of ± SE of independent measurements on three C_6 and three C_1 samples. Means which are followed by the same letter are not significantly different from each other ($P > 0.05$; Duncan's multiple range test). Radicle emergence had occurred within at least 70% of the seeds within 36 h after imbibition.

Hours post-imbibition	C_6/C_1	
12 h	0.372 ± 0.038	a
24 h	0.319 ± 0.033	ab
36 h	0.260 ± 0.043	bc
48 h	0.217 ± 0.024	bc
54 h	0.178 ± 0.018	c
60 h	0.213 ± 0.054	bc
72 h	0.268 ± 0.025	abc

At the time when radicle emergence had occurred in at least 70% of the seeds germinated in liquid culture (36 h post-imbibition), there is an approximately four-fold increase in the free proline concentration (Figure 4.17). The kinetics of the change in proline levels is not matched by changes in the profiles of any of the other amino acids which were assayed (Table 4.13). In seeds of the T_2 generation of the pBI-P5CS1(AS) transformants A5 and B12 germinated under identical conditions in liquid culture, the maximal concentration of free proline was approximately 65% of that observed for WT seeds (Figure 4.17). In both of the antisense lines, this maximum level was reached at the time when radicle emergence was noted in at least 70% of the seeds. Germination occurred approximately 6 h later in seeds of the two pBI-P5CS1(AS) lines than in WT seed, but maximal free proline levels always occurred at the time of radicle emergence in most of the seeds (Figure 4.17). *Arabidopsis* seeds from different stocks are known to display a wide variability in the environmental requirements for germination, owing to differences in the growth conditions of the parent plants (Derkx & Karssen 1993). Throughout the course of the germination studies, some variation was observed between the percentage germination observed with different seed batches from different harvest dates. Nonetheless, the delayed germination response of seeds of the pBI-P5CS1(AS) lines A5 and B12 was confirmed using two separate batches of seed of the T_2 generation of both lines. For both stocks, the seed was collected at the same time as was seed from WT plants grown under identical conditions. In all instances, a slightly reduced

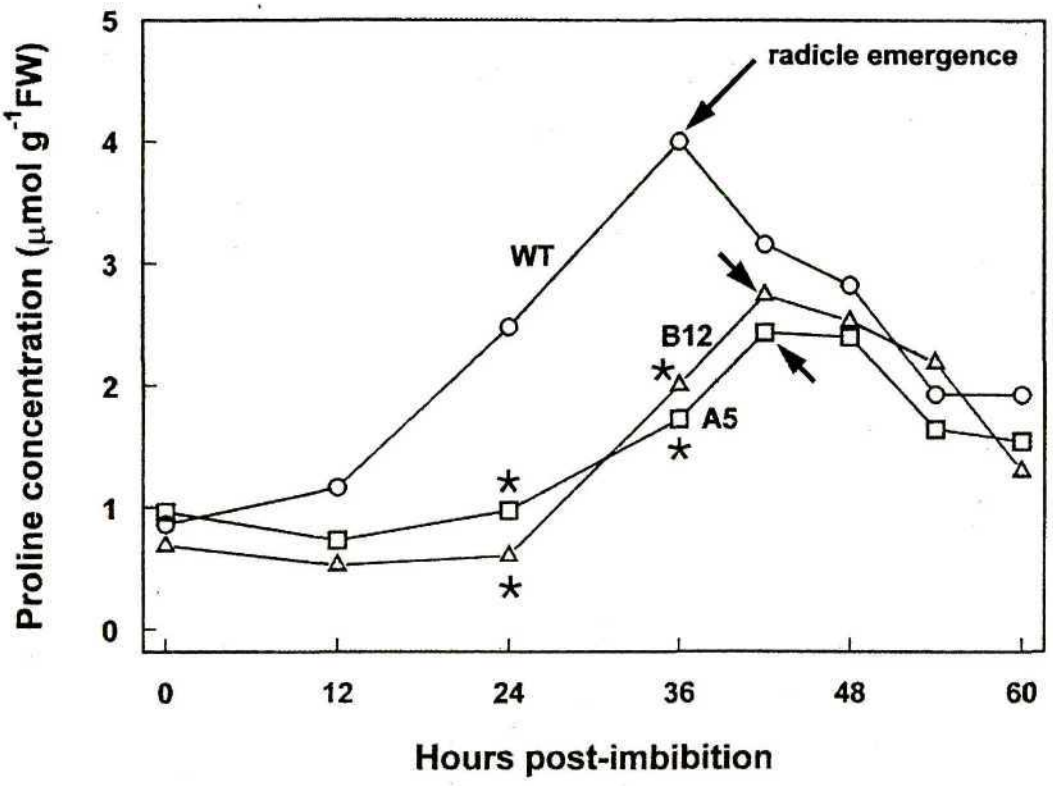


Figure 4.17: Changes in the level of free proline during the course of germination of wild-type *Arabidopsis* and pBI-P5CS1(AS) transformants A5 and B12. Plant material was prepared as described in Section 3.9.1. Proline was assayed essentially as described by Bates et al. (1973), as outlined in Section 3.9.1. Values are the means of nine determinations involving three independent experiments. Arrows indicate the time after which radicle emergence had occurred in at least 70% of the seeds. The different seed types used were harvested from plants grown simultaneously under identical growth conditions. Where the proline content for either of the pBI-P5CS1(AS) lines (□, A5; △, B12) is significantly different ($P < 0.05$; Student's *t*-test) from that of wild-type (o) at the same time point after imbibition, this is indicated by an asterisk.

germination rate correlated with an approximately 35% decrease in the maximal level of proline accumulated at the time of radicle emergence.

An obvious consideration in interpretation of the results presented in Figure 4.17 and Table 4.13 is whether the increase in free proline that is maximal at the time of radicle emergence arises from increased rates of proline synthesis or merely from the degradation of seed storage proteins. Analysis of the amino acid compositions of *Arabidopsis* seed storage proteins indicated that they are characterised by a slightly greater abundance of proline residues than are most *Arabidopsis* gene products (Table 4.14).

Table 4.13: Changes in the concentrations of the major free amino acids and of total amino acids and NH_3 content during *Arabidopsis* seed germination. Germination of seeds of wild-type *Arabidopsis* (ecotype Columbia) and amino acid analysis were conducted as described in Section 3.9.1. Times indicate the number of hours post-imbibition. Radicle emergence had occurred in at least 70% of the seeds by 36 h after imbibition. The data indicate the averages of four determinations. Values in parentheses indicate the standard deviation.

Amino acid	Concentration (µmol g^{-1} fresh weight)				
	0 h	12 h	24 h	36 h	48 h
Asp	**0.893** (0.109)	**0.568** (0.172)	**0.724** (0.084)	**0.561** (0.048)	**0.572** (0.039)
Thr	**0.647** (0.141)	**0.672** (0.222)	**0.811** (0.182)	**1.581** (0.266)	**1.544** (0.265)
Ser	**0.746** (0.146)	**1.281** (0.106)	**1.977** (0.371)	**2.347** (0.138)	**3.479** (0.682)
Glu	**3.116** (0.916)	**2.725** (1.076)	**4.070** (0.894)	**4.897** (0.798)	**6.325** (2.023)
Pro	**1.688** (0.428)	**1.546** (0.462)	**2.927** (0.586)	**4.861** (0.877)	**3.058** (1.303)
Gly	**0.579** (0.085)	**0.353** (0.071)	**0.436** (0.068)	**0.490** (0.072)	**0.604** (0.059)
Ala	**1.059** (0.309)	**1.537** (0.282)	**2.674** (0.792)	**3.066** (1.048)	**2.639** (0.975)
Val	**0.711** (0.067)	**0.563** (0.088)	**1.101** (0.181)	**1.458** (0.282)	**1.375** (0.143)
Met	**0.056** (0.012)	**0.149** (0.064)	**0.232** (0.085)	**0.343** (0.113)	**0.351** (0.137)
Ile	**0.355** (0.087)	**0.289** (0.064)	**0.353** (0.067)	**1.028** (0.311)	**0.755** (0.137)
Leu	**0.271** (0.032)	**0.353** (0.061)	**0.441** (0.082)	**1.233** (0.271)	**0.875** (0.064)
Phe	**0.084** (0.037)	**0.117** (0.027)	**0.109** (0.017)	**0.186** (0.034)	**0.106** (0.008)
Tyr	**0.253** (0.043)	**0.272** (0.045)	**0.292** (0.097)	**0.131** (0.062)	**0.105** (0.049)
His	**0.125** (0.036)	**0.154** (0.019)	**0.308** (0.045)	**0.358** (0.063)	**0.387** (0.049)
Lys	**0.137** (0.039)	**0.167** (0.020)	**0.335** (0.072)	**0.407** (0.047)	**0.415** (0.039)
Arg	**0.319** (0.119)	**0.248** (0.178)	**0.389** (0.186)	**0.609** (0.057)	**0.596** (0.154)
TOTAL	**10.927** (0.812)	**12.526** (0.973)	**20.298** (1.644)	**26.993** (0.889)	**24.862** (3.042)
NH_3	**4.373** (1.016)	**10.763** (2.778)	**9.688** (2.145)	**9.310** (1.134)	**10.652** (3.422)

Table 4.14: The abundance of selected amino acids in *Arabidopsis* seed storage proteins. Values are the percentage contribution of that amino acid to the polypeptide chain of the seed storage protein. The consensus values for the amino acid composition of *Arabidopsis* gene products are shown in parentheses. These were determined by analysis of 515 *Arabidopsis* coding sequences (M Cherry, Department of Molecular Biology, Massachusetts General Hospital, MA). Genbank accession numbers of the sequences used for the analysis are X14312 and X14313 (*Arabidopsis* 12S storage proteins *AtCRA1* and *AtCRB* respectively; Pang et al. 1988) and Z24744 and Z24745 (*Arabidopsis* 2S albumins (*At2S1*, *At2S2*, *At2S3* and *At2S4*; Krebbers et al. 1988).

***Arabidopsis* seed storage protein**	**No. of amino acids**	**Percentage contribution to polypeptide chain**				
		Pro (4.8 %)	**Ser** (7.7 %)	**Arg** (5.3 %)	**Glu** (6.6 %)	**Gln** (3.5 %)
CRA1 (12S)	472	5.51	6.14	6.14	5.51	9.75
CRB (12S)	455	5.49	5.71	5.71	6.37	8.13
2S isoform 1	164	6.71	2.44	4.27	7.32	14.02
2S isoform 2	170	7.65	3.53	1.76	5.88	12.35
2S isoform 3	164	7.32	4.27	6.10	6.10	13.41
2S isoform 4	166	6.63	3.61	5.42	6.02	13.25

Use of SDS-PAGE to monitor the degradation of the major *Arabidopsis* seed storage proteins over the course of the germination process indicated that the peak in free proline levels and subsequent decline in free proline (Figure 4.17; Table 4.13) preceded the complete degradation of the major seed storage proteins (Figure 4.18).

4.8 The effects of exogenous proline on *Arabidopsis* seedling growth

A role for proline synthesis in the regulation of cell division has been reviewed in Section 2.5.2.4. If cycling between proline and its precursors affects plant growth, then one might anticipate that an artificial elevation in the cellular free proline content might disrupt this cycle through inhibition of P5CS activity (Section 2.3.1.1) and that this may be reflected in an altered growth rate. Inclusion of proline in the growth medium at concentrations in excess of 10 mM caused a significant ($P < 0.05$; Student's *t*-test) decrease in the root lengths of 10 d-old *Arabidopsis* seedlings grown *in vitro* either in the presence of light or in darkness (Figure 4.19). Millimolar concentrations of proline inhibited both root and hypocotyl elongation in the dark (Figure 4.19B). For these treatments, seeds were imbibed in the absence of proline at 4 °C for 48 h prior to their

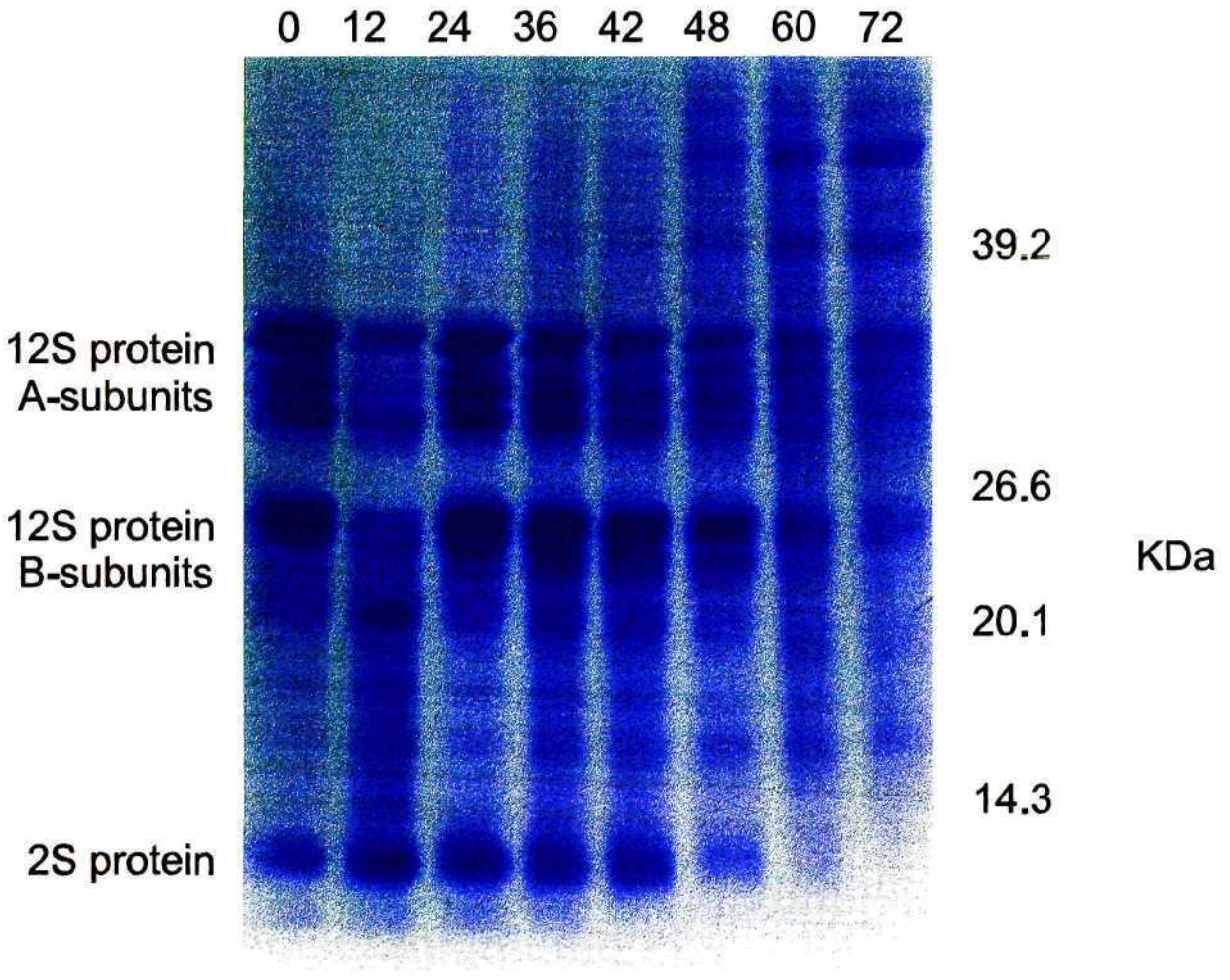


Figure 4.18: Degradation of the major *Arabidopsis* seed storage proteins during germination. Protein extracts were prepared from mature seeds prior to imbibition, and material harvested 12 h, 24 h, 36 h, 48 h, 60 h and 72 h after imbibition and germination under the growth conditions described in Section 3.9.1. The total protein samples were prepared and electrophoresed through a discontinuous SDS-polyacrylamide gel (16% separating gel) as described by Heath et al. (1986). The gel was stained with Coomassie Blue R250 (Sigma). The entire experiment was repeated once, with similar results.

transfer to media containing proline and incubation at 25 °C. Since at least 80% of all seeds plated onto media either with or without proline germinated within a 12 h window period (data not shown), this eliminates the possibility that the dramatically reduced rate of root- and hypocotyl elongation in the presence of exogenous proline measured over a 10 d period arises simply from delayed germination of seeds grown on media containing exogenous proline.

Owing to the short length of the hypocotyls of *Arabidopsis* seedlings grown in the light, it was not practical to quantify the inhibitory effects of exogenous proline on hypocotyl growth in seedlings grown in the light. Nonetheless, as can be seen in Figure 4.20, inclusion of exogenous proline at concentrations of 10 mM or higher caused a substantial decrease in the length of seedling

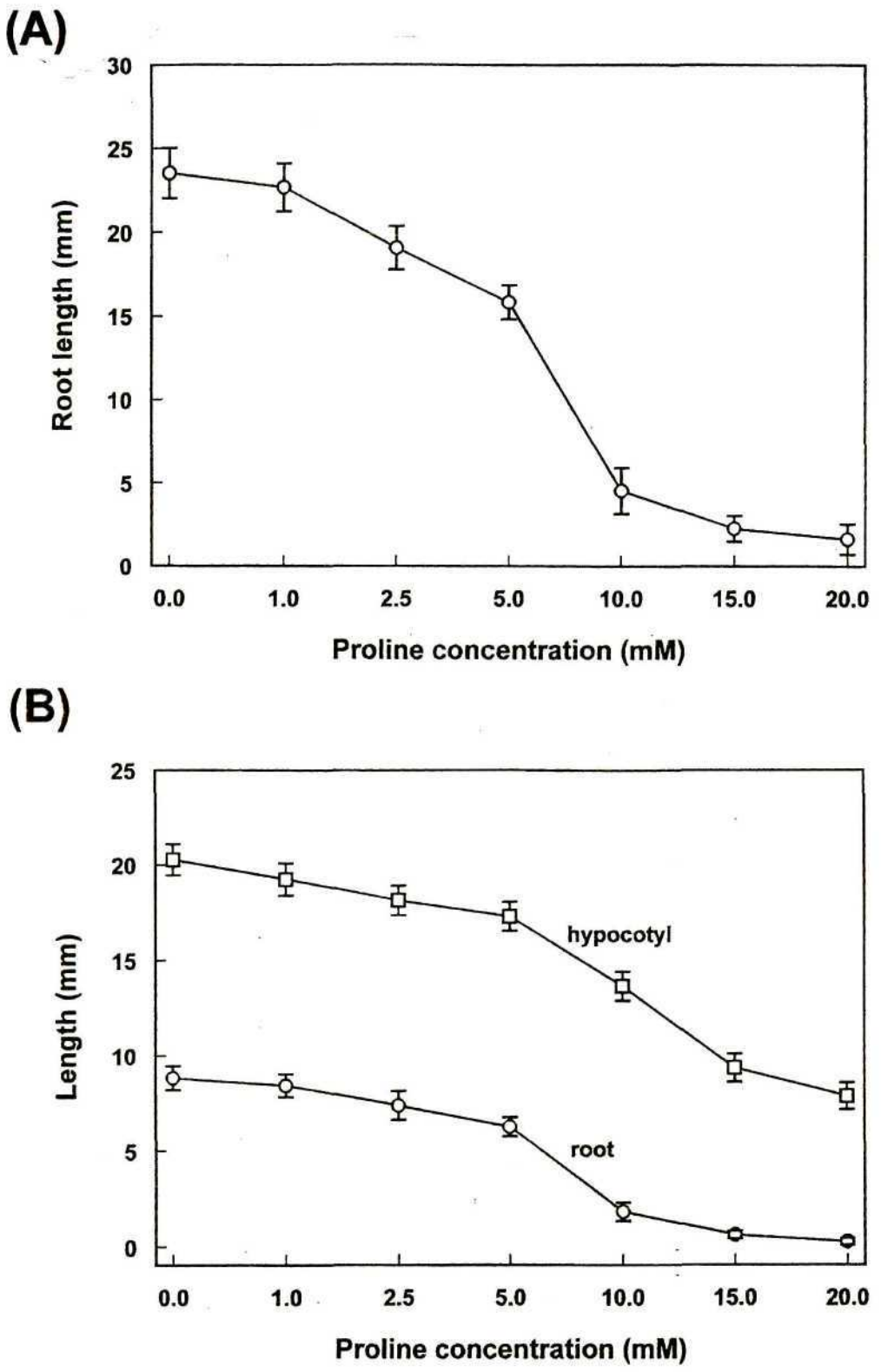


Figure 4.19: The effect of exogenous proline on root and hypocotyl growth in *Arabidopsis* seedlings. **(A)** Root lengths of seedlings grown in the light. **(B)** Root (o) and hypocotyl (□) lengths of seedlings grown in darkness. Surface-sterilised seeds of wild-type *Arabidopsis* (ecotype Columbia) were imbibed for 48 h at 4 °C on MS/2 media containing 5.0 g l^{-1} sucrose before transfer to the same media containing the concentrations of proline indicated and incubation at 25 °C (Section 3.6.2). Root and hypocotyl lengths of at least fifty seedlings per treatment were measured 10 d after germination. Data are the mean root or hypocotyl lengths. Error bars denote the standard error. Similar trends in the inhibition of growth were noted when the experiments were repeated twice ($n > 30$).

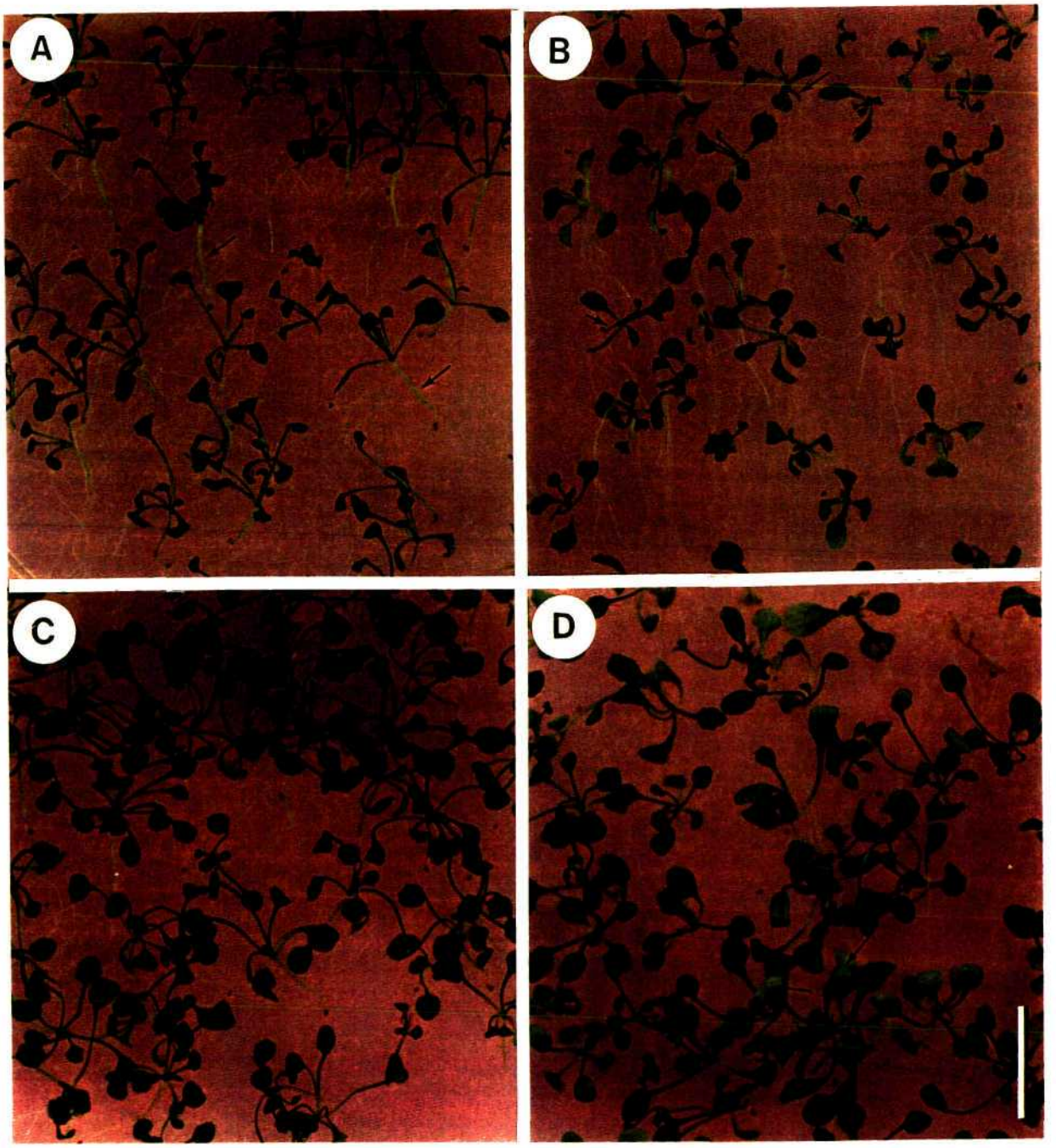


Figure 4.20: The effects of exogenous proline on the appearance of *Arabidopsis* seedlings grown in the light. Seedlings were germinated and grown on MS/2 media containing 5.0 g l^{-1} sucrose with no supplemental proline **(A and C)** or in the presence of 10 mM proline **(B and D)**. Seedlings were photographed either 14 d **(A and B)** or 21 d **(C and D)** after germination. Arrows in (A) and (B) indicate the substantial difference in the length and appearance of the hypocotyls of seedlings grown in the presence of exogenous proline relative to those grown in the absence of proline. Arrows in (D) denote evidence of chlorosis and senescence in 21 d-old seedlings grown in the presence of proline. For (A) to (D), the scale bar represents 10 mm.

hypocotyls. Most strikingly, the hypocotyls of seedlings grown in the presence of 10 mM proline frequently had a dark brown appearance and a reduced diameter. This browning did not appear to affect the viability of the seedlings, at least after 14 d growth in the presence of proline (Figure 4.20). Neither leaf development of 14 d-old seedlings grown in the light (Figure 4.20) nor cotyledon development in 14 d-old etiolated seedlings (Figure 4.21) appeared to be affected by the presence of exogenous proline in the growth medium. However, by 21 d after germination, seedlings grown in the presence of 10 mM proline in the light displayed visible signs of chlorosis (Figure 4.20D). Browning of the hypocotyls of many of the etiolated seedlings grown in the presence of exogenous proline was also noted (Figure 4.21).

The effects of exogenous proline on growth rates (Figure 4.19), chlorophyll loss (Figure 4.20D) and the colouration of hypocotyls prompted several further investigations into the physiological bases of these observations.

The negative effects of exogenous proline on the chlorophyll content was confirmed by analysis of the levels of chlorophylls *a* and *b* as well as total chlorophyll in 21 d-old *Arabidopsis* plants grown *in vitro* in the light either in the presence or absence of three different concentrations of proline (Figure 4.22). Exogenous proline caused a slight decrease in the ratio of chlorophyll *a* to chlorophyll *b*. The mean chlorophyll *a*:chlorophyll *b* ratio is 2.685 for plants grown in the absence of proline and 2.513 for plants grown in the presence of 20 mM proline.

Growth of any plant organ may either occur as a result of cell division, cell expansion or the simultaneous occurrence of both of these processes. As shown by visualisation of the epidermal cells of the hypocotyls using scanning electron microscopy (Figure 4.23), the reduction in hypocotyl growth in the presence of exogenous proline appears to be due, at least in part, to reduced elongation of the cells.

A role for proline synthesis in affecting the synthesis of phenylpropanoids has been proposed (Section 2.5.2.2; Hare & Cress 1997). Certain phenylpropanoids are the precursors for the synthesis of lignin (Figure 2.3; Whetten & Sederoff 1995; Tamagnone et al. 1998). To investigate whether the browning of hypocotyls of seedlings grown in the presence of exogenous proline was related to an accumulation of phenylpropanoids, 14 d-old seedlings grown in either the absence of exogenous proline or in the presence of 5 mM, 10 mM or 20 mM proline were stained with phloroglucinol. A red colouration, which is indicative of a positive histochemical test for lignin or the phenolic precursors of lignin (monolignols), is consistent with elevated levels of phenylpropanoids in the hypocotyls of seedlings grown in the presence of exogenous proline (Figure 4.24).

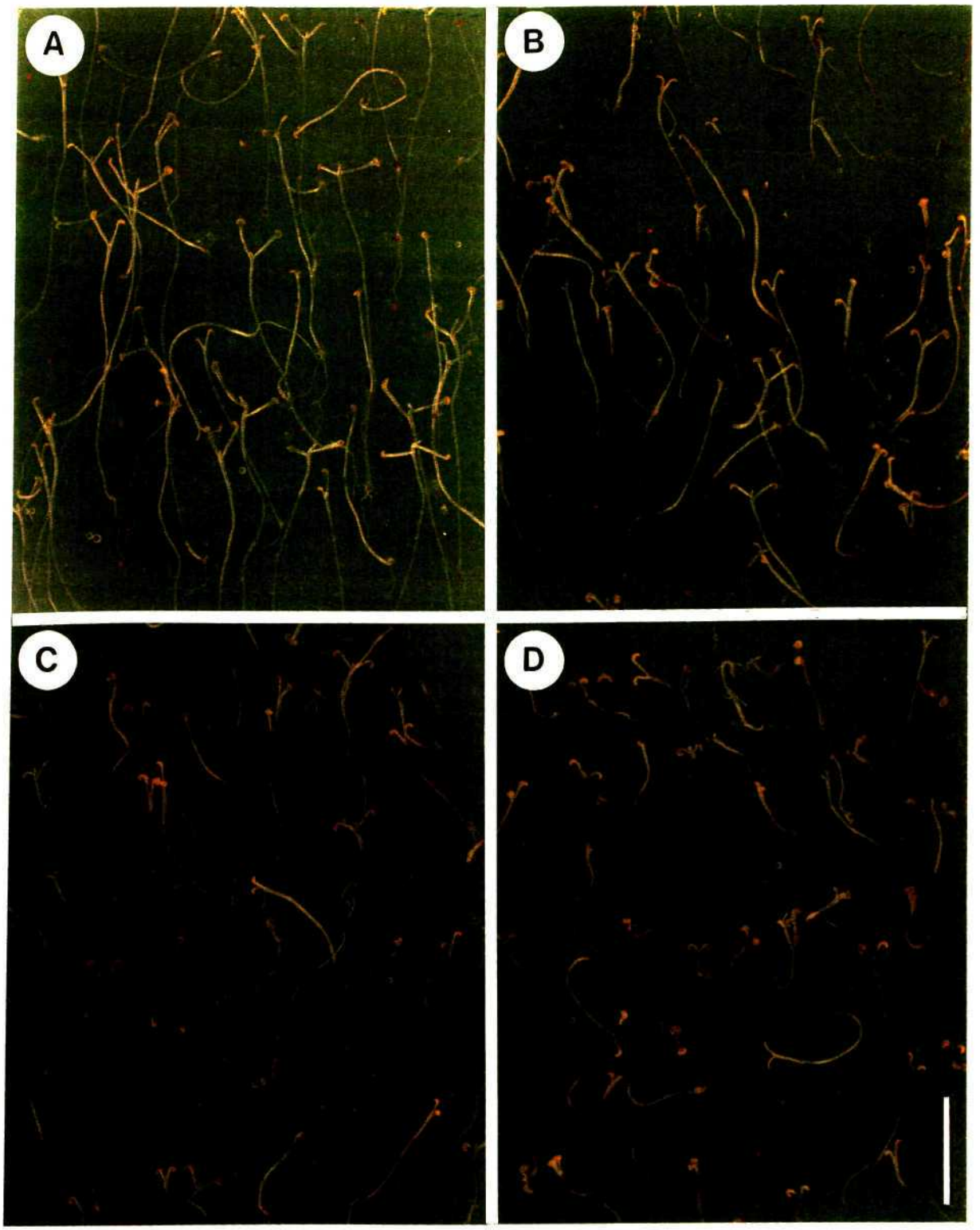


Figure 4.21: The effects of exogenous proline on the appearance of *Arabidopsis* seedlings grown in darkness. Seedlings were germinated and grown on MS/2 media containing 5.0 g l^{-1} sucrose with no supplemental proline **(A)** or containing 5 mM **(B)**, 10 mM **(C)** or 20 mM **(D)** proline. Seedlings were photographed after 14 d growth in darkness. In (B) to (D), arrows indicate visible browning of the hypocotyls which was observed in seedlings grown in the presence of proline, but not when seedlings were grown in the absence of exogenous proline (A). For (A) to (D), the scale bar represents 10 mm.

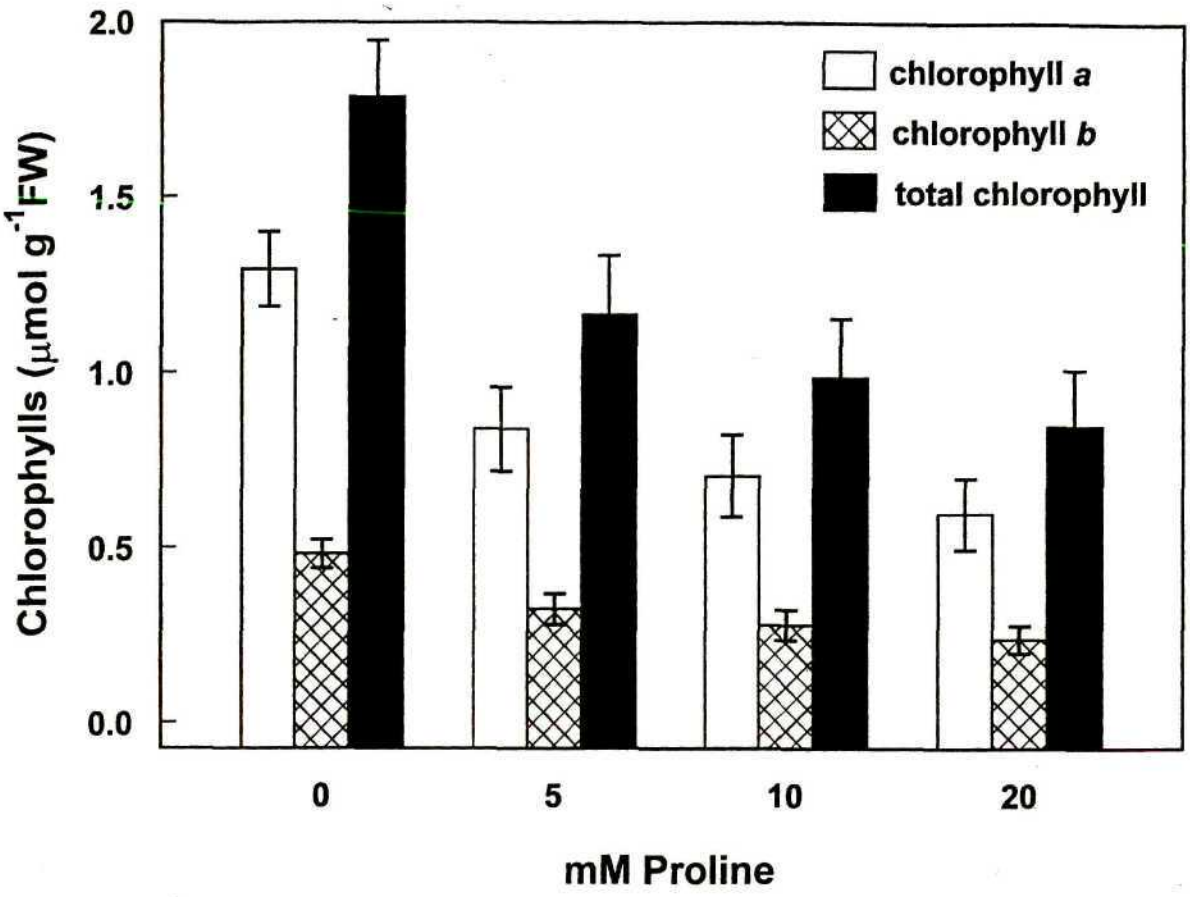


Figure 4.22: The effect of exogenous proline on the chlorophyll content in the rosette leaves of 21 day-old *Arabidopsis* plantlets. Levels of chlorophyll *a*, chlorophyll *b* and the total chlorophyll content were determined as described in Section 3.9.3. Each value is the mean of thirty determinations, involving three separate experiments. Error bars indicate the standard deviation. All data obtained from plants grown in the presence of exogenous proline are significantly different ($P < 0.05$; Student's *t*-test) from the comparable value obtained using plants grown in the absence of exogenous proline.

The report by Chen and Kao (1995) that exogenous proline (10 mM) caused an approximately four-fold induction of ionically-bound peroxidase activity in the roots of rice seedlings prompted investigation of whether a similar proline-mediated induction of peroxidase occurs in *Arabidopsis*. An *in vitro* guaiacol assay of total peroxidase activities in *Arabidopsis* seedlings grown in liquid culture indicated that the addition of proline to a final concentration of 10 mM proline in the growth medium of 8 d-old seedlings resulted in an approximately 75% increase in the total extractable peroxidase activity when assayed 72 h after the addition of proline to the growth medium (Figure 4.25A).

The fact that this induction is highly reproducible, but not as dramatic as that observed by Chen and Kao (1995), led to the investigation of whether proline induces a specific isoform(s) of

Figure 4.23: Comparison of the epidermal cells from the hypocotyls of *Arabidopsis* plants grown in the presence or absence of exogenous proline. Ten d-old hypocotyls of representative seedlings grown in the light **(A to C)** or in darkness **(D to F)** were examined using scanning electron microscopy. **(A)** and **(D)** represent the appearance of hypocotyls from seedlings grown in the absence of exogenous proline. **(B)** and **(E)** represent the appearance of hypocotyls from seedlings grown in the presence of 10 mM proline. **(C)** and **(F)** represent the appearance of hypocotyls from seedlings grown in the presence of 20 mM proline. The scale bar in (F) represents 100 µm for (A) to (F).

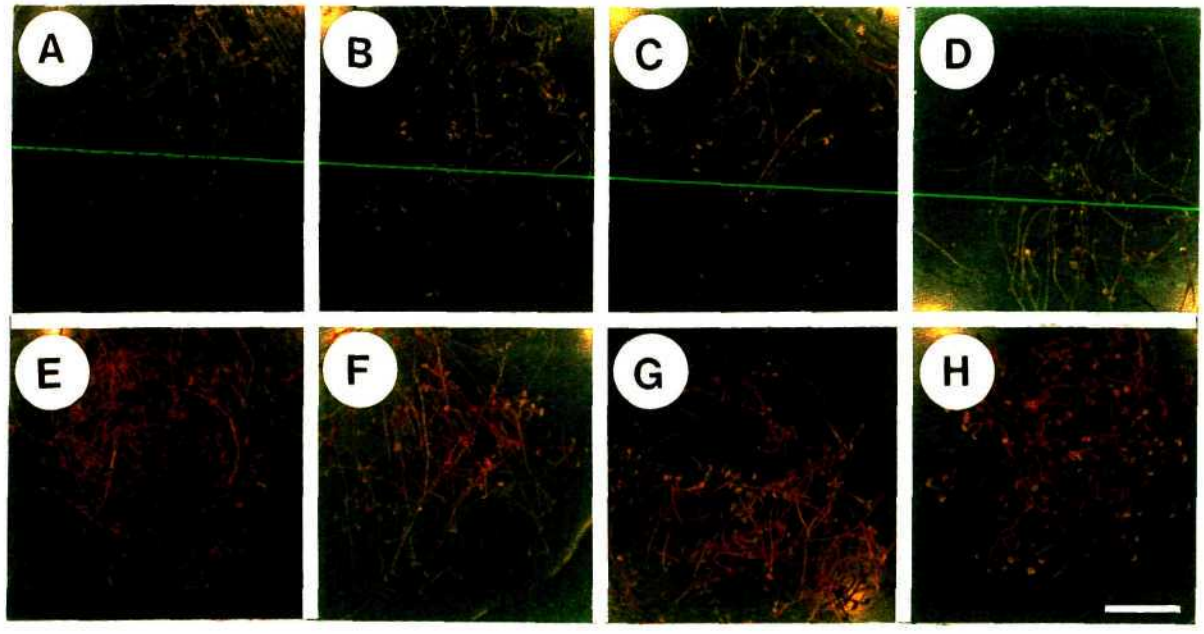


Figure 4.24: The browning of hypocotyls of etiolated *Arabidopsis* seedlings grown in the presence of exogenous proline is correlated with a positive histochemical reaction with phloroglucinol. Fourteen d-old seedlings grown in darkness on MS/2 medium containing 5.0 g l^{-1} sucrose with or without exogenous proline were photographed prior to staining with phloroglucinol **(A to D)** or after incubation in phloroglucinol **(E to H)** as described in Section 3.9.4. **(A)** and **(E)** show seedlings grown in the absence of exogenous proline, **(B)** and **(F)** show seedlings grown in the presence of 5 mM proline, **(C)** and **(G)** show seedlings grown in the presence of 10 mM proline and **(D)** and **(H)** show seedlings grown in the presence of 20 mM proline. The scale bar in (H) represents 10 mm for (A) to (H).

peroxidase. It was reasoned that a relatively small induction of total peroxidase activity might result from a several fold-induction of only one or a few isoforms with a specific physiological function in which proline somehow plays a regulatory role. Analysis of peroxidase isoform profiles confirmed this suspicion. When 150 µg of freshly-prepared protein extract was loaded per well, expression of at least one peroxidase activity increased significantly within at least 48 h of incubation in exogenous proline (Figure 4.25B). Difficulty in resolving individual bands associated with the activities of individual peroxidases prevented an unambiguous conclusion regarding whether the proline-induced peroxidase activity represented the induction of a single peroxidase isoform. However, since peroxidase activity which co-migrates with the proline-induced peroxidase activity was found in seedlings not incubated in proline, proline does not appear to induce this specific peroxidase activity(ies) *de novo*.

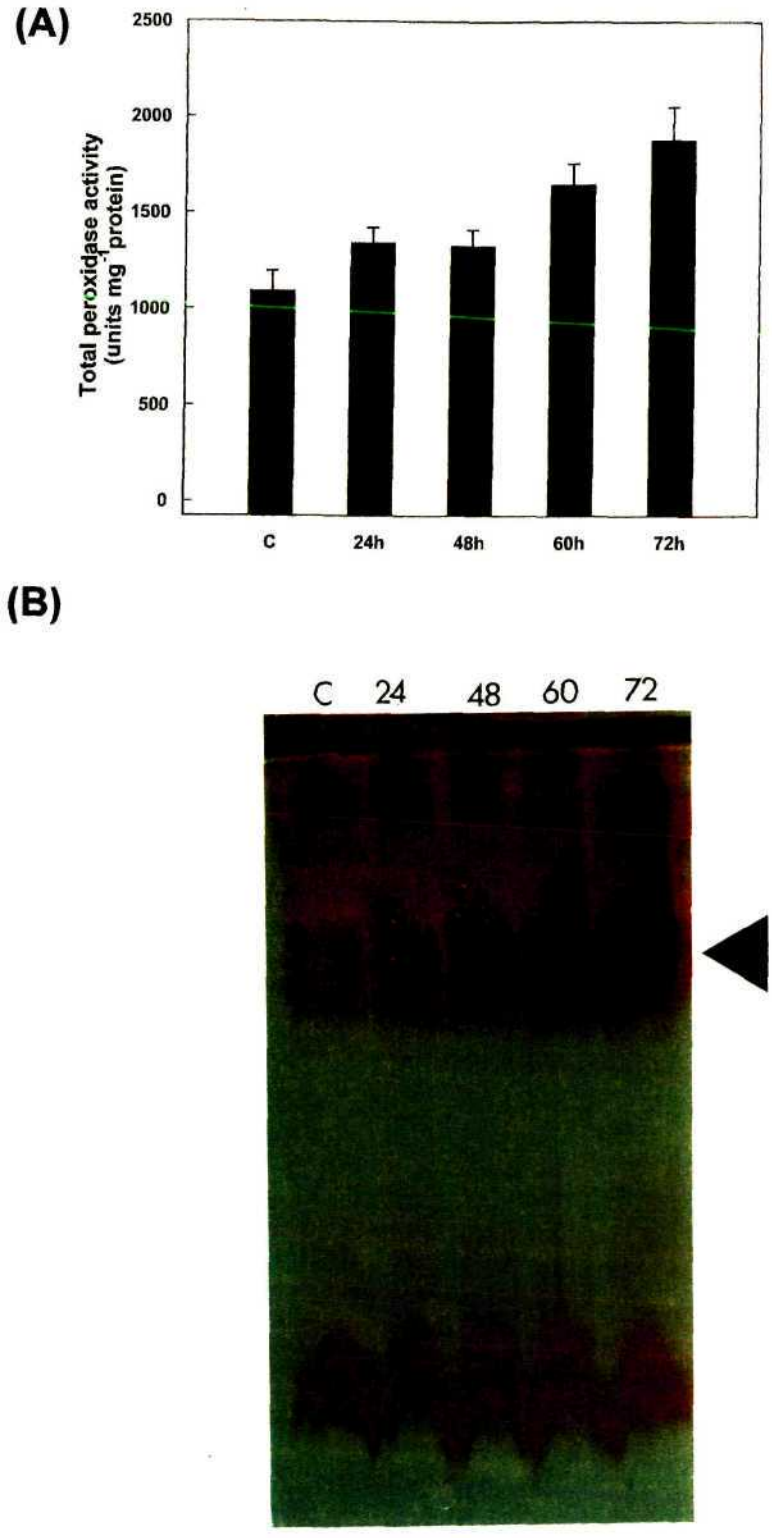


Figure 4.25: Changes in the total peroxidase activity following incubation of *Arabidopsis* seedlings in 10 mM proline. Ten d-old plantlets were grown in liquid MS medium (Murashige & Skoog 1962) containing 10.0 g l^{-1} sucrose to which proline was added to a concentration of 10 mM 24 h, 48 h, 60 h or 72 h prior to harvesting. C denotes the peroxidase activity in plants not exposed to exogenous proline. **(A)** *In vitro* guiacol assay of total peroxidase activity. The times indicate h of incubation in the presence of 10 mM proline. The data are means of three separate experiments, each involving five replicates using two protein extracts per treatment. Error bars denote the standard deviation. **(B)** Resolution of peroxidase isoforms by electrophoresis and staining using 3-amino-9-ethylcarbazole (Section 3.10.2). The arrow indicates the peroxidase activity which increases most rapidly after incubation in proline. The induction of this peroxidase activity by 10 mM proline was reproducibly observed in four separate experiments. In all instances, staining with Coomassie Blue R250 of identical gels containing duplicate samples confirmed that loading of each lane on the gel was equal.

An important aspect of studies in which the effects of exogenous proline on growth and development are tested is the confirmation that proline is indeed taken up by the plant tissue. Addition of three different concentrations of proline to liquid culture medium (MS/2 medium with 5.0 g l^{-1} sucrose) containing 10 d-old *Arabidopsis* seedlings indicated that the plantlets were capable of accumulating exogenously-supplied proline (Figure 4.20). When incubated for 6 h or longer in medium containing 2.5 mM, 5.0 mM or 10 mM proline, endogenous proline levels were significantly higher ($P < 0.01$; Student's *t*-test) than those in seedlings not exposed to exogenous proline (Figure 4.20). Measurement of free proline in seedlings that were not incubated in exogenous proline indicated that proline levels did not fluctuate significantly throughout the period over which proline uptake was monitored.

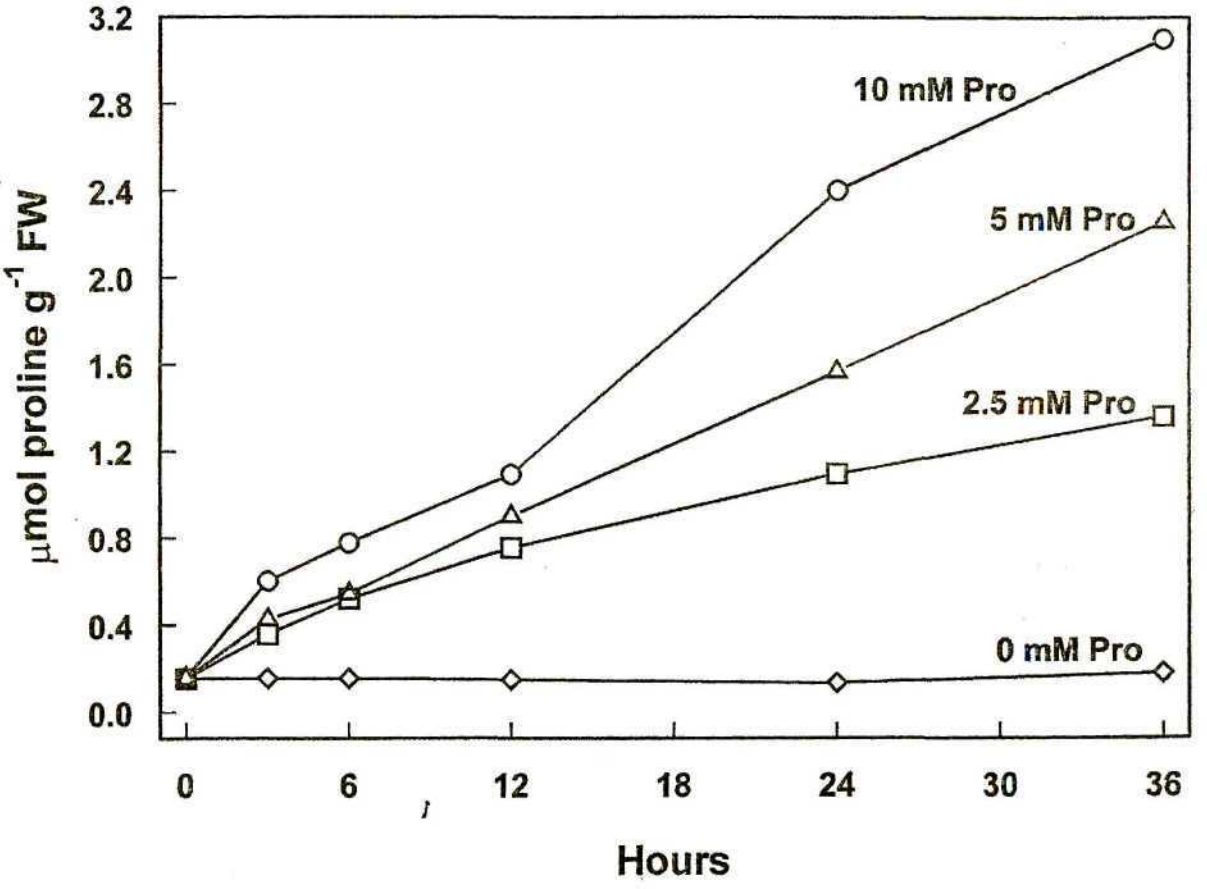


Figure 4.26: Levels of free proline in *Arabidopsis* seedlings grown in liquid culture following their incubation in media containing exogenous proline. Proline was added to the final concentration indicated to 10 d-old plantlets which were germinated and grown in liquid MS/2 medium supplemented with 5.0 g l^{-1} sucrose. At the times indicated, plantlets were removed from the growth medium and washed extensively with water to remove any residual proline associated with the surfaces of the seedlings. Free proline content was measured by slight modification of the method of Bates et al. (1973), as described in Section 3.9.1. All data are the means of three determinations.

4.9 The effects of exogenous proline on cellular ultrastructure

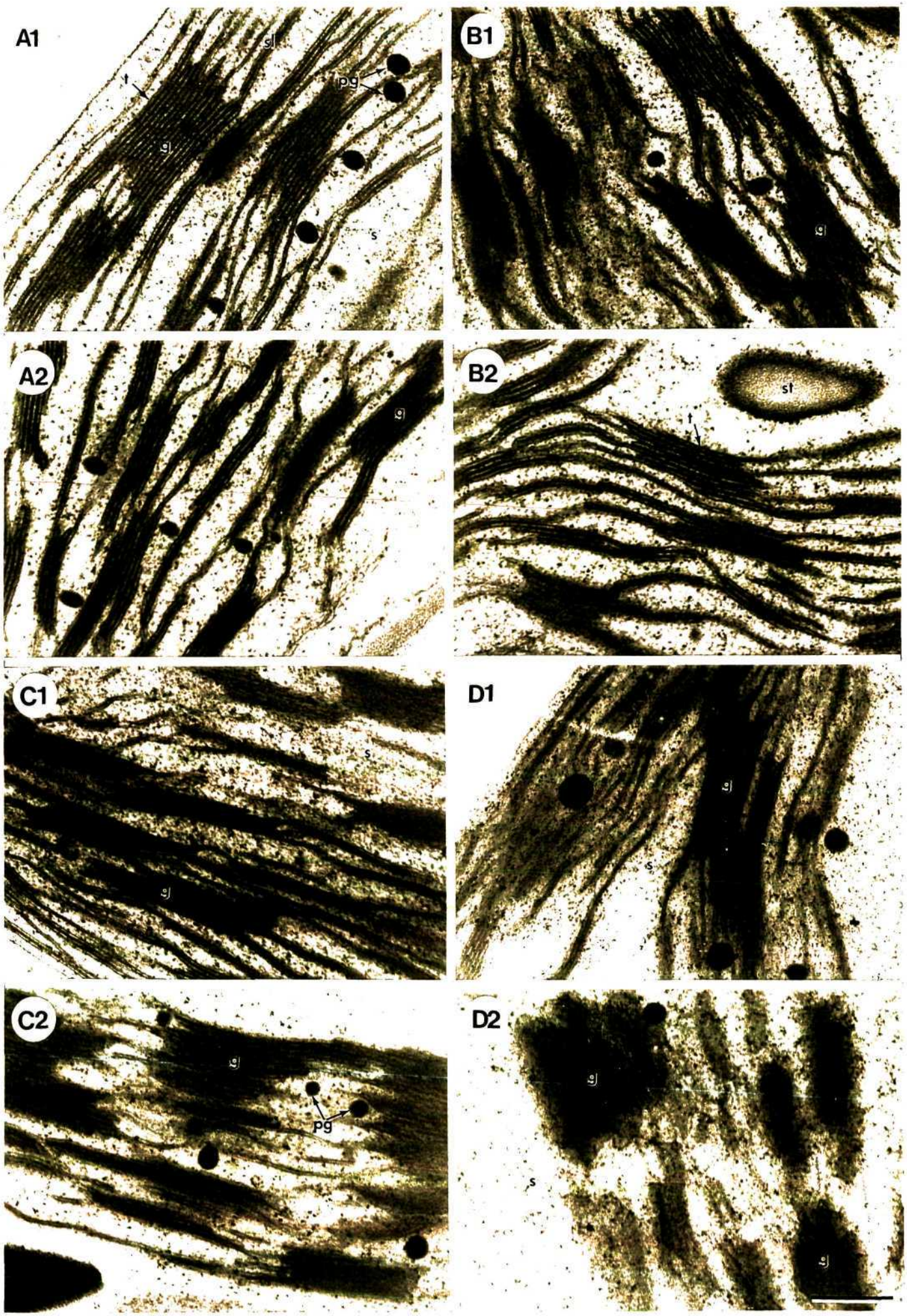
The observation that exogenous proline caused visible chlorosis (Figure 4.20) and a significant loss of chlorophyll in the leaves of 21 d-old *Arabidopsis* plantlets (Figure 4.3) suggested that it might be worthwhile to examine how these effects were reflected at the level of cellular ultrastructure. A central tenet of the hypothesis that osmolytes such as proline act as compatible solutes is that these compounds can be accumulated to high levels without any adverse effects on subcellular structures or plant metabolism. As shown in Figure 4.27, a decrease in the integrity of the thylakoids was noted in the chloroplasts of 21 d-old *Arabidopsis* seedlings grown in the presence of exogenous proline. Chloroplasts from the leaves of seedlings grown in the presence of proline did not appear to have fewer grana than those from control plantlets.

Mitochondrial ultrastructure was also consistently disrupted in the leaves of plantlets grown in the presence of proline (Figure 4.28). The negative effect of exogenous proline on the integrity of the cristae did not appear to be reflected by a disruption of the integrity of the outer mitochondrial membrane.

4.10 The effects of exogenous proline and proline analogues on *in vitro* shoot organogenesis in *Arabidopsis*

The *in vitro* regeneration of shoot buds from undifferentiated callus cells is an important technique in plant biotechnology. While it has long been known that the relative concentrations of auxin and cytokinin in the regeneration medium strongly influence shoot organogenesis, there is little understanding of the mechanisms by which the auxin/cytokinin balance exerts its effects (Hare & van Staden 1997). There is also little appreciation of other factors that may interact with these growth regulators in ensuring a high rate of organogenesis. The observations by others that proline can enhance *in vitro* differentiation of plant tissue explants from several different species (Nuti-Ronchi et al. 1984; Armstrong & Green 1985; Trigiano & Conger 1987; Shetty et al. 1992; Shetty & McKersie 1993; Murthy et al. 1996) led to investigation of whether similar effects could be obtained for shoot bud regeneration from *Arabidopsis* hypocotyl explants.

Inclusion of 1 mM proline, and to a lesser extent 5 mM proline, stimulated shoot organogenesis in *Arabidopsis* hypocotyl explants (Table 4.15). The frequency of shoot bud regeneration ranged from explants which sustained the differentiation of only one shoot bud per explants, to explants which sustained the differentiation of multiple shoot buds (two or more per explant). Multiple shoot


A1
sl
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B1
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C1
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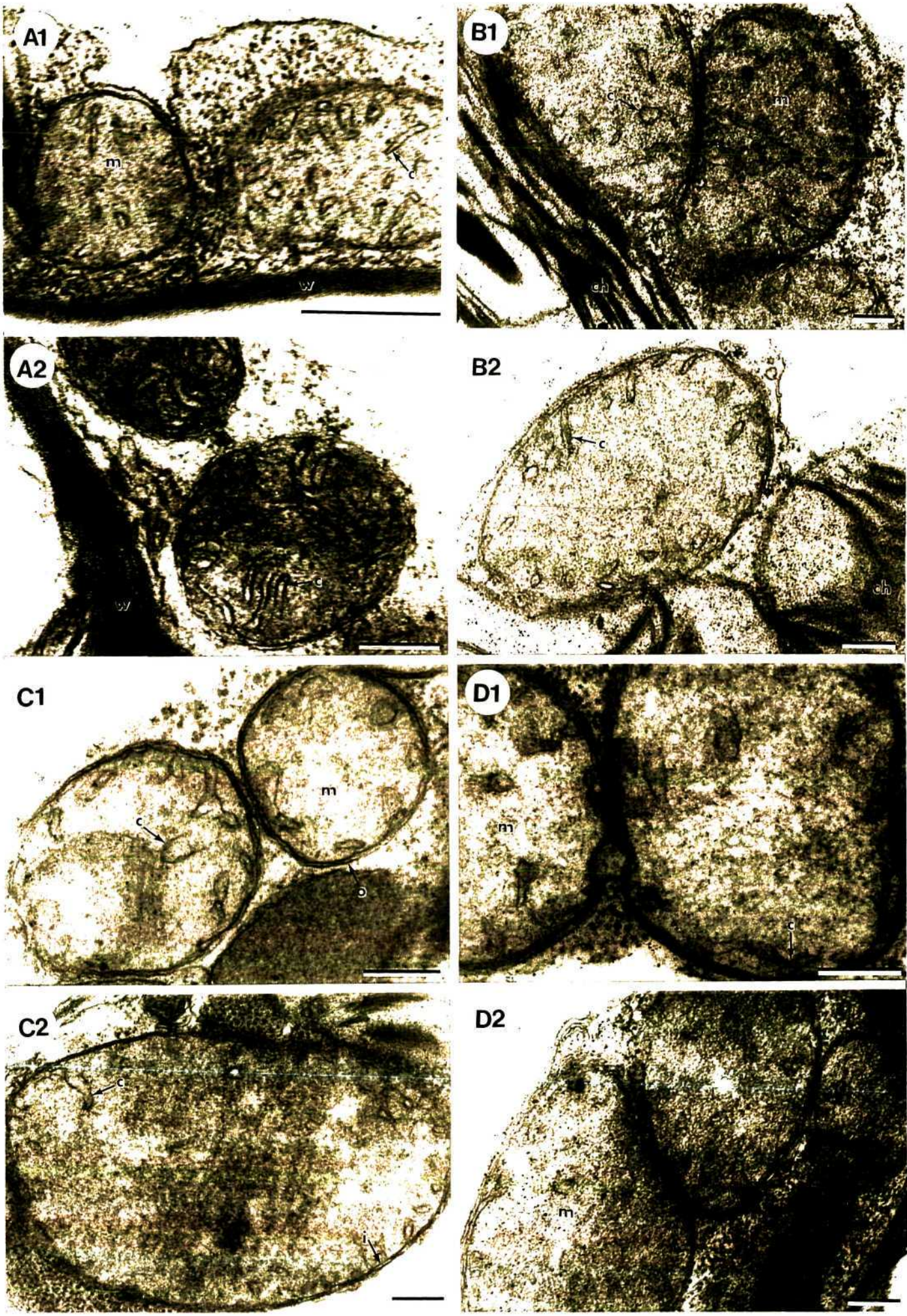
A1
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B1
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A2
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C1
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D1
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c
C2
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m

Table 4.15: The effects of exogenous proline, ornithine, glutamate and proline analogues on *in vitro* shoot organogenesis in *Arabidopsis*. Each value is expressed as the number of explants displaying that response per 40 hypocotyl explants (Section 3.7), taken from a data set comprising 200 explants (n=5). All explants were between 7.0 and 10.0 mm in length. Shoot bud regeneration and callus formation were scored 21 d after transfer of the explants to the SIM-based experimental media (Section 3.7). Unless specified, proline refers to the naturally-occurring L-isomer. Values within the same column which are followed by the same letter are not significantly different ($P > 0.05$; Duncan's multiple range test). The effects of 1.0 mM proline, 5.0 mM proline, 10.0 mM proline and 0.1 mM azetidine-2-carboxylate (AZC) together with 1.0 mM proline were tested in three additional experiments each involving at least 200 explants per treatment. In all experiments, similar trends were obtained to those shown here.

Treatment	Total shoot formation[1]	Multiple shoot formation[2]	Single shoot formation[2]	Callus formation only	No response
Control (no supplements)	14.4 a	9.0 a	5.4 aef	20.0 ab	5.6 abc
1.0 mM proline	31.0 bh	18.6 bf	12.4 b	8.8 cdf	0.2 a
5.0 mM proline	26.8 b	16.4 b	10.4 bc	11.6 di	1.6 a
10.0 mM proline	4.8 cde	3.6 cd	1.2 ad	33.2[3] e	2.0 ab
0.1 mM AZC	1.4 cd	0.0 c	1.4 ade	5.0[3] cf	33.6 ef
1.0 mM proline + 0.1 mM AZC	3.0 cde	0.4 c	2.6 adef	18.8[3] a	18.2 d
0.1 mM thioproline (TP)	2.2 cde	0.8 c	1.4 ade	29.6[3] eg	8.2 c
1.0 mM proline + 0.1 mM TP	7.0 defg	4.2 acde	2.8 adef	25.2[3] bg	7.8 bc
1.0 mM D-proline	7.2 efg	3.8 cde	3.4 adef	10.4[3] cd	22.4 d
0.1 mM D-proline	11.2 afg	7.0 ade	4.2 adef	18.6[3] ah	10.2 c
1.0 mM proline + 0.1mM D-Pro	35.6 h	21.2 f	14.4 b	3.6 f	0.8 a
1.0 mM glutamate	14.6 a	8.8 ae	5.8 ef	16.8 ahi	8.6 c
5.0 mM glutamate	12.0 ag	5.2 acde	6.8 cf	16.6 ahi	11.4 c
10.0 mM glutamate	12.0 ag	6.6 ade	5.4 aef	16.4 ahi	11.6 c
1.0 mM ornithine	6.0 def	2.4 cd	3.6 adef	12.2[3] dih	22.0 d
5.0 mM ornithine	3.4 cde	1.2 c	2.2 ade	7.2[3] cdf	29.4 e
10.0 mM ornithine	0.2 c	0.0 c	0.2 d	2.8[3] f	37.0 f

[1] Total shoot formation is the sum of multiple shoot formation and single shoot formation.
[2] Callus formation was required for shoot formation.
[3] Callus growth was less prolific than that observed for explants grown on the control medium.

buds were often so closely packed together that it was difficult to estimate the precise number of individual shoots contained within them, even when viewed using a dissecting microscope. In contrast to the lower proline concentrations which were tested, 10 mM proline decreased the total number of explants which underwent shoot regeneration as well as the number of explants which displayed multiple shoot bud formation (Table 4.15). The stimulatory effect of 1 mM proline was negated by inclusion of the proline analogues azetidine-2-carboxylate and thioproline in the hormonally-supplemented regeneration medium. When included at a concentration of 0.1 mM, both of these proline analogues caused inhibition of shoot bud formation in the absence of proline, with a stronger inhibitory effect being noted for azetidine-2-carboxylate than for thioproline. Inclusion of a ten-fold higher concentration of exogenous proline in the media was not capable of causing a significant increase in the total number of shoots formed following incubation in the presence of either of the proline analogues (Table 4.15). Inclusion of D-proline, the isomer of L-proline which is not found in proteins, at 1.0 mM caused a significant decrease in the shoot organogenesis response. However, 0.1 mM D-proline did not have a significant effect on the morphogenic response. Indeed, the highest rate of organogenesis was observed with a combination of 1.0 mM proline and 0.1 mM D-proline. However, this response was not significantly better than the response observed with 1.0 mM proline alone (Table 4.15). None of the concentrations of exogenous glutamate which were tested (1.0 mM, 5.0 mM or 10.0 mM) caused a significant increase in the total number of shoots formed. Ornithine had a strong inhibitory effect on both callus formation and shoot regeneration at all of the concentrations which were tested. At 10.0 mM ornithine, almost all of the explants displayed no growth response (Table 4.15).

5. Discussion

The term "environmental stress" remains a relatively broadly defined concept in plant physiology. This arises partly from the need to always view the effects of stress in the context of the age, the physiological state and the previous history of stress exposure of the plant under study. Frequently, consideration of the particular tissue type being studied also warrants consideration. Any factor essential for plant growth can become stressful when a threshold level for the stressor, which can no longer be compensated for the plant, is exceeded. For a single stress factor (e.g. water availability), this threshold will be influenced by additional environmental parameters (e.g. light intensity and temperature). Fluctuations in environmental parameters that can be present at stressful levels occur on a daily basis for most plants grown in their natural environment, yet the capacity for acclimation to these diurnal changes may vary throughout the life-cycle. The capacity for adaptation to stress is also a function both of the severity of the stress and its duration.

Any definition of a stressed state is thus, at best, arbitrary. Regrettably, most popular definitions of stress, e.g. the consequence of "any factor that decreases plant growth and reproduction below the potential of the genotype" (Osmond et al. 1987) or "persistently sub-optimal environmental conditions" (Bohnert & Sheveleva 1998) provide little insight into the *physiological basis* of the stressed state. A central objective of the work described in this document was thus to use the accumulation of a stress-responsive solute as a model system with which to work towards the establishment of a unifying concept of plant stress. This idea is certainly not new: Physiologists have long noted that a multitude of stressors with different modes of action can induce, besides often very specific effects, the same or at least similar overall responses in plants. Thus far, most attempts to justify the establishment of a unified plant stress response system have focused either on the common oxidative component of the response to all adverse conditions (Table 2.5; Section 2.2.1) or changes in the overall balance between the concentrations, or capacity for response to, a range of plant hormones (Hare et al. 1997). Needless to say, these two approaches are not mutually exclusive.

Stated simply, the working hypothesis on which this study was based is that ***any stress encountered by a plant results from a discrepancy between the amount of energy absorbed by a plant and its capacity to use this energy in metabolism.*** The phenomenon of stress-induced proline accumulation was targeted because it is (i) a widely studied response to the imposition of several frequently encountered environmental stresses (Table 2.4), (ii) a relatively simple biochemical trait, and (iii) there is direct evidence for a functional role for proline biosynthesis in increasing plant tolerance to drought and salinity stress (Kavi Kishor et al. 1995)

and a strong correlation between the induction of both proline biosynthetic and degradative capacity in a mutant displaying constitutive freezing tolerance (Xin & Browse 1998). Since the coordinated changes in proline synthesis and degradation qualify as a likely candidate for a general stress response, an understanding of the physiological consequences of these changes and the factors which trigger them may provide valuable insight into how plants may adapt to environmental fluctuations. As has been reviewed in Section 2.5, there is considerable circumstantial evidence that proline and its metabolic interconversions may participate in the integration of several important aspects of intermediary metabolism. Obviously, if we are to gain a more comprehensive view of stress tolerance, these ideas need to be considered in the light of the more widely studied and accepted views that plant redox status (Section 2.2) and endogenous signalling systems (Section 2.4) coordinate the response to adverse environmental conditions.

From the outset of this study, it was appreciated that the paramount role played by environmental fluctuations in shaping the evolution of all plant species strongly suggests that plants may have recruited stress-related responses to normally operate at specific stages throughout the life cycle in anticipation of developmental stages when the capacity to balance the assimilation of energy and its use in metabolism is challenged. Thus, an important goal of this study was also to use the proline metabolic system as a paradigm to illustrate how a phenomenon which has traditionally been viewed primarily as a stress-specific response in vegetative tissues may also play a fundamental role in the regulation of plant growth and development throughout the plant life cycle.

In keeping with the fairly broad scope of these aims, discussion of the findings of this study will be divided into three main parts. These will deal with molecular aspects of the regulation of proline synthesis in *Arabidopsis*, the effects of downregulation of free proline levels in *Arabidopsis*, and physiological studies of the involvement of proline on various aspects of growth and development in this model plant system.

5.1 Molecular aspects of proline biosynthesis from glutamate in *Arabidopsis*

The motivations for the analysis of the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes were three-fold. *Firstly*, we are now in a position to begin to construct primitive models of plant stress-related signal transduction networks (Section 2.4.3). A thorough characterisation of the signalling events that lead to the induction of a selected number of stress-induced genes (e.g. Table 2.11) should provide a basic framework on which to base further elucidation of stress-related signal transmission. Since stress-responsive promoter elements lie at the termini of these

signal transduction chains, examination of the 5'-UTRs in proline biosynthetic genes may enable a tentative assignment of these genes to distinct branches of the signalling network that controls the response to adverse conditions associated with water deficit (e.g. Figure 2.9). This will contribute to our overall appreciation of how the events that regulate stress-induced proline synthesis may fit into the global regulatory network that controls the plant response to environmental fluctuations (Section 2.4.5). The regulation of proline accumulation is subject to multiple regulatory factors that most probably act synergistically, yet we presently have little understanding of how different signals may be integrated. As was mentioned in Section 2.4.5, most of the genes that have been studied thus far in efforts to elucidate stress-related signal transduction appear to be relatively "stress-specific" and are induced *de novo* under adverse conditions (e.g. Table 2.10). Elucidation of the signalling events that regulate proline metabolism may provide an interesting perspective on how stress-related signal transduction is integrated with signalling events downstream of the perception of other cues (e.g. light or nutrient status) or with endogenous developmental programs. *Secondly*, similarities in the types of regulatory mechanisms that induce different genes involved in proline biosynthesis may provide some indication of how these may be coordinately induced and thus open the way for identification of likely "master switches" that control the expression of entire sets of stress-regulated genes (Hare et al. 1996), including those involved in proline metabolism. The power of this approach in increasing agricultural productivity has already been discussed (Section 2.4.1; Hare et al. 1996). *Thirdly*, and as an extension of the last point, it is of interest to investigate the functional significance of the redundancy in P5C synthesis from glutamate in *Arabidopsis*. Strizhov et al. (1997) observed that *AtP5CS1* and *AtP5CS2* have a high overall identity in their coding regions (93% amino acid similarity; Figure 4.2) and suggested that divergence between the transcriptional regulation of the two isoforms may account for the apparent duplication of a progenitor P5CS coding region within the *Arabidopsis* genome. The observation that the coding regions of *AtP5CS1* and *AtP5CS2* cDNAs share 82% sequence homology at the nucleotide level, but only 54% and 53% nucleotide identity in their 5' and 3' untranslated regions (Strizhov et al. 1997) suggests that these genes may have arisen from a fairly recent duplication of an ancestral gene. However, the similarity of the 5'-UTRs of these two genes upstream of the transcription start sites has previously not been assessed. Needless to say, an understanding of how proline synthesis is regulated will be indispensable in assessing the adaptive value of an increase in proline synthesis under adverse conditions.

It is important to note that all of the promoter elements identified in Section 4.2 have been tentatively assigned a function based exclusively on their sequence homologies to regions in the promoters of other genes which have been shown to bind regulatory proteins and/or confer regulated expression on a reporter gene. While this approach by itself is frequently used in

suggesting mechanisms of gene regulation (Chung & Parish 1995; Iturriaga et al. 1996; Kirch et al. 1997; Guan & Scandalios 1998; Velasco et al. 1998), it provides only circumstantial evidence for the involvement of these mechanisms in the regulation of proline biosynthetic capacity *in vivo*. Even complete identity to a promoter element shown to be functional in the regulation of another gene does not confirm its participation in regulation of the gene in question. Nonetheless, identification of these putative regulatory sites provides a basis for future studies using techniques such as fusion of the 5'-UTRs to reporter genes, DNase I footprinting studies, electrophoretic mobility shift assays or microinjection experiments. These may corroborate the proposed functions of these elements.

It is difficult to estimate the likelihood that the presence of any short nucleotide sequence in a 5'-UTR may simply be coincidental. However, considering that, under the assumption that the four bases found in DNA are completely randomly distributed in any sequence, a 5 bp stretch should occur every 4^5 bp (i.e. every 1024 bp), a 6 bp region every 4^6 bp (i.e. every 4096 bp), etc. etc. Using this argument, it seems highly probable that many of the putative promoter elements in the 1.0 - 2.5 kb stretches of 5'-UTRs used in this study (Appendix) may have a regulatory role. However, even further complexity in the interpretation of the results using this rationale is likely to arise from the modular structure of promoters (Benfey & Chua 1990). Many studies have indicated that most short promoter elements require additional elements in order to function and that the overall transcriptional activity is a product of the combinatorial and synergistic interactions of various *cis*-acting elements (Kosugi et al. 1995; Sainz et al. 1997). Thus, while a few plant promoter elements have been shown to be autonomous, the total capacity for promoter expression usually relies on the additive and cooperative interactions of several constituent *cis*-acting elements. The expression of most genes is therefore dependent on the aggregation of several *trans*-acting factors with RNA polymerase II to form a transcriptional complex. Consistent with this knowledge, although it will not be reiterated throughout this discussion, the proximity of any promoter element to the TATA box is likely to be an important consideration in assessment of its likely involvement in transcriptional activation.

A further caveat associated with inferring *in vivo* functionality of a conserved promoter region using sequence homology as a guide is that the abundance of the *trans*-acting factor which binds any promoter element may also limit its participation in gene expression *in vivo* (Neuhaus et al. 1994; Eyal et al. 1995). The expression of many genes encoding transcription factors is regulated by environmental changes (Urao et al. 1993; Gupta et al. 1998; Schaffer et al. 1998; Wang & Tobin 1998) or in a developmental or tissue-specific fashion (Neuhaus et al. 1994). Therefore, once again, even if a promoter region displays significant homology to a known functional *cis*-regulatory element, this may not imply *cis*-regulatory functions for this region if the cognate

transcription factor is not present at a sufficiently high level in the tissue under study or under certain environmental or developmental conditions.

5.1.1 Putative stress-related promoter elements within the 5'-UTRs of *AtP5CS1*, *AtP5CS2* and *AtP5CR*

A simple model which relates stress-related signal transduction pathways to their target promoter elements (Figure 2.9) has been discussed in Section 2.4.3.2. While this is now not accepted to be a perfect reflection of the chain of events that link stress perception to changes in the expression of stress-inducible genes (Ishitani et al. 1997; Xin & Browse 1998), it is nonetheless consistent with much of the available data concerning stress-regulated gene expression (Shinozaki & Yamaguchi-Shinozaki 1997). It also provides a useful starting point to begin to assign the regulation of proline biosynthetic genes to different branches of the network that coordinates the genetic response to dehydrative stresses.

The limitations of the model of Shinozaki and Yamaguchi-Shinozaki (1997) immediately become apparent when attempting to assign the regulation of *P5CS* gene transcription to any of the four pathways defined by these workers. A role for ABA in stress-induced *P5CS* gene expression (Table 2.10) , as well as the requirement for protein synthesis in full induction of this response (Section 2.4.2), tends to implicate the involvement of Pathway I shown in Figure 2.9. Accordingly, Strizhov et al. (1997) observed no induction of either *AtP5CS1* or *AtP5CS2* expression in an ABA-deficient *Arabidopsis* mutant (Table 2.10). However, a complication with the interpretation of an absolute dependence on ABA action for *P5CS* expression arises from the findings of Savouré et al. (1997) that NaCl- and sorbitol-induced *AtP5CS1* expression is not compromised in *aba1* (Table 2.10) and the conclusion by other researchers that ABA action alone cannot account fully for the stress-induced accumulation of proline in *Arabidopsis* (Finkelstein & Somerville 1990; Yoshiba et al. 1995). While most of the focus of this study concerns proline accumulation in *Arabidopsis*, it is worth mentioning that several studies involving other species have failed to indicate a causal link between ABA application and free proline accumulation (Wample & Bewley 1975; McDonnell et al. 1983; Henson 1985; Thomas et al. 1992) or stress induced proline accumulation in ABA-deficient mutants (Stewart & Voetberg 1987).

In keeping with the conclusion (Ishitani et al. 1997) that there is considerable cross-talk between ABA-dependent and ABA-independent signalling cascades, it therefore seems likely that Pathway IV (Figure 2.9) may also participate in stress inducible *P5CS* expression. Accordingly, low temperature treatments have been associated with the induction of *AtP5CS1* expression (Yoshiba

et al. 1995; Savouré et al. 1997; Xin & Browse 1998) and the accumulation of transcript encoding a rice P5CS (Igarashi et al. 1997). It now appears that different stress-induced signalling pathways may converge at a single promoter and target distinct stress-responsive promoter elements (Ishitani et al. 1997). Examination of the 5'-UTRs of proline biosynthetic genes offers one approach to addressing the question of how the transcription of these genes may be regulated and promises to provide some insight into how the various ABA-dependent and ABA-independent pathways may be linked.

As was discussed in Section 2.4.3.2, ABA-mediated induction of several dehydration-inducible genes has been shown to be mediated through binding of bZIP proteins to the ABRE. Transcription factors containing the bZIP motif form a large and diverse family in plants. The involvement of promoter elements containing the G-box (CACGTG) in the induction of gene expression by a wide range of environmental stimuli (Daugherty et al. 1994) introduces further difficulty in establishing what criteria may determine response specificity. Overall, it seems likely that several bZIP factors may interact with any G-box-containing sequence, although the net outcome of such multiple interactions will depend on the relative affinity of each bZIP protein for the particular target sequence, their relative abundance at each stage of development or under the prevailing environmental conditions, as well as the availability of other interacting bZIP-type proteins present in a given tissue. In cereals, ABA responsiveness appears to be dependent on the presence of an ABA response complex comprising coupling elements that flank the ABRE (Shen & Ho 1997).

Perfect matches with the consensus ABRE are found in both the *AtP5CS1* and *AtP5CS2* 5'-UTRs (Table 4.2). The location of one putative ABRE in *AtP5CS1* far upstream of the TATA box (> 2.5 kb) strongly decreases the likelihood that it may participate in ABA-mediated induction of *AtP5CS1* expression. No regions resembling the ABA-specific coupling elements CE1 or CE3 characterised in barley (Shen & Ho 1997) are found in the vicinity of a second putative ABRE in the *AtP5CS1* 5'-UTR found at position -209 upstream of the start codon. In contrast, the likelihood of ABRE-mediated induction of *AtP5CS2* expression is supported by the occurrence of two CE1-like regions within a 70 bp stretch upstream of the putative ABRE (Table 4.2). Nonetheless, the possible significance of this putative ABA response domain is diminished by the observation that exogenous ABA failed to activate expression of a fusion of the full *AtP5CS2* 5'-UTR (Appendix) to a reporter gene (Zhang et al. 1997). Furthermore, *AtP5CS2* induction by NaCl-treatment displays an absolute requirement for protein synthesis (Section 2.4.2; Strizhov et al. 1997), while ABRE-mediated induction of gene expression by ABA is generally considered to be CHX-insensitive (Figure 2.9; Shinozaki & Yamaguchi-Shinozaki 1997). It may be worth noting that putative ABREs have been found in both ABA-inducible and ABA-independent promoters and the

activity of these sequences appears to be determined both by their context within the promoter as well as developmental regulation (Busk et al. 1997). Therefore, although it is difficult to reach an unambiguous conclusion, the ABA-mediated induction of proline synthesis in *Arabidopsis* seems unlikely to rely heavily on a signalling pathway that terminates in ABRE activation. The putative ABA-responsive complex approximately 800 bp upstream of the transcription initiation site in the *AtP5CS2* may be degenerate, as was suggested for an ABRE-like element in the *RAB17* promoter (Busk et al. 1997). Savouré et al. (1997) observed only a weak induction of *AtP5CR* transcript in *Arabidopsis* plants treated with exogenous ABA (Table 2.10). This complicates assessment of the significance of the putative ABA-responsive promoter elements identified in the 5'-UTR of *AtP5CR* (Table 4.2).

Besides CHX-insensitive ABRE-mediated gene expression, ABA-dependent gene activation is also known to sometimes require the synthesis of transcription factors (Figure 2.9). These most commonly belong to the large classes of plant MYB-, MYC- or bZIP-domain containing DNA-binding proteins. In view both of the reversible activation of the DNA-binding capacity of both plant and animal MYB-domain proteins by a thiol-redox control mechanism (Section 2.2.2.3; Myrset et al. 1993; Williams & Grotewold 1997; Martin & Paz-Ares 1997) and the proposed importance of proline synthesis in the regulation of redox potential (Section 2.5.2), the identification of several putative MYB-binding sites in the 5'-UTRs of each of the three proline biosynthetic genes studied (Table 4.1) has considerable appeal. These may provide a direct link between the sensing of cellular redox status and the induction of proline biosynthesis.

Although many of the putative MYB-binding sites in the 5'-UTRs of *AtP5CS1*, *AtP5CS2* and *AtP5CR* lie relatively far away from the sites of transcriptional initiation (Table 4.1), the number of binding sites for a particular transcription factor is considered to be an important determinant of the likelihood of its participation in transcriptional initiation (Benfey & Chua 1990). In this regard, it may be noteworthy that more than one type of functional MYB-binding sequence may occur in a single promoter (Solano et al. 1995) and that where tested, plant MYB-domain proteins may bind to different types of MYB recognition sequences *in vitro* (Li & Parish 1995; Sainz et al. 1997; Uimari & Strommer 1997). In an attempt to explain the observation that many MYB-domain proteins which bind similar or identical sequences *in vitro* exert diverse functions *in vivo*, it has been suggested (Li & Parish 1995; Uimari & Strommer 1997) that MYB proteins may select their target sequences depending on the availability of other factors with which they cooperate. Whether the specificity of binding of a single MYB protein *in vivo* may be affected by other transcription factors is not known, although *in vivo* competition between different MYB proteins for a target site has been reported in animal systems and also appears to occur in plants (Tamagnone et al. 1998). Furthermore, it is worth noting that there is some redundancy in the

different consensus MYB-recognition sites defined by various workers using different MYB-domain proteins (Table 4.1). For instance, the putative recognition sites identified at positions - 630 and - 1368 in the *AtP5CS1* 5'-UTR, and at positions - 218 and - 881 in the *AtP5CR* 5'-UTR are complementary to the consensus c-MYB recognition sequence C(A/C)GTT(A/G) originally identified in mammalian systems (Howe et al. 1990). Although not indicated in Table 4.1, the putative MYB-recognition site at position -1289 in the *AtP5CS2* 5'-UTR also matches the (T/C)AAC(T/C)(A/G) consensus sequence proposed by Shinozaki and Yamaguchi-Shinozaki (1997).

Unfortunately, thus far, all of the studies which have investigated thiol-mediated activation or inactivation of MYB-domain proteins appear to have involved examination of the effects of chemical agents (e.g. 2-mercaptoethanol and DTT) *in vitro*. While the issue of which endogenous molecules may act as physiological redox regulators of MYB activity in eukaryotes remains to be clarified, TRXs (Section 2.2.2.3) may be likely candidates for this role. By analogy, in mammals, reduced TRX plays a direct role in the inactivation of NF-κB (Matthews et al. 1992) and activates AP-1 transcriptional activity more indirectly, through acting as a hydrogen donor to Ref-1 (Hirota et al. 1997), a homologue of which has been identified in *Arabidopsis* (Babiychuk et al. 1994). Clearly, although the evidence in Table 4.1 is indirect, MYB-domain containing proteins appear to play an important role in the regulation of proline biosynthetic capacity. The sensitivity of MYB DNA-binding capacity to cellular redox potential (Myrset et al. 1993; Martin & Paz-Ares 1997; Williams & Grotewold 1997) is of particular interest in view of the postulated link between proline biosynthesis and the maintenance of cellular redox homeostasis (Section 2.5.2). It seems highly probable that the mechanisms of redox sensing and the metabolic systems that control the redox status of the cell are coupled. While the extensive ubiquity of *R2R3-MYB* genes in plants relative to other eukaryotes (Martin & Paz-Ares 1997; Romero et al. 1998) has been regarded as being somewhat enigmatic, it is tempting to speculate that this may be attributed to the particular importance of redox-coupled changes in gene expression in phototrophs (Section 2.2). The induction by ABA of genes encoding MYB-domain proteins does not formally exclude the possibility that cellular redox potential may be the primary determinant of their activities. Guan and Scandalios (1998) have recently proposed that there may be a strong link between ABA action and oxidative stress. While this proposal has yet to be tested at the molecular level, the known effects of ABA on stomatal closure might well cause oxidative stress if there is inadequate capacity for the dissimilation of excess radiant energy.

The MYB-class transcription factors are but one class of regulatory proteins involved in the ABA-dependent branch of stress signalling that requires protein synthesis (Figure 2.9). Plant MYB-domain proteins frequently appear to require direct association with MYC-related DNA-binding

proteins in order to activate transcription of a target promoter (Sainz et al. 1997), although there is no evidence that either factor regulates expression of the other. MYC-type transcription factors have homology to a class of mammalian oncogenes and are characterised by the possession of a basic helix-loop-helix (bHLH) motif in their DNA-binding domains. For example, expression of the maize *C1* gene, which encodes a MYB-type regulator of several genes involved in anthocyanin synthesis, had no effect on anthocyanin production in *Arabidopsis* when expressed by itself. However, hybrid plants which express both *C1* and *R*, a MYC-type transcription factor, are characterised by ectopic anthocyanin accumulation (Lloyd et al. 1992). Similarly, a 67 bp region in the promoter of the *RD22* gene (position -141 to -207 upstream of the transcription start site) which confers dehydration- and ABA-induced gene expression in *Arabidopsis* contains both a MYB- and two MYC-recognition sequences. Mutational analysis has indicated that the MYB site and one of the MYC recognition sequences (found approximately 50 bp upstream of the MYB recognition sequence) are critical *cis*-acting elements required for the dehydration-responsiveness of *RD22* (Abe et al. 1997). These workers cloned a dehydration- and ABA-inducible bHLH domain-containing protein, named RD22BP1, which binds to the critical MYC recognition sequence and have postulated that full activation of *RD22* expression is dependent on the direct association of RD22BP with AtMYB2 to form a transcriptional complex. Transactivation experiments indicated that although either AtMYB2 or RD22BP alone could slightly activate *RD22* expression, coexpression of both proteins increased the expression of a *GUS* reporter gene approximately three times more strongly than when either protein was used alone (Abe et al. 1997).

It is thus of interest that both of the *AtP5CS1* and the *AtP5CS2* 5'-UTRs contain the CACATG motif recognised by RD22BP1 (Table 4.3) and that these occur in close proximity to several putative MYB recognition sequences (Table 4.1). While the large distance of the putative MYC recognition sites in the *AtP5CS1* 5'-UTR from the translation initiation site tends to argue against the likelihood that they play an important role in transcriptional initiation, further investigation of the importance of a transcriptional complex involving MYB and MYC factors at the -750 to - 850 region of the *AtP5CS2* 5'-UTR may be warranted. Following NaCl-stress, the coordinated super-induction of both *AtP5CS1* and *AtMYB2* (proposed to participate in the signalling of osmotic stress; Urao et al. 1993) in both *sos1* (Liu & Zhu 1997a) and *sos2* (Zhu et al. 1998) seems consistent with the involvement of at least one MYB-domain transcription factor in the regulation of *AtP5CS1* expression in response to salinity stress. Investigation of whether *RD22BP1* is also expressed at a higher constitutive level in these NaCl-hypersensitive mutants warrants investigation.

An important objective in examination of the 5'-UTRs of the *P5CS* and *P5CR* genes was to search for common regulatory sites that might ensure coordinated induction of all three proline biosynthetic genes under adverse conditions. The most interesting finding in this regard is the occurrence of an identical 8 bp sequence found in all three genes, which includes the consensus recognition sequence for a cold-induced bZIP-type transcription factor from maize (Table 4.3; Kusano et al. 1995). It may also be worth mentioning that levels of transcript encoding the HD-ZIP protein ATHB-7 (Söderman et al. 1996) are responsive to ABA, desiccation and salinity stress and that this induction is dependent on the action of a pathway which is disrupted by mutation of *ABI1* (Table 2.10). Although there appears to not yet be any direct evidence that ATHB-7 functions as a transcription factor, in view of the possible effects of *abi1* on *AtP5CS1* and *AtP5CS2* expression (Strizhov et al. 1997, but note a contrasting conclusion by Savouré et al. 1997; Table 2.10), it seems a likely candidate for regulation of either or both of the *AtP5CS1* and *AtP5CS2* genes. Mutation of the *ABI1* gene does not appear to have any effect on *AtP5CR* transcript levels (Table 2.10; Savouré et al. 1997).

The DRE/C-repeat motif occurs in a number of drought- and cold-inducible *Arabidopsis* genes, including *RD29A* (Nordin et al. 1993, Yamaguchi-Shinozaki & Shinozaki 1993), *RAB18* (Lång & Palva 1992) and *KIN2* (Kurkela & Borg-Franck 1992). The DRE occurs four times in the 5'-UTR of *RD29A* (Nordin et al. 1993; Yamaguchi-Shinozaki & Shinozaki 1993), but not in the closely related *RD29B* gene which is not responsive to cold stress. Recently, a study which involved overexpression of a DRE-binding protein (CBF1, Stockinger et al. 1997) has demonstrated an important role for the DRE in mediating the induction of *RD29A*, *KIN2* and two other cold-inducible *Arabidopsis* genes which contain DREs in their 5'-UTRs (Jaglo-Ottosen et al. 1998). No perfect matches to the DRE are found in any of the three proline biosynthetic genes which were studied here (Table 4.3). The considerable displacement of DRE-like elements in the 5'-UTRs of *AtP5CS1* and *AtP5CR* from the TATA boxes of these genes discredits their regulation by a pathway that specifically activates dehydration-inducible expression through the DRE. The recent characterisation of the *esk1* mutant (Xin & Browse 1998) seems consistent with this interpretation, at least for *AtP5CS1* expression. Mutation of the *ESK1* locus increases constitutive *AtP5CS1* transcript levels approximately three-fold, but has no effect on the expression of *RD29A*, *KIN2* or two other cold-regulated genes (*COR47* and *COR15a*), which are upregulated in CBF1-overexpressing lines (Jaglo-Ottosen et al. 1998).

As was outlined in Section 2.2.2.3, we presently have almost no appreciation of the extent to which ROIs may directly affect plant gene expression. However, the production of ROIs under adverse conditions suggests that they would be ideal candidates as secondary messengers in mediating the plant response to several environmental stresses. The bacterial OxyR response

element (ORE) which directly links levels of ROIs with the expression of a number of genes encoding defence and repair enzymes in eubacteria (Farr & Kogoma 1991; Zheng et al. 1998), also functions as a redox-dependent enhancer in mammalian cells (Duh et al. 1995). This function results from the interaction of the canonical ORE motif with members of a family of mammalian transcription factors that recognise the Y-box motif (Duh et al. 1995). The finding of these workers suggests that the ORE may reflect a highly conserved mechanism for antioxidative responses that developed early in the evolution of aerobic organisms. While the involvement of Y-box type transcription factors in the regulation of plant gene expression does not appear to have been investigated, Guan and Scandalios (1998) recently reported the occurrence of two Y-box motifs within in the 5'-UTR of a maize *Cu/ZnSOD* gene (positions - 339 and -289 upstream of the start codon). The role of this gene in counteracting oxidative stress seems consistent with the proposal that an ORE/Y-box related mechanism may mediate the response of plant genes to ROI accumulation under adverse conditions (Guan & Scandalios 1998). While the full significance of ORE/Y-box motifs in ROI-mediated induction of plant gene expression has not yet been explored, it is interesting to note that the 5'-UTR of the *AtP5CR* gene contains two very closely spaced ORE elements within 250 bp of the transcription initiation site (Table 4.3). Four ORE/Y-box motifs are found more than 1.0 kb upstream of the start codon within the 5'-UTR of the *AtP5CS1* gene, although this motif does not occur in the *AtP5CS2* 5'-UTR (Table 4.3).

5.1.2 Tissue-specific and developmentally regulated promoter elements

A fairly substantial amount of evidence which supports an important role for proline biosynthesis in supporting a high rate of cell division in plants (Shetty et al. 1992; Hare & Cress 1996; Forlani et al. 1997b; Garcia et al. 1997; Hua et al. 1997; Strizhov et al. 1997) and other eukaryotes (Neuville & Aigle 1992; Downs et al. 1998) has been reviewed in Section 2.5.2.4. The identification of TEF1-like sequences in the 5'-UTRs of the *AtP5CS1*, *AtP5CS2* and *AtP5CR* genes (Figure 4.3) is of interest not only because a TEF1-related regulatory mechanism appears to activate the transcription of several genes in rapidly dividing cells, but also because TEF1 elements have been found in the promoters of several genes involved in redox-regulated processes or in protection against the damaging effects of oxidative reactions (Regad et al. 1995). Indeed, redox status now appears to be an important determinant of cell proliferation not only in animal cells, but also in plants (Section 2.2.2.3; Sánchez-Fernández et al. 1997). Transcriptional regulation of proline biosynthesis by a TEF1-related mechanism may therefore, like the probable involvement of MYB-domain proteins (Table 4.1), be consistent with the proposed importance of cellular redox status as the primary determinant of proline biosynthetic capacity (Section 2.5.2).

Interestingly, Hua et al. (1997) identified a 69 bp element (between nucleotides -212 and -143 upstream from the transcription initiation site) within the promoter of *AtP5CR* which confers a high level of expression to a *GUS* reporter gene both in the root and shoot meristems, as well as in the lateral root and leaf primordia of 10 d-old *Arabidopsis* seedlings. Systematic deletions of the 5'-UTR of *AtP5CR* indicated that the meristematic tissue-specific expression was retained in the fragment that extended to position -212 bp upstream of the transcription initiation site of *AtP5CR*, whereas the promoter activity decreased to background levels when the deletion was extended to position -143 bp (Hua et al. 1997). Thus, it seems that elements in the region between positions -212 and -143 are exclusively responsible for the regulation of *AtP5CR* transcription in actively dividing cells. Rather disappointingly, the putative TEF1 sequence in the 5'-UTR of *AtP5CR* (between nucleotides -126 and -103 upstream from the transcription initiation site) falls outside of the critical 69 bp region responsible for conferring tissue-specificity to *AtP5CR* expression. Nonetheless, the putative TEF-1 site lies proximal to the transcription initiation site (Appendix). Therefore, the findings of Hua et al. (1997) do not eliminate the likelihood that the putative TEF1-regulatory element in the *AtP5CR* promoter (Figure 4.3) contributes to a high level of *AtP5CR* expression in rapidly dividing cells, but they strongly suggest that this region alone is incapable of transcriptional activation. Possibly, *Arabidopsis* TEF1-binding proteins (Regad et al. 1995) may require cooperation with *trans*-acting factors that bind promoter elements in the region 86 bp upstream of the putative TEF1-box (Figure 4.3) in order to form a functional transcriptional complex. Thus far, no convincing candidates for proteins that bind to the 69 bp stretch in the *AtP5CR* promoter have been identified (Hua et al. 1997).

In this regard, it is worth noting that a TELO box-like region (Figure 4.4; nucleotides -176 to -169 upstream from the transcription initiation site) falls within the 69 bp region defined by Hua et al. (1997). The importance of the TELO box in mediating TEF1-dependent functions remains enigmatic. Regad et al. (1995) speculated that TELO-like sequences may antagonise TEF1-dependent activities in quiescent or senescent cells, although remarkably, deletion of a 10 bp region which included the TELO box of the *Arabidopsis EF-1αA1* gene *decreased* the transcriptional activity of the promoter two-fold (Curie et al. 1991). The involvement of the TELO box-like motif within the *AtP5CR* promoter (Figure 4.4) may thus warrant further investigation.

It may also be noteworthy that a putative MYB recognition site (position -208 relative to the transcription start site; Table 4.1) also falls within the 69 bp region defined by Hua et al. (1997). As was discussed in Section 2.2.2.3, the prototypical animal MYB-domain protein c-MYB, has an important promotional effect on mammalian cell division. This effect most likely arises from its ability to activate transcription from the promoter of the mammalian gene which encodes CDC2, a cyclin-dependent kinase (Ku et al. 1993). The involvement of plant cyclin-dependent kinases

in coordinating cell cycle transitions has been reviewed (Hare & van Staden 1997). Although conclusive evidence of a fundamental role for plant *MYB* gene products in plant cell cycle control has yet to be achieved, putative MYB recognition sites have been identified within regions of the promoter of the *Arabidopsis CDC2a* gene that are essential for its maximal expression in actively proliferating plant cells (Chung & Parish 1995).

Most strikingly, as shown in Table 4.3, two overlapping sequences with high homology to the consensus of two *cis*-acting elements which are essential for meristematic tissue-specific expression of a rice gene (Kosugi et al. 1995) are found in the 69 bp stretch identified by Hua et al. (1997). Using DNaseI footprinting and gel retardation analysis, Kosugi et al. (1995) identified two similar elements designated site IIa (TGGGCCCGT, position -197 to -188 upstream from the transcription initiation site) and site IIb (TGGTCCCAC, position -178 to -169) in a rice gene encoding proliferating cell nuclear antigen (PCNA). In eukaryotes, PCNA is an auxiliary protein of DNA polymerase δ. Both of these neighbouring sites are bound by the same nuclear factor *in vitro* and simultaneous disruption of both sites IIa and IIb caused about 80-85% loss of the high meristem tissue-specific activity of this promoter (Kosugi et al. 1995). Both elements were protected by proteins in crude nuclear extracts from both rice and tobacco, thus suggesting that the protein that binds to this region is common to monocotyledonous and dicotyledonous plants (Kosugi et al. 1995). The high homologies (77% and 88% relative to the site IIb sequence) of the two 9 bp elements found on both strands of the *AtP5CR* promoter at around position -200 upstream of the transcription start site (Table 4.3) suggest that they may play an important role in conferring meristematic tissue-specific expression of *AtP5CR*. Regarding the proposed involvement of the putative TEF1 box in the regulation of *AtP5CR* expression, it must be emphasised that double mutation of sites IIa and IIb did not completely abolish the activity of the rice gene encoding a proliferating cell nuclear antigen (Kosugi et al. 1995). The remaining 15-20% of activity in the mutated promoter was ascribed to additional undefined elements. This re-emphasises the frequently observed importance of combinatorial interactions of several different transcription factors in transcriptional activation of most plant genes.

It also seems pertinent to note here that while promoter fusion analyses, such as the one used by Hua et al. (1997), have made substantial contributions to our understanding of plant gene expression, this measure of gene expression has frequently been found to be inadequate (Taylor 1997). Artifactual expression that does not accurately reflect the *in vivo* regulation of the gene of interest may arise from the normal influence of regulatory regions that lie outside of the promoter (Taylor 1997). Thus, a technique such as *in situ* hybridisation (Hare & Cress 1996) should always be used as supportive evidence of the findings of promoter fusion studies. A fair level of consistency is found between the findings of Hua et al. (1997) and Hare and Cress (1996). The

latter study examined the steady state levels of *AtP5CR* transcript in cross-sections of the flowering stem of *Arabidopsis*, since most tissue types are represented within this plant part. Thus, promoter fusion appears to be an adequate measure of *AtP5CR* expression. However, it is worth mentioning that the same caution applies in the interpretation of the results of Zhang et al. (1997) regarding the lack of transcriptional activation of *AtP5CS2* by ABA. As was mentioned above (Section 5.1.1), the presence of a convincing ABA responsive complex in the 5'-UTR of this gene (ABRE with coupling elements) does not seem consistent with the findings of these workers. Notwithstanding the usefulness of the identification of putative promoter elements on the basis of sequence homology alone, it must be noted once again that the presence of any sequence in a promoter should never be regarded as confirmatory evidence of its function *in vivo*.

The occurrence of three regions with weak homology to the site IIa/site IIb consensus (Kosugi et al. 1995; Table 4.3) and two TEF1-like sequences in the 5'-UTR of the *AtP5CS2* gene (Figure 4.3) is of particular interest. Although this gene contributes 20-40% of the total P5CS mRNA in differentiated *Arabidopsis* tissues, it is solely responsible for the synthesis of abundant P5CS mRNA in rapidly dividing cell cultures (Strizhov et al. 1997). The presence of more than one binding site for a transcriptional regulator(s) is widely accepted to indicate its likely importance in the activation of transcription. Accordingly, the tobacco *ParA* gene, which is also actively transcribed in rapidly cycling cultured cells (Takahashi et al. 1990) contains two closely-spaced TEF1 boxes (Figure 4.3). Notwithstanding the possible significance of the two putative TEF1 sequences in *AtP5CS2*, some caution in the interpretation of this finding may be warranted. Alignment of these sequences with the consensus TEF1 box required either the insertion (putative TEF1 box at position -909) or deletion (putative TEF1 box at position -139) of a single nucleotide between the two highly conserved regions within the TEF1 consensus sequence (Figure 4.3). Regad et al. (1995) noted that the universally conserved GG and GT residues in all TEF1 boxes (Figure 4.3) are separated by one turn in the DNA helix. They suggested that this may indicate that the two protein complexes which bind the TEF1 sequence from the *Arabidopsis EF1αA1* and other TEF1-containing promoters (Curie et al. 1991; Regad et al. 1995) may interact with the template at two sites located on the same side of the DNA molecule. The extent to which insertion or deletion of a single nucleotide within the poorly conserved spacer region of the TEF1 box may influence its activity remains to be established. If this is a critical determinant of TEF1 functioning, it may strongly decrease the likely participation of a TEF1-related transcriptional activation mechanism in controlling *AtP5CS2* expression.

The novel identification of a TEF1 box in a plastid-specific ribosomal protein from spinach (Figure 4.3) is consistent with the previous proposal (Regad et al. 1995) that a transcriptional activation mechanism which specifically recognises the TEF1 motif is of importance in priming the cellular

translational apparatus prior to cell division. Li et al. (1995) used footprinting analyses to identify eight DNA elements within the *SoRPS22* gene which interact specifically with spinach leaf nuclear factors. Although the *SoRPS22* promoter is highly active in root meristematic cells, none of these correspond to the putative TEF1 box in the 5'-UTR of this gene (Figure 4.3). Nonetheless, deletion of a region which includes the putative TEF1 box (Figure 4.3) substantially reduced the *in vivo* activity of the promoter (Li et al. 1995a).

The potential involvement of a TEF1-mediated regulatory mechanism in plant responses to adverse conditions does not appear to be have been considered thus far. In view of the likely significance of shifts cellular redox potential as indicators of an energy imbalance resulting from environmental stress (Section 2.5.2.2), the novel identification of a TEF1-like element within the upstream region of the ABA-, drought, NaCl- and cold-inducible *CI7* gene from potato (Kirch et al. 1997; Figure 4.3) may be of some interest. The product of the *CI7* gene has high homology to a class of dehydrins. Although originally characterised as a cold-inducible transcript, *CI7* appears to be general stress responsive gene (Kirch et al. 1997). Accumulation of *CI7* transcript was far more rapid after exposure to ABA or the imposition of dehydration of salt stress than after cold treatment and several putative ABREs and DREs have been identified in the 5'-UTR of *CI7* (Kirch et al. 1997). The observation that the response of the *CI7* gene to low temperature appears to be controlled primarily at a post-transcriptional level (Kirch et al. 1997) complicates interpretation of the significance of the putative TEF1 sequence in the 5'-UTR of this gene (Figure 4.3). However, it is worth noting that genes encoding cold-inducible isoforms of EF-1α have been identified in barley (Dunn et al. 1993) and maize (Berberich et al. 1995) and that a vitronectin-like protein from NaCl-adapted tobacco cells is almost identical to EF1α (Zhu et al. 1994). Regarding the well-documented involvement of redox-mediated changes in gene expression following biotic stress (Sections 2.2.2.1, 2.2.2.3), it is of interest to note that tobacco *str246C*, which was characterised as a gene which is strongly expressed shortly after inoculation with compatible or incompatible pathogens or after elicitor treatment (Froissard 1994), is identical to the *ParA* gene which contains two TEF1-like boxes (Figure 4.3; Regad et al. 1995).

The identification of several AT-rich regions within the *AtP5CS1* and *AtP5CS2* 5'-UTRs which have extensive homology to upstream regulatory regions of genes encoding seed storage proteins and an anther-specific protein (Section 4.2.6) is consistent with the coordination of proline synthesis with certain developmentally-regulated processes. Substantial evidence which implicates a role for proline synthesis in the development of reproductive structures (Chiang & Dandekar 1995; Verbruggen et al. 1993, 1996a; Walton et al. 1998) has already been reviewed (Section 2.5.2.4). While it has been suggested that this relationship may merely be a consequence of the fact that reproductive tissues such as pollen and seeds desiccate naturally

during the course of their maturation (Chiang & Dandekar 1995; Savouré et al. 1995; Hua et al. 1997), the possibility of tissue-specific or developmental regulation of proline biosynthesis in these tissues cannot be formally excluded. A high level of expression of many well-characterised dehydration-inducible genes has been noted in tissues which undergo developmentally programmed desiccation. For example, the promoters of the ABA- and dehydration-responsive *KIN2* and *RAB16A* genes, but not that of *RD29A*, are highly active in both in anthers and the embryos of developing seeds (Wang & Cutler 1995; Ono et al. 1996). Like the proline biosynthetic genes, the possibility that tissue specificity of *KIN2* and *RAB16A* transcription may be separable from dehydration-related influences does not appear to have been investigated. Hua et al. (1997) have noted a high level of transcriptional activation of *AtP5CR* in both seeds and the central septum of *Arabidopsis* siliques only during the early stages of embryogenesis and that this had declined to a nondetectable level while the siliques were still green and had not yet undergone programmed desiccation.

Since the genes which encode seed storage proteins are transcribed at high levels in the endosperm of monocotyledonous seeds and in the cotyledons of dicotyledonous seeds only at a specific stage in the latter half of embryogenesis prior to seed desiccation, they have long been regarded as ideal systems with which to study the developmental and tissue-specific regulation of plant gene expression. Consistent with a high degree of structural similarity between the primary seed storage proteins found in diverse taxonomic groupings, it appears that common regulatory mechanism may direct the tissue-specific and developmentally-controlled expression of seed storage proteins in different angiosperm families (Shirsat et al. 1989; Rerie et al. 1991). Despite considerable research, the mechanisms underlying the tissue-specificity and temporal regulation of seed storage protein synthesis are still poorly understood and there is only limited information concerning the transcription factors involved. Deletion analysis of the *PsLEGA* gene has indicated that an upstream activating sequence(s) located between positions -237 and -549 relative to the transcription initiation site is essential for conferring a high level of regulated expression in transgenic tobacco (Shirsat et al. 1989; Rerie et al. 1991) although the particular protein-binding sequences in this region have not been examined. Since both of the regions identified in Figure 4.7A fall within this range, they may be candidates for directing a high level of temporally regulated seed-specific expression. Accordingly, Rerie et al. (1991) have noted that AT-richness is a conserved feature of the regulatory regions in several seed protein genes which have been identified using gel retardation and DNA footprinting assays. Interestingly, a 20 bp region in the 5'-UTR of *AtP5CS2* bears 90% homology to a region within the promoter of a maize gene encoding a member of the 22 kD class of zein storage proteins (Figure 4.7B). A function for this stretch in the *ZmZA1* promoter does not appear to have been suggested previously, although it occurs downstream of a regulatory region centred around -300 bp which comprises a highly

conserved "prolamin box" in close proximity to the binding site of the OPAQUE-2 transcriptional activator (Schmidt et al. 1992). The OPAQUE-2 and prolamin box binding proteins are believed to work in concert to promote high levels of 22 kD zein gene expression.

Assuming that the results presented in Figure 4.7 reflect some functional relationship between the synthesis of proline and seed storage proteins, then there are at least two interpretations of the possible coordination of both processes. In view of the relative abundance of proline residues in *Arabidopsis* seed storage proteins (Table 4.14), the simplest explanation for the relationship is that elevated free proline levels are required at the time of storage protein synthesis. However, a role for proline synthesis in providing the NADPH required for fatty acid synthesis was discussed in Section 2.5.2.4. Triacylglycerols are an important storage reserve in many oilseed species such as *Arabidopsis*. Upon germination, storage triacylglycerols are mobilised by conversion to carbohydrates for transport to the root and shoot axes of the developing seedling.

Similarly, a primarily metabolic explanation for proline synthesis during flower development can also be invoked for explaining the apparent coordination of proline biosynthesis with the expression of genes involved in anther- or pollen-specific expression (Figure 4.8). In most angiosperms, besides embryos, pollen grains are the only tissues whose protoplasm is able to withstand air-dryness. A high concentration of proline in pollen cells has previously been proposed to function as a readily accessible energy source during pollen tube elongation (Dashek & Harwood 1974). Nonetheless, difficulty in discriminating between whether this relationship is primarily an osmotic effect or whether it reflects developmental regulation arises once again when attempting to interpret the significance of a 24 bp element in the *AtP5CS2* 5'-UTR which has considerable homology to a sequence found within 250 bp of the *LAT52* (late anther tomato) translation initiation site (Figure 4.8A). The *LAT52* gene product affects pollen hydration (Muschietti et al. 1994) and although *LAT52* is expressed primarily in the pollen grain, weak expression is also observed in the endosperm of immature seed (Twell et al. 1991). Pollen specificity is encoded within a 100 bp region upstream of the transcription start site of *LAT52*, although this short proximal region confers only 25% of the total expression provided by the full length *LAT52* promoter (Twell et al. 1991). Eyal et al. (1995) have suggested that redundant or additional tissue specific elements that occur upstream of the -100 bp stretch must modulate the level of *LAT52* expression. The region identified in Figure 4.8A may be an interesting candidate in this regard. This region does not bear homology to any of the previously identified regions within anther- or pollen-specific promoters (Eyal et al. 1995; Weterings et al. 1995). In four-week-old flowering *Arabidopsis* plants, the *AtP5CR* promoter drove strong expression of a reporter gene in pollen grains, but not in the tapetum of anthers (Hua et al. 1997). The failure to identify any putative *cis*-regulatory regions within the *AtP5CR* 5'-UTR which have homology to known anther-

or pollen-specific motifs is consistent with the proposal that there is more than one regulatory system that directs the tissue-specificity of gene expression within the maturing pollen grain (Weterings et al. 1995).

5.1.3 Phytochrome- and hormone-responsive promoter elements

Presently, our understanding of the events that link the perception of light by phytochrome and the induction and repression of light-regulated genes is more advanced than that of any other single plant signal transduction system. Phytochrome signalling has been characterised in some detail both in terms of the macromolecular components that participate in the process as well as in terms of a fairly simple biochemical network involving ubiquitous second messengers (Figure 2.12). However, much remains to be learnt regarding how Ca^{2+} and cGMP operate in the context of the macromolecules involved in phytochrome action (Thomas et al. 1997). Owing to the central role played by light in almost all aspects of plant growth and development, including the response to adverse environmental conditions, the value of using the phytochrome signalling network as a central framework to which stress-induced signalling events can be related has been emphasised (Hare et al. 1997). This study focused on the terminal target sequence elements of individual branches of the phytochrome signalling network which have recently been defined (Wu et al. 1996; Neuhaus et al. 1997). Conceivably, an approximately 50% decrease in steady state *AtP5CS1* transcript levels following transfer to darkness (Strizhov et al. 1997) could reflect either phytochrome-mediated transcriptional activation of this gene in light, repression of its transcription in the dark, or a combination of both processes.

The Box II motif was originally identified as a region that is highly conserved among *RBCS* genes and which occurs within a 166 bp TATA-proximal fragment of the pea *RBCS-3A* promoter which is involved in phytochrome-mediated induction of the transcription of this gene. Reporter gene fusion experiments have demonstrated that Box II acts as a positive element for gene expression in white light (Lam & Chua 1990). The nuclear factor GT-1, which binds to this element *in vitro*, has been studied extensively (Green et al. 1987, 1988). Both deletion and mutation analysis demonstrated a strong correlation between GT-1 binding *in vitro* and *RBCS-3A* light-induced transcriptional activity *in vivo* (Green et al. 1988). These workers defined a functional core sequence GGTTAA which is critical for GT-1 binding to Box II *in vitro*. Methylation interference experiments (Green et al. 1987) and mutational analysis (Green et al. 1988) indicated an absolute requirement for the two G residues found in this sequence for GT-1 binding. In contrast to Unit I found in the *CHS* promoter, which is sufficient to direct light inducible gene expression (Weisshaar et al. 1991), GT-1 binding is necessary but not sufficient for transcriptional activation

(Gilmartin et al. 1990) and apparently requires interaction with other regulated factors for the formation of a stable transcriptional complex (Lam & Chua 1990). Although GT-1 binding sites have been found to participate in light-responsive transcription of many genes (Hiratsuka et al. 1994), including at least of which encodes an enzyme involved in amino acid biosynthesis (Tjaden et al. 1995), the exact role of the Box II element in phytochrome signalling is not yet fully resolved. Complexity in interpretation of the role of GT-1 has arisen through the observation that the sequences of defined GT-1 boxes frequently display considerable variation when compared with the Box II core sequence (Green et al. 1988). Consistent with the suggestion that GT-1 may bind to several related sequences (Gilmartin et al. 1990), it was shown to interact with GATA-motifs present in the 5'-UTRs of several *CAB* and *RBCS* genes (Teakle & Kay 1995). The observation that the nuclear factor(s) capable of binding to Box II binding activity is present in nuclear extracts from light-grown as well as dark-adapted plants (Green et al. 1987, 1988) strongly suggests that although GT-1 is involved in the transduction of the light response to the promoter, it is not through regulation of its DNA binding activity *per se*. It appears that GT-1 activity may be regulated at a post-translational level. In keeping with their demonstration that Ca^{2+} is capable of inducing expression of a fusion of a Box II multimer to a minimal promoter upstream of *GUS*, Wu et al. (1996) suggested that GT-1 may be the substrate of a CaM-dependent protein kinase.

Significantly, both of the G residues which are critical for the interaction of GT-1 with Box II occur within the putative GT-1 recognition site in the *AtP5CS1* 5'-UTR (Figure 4.5A). Mutation of nucleotides to the 3' side of these G's, which display poor conservation in the putative Box II site in the *AtP5CS1* 5'-UTR (Figure 4.5A), caused smaller reductions in the capacity for GT-1 binding (Green et al. 1988). The likely functional significance of the Box II-like element in the 5'-UTR of *AtP5CS2* is diminished firstly by the observation that transfer of light-grown *Arabidopsis* plants to darkness did not decrease *AtP5CS2* transcript levels (Strizhov et al. 1997), and secondly by the absence of a second critical G residue within the Box II core (Figure 4.5A).

Although not explicitly stated by Strizhov et al. (1997), the dual regulation of *AtP5CS1* transcript abundance by both osmotic stress and the availability of light raises the interesting question of whether or not the signalling pathways which are triggered by these different stimuli may interact through common intermediates. The presence of a Box II-like element in the *AtP5CS1* promoter (Figure 4.5A) suggests that if this is a functional phytochrome responsive element, then it is likely to be targeted by the Ca^{2+}/CaM branch of the phytochrome signal transduction network (Figure 2.12). As was discussed in Section 2.4.3.3, the findings of Knight et al. (1997) strongly implicate a role for increases in $[Ca^{2+}]_c$ in the induction of *AtP5CS1* expression. Wu et al. (1997) have noted that at least two model stress-inducible genes (*KIN2* and *RD29A*) do not appear to be responsive to the active form of phytochrome. Thus, further characterisation of the regulation of *AtP5CS1*

may provide an interesting perspective on how a ubiquitous signalling intermediate such as Ca^{2+} may control several different responses. An understanding of how specificity of Ca^{2+} -dependent plant responses to individual stimuli is maintained, while still enabling an appropriate level of "cross-talk" (Section 2.4.5.4) is likely to become a central focus of signal transduction research in the future (McAinsh & Hetherington 1998).

Both of the putative RE3-like elements in the 5'-UTR of *AtP5CS2* (Figure 4.5B) contain the TGGG core motif that is present within all other genes so far characterised as being downregulated by light (Neuhaus et al. 1997). If either of these elements are functionally involved in phytochrome-mediated repression of *AtP5CS2* expression, then by extrapolation of the data of Neuhaus et al. (1997), they are likely to be targeted by a branch of phytochrome signal transmission that relies on activation by both Ca^{2+} and cGMP (Figure 2.12). These workers demonstrated that injection of a RE3 tetramer can prevent downregulation of an *AS1::GUS* fusion by light in WT cells and by activated phytochrome or both Ca^{2+} and cGMP in cells of a phytochrome-deficient mutant. Since a mutated RE3 tetramer in which the TGGG motif was disrupted was ineffective in this regard, these results suggested that injected RE3 is able to compete away a repressor that interacts both this sequence and the same element found within the *AS1* promoter (Neuhaus et al. 1997). At present, it is difficult to assess the significance of the slight increase in *AtP5CS2* transcript abundance following transfer of mature *Arabidopsis* plants to darkness (Strizhov et al. 1997). Comparison of this result with the far more extensive induction of *AS* transcripts after transfer to darkness (Tsai & Coruzzi 1990) indicates that the RE3-mediated phytochrome repression mechanism that regulates *AS1* expression causes a far more extensive than the slight decrease in *AtP5CS2* mRNA levels observed after extended incubation in darkness (Strizhov et al. 1997). However, it is important to note that repression of *AS1* expression by light is not absolute (Tsai & Coruzzi 1990; Neuhaus et al. 1998). Therefore, a fairly substantial level of *AtP5CS2* transcript in light grown plants does not discredit a possible RE3-mediated repression of transcription of this gene in the light. Furthermore, the effects of light on the expression of *AtP5CS* genes have only been examined at a single developmental stage. The sensitivity of *AS1* expression to light varies throughout development and also between different plant organs (Tsai & Coruzzi 1990, 1991). The possibility that *AtP5CS2* expression may be repressed by a phytochrome-regulated pathway therefore requires further investigation.

Yet another interesting issue regarding the signals that control *AtP5CS* gene expression concerns whether or not the involvement of sugars in stimulating proline accumulation (Larher et al. 1993; Pesci 1993) relates to a role for the proposed phytochrome-mediated induction of *AtP5CS1* expression. Glucose, but not glutamate or 2-oxoglutarate, can mimic the enhancing effect of light on stress-induced proline accumulation in detached wheat leaves (Pesci 1993). It has been

suggested (Section 2.5.2.2; Hare & Cress 1997) that this may reflect a limiting requirement for reductant in ensuring maximal rates of proline synthesis, although the involvement of a hexose sensing mechanism (Section 2.4.5.2) in direct control of the expression of the proline biosynthetic genes cannot be formally excluded. Since sugars are the end products of photosynthesis, an interaction between sugar sensing mechanisms and phytochrome-mediated induction of photosynthetic genes seems intuitively obvious. This has recently been demonstrated through the characterisation of several *Arabidopsis sun* (sucrose uncoupled) mutants (Dijkwel et al. 1997). However, the relationship between phytochrome action and sugar signalling pathways is complex, since the expression of certain phytochrome-induced genes (e.g. *CAB* and *RBCS*) is downregulated by sucrose, whereas others (e.g. *CHS* and genes encoding nitrate reductase) are induced by both light and exogenous carbohydrate (Tsukaya et al. 1991; Cheng et al. 1991). In these latter cases, sucrose can replace light in eliciting an increase in gene expression in dark-adapted plants. Sucrose supplementation can also mimic the negative effects of light by repressing *AS* gene expression in both *Arabidopsis* and pea plants (Lam et al. 1994; Ngai et al. 1997). At present, the paucity of information regarding sugar-responsive promoter elements makes it difficult to assess whether or not a hexose-sensing mechanism may affect expression of any of the genes involved in proline synthesis from glutamate, although this issue warrants investigation.

The complexity of phytochrome-mediated changes in plant gene expression is parallelled by the multitude of mechanisms whereby plant hormones appear to elicit their effects at the genetic level. Elucidation of the molecular basis of hormonal effects on gene expression is considerably complicated by the observation that many plant genes are not responsive to a single hormone, but rather can be induced by a variety of agents e.g. several different growth regulators or environmental stimuli (Hare & van Staden 1997). While recent advances in our understanding of auxin-induced gene expression have provided some insight into the mechanisms of auxin action, all of the available evidence indicates the involvement of different combinations of fairly conserved AuxREs operate in conjunction with less widespread promoter elements to confer fairly unique modes of transcriptional regulation on each of a wide range of auxin-inducible genes. This diversity in auxin-responsive domains is consistent with the involvement of auxins in the activation of a broad spectrum of genes that are likely to function in very different auxin-regulated processes (e.g. cell elongation, cell division and cell differentiation).

Since AuxREs may be functionally distinguished by the types of responses in which they participate, they may provide some indication of how different responses to auxin may be regulated independently of one another. For example, the association of a high level of proline biosynthesis with raid rates of cell division has already been discussed extensively. The failure

to identify sequences with homology to the Domain A consensus sequence [(T/G)GTCCCAT] in the auxin-inducible *AtP5CS* genes (Strizhov et al. 1997) may be consistent with the observation (Ballas et al. 1993) that all of the genes which contain this motif are specifically induced by auxin in the elongating cortical and epidermal cells of various plant species. Auxin-induced genes which are known to be expressed prior to cell division do not appear to contain this motif (Ballas et al. 1993). In contrast, another type of AuxRE with a $TGACG(N)_7TGACG$-like signature (AS-1 type element) appears to be involved in the auxin-mediated transcriptional activation of genes that are predominantly expressed in actively dividing cells (Liu & Lam 1994; Droog et al. 1995).

The relatively high homology of several regions within both of the *AtP5CS* 5'-UTRs to the 16 bp consensus Domain B sequence (Ballas et al. 1993) may account for the induction of both of these genes by exogenous auxin (Strizhov et al. 1997). Analysis of the *PS-IAA4/5* promoter has indicated that although a Domain A-like element appears to be the primary switch that potentiates the induction of *PS-IAA4/5* transcription by auxin, Domain B has characteristic features of an enhancer element. However, the demonstration that tetramerisation of Domain B can compensate for a loss of Domain A function suggests that Domain B may contain a less efficient AuxRE, whose activity in amplified by multimerisation (Ballas et al. 1995). The activity of Domain B is orientation- and position independent and it can function over a long distance from the transcription initiation site (Ballas et al. 1995). Chung and Parish (1995) identified two regions within the 5'-UTR of the *Arabidopsis CDC2a* gene which have homology to Domain B. Both of the putative elements identified by these workers are more than 1 kb upstream from the transcription initiation site. The involvement of *CDC2* genes as key regulators of progress through the cell cycle (Hare & van Staden 1997) suggests that if these regions in the *CDC2a* 5'-UTR are functional, Domain B-like motifs in the *AtP5CS2* 5'-UTR may contribute to the high level of expression of this gene in rapidly dividing cells (Strizhov et al. 1997).

The presence of a region within the *AtP5CS2* 5'-UTR which has homology to the consensus AS-1 type binding site for ASF-1 (Figure 4.6C) may also be of some significance regarding the high level of expression of this gene in meristematic tissues. The expression pattern observed with promoters containing a single AS-1 type element appears to correspond to regions which are known to have higher mitotic activity such as root tips, emerging lateral roots and the shoot apex (Benfey et al. 1989; Fromm et al. 1989). In contrast, the involvement of Hex-1 type ASF-1 binding motifs in driving high rates of transcription in rapidly dividing cells does not appear to have been reported. A single AS-1 type element is both necessary and sufficient for root-specific gene expression of a truncated CaMV35S promoter (Lam et al. 1989). In leaf tissue, a low level of expression from this element appears to result from limiting concentrations of its cognate transcription factor (Lam et al. 1989; Lam & Chua 1990; Neuhaus et al. 1994). Comparable levels

of *AtP5CS2* transcript in both root and leaf tissues from *Arabidopsis* (Strizhov et al. 1997) do not support a role for the putative AS-1 type element in the upstream regulatory region of this gene in conferring tissue-specificity of *AtP5CS2* expression.

Regarding assessment of the physiological significance of auxin-mediated induction of *AtP5CS* gene expression through the *as-1* box-containing motifs of these genes (Figure 4.6C), it is worth noting that there is some controversy regarding whether or not TGACG-containing elements are normally targeted by an auxin specific signalling cascade. The observations that auxin-responsiveness of AS-1 type elements is restricted to relatively high, non-physiological concentrations of 2,4-D (50 - 100 µM) and are activated by inactive auxin analogues as well as by other growth substances, has led to the suggestion that these elements may simply be the targets of a general stress-related signal transduction pathway (Liu & Lam 1994). Besides auxins, salicylic acid (SA), methyl jasmonate (MJ) and H_2O_2 also activate transcription through an AS-1 type element (Xiang et al. 1996). In contrast, neither ABA or cytokinin (Liu & Lam 1994) nor ethylene or GSH (Xiang et al. 1996) are effective in activation of an AS-1 type element. Cadmium and heat shock had less activation capacity than auxin, SA and MJ (Xiang et al. 1996). The full significance of ASF-1 binding in normally mediating the effects of several different growth regulators does not yet appear to have been resolved. Similarity in the induction kinetics of *as-1* by auxin, SA and MJ, together with the absence of any additive effect when two or more of the hormones were applied consecutively led to the conclusion that AS-1 type elements and the associated proteins that mediate their activity may be a point of convergence of several hormonally-triggered signal transduction pathways (Xiang et al. 1996). A scenario which implicates the serial induction of the synthesis of any of these hormones by another seems unlikely, since this mechanism would most likely be reflected by differences in the induction kinetics of the different growth regulators. The conclusion that auxin, SA and MJ act via a common set of *trans*-acting factors which bind to AS-1 type elements is also consistent both with a lack of any additive effects when more than one of the hormones is added at a saturating level (Xiang et al. 1996) and the CHX-insensitivity of AS-1 activation by either auxin, SA or MJ (Liu & Lam 1994; Xiang et al. 1996). The apparent broadness of specificity of AS-1 dependent transcription activation serves to emphasise the long-standing challenge in plant hormone physiology of establishing whether or not the effects of exogenous application of a growth regulator necessarily reflect its normal involvement in the physiological response being investigated. An auxin concentration of 1 mg l^{-1} (approximately 4.5 µM), which was the concentration of 2,4-D used by Strizhov et al. (1997), does not seem to constitute an unnaturally high concentration of this growth regulator. While the kinetics of auxin-induction of *AtP5CS2* seem comparable to the 2,4-D-mediated induction of a -90 CaMV35S construct by 50 µM 2-4-D in tobacco seedlings (Liu & Lam 1994), it was found that 2 µM 2,4-D had little effect on AS-1

mediated activation of transcription (Liu & Lam 1994). Despite the difficulties in direct comparison of both of these studies, it seems unlikely that the fairly rapid induction of *AtP5CS2* transcript accumulation by auxin (Strizhov et al. 1997) can be ascribed exclusively to an ASF1-dependent mechanism. Nonetheless, given the view that *as-1* containing elements may represent a class of AuxRE with relaxed auxin specificity, it may be of interest to discover whether or not SA is capable of inducing expression of any of the genes involved in proline biosynthesis from glutamate. Induction of proline accumulation by the application of MJ was recently reported (Fedina & Tsonev 1997), although the effects of jasmonates on the expression of proline biosynthetic genes do not appear to have been investigated. In pea seedlings, the effects of exogenously applied MJ and salt stress on free proline accumulation were not additive (Fedina & Tsonev 1997). This finding can be interpreted to indicate that exposure to either of the two stimuli does not desensitise the response to the other stimulus. Thus, MJ may normally play a role in NaCl-induced proline accumulation. While little is known about jasmonate-responsive promoter elements, promoter regions of proline biosynthetic genes which contain either of the AS-1 or Hex-1 type ASF-1 recognition sites may be candidates for their regulation by this stress-related growth regulator.

The recent identification of cAMP as an intermediate in auxin-triggered cell division (Ichikawa et al. 1997) may be of some significance with regard to elucidating the somewhat enigmatic involvement of this growth regulator in affecting proline synthesis. Verbruggen et al. (1993) identified six sequences identical to human cAMP-regulated enhancer sites in the 5'-UTR of *AtP5CR*. At that time, interpretation of this finding was confounded by the failure of plant physiologists to have convincingly identified a role for cAMP in plant gene regulation. The consensus cAMP-regulated enhancer motif (CGTCA) also occurs once within each of the 5'-UTRs of *AtP5CS1* (position -443; Appendix) and *AtP5CS2* (position -811; Appendix). Ichikawa et al. (1997) have recently ended over 25 years of controversy concerning whether or not plants use cAMP by identification of a functional plant adenylyl cyclase and the demonstration that tobacco protoplasts treated with cAMP, or the adenylyl cyclase activator forskolin, no longer require auxin to divide. This finding, together with the observation that the adenylyl cyclase inhibitor dideoxyadenosine inhibits cell proliferation in the presence of auxin, suggests that cAMP is involved in auxin-triggered cell division in higher plants. Several plant transcription factors with significant homology to animal cAMP responsive element binding proteins have been identified (Assmann 1995). Whether any of these may bind to the putative cAMP response elements in proline biosynthetic genes, and thereby confer responsiveness to auxin, remains to be investigated.

Besides the analysis of auxin-responsive promoters, the effects on auxin-responsive gene expression by the mutation of macromolecules that are involved in transmitting the auxin signal also provides some indication of how different aspects of auxin-regulated signalling may be coordinated at the genetic level. Although it was not mentioned by Strizhov et al. (1997), there is a parallel between the observed ability of an *axr2* mutation to reduce both *AtP5CS1* and *AtP5CS2* mRNA accumulation during NaCl stress but the absence of any effect of *aux1* on this process (Strizhov et al. 1997), with the effects of *axr2* and *aux1* mutations on the induction of *Arabidopsis ASC4*. This auxin-inducible gene encodes an isoform of 1-aminocyclopropane-1-carboxylate synthase (EC 4.4.1.14), the key regulatory enzyme in ethylene biosynthesis. Abel et al. (1995) reported that auxin-inducibility of *ASC4* is abolished in an *axr2* mutant, but is retained in a mutant disrupted in AUX1 action. Both of the *AtP5CS* genes and *ASC4* may thus be regulated by a similar auxin-related signalling mechanism. This question requires further investigation since the effects of *axr2* and *aux1* on auxin-inducibility of the *AtP5CS* genes were not examined by Strizhov et al. (1997). The observation that osmotic shock had no effect on the steady state levels of *ASC4* mRNA (Abel et al. 1995) suggests that an auxin-regulated pathway that regulates both of the *AtP5CS* genes as well as *ASC4* may branch downstream of AXR2 action. The *AUX1* gene product bears considerable homology to amino acid permeases (Bennett et al. 1996). Since IAA, the major naturally occurring auxin in higher plants, is structurally similar to tryptophan, it was suggested that AUX1 participates in the uptake of auxin after its transport from the shoot apical meristem, which is the primary site of auxin synthesis. It is tempting to speculate that the absence of any effect of mutation of *AUX1* on stress-induced *AtP5CS* gene expression (Strizhov et al. 1997) indicates that if auxin normally plays a role in this process, then the long-range transport of this hormone is unlikely to be an importance determinant of *AtP5CS* gene induction. Instead, auxin effects on *AtP5CS* gene expression may be restricted to the vicinity of the site of auxin synthesis.

A molecular interpretation of the effects of cytokinins (CKs) on free proline accumulation and *AtP5CS* gene expression (Section 2.4.3.4) is difficult, owing largely to the paucity of understanding regarding how the CK signal is transmitted (Hare & van Staden 1997). Cytokinin-responsive promoter elements have yet to be identified. Consistent with the requirement of both auxin and CK to stimulate cell division, at least in cultured plant tissues, several CK-responsive genes are also induced by auxin (Hare & van Staden 1997). However, antagonism between auxin and CK in the regulation of gene expression has also frequently been noted (Hare & van Staden 1997). Examination of the results of Strizhov et al. (1997) does not permit an unambiguous conclusion regarding whether the effects of auxin and CK on *AtP5CS* gene expression may be attributed to a common pathway that monitors the ratio of endogenous auxin:CK concentrations.

Cytokinins are able to recapitulate many of the effects of light on plant morphology (photomorphogenesis) and gene expression (Hare & van Staden 1997; Thomas et al. 1997). In view of the central role played by heterotrimeric guanine nucleotide-binding proteins in phytochrome signalling (Figure 2.12), it was proposed that a heterotrimeric G-protein(s) may represent a nexus between the two signals (Thomas et al. 1997). In mammals, more than a hundred heterotrimeric G-proteins (comprising three subunits designated α, β and γ) act as molecular switches which link the binding of a wide range of ligands to their cognate membrane-bound receptors with the activation of effector proteins of signal transduction pathways (Simon et al. 1991). Since G-proteins are generally associated with signal transmission from cell surface receptors with seven membrane spanning domains, it is of interest that a decrease in levels of expression of the *Arabidopsis GCR1* gene, which encodes a protein with features of a receptor with seven transmembrane domains, reduced the sensitivity of root and shoot growth to normally inhibitory concentrations of exogenous CKs, but not of high levels of auxin, gibberellins or ethylene (Plakidou-Dymock et al. 1998). The observation that antisense suppression of *GCR1* expression reduced the ability of exogenous CK to suppress hypocotyl elongation in the dark seems consistent with the notion that GCR1 may participate in phytochrome signal transduction, since this is one of several aspects of the phytochrome-mediated de-etiolation response which can be mimicked by exogenous CK (Thomas et al. 1997). As more information on the enigmatic overlap between phytochrome and CK actions becomes available, so it may be possible to establish whether or not the effects of exogenous CK on the expression of *AtP5CS* gene expression may at least partially owe their effects to interference with the proposed phytochrome-mediated regulation of these genes.

5.1.4 Functional redundancy in P5C synthesis from glutamate

Promoter analysis of the *AtP5CS1* and *AtP5CS2* genes provides a valuable approach to assessment of the extent of functional overlap between the activities of these two genes. The significance of duplication of a large number of plant enzymes has long been a poorly resolved issue in plant metabolism. Many enzymes which play a central role in intermediary metabolism are occur as both cytosolic and plastidic isoforms (Emes & Dennis 1997). Standard biochemical techniques have been of limited use in assessment of whether different isoforms have specific roles because it is often difficult to distinguish between isoenzymes at the protein level and organelle isolation can be subject both to contamination and a loss of enzyme activities. The availability of nucleic acid probes and dissection of the regulatory mechanisms that control the expression of genes encoding isoenzymes holds considerable potential in investigating whether or not isoforms have overlapping functions or whether they are subject to differential regulation

at the developmental, environmental or tissue-specific levels. For enzymes that play a central role in primary metabolism, such as those involved in amino acid synthesis, this problem is compounded by the fact that they normally have a "housekeeping" function. Thus, any additional regulation is likely to be superimposed upon a basal level of fairly constitutive expression.

Somewhat paradoxically, despite considerable divergence of the nucleotide sequences of the 5'-UTRs of *AtP5CS1* and *AtP5CS2* (Figure 4.9A), both of the promoter regions appear to contain similar types of promoter elements. Obviously, this may merely be a reflection of the various classes of regulatory motifs that have been searched for in this study. While functionality of any of these promoter regions has yet to be demonstrated, if similar or identical *trans*-acting factors are capable of binding to the 5'-UTRs of both of the *AtP5CS* genes, then their ability to activate transcription of either of the two P5CS isoforms is likely to be determined by the overall context of the target sequence within the 5'-UTR. The study of Strizhov et al. (1997) has clearly provided convincing evidence of a divergence in the regulation of both of the *AtP5CS1* and *AtP5CS2* genes. The evidence that both genes are under very different control mechanisms is reflected by the observations that:

i) CHX has different effects on the induction of *AtP5CS1* and *AtP5CS2* expression following exposure to NaCl-stress: early transcriptional activation of *AtP5CS1* does not require protein synthesis, whereas stress-induced *AtP5CS2* expression displays an absolute dependence on the synthesis of *trans*-acting factors which bind to its promoter;
ii) the induction of *AtP5CS1* by ABA is more rapid and extensive than that observed for *AtP5CS2*;
iii) basal levels of expression of both genes in vegetative tissues, callus and rapidly dividing cultured cells were different,
iv) there is a considerable difference both in the tissue-specificity and absolute levels of induction of both genes following exposure to exogenous auxin or CK;
v) *AtP5CS1* and *AtP5CS2* transcript levels display different sensitivities to the availability of light.

Of these findings, promoter analysis of the *AtP5CS1* and *AtP5CS2* 5'-UTRs in this study potentially sheds some insight only regarding the different effects of light on the expression patterns of both genes. The identification of a putative Box II element in the *AtP5CS1* 5'-UTR and two RE3-like elements in the *AtP5CS2* 5'-UTR suggests that these elements, which elicit opposite effects of phytochrome activation of gene expression, may account for a divergence in the regulation of both of the genes by light.

Although the physiological *raison d'être* for the redundancy in P5C synthesis from glutamate remains enigmatic, it is tempting to draw some parallels between this important step in proline

synthesis and recent advances in our understanding of glycerol synthesis in *Saccharomyces cerevisiae*. In yeast cells, glycerol has long been accepted to be the primary compatible solute which counters the dehydration and loss of cell volume that accompanies osmotic stress. However, as has been proposed for proline synthesis in higher plants (Section 2.5.2; Hare & Cress 1997), glycerol production in *S. cerevisiae* also serves as a redox valve to dispose excess reducing power, for example under anaerobic conditions, when there is an increased demand for endogenous electron acceptors (Ansell et al. 1997). In yeast, two homologous genes *GPD1* and *GPD2* encode isoenzymes of NAD^+-dependent glycerol-3-phosphate dehydrogenase. Expression of *GPD1* is induced by osmotic stress and mutants lacking *GPD1* are osmosensitive. In contrast, expression of *GPD2* is not affected by changes in external osmolarity, but is stimulated by anoxic conditions. Mutants lacking *GPD2* show poor growth under anaerobic conditions. Whereas osmotically-induced *GPD1* expression is regulated by the well-characterised HOG (high-osmolarity glycerol) osmosensing signal transduction cascade, induction of *GPD2* under anaerobic conditions is mediated by a novel oxygen-independent signalling pathway which senses changes in NAD^+/ NADH ratio (Ansell et al. 1997).

The most salient point arising from the study of Ansell et al. (1997) is that despite these differences in the transcriptional regulation of both *GPD1* and *GPD2*, the products of these two genes can replace each other under anaerobic salt stress. Thus, the anaerobic growth defect of a GPD2-deficient strain does not appear in cultures containing NaCl, while conversely, anaerobic incubation of a *gpd1Δ* mutant strain relieves the osmotic constraints on growth. This was further validated by the demonstration that overexpression of either of the isogenes can relieve the growth defect of a *gpd1Δ gpd2Δ* double mutant on high salinity media or under anoxic conditions (Ansell et al. 1997). Apparently, during the evolution of yeasts, there has been selection for duplication of a progenitor *GPD* gene and diversification of regulation of two separate GPD isoforms, rather than placing the control of a single GPD-encoding gene under the regulation of different signalling cascades which are activated by different physiological triggers. Nonetheless, mutational analysis clearly indicates some functional overlap between the two GPD isoforms.

Analysis of the stress-induced, hormonal and tissue-specific regulation of *AtP5CS1* and *AtP5CS2* (Strizhov et al. 1997), as well as examination of their 5'-UTRs (Section 4.2) indicates that the evolution of two P5CS isoforms for distinct physiological purposes may not be as straightforward as appears to be the case for glycerol synthesis in yeast. A simple interpretation of the redundancy in P5C synthesis from glutamate in plants may be more complex owing to the developmentally-regulated contribution of OAT to the P5C pool (Roosens et al. 1998). There appears to be a substantial functional overlap between the two *Arabidopsis* P5CS isoforms, and elucidation of the redundancy in P5C synthesis from glutamate requires further investigation.

5.1.5 Conclusion

In closing this section, it must be emphasised that focus on the transcriptional regulation of P5CS and P5CR activities alone is unlikely to account fully for the regulation of proline accumulation. The analysis presented here has not dealt with transcriptional regulation of *AtPDH* gene expression. A plant genomic PDH sequence has yet to be reported, although interestingly, three consecutive stretches spanning 2.2 kb of a genomic sequence from chromosome 5 of *Arabidopsis* (Genbank Accession No. AB011478) bear high nucleotide homology (73 - 78%) to the mRNA sequence of the presently characterised *Arabidopsis AtPDH* (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996a). Since the presently characterised *AtPDH* sequence maps to chromosome 3 of *Arabidopsis* (Verbruggen et al. 1996a), this introduces the possibility that there may be some redundancy in proline oxidation in *Arabidopsis*. The *AtPDH*-like sequence on chromosome 5, reported here for the first time, may merely represent a pseudogene. However, assuming that it is transcribed, this may reflect a complex regulation of proline degradation which parallels the apparent complexity of P5C synthesis from glutamate.

Besides examination of the regulation of proline catabolism, the apparent presence of a proteinaceous inhibitor of P5CS in plant cells (Kavi Kishor et al. 1995; Zhang et al. 1995) introduces the possibility that this may be an important component in regulating proline synthesis at the post-translational level. Also, the regulation of inter- and intracellular proline transport (Section 2.3.4) and the contribution of ornithine to P5C synthesis (Section 2.3.2) should ideally also be considered if we are to gain a global view of how regulated shifts in proline metabolism are controlled at the level of gene expression.

5.2 Antisense suppression of *AtP5CS* and *AtP5CR* expression in *Arabidopsis*

At the outset of this study, it was clear that many of the pertinent questions relating to the functionality of proline accumulation in stressed plants could not be answered by purely biochemical or physiological studies. The observation that research efforts over the past four decades have failed to identify an unequivocal solution to the problem indicated that classical physiological approaches are unlikely to enable resolution of this issue. Following the demonstration by Kavi Kishor et al. (1995) that an increase in proline biosynthetic capacity in transgenic tobacco plants was associated with improved tolerance of hyperosmotic stress, but the failure to resolve the physiological basis of this effect (Blum et al. 1996), it became increasingly apparent that a valuable approach to resolving the functionality of free proline accumulation in stressed plants might be to examine the effects of silencing proline biosynthesis.

5.2.1 The rationale which underlied attempts to suppress proline synthesis using an antisense strategy

While a vast number of recent advances in our understanding of plant growth and development have resulted from the screening of mutagenised populations of *Arabidopsis* seedlings, this approach to assess the effects of modifications in gene expression is limited to situations where impairment of the function of the gene of interest yields a clear-cut phenotype, while still yielding viable plants. Classical mutagenesis is not suitable for the disruption of genes which are members of multigene families, when the expected phenotype of a mutant cannot be predicted, or when mutation of a gene is lethal. The expression of an antisense transcript is a useful alternative to investigate the effects of a targeted disruption of gene expression. The ability to specifically suppress plant gene expression with antisense transcripts is now well documented (Bourque 1995). Perhaps the two most graphical and well known demonstrations of antisense inhibition of plant gene expression have involved the manipulation of tomato ripening (Picton et al. 1995) and the pigmentation of flowers (van der Krol et al. 1988, 1990). Besides these practical applications, the widespread use of antisense technology in basic research has opened up an important area of continuity between molecular biology, biochemistry and whole plant physiology. For instance, antisense suppression can be used not only to assess the *in vivo* function of a specific gene (Plakidou-Dymock et al. 1998), but also to quantify the contributions of individual enzymes to carbon flux through metabolic pathways (Frommer & Sonnewald 1995).

There are two primary features of the antisense approach which make it an attractive experimental system to examine the consequences of silencing proline synthesis. Firstly, a vast number of studies have indicated that the antisense strategy is not reliable for the production of null mutants, although a partial inhibition is often adequate to produce a mutant phenotype (Bourque 1995). Generation of "leaky" mutants which are only partially blocked in their capacity for proline synthesis addresses the concern that severe disruption of any step in proline synthesis associated with a null mutation might be lethal. In most cases, provided that a sufficient number of transgenic lines which express the antisense transcript are generated, variability between the individual transgenic lines in the level of suppression of the endogenous gene provides a range of plants from which a few, with an optimal degree of inhibition, can be selected for further analysis. This variability, which most probably arises through differences in the chromosomal insertion site, has frequently been found to be useful in the interpretation of antisense-mediated phenotypic effects since it provides a means of testing whether phenotypic effects can be correlated with different levels of inhibition of the expression of the gene under investigation. A second advantage of the antisense approach is that it is capable of simultaneously targeting different members of a multigene family, provided that substantial divergence of the isogenes has

not occurred (Bourque 1995). Although at the time of commencement of the antisense studies in this project, redundancy in P5C synthesis from glutamate had not yet been identified, indirect evidence for multigenicity in the enzymatic reduction of P5C to proline had been reported (Rayapati et al. 1989; Szoke et al. 1992).

Characterisation of the pBI-P5CS1(AS) and pBI-P5CR(AS) transformed lines was intended to serve three purposes. The first was to establish whether free proline levels could be substantially reduced in transgenic lines without a loss of plant viability in the absence of exogenously supplied proline. Secondly, it was anticipated that either or both sets of transgenic lines might provide valuable insight regarding the regulation of proline synthesis and related metabolic pathways. The third objective was to establish whether or not a decrease in free proline negatively affects stress tolerance in a manner that can be distinguished from the essential role of the imino acid in normal growth and development.

5.2.1.1 Mechanisms of antisense RNA-mediated gene suppression

The precise molecular basis of antisense-mediated inhibition of gene expression remains unclear. As with most investigations which involve antisense suppression, this study primarily concerned the use of antisense suppression as a means to generate a series of plant lines with "leaky" mutations in either P5CS or P5CR activities rather than investigation of the molecular basis of the antisense effect. Nonetheless, some description of the mechanisms of antisense-mediated gene suppression is necessary to provide a background for discussion of the results described in Sections 4.5 and 4.6. Both the gene-specific nature of the antisense effect and the frequent observation that levels of both the antisense transcript and its naturally-occurring complement are usually decreased in plants which display an efficient antisense response, strongly suggest that the effect is associated with direct interaction between the sense and antisense transcripts *in vivo*. The view that transcription of the antisense gene is required for the inhibitory effect is supported by the demonstration that, where tested, promoter-less antisense constructs have been found to be inactive (de Lange et al. 1993). The formation of an mRNA duplex between the transgene transcript and the target mRNA could conceivably interfere with several events in the process of gene expression. For instance, it has been suggested that sense:antisense RNA duplexes may be rapidly degraded by double stranded RNA-specific nucleases, that duplex formation may prevent the normal splicing of introns out of the pre-mRNA of the endogenous gene, that RNA duplexes may not be effectively transported from the nucleus to the cytoplasm, or that they prevent ribosome binding and thus block translation of the endogenous target mRNA. Hybridisation of the antisense transcript to the DNA template may also interfere with transcription

of the endogenous gene, although where run-on transcription assays have been used to measure the rates of transcription of target genes in antisense lines, an unaffected level of transcription suggests that the antisense effect is mediated at a post-transcriptional level (Bourque 1995). None of these mechanisms are mutually exclusive. Examination of the effects of expressing antisense copies specific to different regions of various transcripts (3'-termini, 5'-termini or sequences that do not contain intron splice sites) have failed to identify any specific feature of an antisense mRNA which is required for its effectiveness in silencing gene expression (Bourque 1995). However, once again, the lack of requirement for a 5'-specific antisense region discredits a role for antisense transcripts in disruption of transcriptional initiation of the target gene. It appears that any part of an mRNA can be used effectively as an antisense transcript, although the possibility that this generalisation may not apply to all target genes cannot be formally dismissed. The high degree of variability in the effectiveness of the antisense approach in different applications and the likelihood that antisense transcripts may interfere with different steps in the gene expression process complicates comparison of different antisense studies. Antisense fragments of 100 bp or less have been shown to be as effective as full length antisense transcripts in strongly suppressing the accumulation of a target sequence (Flachmann & Kühlbrandt 1995). Therefore, the length of the antisense transcripts used in this study seem more than adequate to disrupt expression of the endogenous *Arabidopsis AtP5CS* and *AtP5CR* mRNAs. Nevertheless, as long as it is not established how antisense transcripts exert their function *in vivo*, each antisense gene should ideally be tested empirically to determine which portion is most effective in inhibiting expression of the target gene. Some full-length antisense cDNAs are less effective than truncated antisense transcripts (de Lange et al. 1993). This most probably arises through their ability to form a stable secondary conformation that prevents interaction with the target gene transcript.

5.2.1.2 The choice of CaMV35S-driven expression of AtP5CS1 *and* AtP5CR *antisense transcripts*

Consistent with the inability to define a central unifying mechanism by which antisense RNA-mediated ablation of gene expression occurs, considerable variability in the effectiveness of this technique has been noted between studies which have aimed at the downregulation of different target genes (Bourque 1995). As has been found with all aspects of plant transformation, within any single study involving antisense suppression, there is often a wide range of variation between individual transformants in the "strength" of the antisense effect. This effect is commonly attributed to variations in the chromosomal site of insertion of the transgene. It is believed that the local chromosomal context may have a strong influence on the level of gene transcription. The

number of copies of the antisense gene has frequently been suggested to be an important determinant of the level of target gene suppression. However, comparison of the effectiveness of antisense suppression, using different genes targeted in a range of species, has failed to indicate an absolute correlation between antisense gene copy number and silencing of the targeted gene (Bourque 1995).

By far the greatest number of antisense studies have used the CaMV35S promoter (Bourque 1995). This viral promoter is highly active in most cell types, albeit to varying degrees (Benfey et al. 1989). It appears that a large excess of antisense transcript is required for an efficient antisense effect since the inability to express sufficient levels of antisense RNAs in a cell has often led to incomplete and ineffective gene suppression (Bourque 1995). For instance, an early study (Ecker & Davis 1986) found that whereas the fairly strong nopaline synthase and CaMV35S promoters conferred a high level of suppression on chloramphenicol acetyltransferase expression in carrot protoplasts, a *PAL* gene conferred much reduced inhibition of reporter gene expression. The apparent requirement for a fairly high level of transcription of the antisense gene is also reflected by the observation that, where investigated, levels of antisense transcript are generally not higher than those of the endogenous target mRNA. This has been noted even in instances the normal level of expression from the CaMV35S promoter is much higher than that for the mRNA being targeted (Bourque 1995). The requirement for a molar excess of antisense transcript over sense gene transcript in order to obtain an appreciable reduction in target gene expression is consistent with the view that the mechanism of antisense inhibition is likely to rely on the formation of an RNA duplex. The likelihood that a high rate of antisense RNA synthesis may be an important parameter in ensuring the silencing of an endogenous gene led to the choice of the CaMV35S promoter to direct the expression of the antisense transgenes used in this study.

It should nevertheless be noted that when dealing with a gene which has an important "housekeeping" function, a caveat associated with the use of CaMV35S is that the strength of this promoter and its poor tissue-specific and temporal regulation may cause such dramatic phenotypic modifications as to prevent the regeneration of viable transformants. While this was appreciated at the outset of this study, it was anticipated that the requirement for proline in shoot regeneration and transformant establishment could be satisfied by inclusion of exogenous proline in the regeneration media. While antisense suppression of any enzyme involved in proline synthesis has not been reported previously, at least three studies have used CaMV35S-driven antisense transcripts to disrupt different steps in amino acid biosynthesis (Temple et al. 1993; Li et al. 1995b; Kozaki & Takeba 1996). In all three instances, a decrease in the level of expression of the target gene was noted without any apparent symptoms of amino acid auxotrophy in the mature transformants.

An alternative to the use of a strong, almost constitutive promoter to drive antisense transgene expression is the use of an inducible gene expression system. Regulated expression enables the specific induction of transgene expression, theoretically in a single tissue-type. A number of alternative strategies have been described for the regulation of transgene expression in plants. The best characterised approaches employ heat-shock promoters or chemically inducible gene expression systems (Mett et al. 1993; Gatz 1996). For many years, the development of a "perfect" inducible gene expression system has evaded plant molecular biologists. Plant-derived inducible promoters have frequently been shown to give background expression in the absence of inducing conditions. Furthermore, significant changes in whole plant physiology are often caused by the nature of the environmental signal required for their activation. Recent advances using synthetic promoters that respond to chemical inducers (Aoyama & Chua 1997; Caddick et al. 1998) apparently address these limitations. At the time of commencement of this study, most of the available plant inducible gene expression systems had only been tested using tobacco. The tetracycline-inducible gene expression system is not effective in *Arabidopsis* (Gatz 1996) and the successful use of a Cu^{2+}-inducible gene expression system (Mett et al. 1993) in this species has not been reported. Somewhat surprisingly, the effectiveness of an inducible antisense gene in mediating gene suppression does not appear to have been investigated. While the requirement for expression of an antisense gene by a strong promoter has been contested (van der Krol et al. 1990; Flachmann & Kühlbrandt 1995), the general view that a large excess of antisense transcript relative to sense transcript abundance may account at least partly for a hesitance to test the efficacy of inducible antisense suppression. A several-fold increase in the activity of an inducible promoter may be deceptive if one considers its overall strength after induction. For example, Holtorf et al. (1995) compared the strength of a heat-shock inducible promoter with that of CaMV35S. While the heat-shock promoter was inducible by up to 18-fold in the most highly-expressing *Arabidopsis* transgenic lines, the maximum levels of expression driven by this inducible promoter following a single heat shock was approximately one thousand-fold lower than that observed for the most highly-expressing CaMV35S lines (Holtorf et al. 1995).

5.2.2 Assessment of the success of the antisense approach in elucidating a stress-related role for proline accumulation in *Arabidopsis*

The standard means of *Agrobacterium*-mediated transformation, *viz. in vitro* co-cultivation of tissue explants with an *Agrobacterium* strain carrying the T-DNA insert of interest, was used in this study since previous workers (Valvekens et al. 1988; Akama et al. 1992) had tested the suitability of various types of *Arabidopsis* tissue explants for infection by *Agrobacterium* and established reliable protocols for the regeneration of transgenic *Arabidopsis* lines. As was

reported by these workers, who used different constructs to those use here, the T-DNAs containing the antisense copies of both of the partial *AtP5CS1* and *AtP5CR* cDNAs were stably integrated into the transgenic lines (Figure 4.13). Furthermore, although poor seed set under *in vitro* conditions prevented a reliable analysis of segregation ratios, many of the plants appeared to contain multiple copies of the foreign constructs. Both Valvekens et al. (1988) and Akama et al. (1992) found that a substantial proportion of their *Arabidopsis* transformants contain multiple T-DNA insertions. Analysis of the kanamycin resistance in the T_1 progeny of the primary transformants (T_0 generation) generated in this study suggests that pBI-P5CS1(AS) transformants B6 and C6, as well as pBI-P5CR(AS) transformants A9 and D2 may have a single dominant insert, based on the observation that their segregation ratios (Kan^r:Kan^s) approach the theoretical value of 3:1 expected for a single segregating allele (Table 4.4). The other transformants are likely to have multiple inserts since their segregation ratios are closer to those expected for two independent segregating alleles (15:1) or three segregating alleles (63:1). An estimation of two T-DNA integrations per genome seems valid for most of the lines [e.g. pBI121 transformants A and C, pBI-P5CS1(AS) transformants A2, A8, A9 and B7 as well as pBI-P5CR(AS) transformants E1 and F1]. Estimation of the number of T-DNA inserts in lines which displayed 10-20% kanamycin sensitivity in the T_1 generation [e.g. pBI-P5CS1(AS) transformant A5 and pBI-P5CR(AS) transformant D2] is more difficult. In this regard, it is worth noting that deviations from the theoretically expected segregation ratios for kanamycin resistance in *Arabidopsis* have often been reported. This deviation has been ascribed to non-Mendelian behaviour of the *NPTII* transgene (Kilby et al. 1992). In any event, the low number of T_1 seeds which were recovered precludes accurate assessment of this. The high degree of variability in seed set under *in vitro* conditions did not appear to be a consequence of transformation, since none of the transgenic lines displayed a significant reduction in the number of siliques or seeds per silique when the T_1 generation was grown to maturity under greenhouse conditions. Likewise, although some reduction in the frequency of transformation when using either of the pBI-P5CS1(AS) or pBI-P5CR(AS) constructs relative to pBI121 (control) regeneration rates was noted, this observation may need to be interpreted cautiously. There was considerable variability in the efficiencies observed in individual transformation experiments and far more effort was made in the regeneration of pBI-P5CS1(AS) and pBI-P5CR(AS) transformants than in the production of a large number of pBI121 control lines.

All of the transgenic plants grew well both under growth room and greenhouse conditions. For the *Arabidopsis* transformants, there were no gross differences in morphology between wild-type (WT) plants or pBI121 controls and lines carrying either of the pBI-P5CS1(AS) or pBI-P5CR(AS) constructs. All of the studies involving examination of root growth and proline accumulation in the transgenic lines concerned plants of the T_2 generation. There often appears to be a gene dosage

effect for antisense-mediated suppression (Picton et al. 1995; Lagrimini et al. 1997b). Plants hemizygous for an antisense transgene (single copy of the antisense gene per diploid genome) have occasionally been found to show less severe phenotypic effects than homozygous plants (two copies per diploid genome). However, examination of the growth capacity of the most severely affected pBI-P5CS1(AS) lines A5 and B12 in the T_3 and T_4 generations did not suggest a more significant reduction in growth rates relative to the WT than were observed in the T_1 generation (data not shown). Like their *Arabidopsis* counterparts, transgenic tobacco plants transformed with either of the two antisense constructs did not display a severe retardation of growth that would be anticipated for a reduced capacity for proline synthesis. As was noted in Section 4.6, a much larger number of tobacco transformants carrying either of the two antisense constructs was obtained than were *Arabidopsis* transformants. Owing to time constraints, it could not be unequivocally established whether or not the morphological defects observed in many of the tobacco antisense transformants could be correlated with a decreased capacity for proline synthesis in these plants. Somaclonal variation is a poorly understood effect that is frequently associated with either de-differentiation or re-differentiation during the course of *in vitro* regeneration of plants. However, the abnormal development of leaves and flowers in certain of the tobacco pBI-P5CS1(AS) plants and similar floral abnormalities in at least three of the tobacco pBI-P5CR(AS) plants does not appear to be a general feature associated with passage of tobacco through tissue culture. Neither of these morphological aberrations were found in all of the leaves or flowers of the tobacco plants. Since the tobacco plants did not seem less robust than controls, it seems unlikely that they were severely compromised in their capacity for proline synthesis. Although the focus of this study concerned analysis of proline synthesis in *Arabidopsis*, analysis of the consequences of transformation of tobacco with the pBI-P5CS1(AS) and pBI-P5CR(AS) constructs provided confirmation of the lack of severe growth defects in pBI-P5CS1(AS) or pBI-P5CR(AS) *Arabidopsis* transformants.

Importantly, demonstration of the incorporation of a transgene into the genome (e.g. Figure 4.12) does not confirm its expression *in vivo*. While direct evidence has yet to be obtained that any of the antisense lines do in fact contain reduced levels of the endogenous *AtP5CS1* or *AtP5CR* transcripts, histochemical GUS staining provides indirect evidence that suggests that this may be the case. A useful feature of the use of the GUS reporter gene system is that *GUS* has frequently been shown to tolerate large N-terminal additions and remain enzymatically active (Jefferson et al. 1987; Pua & Lee 1995). As is evident in Figure 4.13, fusion of either of the 105E5T7 or YAP057 cDNAs in an antisense orientation to *GUS* and the expression of a chimeric antisense::*GUS* transcript did not prevent the expression of GUS activity in at least certain of the transformants carrying either construct. Interestingly, at least for pBI-P5CS1(AS) transformants A5 and B12, as well as pBI-P5CR(AS) transformants D1 and D2, there was a qualitative

correlation between GUS activity and seedling root growth rates (Table 4.4; Figure 4.14; Table 4.5). Since the antisense copies of the 105E5T7 and YAP057 are expressed from the CaMV35S promoter as a fusion to *GUS*, it might be anticipated that downregulation of either of the endogenous proline biosynthetic genes might be associated with attenuation of GUS activity in both classes of antisense lines. Pua and Lee (1995) have demonstrated the validity of this approach to inferring an efficient antisense effect. However, it is important to note that position effects on transgene expression cannot be formally excluded. In other words, a reduced level of GUS expression in pBI-P5CS1(AS) lines A5 and B12 could arise simply from the incorporation of the transgene into a chromosomal region that is not actively transcribed. Since strong phenotypic effects were observed in T_2 generation plants of both of these lines (Figures 4.14 and 4.15), the apparent absence of GUS activity does not appear to have resulted from transgene loss or inactivation. The data presented in Table 4.5 indicates that the same argument can be applied for the pBI-P5CR(AS) lines D1 and D2.

It is important to note that although genetic manipulation is a precise process, there are always two processes that may occur when a gene is introduced into a plant. Firstly, the introduced gene may be expressed. Secondly the genome will be disrupted by the integration of the introduced gene. It was thus always necessary to demonstrate as adequately as possible that the phenotypic changes which are observed in any transgenic line are not merely the consequence of disruption of the expression of a gene unrelated to the process under investigation. The demonstration that two independent pBI-P5CS1(AS) lines display at least qualitatively similar phenotypic effects strongly suggests that the effects observed in lines A5 and B12 do not simply arise as an artifact resulting from accidental gene disruption or somaclonal variation. However, a more significant demonstration that the lines have an attenuated proline biosynthetic capacity is the demonstration that their growth can be restored to WT levels in the presence of exogenous proline at a concentration that has little effect on growth rates in the WT (Table 4.6). Furthermore, both of these transgenic lines were characterised by significantly lower free proline contents both in the absence of stress and after the imposition of either exposure to elevated concentrations of NaCl (an ionic stress), sorbitol (to mimic water deprivation) or cold stress (Figure 4.15). As shown in Figure 4.17, both of the lines displayed a delayed germination response, which once again correlated with a significantly decreased maximal concentration of free proline at the time of radicle emergence. As with the inhibitory effects of exogenous proline on radicle emergence in WT seeds (Table 4.8), these effects could be at least partially overcome by pre-incubation of the seeds in the dark at 4 °C for at least 60 h. When the WT and antisense lines used in the analysis of root growth were subjected to this treatment, radicle emergence of all lines occurred within a 12 h period. Therefore, when monitored over 10 d, the decreased root growth in the pPBI-P5CS1(AS) lines A5 and B12 cannot be attributed to a slightly delayed germination rate.

5.2.3 Interpretation of the results of attempts to suppress proline synthesis in *Arabidopsis*

An important consideration in interpretation of the rather moderate effects observed following transformation with either of the antisense constructs is consideration of why the apparent suppression of P5CR (Table 4.5) and P5CS (Figure 4.14; Table 4.6; Figure 4.15) activities in certain of the lines was not associated with more severe phenotypic effects. There are several explanations which may account for these observations. Briefly, it seems possible that:

i) a high level of expression of an antisense copy of a proline biosynthetic gene driven by the strong and almost constitutively expressed CaMV35S promoter may be a lethal modification which eliminates transformants capable of high levels of expression at an early stage in the regeneration process;

ii) the transgenic plants may have compensated for a decrease in either of the enzymatic activities by adjusting the levels or activities of other enzymes involved in the synthesis or reduction of P5C or by downregulation of proline degradative capacity;

iii) if plants possess excess amounts of either P5CS or P5CR activities, an antisense-mediated decrease in the level of either enzyme may not be adequate to exhibit a phenotypic effect under optimal environmental conditions;

iv) post-transcriptional events responsible for the regulation of proline synthesis from glutamate may mask the effects of a slight decrease in the abundance of transcripts which encode proline biosynthetic genes.

Unfortunately, on the basis of the results obtained thus far using the antisense lines, it is not possible to establish whether the antisense approach used may have been too effective to permit regeneration of viable plants, or whether it may have been of only minimal effectiveness. The different possible explanations for the relatively minor phenotypic effects observed in the antisense lines will be discussed separately, and in relation to other studies which have failed to observe dramatic phenotypic effects associated with antisense transformation. Obviously, it is possible that more than one of these explanations might account for the results obtained using the antisense lines. Irrespective of whether any of these interpretations are valid, characterisation of the pBI-P5CS1(AS) transformants A5 and B12 strongly suggests that *Arabidopsis* plants can tolerate as much as a 35% reduction in their free proline levels without any substantial effects on their growth under optimal conditions (Figure 4.15). It seems likely that this level may define a threshold concentration of free proline which is required for plant viability.

While it is not feasible to review in detail a vast number of studies which have involved assessment of the effects of antisense-mediated disruption of various steps in carbohydrate

metabolism in plants, two major problems associated with the manipulation of single steps in metabolism have emerged from these investigations (Frommer & Sonnewald 1995). First, if several enzymes are involved in the same reaction, or if other steps enable the targeted reaction to be bypassed, it is often impossible to manipulate metabolism in the desired way by altering levels of only one enzyme. Furthermore, when an enzyme is regulated, it can be difficult to block the reaction it catalyses, since the remaining protein can be activated and thereby compensate for the missing protein. Studies which have involved antisense suppression of steps in amino acid biosynthesis in plants are limited. Temple et al. (1993) demonstrated that introduction of an antisense copy of a cDNA encoding alfalfa GS_1 (a cytosolic isoform of glutamine synthetase, GS) into tobacco resulted in an up to 40% reduction in the total leaf GS activity per unit leaf area and a decrease in total soluble leaf protein to a similar magnitude. The antisense transcript appeared to suppress both the GS_1 (encoding cytosolic GS) and GS_2 (encoding plastidic GS) isoforms. Surprisingly, despite the 40% decrease in total soluble protein, no adverse effects on plant growth in the antisense lines were reported (Temple et al. 1993). Hirel et al. (1997) have reported preliminary results of experiments which aimed to use a phloem-specific promoter to block GS activity specifically in this tissue. The GS_1 isoform is expressed only in the vicinity of the phloem, where it functions in the generation of glutamine for nitrogen transport. Plants with 20-30% less GS activity in stems and midribs have been identified, although no evident phenotypic effect has been observed in these lines (Hirel et al. 1997). These workers also reported that tobacco plants transformed with an antisense copy of a Fd-dependent glutamate synthase (GOGAT, EC 1.4.7.1) cDNA contained levels of Fd-GOGAT activities which varied between 100% and 40% of the WT levels. The plants were generated in a CO_2-enriched atmosphere to avoid toxic photorespiratory ammonia accumulation during the selection procedure. When grown in air, symptoms of ammonia toxicity were observed in only two lines containing less than 60% of the Fd-GOGAT activity found in controls. None of the Fd-GOGAT antisense lines showed a substantial decrease in the content of total amino acids when grown under high CO_2 concentrations (Hirel et al. 1997). Kozaki and Takeba (1996) used a rice cDNA which encodes GS_2 to suppress GS_2 levels in tobacco plants. Differences in the certain metabolic parameters associated with a diminished capacity for photorespiration and an increased susceptibility to photoinhibition were observed in the antisense GS_2 lines, although it appears that these effects were only evident under conditions of high light intensity (Kozaki & Takeba 1996). A consistent feature of all of these reports (Temple et al. 1993; Kozaki & Takeba 1996; Hirel et al. 1997) is a failure to detect severe phenotypic effects that might be anticipated for the disruption of reactions that play a central role in plant nitrogen metabolism. Another common theme that has emerged from the use of antisense technology in the manipulation of plant metabolism is that often, the effects of antisense suppression only become evident following specific modifications of the environmental conditions.

5.2.3.1 Is suppression of either step in proline synthesis from glutamate lethal?

One explanation for the failure to regenerate plants that display an absolute requirement for exogenously supplied proline is that the magnitude of the change in either P5CS or P5CR enzymatic activity mediated by CaMV35S-driven antisense suppression of these gene products may have been in excess of a level that is suitable for investigation of the role of a proteinaceous amino acid in mediating plant stress tolerance. Proline auxotrophic calli may have grown significantly more slowly than the prototrophic calli, even when exogenous proline was included in the regeneration media. If a severe disruption of proline synthesis has a negative effect on shoot regeneration, this may have prevented the recovery of proline auxotrophs from Kan^r calli. An involvement of proline with *in vitro* shoot organogenesis in *Arabidopsis* (Section 4.10) seems consistent with this view. If this interpretation is correct, then given the presence of proline in the regeneration medium, it would be consistent with the view that the synthesis of proline, and not merely its availability, is of importance in *in vitro* shoot regeneration.

The lethality of a severe suppression of amino acid biosynthesis has previously been suggested to account for the failure to regenerate tryptophan auxotrophs when *Arabidopsis* lines carrying a antisense copy of a phosphribosylanthranilate isomerase (PAI) cDNA were regenerated (Li et al. 1995b). These workers observed that the expression of antisense *Arabidopsis PAI1* mRNA significantly reduced the immunologically observable PAI protein and enzyme activity in transgenic *Arabidopsis* plants. The observation that the antisense plants were resistant to 6-methylanthranilate, the precursor to a toxic tryptophan analogue, clearly indicated that the plants were disrupted in tryptophan synthesis. Furthermore, the observation that they fluoresced blue under UV illumination suggested that they accumulated anthranilate derivatives (Li et al. 1995b). However, the growth rate and morphology of even the most severely affected transgenic lines were identical to control plants both in the presence and the absence of exogenous tryptophan.

5.2.3.2 Functional redundancy in the synthesis and reduction of P5C

Plant metabolism is characterised by a high degree of flexibility. This plasticity is likely to be of particular importance in ensuring tolerance of the large fluctuations in environmental parameters such as light intensity and water availability that plants will normally have to deal with over relatively short periods of time. There are at least three mechanisms that might be implicated in an attempt to overcome a block in proline synthesis. Firstly, in view of the likelihood that proline and its precursors are continuously interconverted (Figure 2.14), there is likely to be some latitude in the extent to which flux through this cycle can be decreased. Conceivably, a relatively mild

disruption of proline synthesis could result in modulation of proline degradative capacity to ensure stabilisation of the size of the pool of cytosolic free proline. Secondly, other activities capable of catalysing functionally equivalent metabolic conversions may have masked even a dramatic reduction in the activities of either of the two targets enzymes. Finally, if the degree of inhibition in the plants selected was not high enough, a minor amount of residual activity of either enzyme might be sufficient to confer a phenotype not significantly different from that of the WT.

At least for the pBI-P5CS1(AS) lines, a disruption in P5C synthesis from glutamate could conceivably be overcome to a large extent by an increased contribution of OAT activity to the P5C pool. In view of the recent report by Roosens et al. (1998) that the contribution of ornithine to the P5C pool is developmentally regulated, it seems possible that the choice of seedlings as the subject of investigation to test the effects of transformation with pBI-P5CS1(AS) may not have been the best choice. These workers found that under both optimal growth conditions and upon exposure to stressful NaCl concentrations, OAT activity and levels of the cognate transcript were 10-fold higher in 12 d-old plantlets than in 28 d-old plants. In four week-old plants, salt stress had no effect on OAT activity and mRNA encoding OAT was below the limits of detection (Roosens et al. 1998). Future studies might thus involve examination of the stress responses of mature pBI-P5CS1(AS) A5 and B12 plants, although under optimal growth conditions, neither of the lines appeared to display a substantial reduction in vigour at later stages in the life-cycle. Developmental and environmental differences in the efficiency of antisense-mediated inhibition have been reported (van der Krol et al. 1988). These most likely arise from the likelihood that the use of two different promoters to drive the expression of the antisense and sense genes does not permit co-regulation of the abundances of the both the sense- and antisense transcripts. Delauney et al. (1993) suggested that the relative contributions of P5CS and OAT activities to the increase in the size of the P5C pool under stress may be affected by the plant nitrogen status. In an attempt to investigate whether only a slight reduction in growth of the two most severely affected pBI-P5CS1(AS) lines might result from an increased participation of OAT in P5C synthesis, their growth under conditions of nitrogen deprivation was assessed. As shown in Figure 4.14, this approach did not provide any evidence to suggest that a disruption of seedling nitrogen status had any effect on proline levels in the antisense lines. It remains to be established whether or not P5C synthesis from ornithine can be decreased under conditions of inadequate nitrogen status in *Arabidopsis*, as was suggested to occur in *Vigna aconitifolia* (Delauney et al. 1993).

A similar argument for functional redundancy in P5CR activity might also be applicable. In keeping with the existence of two forms of OAT in bacteria (Heimberg et al. 1990), radiolabelling experiments have suggested that in plants, the transamination of ornithine can occur not only via δ-OAT (referred to in this document merely as OAT), but also via an α-OAT (Mestichelli et al.

1979). Until now, only δ forms of OAT have been cloned from plants, mammals, yeasts and bacteria (Roosens et al. 1998). The radiolabelling data of Mestichelli et al. (1979) suggest that the synthesis of proline from ornithine can be catalysed not only through the reduction of P5C, but also *via* an alternative pathway which involves Δ^1-pyrroline-2-carboxylate (P2C) as the intermediate. The evidence in favour of the alternative route of proline synthesis catalysed by α-OAT and P2C reductase has been discussed elsewhere (Hare 1995). This pathway has been suggested to occur in *Arabidopsis* (Chiang & Dandekar 1995). It is also worth emphasising that the complementation strategy used by Delauney et al. (1993) in the cloning of plant genes with OAT activity precluded the identification of a plant α-OAT (Hare 1995). Roosens et al. (1998) identified an *Arabidopsis* EST as a possible clone of OAT based exclusively on its sequence homology to the previously reported genes encoding δ-OAT. Thus, while there is no molecular genetic evidence to implicate an alternative route of proline synthesis via P2C, the likelihood of functional redundancy in P5CR activity in plants cannot be fully excluded. If this is the case, it might provide a bypass reaction which can overcome an antisense-mediated depression in P5CR activity. It is also worth noting that although genomic copy number studies do not suggest the existence of P5CR isoforms in *Arabidopsis* (Verbruggen et al. 1993; Hare & Cress 1996), evidence for the existence of plastidic and cytosolic isoforms of P5CR has been obtained using tobacco (Rayapati et al. 1989; Szoke et al. 1992).

Besides different enzymatic activities, isoforms of the same enzyme may not be targeted by an antisense transcript to the same extent. It is difficult to estimate the extent to which the antisense *AtP5CS1* transcript may have decreased the expression of the *AtP5CS2* gene. Using BLAST analysis, the highest nucleotide homology of the *AtP5CS2* cDNA to the partial *AtP5CS1* sequence found in the pBI-P5CS1(AS) construct is 84% over a 667 bp stretch of the *AtP5CS2* mRNA. While it is impossible to predict the effectiveness of the 105E5T7 antisense transcript in mediating the coordinated downregulation of both of the *AtP5CS* isogenes, an antisense gene homologous to the cDNA encoding an isoform of CHS from petunia inhibited the expression of other members of the CHS gene family in this species, and could also decrease *CHS* expression in tobacco (van der Krol et al. 1988). In these cases, the overall nucleotide similarity between the homologous genes was as low as 75%. The failure of antisense RNAs which suppressed the expression of specific anionic peroxidases in tomato (Sherf et al. 1993) and tobacco (Lagrimini et al. 1997b) to affect wound-induced suberisation or lignification has been attributed to the ability of other peroxidase isoenzymes to substitute for the missing enzyme.

Regarding the pBI-P5CR(AS) lines, a likely explanation for the lack of any effects of the antisense YAP057 transgene may largely be ascribed to the apparent ubiquity of P5CR in plant cells. As much as a 50-fold increase in P5CR activity in tobacco plants which expressed functional

soybean P5CR did not cause substantial increases in free proline levels in these transgenic lines (Szoke et al. 1992). Although some of the transgenics did show slightly elevated levels of proline after salt stress, multiple sample analysis did not suggest that the difference was significant. Furthermore, LaRosa et al. (1991), working with NaCl-adapted and non-adapted tobacco cell cultures, demonstrated that although the salt-adapted cells accumulated up to 80-times as much proline as non-adapted cells, there was no difference in the total extractable P5CR activity from both of these sources. It was estimated that the specific activity of P5CR in the unadapted cells was between 1053- and 4227-fold in excess of what was required for the observed level of proline synthesis. Therefore, in tobacco at any event, it appears that P5CR activity *in vivo* is limited by P5C availability and that the enzyme functions at only a small fraction of its V_{max}. The subsequent report that an increase in P5CS activity increased free proline levels (Kavi Kishor et al. 1995) substantiates this viewpoint that P5CR activity is limited by substrate supply. Although the pBI-P5CR(AS) lines need to be characterised in greater detail, they seem to suggest that a similar situation might apply in *Arabidopsis*. If this is the case, then it seems reasonable to anticipate that very severe levels of suppression of P5CR activity may be required in order to observe any phenotypic effects related to a decrease in free proline content. In many respects, it is curious that natural selection has resulted in the maintenance of certain enzymes in a large excess relative to the level that is required for growth. A classic example of such an enzyme is RUBISCO. Extensive investigations on the impact of antisense-mediated decreases in RUBISCO activities on photosynthesis have noted that the protein can be reduced to at least half of the level normally found in WT plants with little effect on photosynthesis (Quick et al. 1991). It is tempting to speculate that for certain enzymes, their maintenance at levels in excess of what those adequate to catalyse the observed reaction rate may reflect a particularly important role for these enzymes in have a buffering effect on metabolic fluxes following environmental fluctuations.

Clearly, a number of arguments can be invoked which support a mechanism by which endogenous "bypass" reactions could overcome a suppression of either P5CS or P5CR activities in *Arabidopsis*. Remarkably, antisense studies have revealed the somewhat controversial view that plants might even have the capacity to circumvent the disruption of gene expression by the expression of novel, but functionally equivalent, gene products. The observation that expression in tobacco of an antisense gene which encodes the A isoform of an antifungal class-I isoform of β-1,3-glucanase (βGlu) specifically blocked the induction of its expression in response to ethylene treatment or infection with a pathogenic fungus or virus, but had no consistent effects on the time course or the severity of disease symptoms, led to the discovery that the antisense plants compensated for a deficiency in βGlu activity by producing a serologically distinct, but functionally equivalent, βGlu activity (Beffa & Meins 1996). This compensatory (referred to as "ersatz") activity was not detectable in comparable healthy or infected leaf tissues of control plants. Beffa and

Meins (1996) proposed that one or more genes which encode "ersatz" enzymes, are not normally expressed in leaves, but can be specifically induced to circumvent an artificially-induced loss of class-I βGlu activity. Until these "ersatz" enzymes have been isolated and their putative genes characterised, the possibility that they may merely arise from modifications of known βGlu arising through alternative splicing or post-translational modification cannot be dismissed. Similar reports of "ersatz" activities involved in intermediary metabolism do not appear to have been reported, and it remains to be seen whether "ersatz" activity merely a fairly specific feature of βGlu gene expression.

5.2.3.3 Is an antisense approach suitable for the suppression of proline synthesis?

A third approach to explaining the absence of severe phenotypic effects observed following transformation with either of the antisense constructs is to consider whether antisense suppression may simply not be a viable means to suppress proline synthesis. At least two studies have been reported where reduced expression of a specific RNA by antisense RNA is not correlated with a reduction of the cognate protein. Palomares et al. (1993) found that transgenic antisense plants that exhibit 10% or less of the WT levels of mRNAs encoding either of two components of the oxygen-evolving complex, or the Rieske iron/sulphur protein, do not have reduced levels of the proteins encoded by these transcripts. Since normal development of the antisense lines progressed without a detectable change in rates of oxygen evolution, it was suggested that the abundances of these proteins are primarily regulated at a post-transcriptional level (Palomares et al. 1993). Similarly, transgenic antisense *CAB* plants had severely reduced *CAB* mRNA levels (as low as 5% of those found in the WT tobacco), although the targeted light-harvesting complex protein accumulated to the levels found in WT controls (Flachmann & Kühlbrandt 1995). Biochemically and physiologically, the *CAB* antisense lines appeared to be indistinguishable from their WT parents (Flachmann & Kühlbrandt 1995). Once again a post-transcriptional process was proposed to be the rate-limiting step in translation of the PSII light-harvesting complex from the endogenous pool of *CAB* mRNA transcripts. While these two studies contrast with a far more extensive list of reports concerning a strong positive correlation between mRNA abundance and levels of the protein being targeted in antisense plants (Bourque 1995), the suitability of antisense suppression as a universally applicable approach to gene suppression may be overestimated. Positive results find their way into the published literature more easily than do negative ones! Since post-transcriptional control mechanisms may circumvent, at least partly, the effects of a reduction in mRNA levels, the findings of both Palomares et al. (1993) as well as those of Flachmann and Kühlbrandt (1995) emphasise a useful, although often neglected application of antisense technology *viz.* assessment of the importance of post-transcriptional regulation of gene expression.

Although most effort in understanding the regulation of plant gene expression has been devoted to transcriptional mechanisms, more downstream events in the gene expression process have also frequently been found to be important sites of control (Gallie 1993; Sullivan & Green 1993). Post-transcriptional regulation can occur at the levels of mRNA processing, the regulation of transcript stability or translational initiation as well as through protein modification or alterations in the rates of protein turnover. This wide range of possibilities allows for increased flexibility and more rapid responses than can be achieved through transcriptional regulation alone. In view of the proposed role of proline synthesis in the buffering of cellular redox potential (Hare & Cress 1997), post-transcriptional control mechanisms may be of particular importance in the regulation of proline synthesis. Following environmentally-induced shifts in redox potential, the initiation or termination of transcription of proline biosynthetic genes would be unlikely to assist in the maintenance of redox homeostasis in the short term. Transcripts whose steady-state levels need to be adjusted rapidly in response to environmental fluctuations have often been found to be unstable (Sullivan & Green 1993). This ensures that changes in steady-state mRNA levels can be achieved rapidly. Post-transcriptional control mechanisms of the expression of proline biosynthetic genes may represent another mechanism by which plants may compensate for a less than absolute reduction in levels of transcript encoding P5CS or P5CR.

The likelihood that regulation of the expression of either of the genes encoding P5CS in *Arabidopsis* may be mediated at least partially at a post-transcriptional level has already been mentioned (Section 2.4.3.1). Comparison of the results of Zhang et al. (1997) and Strizhov et al. (1997) concerning the promotive effect of ABA on *AtP5CS2* transcript accumulation suggests that the stimulative effects of ABA on the abundance of *AtP5CS2* mRNAs may be mediated through their stabilisation, since ABA did not activate the *AtP5CS2* promoter when fused to a reporter gene (Zhang et al. 1997). Post-transcriptional regulation of plant P5CR activity also seems to occur since Mattioni et al. (1997) failed to correlate an increase in the specific activity of P5CR following water deprivation or salt stress with an increase in the abundance of its mRNA. The importance of post-transcriptional regulation of any of the proline biosynthetic genes has yet to be assessed directly. The demonstration that both P5CR (Szoke et al. 1992) and P5CS (Kavi Kishor et al. 1995) protein levels can be increased by the expression of sense copies of these genes under CaMV35S regulation clearly indicates that the efficiency of translation or regulation of mRNA stability cannot exert an absolute limitation on their levels of expression. It nonetheless seems possible that post-transcriptional regulatory mechanisms may be superimposed on those which regulate rates of P5CS and P5CR synthesis at the level of transcriptional initiation.

A role for post-transcriptional regulation in the control of *AtP5CS1* expression has been proposed by Savouré et al. (1997). A diagrammatic representation of the findings of these workers is shown

in Figure 5.1. As was described in Section 2.4.3.1 and can be seen from the summary presented in Table 2.10, these workers reported that proline accumulated to a greater extent following osmotic stress than after the application of ABA or the imposition of cold stress. Examination of the responses of the *aba1* mutant indicated that both *AtP5CS1* expression and proline accumulation is independent of endogenous ABA concentrations following cold treatment or exposure to sorbitol-mediated dehydration, but that the endogenous ABA content apparently affects proline accumulation following salt stress. The observation that mutation of ABI1 affected NaCl-induced proline levels, but had no apparent effect on *AtP5CS1* transcript abundance led Savouré et al. (1997) to conclude that ABI1 participates in an ABA-dependent signalling pathway which stimulates proline accumulation without affecting *AtP5CS1* transcript abundance (Figure 5.1).

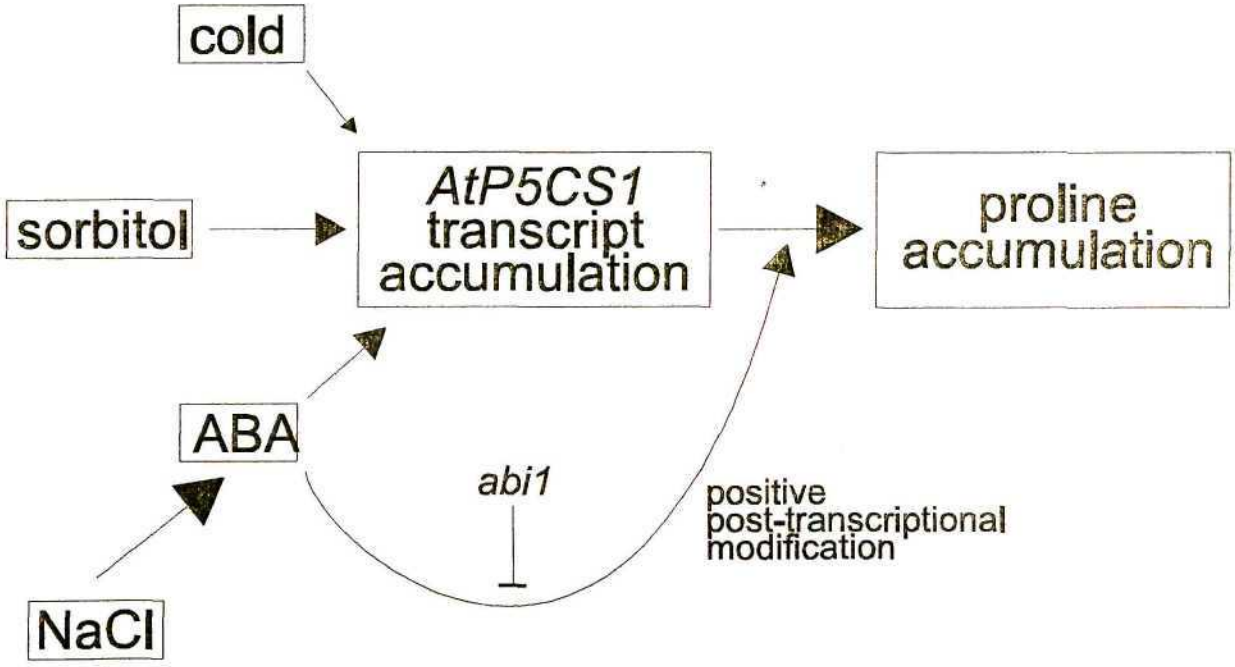

Figure 5.1. A model for the differential effects of isosmotic concentrations of sorbitol and NaCl on proline accumulation in *Arabidopsis* seedlings. A positive post-transcriptional modification, which does not affect *AtP5CS1* transcript abundance and is disrupted by mutation of ABI1, mediates the induction of proline accumulation by NaCl, but not by sorbitol or cold-treatment. The model is based exclusively on the findings of Savouré et al. (1997).

The multipartite nature of salinity stress complicates direct comparison of its effects with those associated with water deprivation. Although dissolved salts reduce the effective concentration of water, additional effects which are associated with salinisation arise from the mineral toxicity of the salt as well as interruptions of the mineral nutrition of the plant. Since Savouré et al. (1997)

used isosmotic concentrations of NaCl and sorbitol in their experiments, their results suggest that the ionic toxicity component of salinity stress triggers a pathway that is distinct from that induced merely by cellular dehydration. The specific roles of *SOS* gene products (Ding & Zhu 1996; Liu & Zhu 1997b; Zhu et al. 1998) in the tolerance of the ionic, but not the osmtic component of salt stress, strongly supports the existence of ionic stress-specific signalling involving a calcineurin-related pathway (Liu & Zhu 1998). As was mentioned in Section 2.4.3.1, de Bruxelles et al. (1996) noted an almost 30-fold increase in the level of ABA in roots and an approximately five-fold increase in the shoots of 28 d-old *Arabidopsis* plants within 12 h of exposure to 600 mM mannitol. This stress is comparable to the sorbitol treatment used in this study and that of Savouré et al. (1997) involving 14 d-old and 10 d-old seedlings respectively. Lång et al. (1994) observed a two- to four-fold transient increase in shoot ABA content in *Arabidopsis* plants grown at 4 °C. Irrespective of these findings, it is still possible that an increase in endogenous ABA levels may not be causally related to proline synthesis following sorbitol-mediated dehydration or exposure to low temperatures. Differential effects of treatment with isosmotic concentrations of sorbitol and NaCl on proline contents in both WT plants and the pBI-P5CS1(AS) lines A5 and B12 are evident in Figure 4.15. In contrast to the findings of Savouré et al. (1997), it was found that 550 mM sorbitol consistently resulted in a greater stimulation of proline accumulation than did an isosmotic concentration of sorbitol. While the reason for this discrepancy is unclear, it may be worth noting that in contrast to the finding that 250 mM NaCl was more effective in the induction of *AtP5CS1* transcript accumulation than an isosmotic concentration of sorbitol (Savouré et al. 1997), Knight et al. (1997) observed that in 7 d-old *Arabidopsis* seedlings, 660 mM mannitol caused a much larger increase in steady state *AtP5CS1* transcript levels than did an isosmotic concentration of NaCl. Werner and Finkelstein (1995) reported that a 24 h exposure of 14 d-old *Arabidopsis* plants to 600 mM sorbitol caused an accumulation of proline to levels almost two-fold higher than those found after a 24 h incubation in 400 mM NaCl. The former treatment caused a much more extensive decrease in the cellular water content than did treatment with NaCl (Werner & Finkelstein 1995). The view that polyols may be absorbed less efficiently than NaCl, and the likelihood that Na^+ uptake may normally constitute an important aspect of reducing the internal osmotic potential in order to ensure continued water uptake in *Arabidopsis* (Liu & Zhu 1997a) seem consistent with the observation that incubation in sorbitol may be a more stressful osmotic treatment than exposure to an isosmotic concentration of NaCl (Figure 4.15). Chiang and Dandekar (1995) observed a massive accumulation of Na^+ and Cl^- ions in 14 d-old NaCl-stressed *Arabidopsis* plants.

Perhaps the most informative aspect of the physiological characterisation of both of the pBI-P5CS1(AS) lines A5 and B12 is the demonstration that a reduction in stress-induced free proline accumulation in both of the antisense lines relative to the WT controls was more evident following

sorbitol-mediated dehydration, than after exposure to an isosmotic concentration of NaCl. For example, in the pBI-P5CS1(AS) transformant B12, proline levels following a 24 h exposure to NaCl stress were 76% of those found in the comparable WT plants. In contrast, following sorbitol stress, levels were 53% of those found in comparable WT seedlings (Figure 4.15). The more dramatic antisense-mediated suppression of proline accumulation observed following sorbitol-mediated dehydration than after salinisation is consistent with the view that a post-transcriptional event is associated with NaCl-mediated, but not sorbitol-mediated proline accumulation (Savouré et al.; Figure 5.1). A post-transcription site of control is likely to render a partial suppression of *AtP5CS1* abundance less evident. Although the differential effects of isosmotic concentrations of sorbitol and NaCl on the relative levels of proline accumulation in WT and pBI-P5CS1(AS) lines requires further characterisation at the molecular level, it seems reasonable to propose that the results presented in Figure 4.15 reveal the involvement of an endogenous post-transcriptional effect in overcoming the effects associated with a less than absolute elimination of *AtP5CS1* transcript. The apparent involvement of a proteinaceous inhibitor of P5CS activity (Zhang et al. 1995; Kavi Kishor et al. 1995) in mediating this effect merits further investigation.

5.2.4 Conclusion

Clearly, the use of a strong and almost constitutively expressed promoter to drive antisense copies of cDNAs which encode either a P5CS or P5CR has been found to be of limited usefulness in discerning a stress-related role for proline synthesis. As the first reported attempt to use this approach, this study nonetheless suggests several possible directions that future efforts to silence proline synthesis might take. Although tedious, the best approach might be to empirically analyse each of these alternatives individually. If the notion that P5CS activity can be masked by OAT, or that there is functional redundancy in the reduction of P5C to proline is valid, then it may be desirable to consistently knock out the expression of genes involved in proline synthesis from glutamate. Despite its usefulness in certain instances, the considerable variability in antisense effects and the failure to identify a means by which the technique can be standardised represent major limitations of this approach. Recently, Angell and Baulcombe (1997) reported a new strategy with which to achieve consistent inactivation of gene expression in transgenic plants. It was shown that when a transgene is integrated into a cDNA of replicating potato virus X RNA (amplicon) and plants are transformed with this construct, there is strong and consistent suppression of the endogenous gene that does not appear to be influenced by chromosomal position effects. Although the usefulness of this recently-described strategy to trigger gene silencing requires further analysis by others, amplicon-mediated gene suppression promises to provide one approach to examine the effects of strong and consistent ablation of

proline biosynthetic genes. Targeted disruption of gene expression by homologous recombination has recently been found to be another approach to create a directed null mutation in *Arabidopsis* (Kempin et al. 1997), although presently, this technique appears to be very labour-intensive.

While it is still not possible to predict whether any of the proline biosynthetic genes are indispensable for the regeneration of viable plants, controlled expression is one way of eliminating concern about the potentially detrimental effect of high levels of suppression on proline synthesis during regeneration or that it may severely hinder subsequent growth and development. Further studies might investigate the use of inducible plant gene expression systems in regulating a lower level of antisense gene expression. Moore et al. (1998) have recently developed a gene expression system that can be used to generate transgenic plants where the transgene is not expressed in the primary transformant. Hybrid progeny, produced by crossing the T_0 generation with a second transgenic line carrying a specific transcriptional activator, are used to investigate the effects of the expression of the transgene under investigation. The main advantage of this approach is that it enables investigation of the effects of transgenes that might be so detrimental to plant growth and survival that they preclude the generation and propagation of viable transgenic lines. Furthermore, simple Mendelian segregation of the target transgene and activator transgene can be used to reverse gene activation.

Since an important requirement for the use of transgenesis in the study of plant metabolism is often the ability to regenerate a large collection of transgenic lines, it may be necessary to generate a greater number of transgenic plants than was accomplished in this study. In this regard, future studies might investigate the creation of transgenic plants using vacuum infiltration transformation of *Arabidopsis* (Bechtold et al. 1993). This approach, which has now been widely adopted to create transgenic *Arabidopsis* lines, involves submersion of flowering plants in a suspension of *Agrobacterium* and subjecting them to a vacuum. The *Agrobacterium* enters the plant and probably transforms the egg cells, or their progenitors, with the result that transformants can be screened by germination of selfed progeny. Another recent advance in increasing the efficacy of *Agrobacterium*-mediated transformation is the important, although until recently poorly appreciated, role of temperature in enabling pilus assembly (Fullner et al. 1996) and thus efficient transformation frequencies (Dillen et al. 1997). Systematic investigation of the effect of temperature on the efficiency of T-DNA transfer to plants indicated that this was strongly decreased when the temperature was raised above 22 °C and became negligible at temperatures of 27 °C or higher (Dillen et al. 1997). It seems possible that a larger number of transgenic *Arabidopsis* lines might have been recovered in this study if the factor of temperature had been considered in attempts to ensure a high rate of plant transformation.

5.3 A regulatory role for proline metabolism in *Arabidopsis*

As was proposed in Section 2.5, a central feature of the role of disruptions in proline metabolism under adverse conditions appears to be the modulation of cellular redox balance to ensure metabolic homeostasis. Given the central role played by pyridine nucleotides and adenylates in metabolism, one might anticipate that regulated changes in nucleotide ratios might be affected by a disruption in proline metabolism and that this may be reflected by metabolic alterations that affect growth and development. In this study, seed germination, seedling growth and development and *in vitro* shoot organogenesis in *Arabidopsis* were selected as experimental systems to examine whether or not an artificially-induced increase in cellular free proline might affect physiological processes in the absence of stressful conditions. Although the effects of exogenous proline on these processes will be discussed individually, in all three instances, the evidence obtained is consistent with the view that an artificial elevation of endogenous free proline levels activates metabolic responses which involve substrates unrelated to intermediates in proline synthesis and degradation. It is important that this criterion be met if the proline metabolic system is to qualify as a mechanism which has true regulatory capacity.

A central tenet of the classical view of proline as a compatible solute is that this imino acid can be accumulated to high cytosolic concentrations with minimal effects on cellular metabolism. Therefore, a critical aspect of testing this hypothesis is to examine whether or not exogenously-applied proline is without effect on cellular ultrastructure. Increasing evidence suggests that besides their roles in carbon exchange reactions, many metabolic interconversions trigger changes in gene expression. It is becoming increasingly apparent that the effects of the proline metabolic system appear to be exerted not only at the level of intermediary metabolism, but also through disruption of a signalling pathway(s) that controls the expression of certain plant genes (Section 2.5.3; Hare et al. 1998). If the metabolic regulatory effects of changes in proline synthesis and degradation are on a continuum with the apparent signalling potential of changes in flux through the proline biosynthetic and catabolic pathways (Hare et al. 1998; Iyer & Caplan 1998), then it is also necessary to assess how changes in proline-regulated gene expression may be mediated. The observation that exogenous proline increases peroxidase activity (Figure 4.25; Chen & Kao 1995) potentially offers a molecular explanation for many of the effects of proline on *Arabidopsis* seed germination and seedling growth as well as *in vitro* shoot organogenesis in this species.

5.3.1 The effects of proline on *Arabidopsis* seed germination

Plant stress responses should always be considered in a developmental context. For many plant species, germination and the post-germinative events that terminate in the establishment of autotrophy constitute amongst the most stressful stages of the life cycle. In order to ensure that these critical transitions are successful, and thus guarantee species survival, seeds must integrate endogenous signals with a variety of environmental cues in order to prevent making a foolish commitment to the mobilisation of their nutrient reserves which are required to support initial seedling growth.

Owing to our poor appreciation of the molecular basis of dormancy breaking, it is not yet clear whether all seeds possess a universally conserved signalling cascade that coordinates the events triggered by the perception of environmental conditions suitable for embryo growth, or whether the signalling events that result in seed germination may differ between species. However, consistent with the importance of cellular energy status in ensuring seedling establishment, a special role for the oxidative pentose phosphate pathway (OPPP) in stimulating germination has been proposed for many years (Roberts 1973; Botha et al. 1992). In heterotrophic tissues, this cyclical pathway not only provides NADPH needed for reductive biosynthetic reactions, but also contributes both to the synthesis of pentoses (precursors for the production of nucleic acids and cell wall components) as well as their catabolism (by their conversion to hexose phosphates and triose phosphates for entry into glycolysis).

As was mentioned in Section 2.2.3, support for an important role for the OPPP in the regulation of germination has never been universal (Botha et al. 1992). In part, the enigma which surrounds the apparent importance of the OPPP in seed germination has arisen from a failure to identify a functional role for OPPP activity in the germinating seed. Since elevated OPPP activity occurs in actively dividing or differentiating tissues, special significance of the OPPP during germination and early seedling establishment might be attributed to the provision of NADPH and pentoses for anabolic reactions at a time when photosynthesis is not possible. Evidence for an important role for TRX *h*, which is reduced enzymatically by NADPH generated by the OPPP, in the mobilisation of carbon and nitrogen reserves during the germination of cereals has already been reviewed (Section 2.2.3; Figure 2.6; Table 2.8). It was also suggested that the provision of NADPH by the OPPP may ensure an efficient antioxidative response to counteract ROI production associated with high metabolic rates in germinating seeds (Section 2.2.3). Nonetheless, an exclusively metabolic role for the OPPP during germination through the generation of reductant seems unlikely, since agents which maintain the NADP pool in the oxidised state have frequently been shown to stimulate the loss of seed dormancy (Roberts 1973). Furthermore, rates of nucleic acid synthesis in dormant and nondormant seeds of *Avena fatua* are comparable (Roberts 1973).

In many respects, *Arabidopsis* is well suited for investigation of the physiological mechanisms of seed dormancy and germination. The seeds of this model plant are small and numerous. Furthermore, as an undomesticated species, in contrast to many crops, all of the regulatory mechanisms for dormancy control are still intact (Koornneef & Karssen 1994). Given the likely involvement of the proline biosynthetic pathway in regulating flux through the OPPP (Section 2.5.2.2), the dose-dependent inhibitory effect of exogenous proline on radicle emergence in *Arabidopsis* (Table 4.7) may relate the disruption of proline-linked OPPP activity within imbibed seeds. The involvement of the OPPP in facilitating radicle emergence in *Arabidopsis* does not appear to have been directly investigated by others. Nitrate, which is commonly attributed with the properties of an electron acceptor capable of oxidising the NADP pool, has a promotive effect on radicle emergence in *Arabidopsis* (Derkx & Karssen 1993). Consistent with what is been found in many, but not all species (Botha et al. 1992), there is an increase in the specific activities of both glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) during the course of *Arabidopsis* seed germination (Figure 4.16). These $NADP^+$-dependent dehydrogenases catalyse the rate-limiting steps of the OPPP. During germination, activation of OPPP activity relative to glycolytic flux is indicated by a decrease in the ratio of ^{14}C-labelled CO_2 released from $^{14}C_6$-labelled glucose to that respired from $^{14}C_1$-labelled glucose. At the time of radicle emergence, the C_6/C_1 ratio is significantly lower than when seeds have been imbibed for only 12 h (Table 4.12). The decrease in the C_6/C_1 ratio, which is maximal 18 h after radicle emergence (Table 4.12) correlates well with the broad optima of G6PDH and 6PGDH activities which occur between 36 h and 54 h after imbibition (Figure 4.16). If flux through the proline biosynthetic pathway from glutamate is coupled to OPPP activity, then conceivably, feedback inhibition of P5CS by high levels of exogenous proline would prevent the generation of adequate $NADP^+$ to support a high rate of activity of the two NADPH-inhibited dehydrogenases which limit the entry of carbon into the OPPP. In particular, the demonstration that the auto-oxidants methylene blue and phenazine ethosulphate can overcome the inhibitory effects of proline (Table 4.9) strongly supports the proposal that the effect of exogenous proline on seed germination is at least partly a redox-related phenomenon.

Where investigated, the pool of NADP in dry seeds has been found to be primarily in the reduced state, and thus requires oxidation in order to relieve the inhibitory effect of NADPH on OPPP activity (Botha et al. 1992). Roberts (1973) suggested that an inadequate NADPH oxidising system may be the primary restraint to germination in dormant seeds. The notion that a cycle of proline synthesis and degradation may normally participate in this role is substantiated by several observations. *Firstly*, proline is found at extremely high levels in dry seeds when compared to the levels of other amino acids (Table 4.13). Although levels of asparagine, glutamine, cysteine and ornithine were not determined during total amino acid analysis (Table 4.13), the finding that free

proline constitutes approximately 15.4% of the total amino acids which were quantified compares favourably with the report by Chiang and Dandekar (1995) that free proline constitutes approximately 16.7% of the free amino acid pool in mature *Arabidopsis* seeds. In contrast, these workers found that proline constitutes only 1-3% of the total free amino acid pool in vegetative tissues of *Arabidopsis* (leaves and roots). *Secondly,* a three- to four-fold increase in free proline levels accompanies radicle emergence in *Arabidopsis* (Figure 4.17; Table 4.13). The demonstration that this maximum proline level occurs at least 24 h prior to a time when seed storage protein degradation is complete (Figure 4.18) indicates that the increase in free proline cannot be ascribed exclusively to the breakdown of seed storage proteins. Accordingly, Heath et al. (1986) observed detectable levels of 12S proteins in fully emerged cotyledons of seedlings three days after the imbibition of *Arabidopsis* seeds. Degradation of these proteins was complete within an additional two days (Heath et al. 1986). Thus, the inhibitory effect of exogenous proline (a treatment likely to inhibit proline synthesis) on seed germination apparently reflects a normal involvement of proline synthesis in the regulation of embryo growth. *Thirdly,* the occurrence of high levels of transcripts encoding P5CR (Verbruggen et al. 1993) and PDH (Verbruggen et al. 1996a) in *Arabidopsis* seeds relative to most vegetative tissues is consistent with the proposal that cycling between proline and P5C or glutamate might be important in simultaneously stimulating OPPP and TCA cycle activities during the rapid growth and differentiation associated with seedling establishment. Although such cycling is futile in terms of carbon exchange, it might have considerable impact on the level of reduction of the pyridine nucleotide pools and the cellular adenylate charge (Figure 2.14; Hare & Cress 1997). *Fourthly*, an approximately 35% reduction in the maximal level of free proline accumulated at the time of radical emergence in the T_2 seeds of two pBI-P5CS1(AS) lines and a delayed rate of proline accumulation during the germination of seeds likely to have a reduced capacity for P5C synthesis from glutamate correlates with a delayed germination rate in both of these antisense lines (Figure 4.17). *Finally*, a 48 h chilling pre-treatment immediately after imbibition can partly overcome the inhibitory effect of exogenous proline on radicle emergence (Table 4.8). It is tempting to speculate that this may relate to the observation that the breaking of seed dormancy by stratification (low-temperature treatments of imbibed seeds) has been associated with an increase in the contribution of the OPPP to overall carbohydrate metabolism (Roberts 1973). Chilling of *Corylus avellana* seeds under moist conditions caused increases in the activities of both G6PDH and 6PGDH which were associated with the breaking of seed dormancy in this species (Gosling & Ross 1980).

When *Arabidopsis* seeds are germinated in liquid culture, the maximum concentration of free proline accumulated during the germination process (Figure 4.17; Table 4.13) is reached some time (approximately 18 h) before the apparent maximal stimulation of OPPP activity (Figure 4.16; Table 4.12). In this regard, it seems worthwhile to point out that free proline levels themselves

may not be an accurate measure of flux through the proline biosynthetic pathway. Proline accumulation may be a symptom of uncoupling between the cycle of proline synthesis and degradation which is proposed to support a high rate of OPPP activity (Figure 2.14). The increase in free proline level during the course of germination does not appear to be at the expense of free glutamate. A slight decrease in glutamate levels was noted 12 h after imbibition (Table 4.13), but this preceded the increase in proline levels which is evident 24 h after imbibition of WT seeds (Figure 4.17; Table 4.13).

As has been suggested for the transient accumulation of proline during germination, it is tempting to speculate that a consistent increase in free serine content (a level approximately five-fold of that found in dry seed is found 48 h after imbibition; Table 4.13) may also be symptomatic of elevated OPPP activity during germination. Examination of the effects of selenite-induced oxidative stress on amino acid levels in the lens of the mammalian eye indicated that an increase in flux through the OPPP was accompanied by an approximately four-fold increase in free proline concentration and as much as a ten-fold increase in the abundance of serine (Mitton et al. 1997). These workers attributed the increase in lens free serine content to an increased flux of carbon through the OPPP, since glyceraldehyde-3-phosphate, a product of the non-oxidative portion of the OPPP (Figure 2.14), contributes to serine synthesis in heterotrophs. Unfortunately, most of our knowledge of serine synthesis in plants has been concerned with photorespiration. In the glycolate pathway, two molecules of glycine are converted into one molecule of serine by the concerted actions of glycine decarboxylase and serine hydroxymethyltransferase. In heterotrophic plant tissues, serine is believed to be synthesised by two alternative routes which start with 3-phosphoglycerate instead of glycine (Ireland & Hiltz 1995). The "phosphorylated pathway" of serine synthesis from 3-phosphoglycerate starts with reduction of 3-phosphoglycerate to phosphohydroxyypyruvate, which is transaminated to phosphoserine, and then dephosphorylated to serine. The "unphosphorylated pathway" involves the dephosphorylation of 3-phosphoglycerate to glycerate, followed by the reduction of glycerate to hydroxypyruvate, which is transaminated to serine (Ireland & Hiltz 1995). While the relative contributions of these two pathways to serine synthesis in different heterotrophic plant tissues remains to be clarified, characterisation of the expression of a gene encoding spinach phosphoserine aminotransferase suggests that the synthesis of serine in rapidly proliferating tissues of low photosynthetic activity is likely to occur via phosphoserine (Saito et al. 1997). The observation that serine is not particularly abundant in *Arabidopsis* seed storage proteins (Table 4.14) discredits the notion that a relatively high rate of serine accumulation during the course of germination might arise from the breakdown of nitrogenous seed reserves. The observation that proline contributes more to the polypeptide backbones of seed storage proteins than it does to an "average" *Arabidopsis* gene product (Table 4.14) is intriguing. This might relate to the high level of respiration during early seedling growth,

although the possibility that it may simply reflect a structural feature of seed storage proteins cannot be excluded. Proline residues are known to cause turns in polypeptide chains and thus affect the secondary structure of proteins.

Previous investigations regarding changes in nitrogen metabolism during seed germination appear to have disregarded the importance of changes in the contents of free proline and serine. However, the assimilation of arginine into biosynthetic and catabolic pathways has frequently been proposed to be of particular importance in the nitrogen nutrition of seedlings (King & Gifford 1997). A ten-fold increase in seed arginase activity occurs during the first 6 d after germination of *Arabidopsis* seeds (Zonia et al. 1995). Although ornithine and urea are the products of arginase activity, the increase in ornithine which results from elevated arginase activity does not appear to influence proline levels (Polacco & Holland 1993). Rather, most of the glutamate semialdehyde generated by OAT activity is oxidised to glutamate via P5CDH. Most of the glutamate thus generated is believed to contribute to new carbon constituents after entry into the TCA cycle via 2-oxoglutarate (Polacco & Holland 1993). Surprisingly, *Arabidopsis* seed storage proteins do not appear to be particularly rich in arginine (Table 4.14). In contrast, glutamine may be a more important repository of nitrogen in the mature *Arabidopsis* seed.

If flux through a metabolic cycle involving proline synthesis and degradation does play an important role in driving OPPP activity and thereby stimulating seed germination, then one might anticipate that such cycling may be stimulated by low concentrations of proline which are not inhibitory to P5CS activity. In this regard, it is interesting to note that low concentrations of exogenous proline can alleviate the innate dormancy of seeds of the succulent forb *Zygophyllum simplex* (Khan & Ungar 1997). In the absence of salt stress, 0.1 mM proline increased seed germination rates of this species from 12% to approximately 60%, while a 42% germination rate was obtained in the presence of 1 mM proline. Both concentrations of proline were effective in alleviating the inhibitory effect of 25 mM NaCl on germination, although 0.1 mM proline was more effective than 1.0 mM (Khan & Ungar 1997). Neither of these two proline concentrations was effective in stimulating germination at 75 mM or 125 mM NaCl (Khan & Ungar 1997). Although these workers suggested a role for proline as a compatible solute, two arguments suggest that it may be acting as a germination-inducing signal in *Zygophyllum simplex*. Firstly, the low concentrations used almost certainly preclude an osmotic mode of action. A purely osmotic explanation is also inconsistent with the observation that 0.1 mM proline was more effective than 1.0 mM proline in the breaking of dormancy. Secondly, stimulation of germination was most pronounced in the *absence* of salt stress, and no ameliorative effect of proline at the concentrations used was noted at 75 mM or 125 mM NaCl. These data thus corroborate the proposal that a signal related to flux through the proline synthetic and degradative pathways may

affect germination. Low concentrations of proline would conceivably stimulate cycling between proline and its precursors, without blocking this process, as occurs at millimolar concentrations. Although no stimulation of seed germination by low concentrations of proline was noted in *Arabidopsis* (Table 4.10), this may be because the high rates of germination that normally occur in this species might mask the detection of a slight stimulatory effect. Nonetheless, when the rates of germination of *Arabidopsis* seeds were substantially decreased by the imposition of hyperosmotic stress, neither 0.1 mM proline nor 1.0 mM proline appeared to be capable of causing a significant increase the rate of radicle emergence (Table 4.10). When germination was scored 60 h after imbibition, 0.1 mM proline could significantly increase germination rates in the presence of 200 mM sorbitol to a level comparable to seeds which had been incubated in the absence of hyperosmotic stress. However, this ameliorative effect was not noted when germination of seeds imbibed in the presence of both sorbitol and 0.1 mM proline was scored earlier in the germination process (Table 4.10). The failure of exogenous proline (0.1 mM) to alleviate the inhibitory effect of 100 mM NaCl on radicle emergence in *Arabidopsis* contrasts with the observation of Bar-Nun and Poljakoff-Mayber (1977) that 0.1 mM proline could significantly counteract the inhibitory effects of 120 mM NaCl on the germination of pea seeds. However, an ameliorative effect of exogenous proline on salinity-induced suppression of germination has not been reported for all species tested. For seeds of the halophyte *Kosteletzkya virginica*, application of 10.0 mM proline was ineffective in alleviating the effects of salinity stress on germination or in breaking innate dormancy (Poljakoff-Mayber et al. 1994). Likewise, 1.0 mM proline was incapable of alleviating the innate and salinity-induced dormancy of another halophyte *Arthrocnemum indicum* (Khan et al. 1998).

The failure of over three decades of research to identify a functional role for the OPPP in the stimulation of radicle emergence introduces considerable difficulty in unambiguously assigning a role for high levels of exogenous proline in inhibiting seed germination through a disruption of OPPP activity. The rationale behind the inclusion of experiments which investigated the effects of glycerol on radicle emergence in *Arabidopsis* (Table 4.10) was based on the demonstration by Aubert et al. (1994) that replacing sucrose in the culture medium of sycamore (*Acer pseudoplatanus*) cells with 50 mM glycerol resulted in non-functioning of the cytosolic and plastidic OPPPs (Aubert et al. 1994). A large decrease in the $NADPH/NADP^+$ ratio in tobacco cells which were cultivated for 14 d in the presence of 50 mM glycerol in place of sucrose was also attributed to a decrease in OPPP activity (Pugin et al. 1997). Aubert et al. (1994) demonstrated that the rapid accumulation of *sn*-glycerol-3-phosphate in the cytosol of glycerol-fed cells competitively inhibits glucose-6-phosphate isomerase with respect to fructose-6-phosphate and thereby prevents the rapid recycling of triose phosphates back to hexose phosphates. A rapid cycling between cytosolic triose phosphates and hexose phosphates has been shown to occur

in many heterotrophic plant tissues, including maize endosperm (Hatzfeld & Stitt 1990). Pyrophosphate:fructose-6-phosphate phosphotransferase (EC 2.7.1.90) has been suggested to catalyse the recycling of cytosolic triose phosphates back to hexose phosphates, which occurs at a rate that is considerably larger than the net glycolytic flux (Hatzfeld & Stitt 1990). It was suggested that the OPPP cannot function in heterotrophic suspension cells which are maintained in medium containing glycerol as a unique carbon source because *sn*-glycerol-3-phosphate prevented the flowing back of carbon from triose phosphates to glucose-6-phosphate (Aubert et al. 1994). The demonstration that inhibition of *Arabidopsis* seed germination by 200 mM glycerol was no more inhibitory than an isosmolar concentration of sorbitol (Table 4.10) indicates that in this system, the metabolism of carbohydrate and lipid reserves is likely to mask any negative effects of exogenous glycerol on glucose-6-phosphate availability and that the inhibitory effect of glycerol on radicle emergence is likely to be primarily an osmotic effect. At lower concentrations (25 mM, 50 mM and 100 mM), glycerol was not found to significantly inhibit *Arabidopsis* seed germination (data not shown). These results indicate that the imbibition of seeds in the presence of glycerol is not a useful approach towards substantiating a significant role for the OPPP in stimulating seed germination.

Regarding the speculation which continues to surround the apparent importance of the OPPP in seed germination, it may be worth noting that examination of changes in the levels of reduction of pyridine nucleotides, C_6/C_1 ratios and the specific activities of the two dehydrogenases which participate in the oxidative portion of the OPPP (Botha et al. 1992) has been at the expense of assessment of the involvement of the intermediates in the nonoxidative segment of the pathway. The nonoxidative reactions of the OPPP involve the sugar interconversion reactions catalysed by phosphoribose isomerase, phosphopentose epimerase, transaldolase and transketolase (Figure 2.14). Fairly recently, flux through the nonoxidative portion of the OPPP has been implicated in the regulation of a number of genes in animal systems which require the hexokinase (HXK)-mediated phosphorylation of glucose. Xylulose-5-phosphate induces the expression of genes encoding pyruvate kinase (Doiron et al. 1996), glucose-6-phosphatase (Massilon et al. 1998), fatty acid synthase and malic enzyme (Hillgartner & Charron 1998), and represses the expression of a gene encoding phospho*enol*pyruvate carboxykinase (Massilon et al. 1998). Xylulose-5-phosphate and ribose-5-phosphate are substrates for transketolase, which limits flux through the nonoxidative branch of the OPPP in human erythrocytes (Berthon et al. 1992). As in plants (Section 2.4.5.2; Jang et al. 1997), the role of HXK as a sugar sensor in mammalian systems is well documented (Smeekens & Rook 1997).

The emerging view that in animal cells, the effects of hexose abundance on gene expression arise not only through the perception of flux through the HXK-catalysed phosphorylation of

glucose but also through an increase in the concentration of xylulose-5-phosphate, suggests that future studies should consider whether pentose phosphate availability might also alter gene expression patterns in plants. It is generally accepted that plants possess several mechanisms of sensing sugar availability which are independent of HXK action (Jang & Sheen 1997; Smeekens & Rook 1997). It is also worth mentioning that previous studies to elucidate the effects of hexoses on plant gene expression appear to have considered only glycolytic intermediates and have disregarded the possible involvement of flux through the OPPP in mediating the transduction of a signal related to hexose abundance to the nuclear transcriptional apparatus (Jang & Sheen 1997).

The notion of a pentose sensing mechanism in plants is particularly attractive since it may enable monitoring of the availability of pentose-, hexose- and triose-phosphates and thereby permit the integration of carbohydrate status with environmental and other endogenous cues which regulate plant growth and development. In view of the limitations of explanations based exclusively on the regulation of redox potential and/or the provision of pentoses in accounting for an important role for the OPPP in triggering germination (Roberts 1973), it is tempting to speculate that increased carbon flux through the OPPP may activate a signalling cascade which coordinates the changes in gene expression which result in the emergence of the embryo through the seed coat. A signalling role for flux through the OPPP might account for the ability of electron acceptors to act synergistically with, or partly substitute for, the action of phytochrome in the breaking of seed dormancy in many species (Hilhorst and Karssen 1988; Giba et al. 1994). Signals triggered by changes in the cellular redox state associated with altered flux through the OPPP might interact with events downstream of the activation of phytochrome through the initiation of a common cascade of events that results in radicle emergence. The view that the light-mediated activation of phytochrome may owe many of its actions to a signalling pathway(s) that is sensitive to the cellular redox potential has previously been suggested to occur in autotrophic tissues, where a signal which transduces the level of reduction of the plastoquinone pool probably interacts with phytochrome signal transduction in regulating the expression of genes involved in photosynthesis (Mustilli and Bowler 1997).

Interestingly, a potent stimulatory effect of xylulose on the germination of *Striga hermonthica* seeds was reported almost fifty years ago (Brown et al. 1949). The demonstration that both pea meal and wheat germ possess high levels of xylulokinase activity (Zahnley & Axelrod 1965) suggests that *Striga* seeds most probably possess the ability to phosphorylate exogenous xylulose to xylulose-5-phosphate. Irrespective of this speculation regarding a mechanism by which increased OPPP activity may be an important cue for germination to occur, the demonstration that levels of exogenous proline capable of feedback inhibiting P5CS activity can retard *Arabidopsis*

seed germination is consistent with the view that if the OPPP does play a regulatory role in controlling germination, then the coupling of proline synthesis with OPPP activity may regulate radicle emergence in *Arabidopsis*.

5.3.2 The effects of proline on *Arabidopsis* seedling growth and development

It seems feasible to propose that the inhibitory effects of exogenous proline on root and hypocotyl growth in *Arabidopsis* seedlings (Figure 4.19) may also arise from a disruption of OPPP activity. In maize root tips, OPPP activity was estimated to consume 27% of the glucose entering the tissue, and 38% of the hexose phosphates metabolised in triose phosphate pathways (Dieuaide-Noubhani et al. 1995). A high rate of NADPH production by the OPPP is required for biosynthesis in rapidly growing tissues. As has been proposed above, high levels of exogenous proline capable of feedback inhibiting P5CS activity might block the synthesis of proline. If the $NADP^+$ generated by proline synthesis normally provides substrate for G6PDH and 6PGDH, then an increase in the level of reduction of the NADP pool might result in reduced flux through the OPPP.

Circumstantial evidence which supports a role for a high rate of proline synthesis in root growth has already been presented. It is difficult to discriminate between the role of proline synthesis in providing adequate levels of the imino acid for protein synthesis and the putative role of the process in regulating unrelated metabolic pathways through the modulation of $NADP^+$ availability. However, incorporation of an antisense copy of a partial cDNA encoding P5CS reduced root growth rates both in the absence and presence of salinity stress (Figure 4.14). The presence in the 5'-UTRs of proline biosynthetic genes of sequences with homology to promoter elements shown to confer a high level of gene expression in rapidly dividing cells (Table 4.3; Figure 4.3) has already been discussed (Section 5.1.2). High levels of transcript encoding P5CR have been noted in the vascular cambium in the flowering stems of mature *Arabidopsis* plants (Hare & Cress 1996). Furthermore, in ten d-old *Arabidopsis* seedlings, the *AtP5CR* promoter is particularly active in the root tips as well as in lateral root primordia (Hua et al. 1997). An involvement of high rates of proline synthesis in playing a special role in supporting root growth is also suggested by the observation that tobacco plants with elevated P5CS activity displayed vastly improved root length and an approximately 150% increase in dry root weight following growth in the presence of 0.5 M NaCl (Kavi Kishor et al. 1995). A role for activation of a hexokinase-mediated sugar sensing pathway in the regulation of photoassimilate partitioning between root and shoot tissues has recently been proposed to account for the improved root growth observed in transgenic lines which overexpress genes involved in the synthesis of other osmolytes such as mannitol, trehalose and fructans (Hare et al. 1998). As was mentioned in Section 2.2.2.3, the observations of

Sánchez-Fernández et al. (1997) that exogenous reductants stimulate *Arabidopsis* root growth, suggest an important role for redox potential in the modification of root growth in order to cope with environmental fluctuations. Since an inhibition of proline synthesis is likely to disrupt cellular redox homeostasis, an inhibition of root growth by artificially-induced elevations of cellular proline may reflect an oxidative stress. Improved root growth in transgenic plants which have an increased capacity for proline synthesis may result from a stimulatory effect of proline synthesis on pathways that generate NADPH. It is also interesting to note that Voetberg and Sharp (1991) reported a particularly high accumulation of proline in the apical few millimetres of maize roots grown at low water potentials. At a low water potential (-1.6 MPa), proline constituted approximately 70% of the free amino acid pool, compared with only 7% at a water potential of -0.03 MPa (Voetberg & Sharp 1991). The high level of proline in the maize root apex at low water potentials correlates with the observation by Sacks et al. (1997) that water stress reduces the rates of cell division within the meristem of the primary root of maize seedlings (Sacks et al. 1997). The largest stress-induced decreases in cell division rates were shown to occur towards the basal end of the meristem of the maize primary root. Sacks et al. (1997) proposed that the high level of proline accumulated in the maize root apex following the imposition of water deficit arose from transport of proline to the meristematic cells rather than an elevated rate of proline synthesis in the root tip.

Examination of the effects of exogenous proline on the lengths of the epidermal cells of *Arabidopsis* hypocotyls (Figure 4.23) strongly suggests that the inhibitory effects of exogenous proline on the growth of this tissue arise from an inhibition of cell elongation. Gendreau et al. (1997) have demonstrated that growth of the *Arabidopsis* hypocotyl either in the light or in the dark does not involve significant cortical or epidermal cell divisions. No significant difference was observed between the number of individual cells in a single cell file in embryos and in the hypocotyls of light-grown seedlings before and after the exponential phase of *Arabidopsis* hypocotyl growth (Gendreau et al. 1997). Regarding the inhibition of root growth by exogenous proline, simultaneous determination of the growth rates and sizes of the root cells from plants grown in the presence and absence of proline should indicate whether the effects of proline on the growth of this organ are likely to arise exclusively from a decrease in cell elongation or whether an exogenous proline also affects cell division rates. In the absence of exogenous proline and in the presence of less than 10 mM exogenous proline, *Arabidopsis* root growth was far more extensive in the light than in darkness (Figure 4.19). In most plant species, light inhibits root elongation (Kurata & Yamamoto 1997). These workers used *Arabidopsis phyA* and *phyB* mutants to demonstrate that the stimulative effect of light on *Arabidopsis* root growth is dependent on the actions of both of these phytochromes. It was proposed that the demonstration that DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], an inhibitor of photosynthetic electron transport,

completely inhibited the stimulation of root growth in the light indicated an essential role for the production of sugars in mediating this response (Kurata & Yamamoto 1997). Given the disruptive effect of DCMU on the level of reduction of the plastoquinone pool (Karpinski et al. 1997), and the likely overlap between phytochrome action and a redox-related signalling pathway controlled by the abundance of reduced plastoquinone (Figure 2.12; Mustilli & Bowler 1997), it is tempting to speculate that the findings of Kuarata et al. (1997) might further support the importance of redox potential in the regulation of *Arabidopsis* root growth.

Histochemical staining with phloroglucinol (Figure 4.24) suggests that the browning of hypocotyls from seedlings grown in the presence of exogenous proline both in the light (Figure 4.20) and in darkness (Figure 4.21) may be associated with the accumulation of monolignols. While the precise nature of the compounds responsible for the browning of the hypocotyls awaits identification, this suggestion would be consistent with the notion that flux through the proline biosynthetic pathway might stimulate OPPP activity and thus the synthesis of erythrose-4-phosphate, which is a precursor in the shikimate pathway (Section 2.5.2.2). Given the likely importance of MYB-type transcription factors in the transcriptional activation of proline synthesis (Table 4.1; Section 5.1.1) and the well-documented involvement of MYB-type transcription factors in the induction of many genes involved in phenylpropanoid synthesis (Grotewold et al. 1994; Sablowski et al. 1994; Uimari & Strommer 1997; Tamagnone et al. 1998), it is tempting to speculate that placing both classes of biosynthetic genes under a common regulatory mechanism would be an efficient means of coordinating proline synthesis with the synthesis of phenylpropanoids.

Clearly, a caveat associated with the interpretation of the effects of high concentrations of exogenous proline in stimulating phenylpropanoid synthesis through activation of the OPPP is that this is not consistent with the interpretation that following its uptake (Figure 4.26), exogenous proline is likely to elevate the cytosolic proline content to a concentration that would normally feedback inhibit P5CS activity and thus block cycling between proline and its precursors. An advance in the technology to quantify metabolic fluxes in extended networks may be necessary before it is possible evaluate the significance of a coupling between proline synthesis and OPPP activity and exactly how an increase an artificially-induced increase in cellular proline content might affect this interaction. The usefulness of two-dimensional representations of the relationships between metabolic pathways such as the one proposed in Figure 2.14 is limited by the inability to represent the quantitative contributions of different metabolic interconversions. Owing to the central role played by pyridine nucleotides in the coordination of different aspects of intermediary metabolism, it will be essential to quantify the relative rates of flux through both the OPPP and proline biosynthetic pathways before the contribution of proline synthesis to a stimulation of OPPP activity through $NADP^+$ regeneration can be assessed.

The importance of quantifying the relative significance of different metabolic reactions which contribute to the overall cellular redox potential is reflected by the apparent importance of redox cycling (Babiychuk et al. 1995) in ensuring metabolic homeostasis under adverse conditions. At first glance, the proposal by these workers that enzymes which oxidise NADPH can play an adaptive role in counteracting oxidative stress, might seem ironical if the cell is viewed as a "bag of soluble enzymes" which compete for a common pool of reduced pyridine nucleotide cofactors. However, it now appears that soluble enzymes which participate in the same metabolic pathway form "metabolons" or metabolic clusters of enzymes that enable the rapid channelling of intermediates from one enzyme to another (Srere 1987; Hrazdina & Jensen 1992). The physical associations of enzymes which act sequentially in a pathway both ensures that pathway intermediates need not be in equilibrium with identical molecules in the bulk medium. Since cells are limited by their solvent capacity (Atkinson 1977), there is likely to have been strong selection for mechanisms that facilitate high catalytic rates without placing a substantial osmotic burden on the cell. The direct transfer of pyridine nucleotide cofactors between certain enzymes, rather than their free diffusion throughout the cytoplasm may be of particular importance, since the reduced and oxidised forms participate in many different reactions. Obviously, some release of these intermediates into the aqueous phase is essential, since not all enzymes which use NADPH generated by the OPPP can be physically associated with the two dehydrogenases responsible for the reduction of $NADP^+$. Debnam et al. (1997) have recently presented evidence that intermediates of the OPPP are channelled in yeast, soybean and pea. The observation that the K_m values of purified ribulose-5-phosphate epimerase and transketolase from spinach chloroplasts are much higher than the chloroplastic concentrations of their respective substrates supports the notion that plastidic OPPP activity relies on substrate channelling (Teige et al. 1998). A fairly recently-developed tool in yeast genetics, the yeast two-hybrid system, provides a means of testing physical interactions between polypeptides encoded by cloned cDNAs (Phizicky & Fields 1995). Two-hybrid screening using either of the P5CS or P5CR cDNAs has significant potential for future investigations regarding whether or not the proline biosynthetic enzymes might be physically associated with this "metabolon" in which intermediates of the OPPP are preferentially channelled between consecutive enzymes of the OPPP.

While this is highly speculative, it seems possible that a negative effect of exogenous proline on cell elongation (Figure 4.23) may relate to the importance of NADPH oxidase activity in driving peroxidase-mediated cell wall tightening (Figure 2.3; Ogawa et al. 1997). Pugin et al. (1997) have demonstrated activation of NADPH oxidase activity by the OPPP. If the amounts of NADPH generated by the OPPP are not stoichiometrically identical to the levels of $NADP^+$ generated by the NADPH oxidase, then the generation of additional $NADP^+$ by proline synthesis might be important in maintaining redox homeostasis through ensuring that all NADPH-consuming

reactions are provided with an adequate amount of reductant. Lignin is a complex aromatic polymer derived mainly from the polymerisation of three different hydroxycinnamyl alcohols: *para*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Whetten & Sederoff 1995). A link between proline synthesis and xylogenesis (Roberts & Baba 1963; Bornman & Huber 1979; Hare & Cress 1996) is consistent with the involvement of proline synthesis in lignification, since lignin synthesis is one of the major processes associated with xylogenesis. If the increased histochemical reaction with phloroglucinol observed in seedlings grown in the presence of exogenous proline (Figure 4.24) reflects an increase in any of the three lignin precursors, then a link between proline synthesis and lignification may be multifaceted. Specific isoenzymes of cell wall-localised peroxidases are accepted to catalyse the oxidative dehydrogenation of monolignols in the final enzymatic step in lignification. Therefore, the induction of peroxidase activity by exogenous proline (Figure 4.25) is yet another possible explanation for the inhibition of exogenous proline on seed germination and seedling growth. The possibility that proline-mediated induction of peroxidase activity causes cell wall rigidification, and thus limits growth by cell expansion, will be discussed in Section 5.3.5.

5.3.3 The effects of proline on *in vitro* shoot organogenesis in *Arabidopsis*

In vitro shoot regeneration results from a series of cellular changes that cause the transition of a group of disorganised and undifferentiated callus cells to a form an shoot bud which comprises differentiated cells. An understanding the physiological processes that govern the totipotency of plant cells is important for the understanding of plant growth and development, as well as for the improvement of micropropagation, regeneration and other plant cell manipulations. Although much research has been undertaken to characterise the switch from unorganised to organised growth, the precise mechanism(s) which causes certain cells within a heterogenous tissue explant to complete a differentiation program are still poorly understood. The duration of this regeneration response, which usually spans at least several days, and the inability to predict which cells within a multicellular explants may undergo organogenesis, have retarded progress in elucidating the biochemical basis of shoot regeneration.

Cytokinins are considered to be key regulators of the formation of vegetative buds, both when they are provided *in vitro* (Skoog & Miller 1957) and when they are synthesised endogenously (Estruch et al. 1991). Thus, shoot regeneration is normally stimulated by an increase in the cytokinin:auxin ratio, while callus proliferation predominates when this ratio is decreased. It is assumed that the requirement for exogenous plant growth regulators in mediating this process reflects the involvement of these hormones in processes that normally play a role in *in vivo*

development. However, the observation that usually only a limited number of cells within a heterogenous tissue explant undergo a differentiation program (organogenesis or embryogenesis) even when the cytokinin:auxin ratio is optimal suggests that other factors may play important roles in determining the ability of callus cells to become organised into a shoot bud or embryo. Besides growth substances, the availability of nutrients and light are also likely to affect cell differentiation under *in vitro* conditions.

Several studies involving different plant species have found that inclusion of exogenous proline or proline analogues can increase the number of cells within an explant to undergo a developmental program that results in embryogenesis (Nuti-Ronchi et al. 1984; Armstrong & Green 1985; Trigiano & Conger 1987; Shetty & Asano 1991; Shetty & McKersie 1993; Murthy et al. 1996). Shetty et al. (1992) observed that inclusion of 10 mM proline in an hormonally-supplemented regeneration medium significantly increased the *in vitro* shoot organogenesis in *Cucumis melo*. A strong inhibition of bud regeneration was noted at concentrations in excess of 20 mM proline (Shetty et al. 1992). Examination of the effects of exogenous proline on shoot bud regeneration in *Arabidopsis* indicated that a similar stimulation of shoot organogenesis at lower concentrations (1 mM and 5 mM proline) and an inhibition of shoot bud formation in the presence of higher concentrations of exogenous proline (10 mM). Both the concentration at which stimulation of bud formation was optimal and the concentration which became inhibitory to *Arabidopsis* shoot organogenesis were lower than those observed for *Cucumis melo* (Shetty et al. 1992).

The results presented in Table 4.15 suggest that proline may couple the hormonal signals which trigger differentiation with the metabolic processes that support this developmental transition. Although little is known about the actual triggers of morphogenic processes *in vitro*, a high rate of carbon flux through the OPPP relative to glycolysis is considered to support an elevated rate of biosynthesis in an actively dividing or differentiating tissue. The suggestion by Shetty et al. (1992) that the stimulative effects of proline on *in vitro* shoot organogenesis in *Cucumis melo* might arise through accelerated flux through both the proline biosynthetic and degradative pathways, which in turn increases purine synthesis through activation of the OPPP and simultaneously provides reductant for mitochondrial respiration to support the energy requirements for differentiation, seems equally valid for interpretation of the effects of 1 mM and 5 mM proline on shoot regeneration in *Arabidopsis* (Table 4.15). The view that proline may merely be a source of reduced nitrogen does not seem valid, given that neither glutamate nor ornithine can mimic its effects on morphogenesis (Table 4.15). Although Shetty et al. (1992) did not suggest an explanation for the inhibitory effects of supraoptimal concentrations of proline on shoot bud formation, feedback inhibition of P5CS by proline (Hu et al. 1992; Zhang et al. 1995;

García-Ríos et al. 1997) is one explanation for this observation. This situation does not normally arise under adverse conditions when feedback inhibition of P5C synthesis by proline is lost (Boggess et al. 1976a). It is also tempting to speculate that since CKs are adenine-based growth regulators, and purine synthesis is likely to be affected by increased flux through the proline-linked OPPP, low levels of exogenous proline might increase the level of endogenous CKs. Nonetheless, there is little understanding of the mechanism by which CKs are synthesised and no conclusive evidence that adenine is incorporated into endogenous CKs (Prinsen et al. 1997). A promotive effect of chilling treatments on activation of the OPPP has already been mentioned in relation to the stimulation of the germination of the seeds of many species by stratification (Section 5.3.1). Márton & Browse (1991) reported that cold treatment (5 °C) of *Arabidopsis* plants used as the source of root explants promoted shoot regeneration by approximately three-fold. This may constitute indirect evidence of the significance of elevated OPPP activity in ensuring the transition of undifferentiated callus cells to differentiated shoots. This interpretation is consistent with the proposal that G6PDH activity is a reliable marker for determining the regenerability and recalcitrance of callus lines (Gahan et al. 1997).

The rationale behind examination of the effects of D-proline on shoot organogenesis was based on the observation that this isomer of the biologically active form of proline is a potent inducer of *AtPDH* gene expression in 28 d-old *Arabidopsis* plants as well as in cultured *Arabidopsis* cells (Kiyosue et al. 1996). The more stable expression of *AtPDH* which was induced by D-proline than its naturally-occurring isomer was suggested to arise from the inability of cells to metabolise D-proline. A slight, albeit not statistically significant increase in shoot organogenesis in the presence of 1 mM proline by supplementation with 0.1 mM D-proline may be consistent with the notion that simultaneous stimulation of proline biosynthetic as well as proline degradative capacity may be enhance the rate of flux through the proline biosynthetic and degradative pathways. The observation that increasing concentrations of exogenous proline in the shoot-inducing medium cause progressively greater reductions in the frequency of shoot bud regeneration may be consistent with a block in the cycling arising from feedback inhibition of P5CS. Thioproline, and to a lesser extent azetidine-2-carboxylate (AZC), are both inhibitors of PDH activity in etiolated barley shoots (Elthon & Stewart 1984). Inclusion of proline in the regeneration media overcame the inhibitory effects of both of these proline analogues in a manner reminiscent of the observations of Jost et al. (1988) that exogenous proline can overcome the inhibitory effect of AZC on differentiation of Leydig cells in the rat fetal testis. Unfortunately, it is difficult to distinguish between whether the inhibitory effect of AZC on shoot regeneration may not arise simply from its incorporation into proteins. Azetidine-2-carboxylate is toxic to most organisms due to the incorporation of AZC into proteins instead of proline. This results in an altered tertiary structure (Verbruggen et al. 1996b). However, AZC also inhibits the growth of *Escherichia coli* by

acting as a false feedback inhibitor of proline biosynthesis (Verbruggen et al. 1996b). The failure of thioproline to affect the incorporation of ^{14}C-labelled proline into protein (Elthon & Stewart 1984) suggests that this proline analogue is not incorporated into proteins.

5.3.4 The effects of proline on cellular ultrastructure in *Arabidopsis* leaves

The reduction in chlorophyll observed in 21 d-old *Arabidopsis* plants grown in the presence of exogenous proline (Figure 4.20; Figure 4.22) prompted investigation of whether or not this effect might be reflected at the ultrastructural level by a reduction in the number of grana in the chloroplasts. A negative effect of exogenous proline on cellular chlorophyll levels is consistent with the findings of others. Kiyosue et al. (1996) noted that green cultured *Arabidopsis* cells that were grown in the presence of 100 mM proline for 10 d, turned yellowish and seemed to be dead. Garcia et al. (1997) reported that millimolar concentrations of exogenous proline accentuated the loss of chlorophyll caused by incubating rice plants in 1% NaCl (approximately 170 mM) for 3 d. Proline had a more negative effect when included at 1 mM or 5 mM than when it was included at 10 mM (Garcia et al. 1997). The results presented in Figure 4.22 indicate that in *Arabidopsis*, prolonged exposure to exogenous proline has a dose-dependent negative effect on chlorophyll abundance in the absence of hyperosmotic stress.

Visualisation of the cellular ultrastructure of the leaves of 21 d-old *Arabidopsis* plantlets using transmission electron microscopy did not indicate an difference in the number of chloroplasts or in their size and shape. As is reflected in Figure 4.27, the chloroplasts of 21 d-old *Arabidopsis* plantlets grown in the presence of millimolar concentrations of exogenous proline do not contain substantially fewer grana, although evidence of disruption of thylakoid membrane integrity was consistently found in plantlets grown in the presence of proline. This was particularly evident at concentrations in excess of 5 mM proline (Figure 4.27). Chlorophyll loss and thylakoid lipid peroxidation are classic symptoms of photoinhibition. While it is not possible to provide an unambiguous explanation for the ultrastructural damage to chloroplasts following prolonged exposure to exogenous proline, the well-documented effects of free radical processes on membrane damage suggest that this may account for the disruption of thylakoid integrity in the chloroplasts of leaf mesophyll cells from *Arabidopsis* plants grown for 21 d in the presence of exogenous proline (Figure 4.27).

A model which might help to explain proline-mediated thylakoid membrane damage is presented in Figure 5.2. A simplified representation of photosynthetic electron transport is shown. For further details regarding our current understanding of photophosphorylation, the reader is referred to

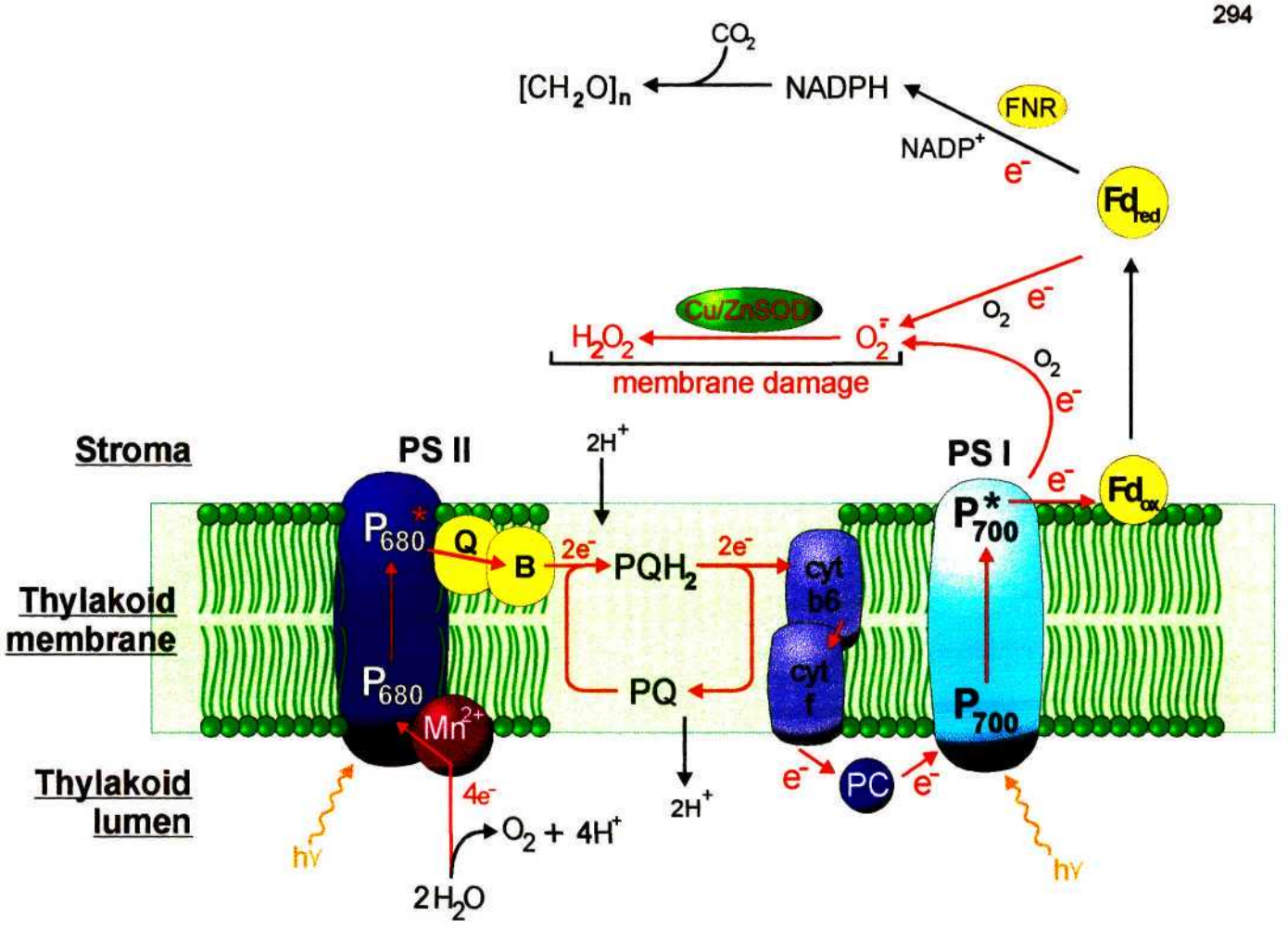

Figure 5.2: Sites of oxygen photoreduction in the photosynthetic electron transport chain. The site of water oxidation is considered to contain a cluster of four manganese atoms. Electrons (e^-) are passed from this manganese cluster to the primary electron donor (P_{680}) of photosystem II (PSII). Following excitation of P_{680} (conversion to P_{680}^*), one electron is passed from P_{680}^* to a bound quinone (Q), which passes its reducing equivalents to plastoquinone (PQ) via the B protein. It is believed that B accumulates two electrons before passing the electron pair onto PQ. When B accepts two electrons, it also takes up two protons from the stroma and both electrons and protons are passed along the electron transport chain when B is subsequently oxidised by plastoquinone. When plastoquinol (PQH_2) donates two electrons to the cytochrome b_6/cytochrome *f* (cyt *b*/*f*) complex, two protons are released into the thylakoid lumen. The light-driven accumulation of protons in the lumen drives ATP formation by the chloroplastic ATP synthase (CF_0-CF_1 complex), which is not shown here. Cytochrome *f* reduces plastocyanin (PC), which then reduces the P_{700} reaction centre donor chlorophyll of photosystem I (PSI). After excitation of P_{700}, the electrons are transferred individually to ferredoxin (Fd), which in turn normally reduces $NADP^+$ with the aid of stromal ferredoxin-NADP reductase (FNR). The NADPH thus generated is used for the assimilation of CO_2 for carbohydrate ($[CH_2O]_n$) production. Importantly, *if the availability of $NADP^+$ is limiting*, photoreductant can alternatively be used for the activation of molecular oxygen, which is found at a higher concentration in chloroplasts than in the surrounding atmosphere. In the presence of Cu/Zn-dependent superoxide dismutase (Cu/ZnSOD), $O_2^{\cdot-}$ may be converted to H_2O_2. Both $O_2^{\cdot-}$ and H_2O_2 are capable of destroying thylakoid membrane integrity. Direct interaction of O_2 with PSI is believed to make a far less significant contribution to $O_2^{\cdot-}$ production than the reaction of Fd_{red} with O_2 (Badger 1985).

The diagram is based on information presented by Badger (1985), Robinson (1988) and Prézelin & Nelson (1997).

Prézelin and Nelson (1997). Essentially, electrons derived from the oxidation of water are passed sequentially through the two photosystems to achieve the light-driven reduction of $NADP^+$ with the concomitant production of a proton gradient, which is used to generate ATP. The NADPH and ATP produced by electron transport and photophosphorylation are consumed during CO_2 assimilation by the reductive pentose phosphate (Calvin) cycle.

There is little doubt that appreciable rates of O_2 photoreduction, which is distinct from photorespiration, occur in intact leaves as well as in isolated chloroplasts and thylakoids (Smirnoff 1993). Oxygen photoreduction is largely dependent on whole chain electron transport and can thus be attributed to photosystem I (PSI). The rate of photoreduction under normal conditions has been estimated as 10-40 μmol O_2 mg^{-1} chlorophyll h^{-1}, which is of the order of 10% of total oxygen evolution (Badger 1985; Robinson 1988). The main mechanism of oxygen photoreduction associated with PSI is autoxidation of reduced ferredoxin, although direct autoxidation of the primary PSI electron acceptor can also occur (Figure 5.2; Badger 1985). The primary product of O_2 photoreduction by either of these mechanisms is $O_2^{\cdot-}$, which then has the possibility of forming H_2O_2 through superoxide dismutase action (Figure 5.2) and hydroxyl radicals by the Haber-Weiss reaction (Section 2.2.1). If the production of ROIs exceeds the capacity of the chloroplastic antioxidant apparatus for their destruction, this results in photooxidation of chloroplast pigments and to symptoms of chlorosis and necrosis.

Normally, continuous oxidation of NADPH by plastidic processes (e.g. the assimilation of CO_2, the reduction of nitrite to ammonia or the reduction of glutathione) regenerate sufficient $NADP^+$ to ensure a high efficiency of electron transfer to $NADP^+$ (Robinson 1988). Proline synthesis, which may occur in chloroplasts (Rayapati et al. 1989; Szoke et al. 1992) as well as in the cytosol, has been proposed to ameliorate the imbalance between light energy absorbed by the photosystems and the reduced capacity for energy use under stressful conditions (Section 2.5.2.1; Hare & Cress 1997). Chlorophyll loss and increased lipid peroxidation resulting from ROI-mediated damage are classic symptoms of photoinhibition. In the absence of stress, an increase the $NADPH/NADP^+$ ratio following feedback inhibition of proline synthesis by exogenous proline might increase rates of reaction of reduced ferredoxin with O_2 , thus causing increased rates of ROI production.

Chloroplasts have multiple repair and protection systems to deal with a high level of ROIs produced as a result of the high oxygen concentration that results from O_2 production by PSII. Besides the ascorbate-glutathione cycle (Figure 2.1), mechanisms that protect against photodamage implicate either increased dissipation of absorbed light energy as heat (Pfündel & Bilger 1994) or the diversion of assimilatory power to alternative electron sinks such as photorespiration (Kozaki & Takeba 1996). The dissipation of excess excitation energy as heat is

measured as non-photochemical quenching of chlorophyll fluorescence , and is correlated with the amounts of zeaxanthin and antheraxanthin formed from violaxanthin by the operation of the xanthophyll cycle. Although the de-epoxidation of violaxanthin to zeaxanthin is a very rapid response to photoinhibitory conditions, characterisation of two mutants of *Chlamydomonas reinhardtii* which are defective in the xanthophyll cycle indicated that excessive light did not impair their growth relative to WT controls (Niyogi et al. 1997). Thus, at least in this alga, processes in addition to the xanthophyll cycle are involved in the dissipation of excess absorbed energy and survival under photoinhibitory conditions (Niyogi et al. 1997). A similar conclusion was reached using *Arabidopsis aba1* mutants, which are deficient in zeaxanthin epoxidase activity (Hurry et al. 1997). Less rapid responses to photoinhibitory conditions, such as an increase in the rate of proline synthesis, may constitute additional mechanisms of permitting continued operation of the photosystems under adverse conditions by preventing over-reduction of the electron acceptor pools.

Unexpectedly, a disruption of mitochondrial ultrastructure was also noted in the mesophyll cells of leaves from *Arabidopsis* plantlets grown for 21 d in the presence of exogenous proline (Figure 4.28). It is also tempting to propose that a proline-mediated disruption of mitochondrial electron transport may account for the visible damage to the cristae of the mitochondria within these cells. In heterotrophic tissues, the respiratory chain of mitochondria is the most significant source of $O_2^{\cdot-}$ and H_2O_2 (Cadenas 1989). A simplified representation of mitochondrial electron transport is provided in Figure 5.3. As for photosynthetic electron transport, the electrochemical proton gradient generated across the inner mitochondrial membrane is used to drive ATP synthesis. Usually, an estimated 1-2% of oxygen in the mitochondrial matrix is subject to one-electron reduction (Boveris 1984), although Rich and Bonner (1978) estimated that the production of water by cytochrome *c* oxidase accounts for the consumption of only approximately 95% of the oxygen used by higher plant mitochondria. The mitochondrial respiratory electron transport chain produces $O_2^{\cdot-}$ at two sites: firstly at the flavoprotein NADH dehydrogenase and secondly, at the ubiquinone-cytochrome *b* region, most probably by autoxidation of ubisemiquinone (Rich & Bonner 1978). At pH 7.4, NADH dehydrogenase contributes about one-third and ubisemiquinone about two-thirds of the total $O_2^{\cdot-}$ production by mammalian mitochondria (Boveris 1984). The demonstration that there is a linear relationship between quinone content and H_2O_2 production when mitochondrial membranes depleted of endogenous ubiquinone are reconstituted with ubiquinone, confirms the production of ROIs at the ubiquinone-cytochrome *b* region of the respiratory electron transport chain (Purvis & Shewfelt 1993).

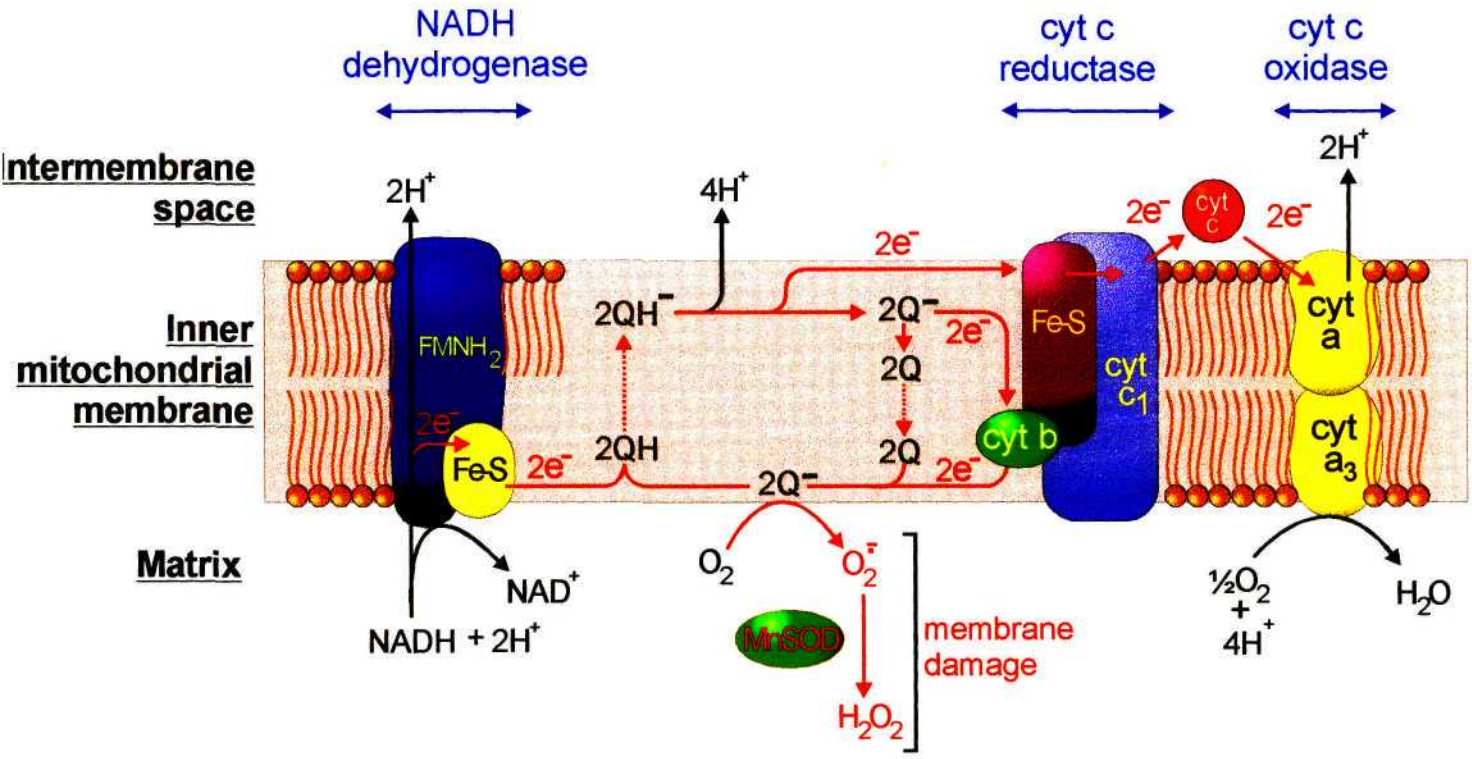

Figure 5.3: Elevated rates of mitochondrial electron transport are likely to cause oxidative stress. The transfer of electrons from NADH to the NADH dehydrogenase complex is coupled to the removal of two protons from the matrix, which are released into the intermembrane space upon donation of electrons to ubiquinone. Ubiquinones are closely associated with the cytochrome *c* reductase complex, which contains cytochromes *b* and c_1 and an iron-sulphur (Fe-S) centre, the Rieske protein. The mechanism of electron transfer, which is coupled to the translocation of four protons per electron pair from the matrix to the intermembrane space is poorly understood, although it is believed that a "Q cycle" permits proton translocation (Lambers 1997). In this process, electrons from the NADH dehydrogenase complex are donated to ubisemiquinone (Q^-). The reduction of Q^- to ubiquinol (QH_2) requires two protons per electron, which are taken up from the matrix. The ubiquinol then diffuses to the intermembrane side of the inner membrane, where one electron is donated to the Rieske protein, producing Q^-. Two protons are simultaneously released into the intermembrane space. Ubisemiquinone donates an electron to cytochrome *b*, forming ubiquinone (Q), which diffuses to the matrix side of the inner membrane where it accepts an electron from cytochrome *b* to regenerate Q^-. Thus, one electron traverses the electron transport chain and one cycles through cytochrome *b*. In the process, two protons are pumped for every electron passing through the cytochrome *c* reductase complex. The electron transferred to the Rieske protein is transferred sequentially to cytochrome c_1 and then to cytochrome *c*. The terminal oxidase of the cytochrome pathway, cytochrome *c* oxidase comprises cytochromes *a* and a_3. Cytochrome *a*, which faces the intermembrane space, accepts electrons from cytochrome *c*. The electrons are then donated to cytochrome a_3, which reacts with O_2. During this process, two additional protons are extruded to the intermembrane space. Succinate dehydrogenase, four rotenone-insensitive NAD(P)H dehydrogenases and the alternative oxidase, which are also present in the inner membrane of plant mitochondria, are not depicted. Importantly, a low level of autoxidation of ubiquinone is believed to normally generate $O_2^{\cdot-}$, which can be dismutated to H_2O_2 by Mn-dependent superoxide dismutase (MnSOD) found in the mitochondrial matrix. The direct reduction of oxygen to $O_2^{\cdot-}$ at the flavoprotein region of the NADH dehydrogenase segment of the respiratory chain (Rich & Bonner 1978) is not shown.

The diagram is based on information presented by Rich & Bonner (1978), Purvis & Shewfelt (1993) and Lambers (1997).

The ability of proline to stimulate oxygen uptake by mitochondria under *in vitro* conditions is well documented (Boggess & Koeppe 1978; Huang & Cavalieri 1979; Sells & Koeppe 1981; Elthon & Stewart 1981, 1982). Proline oxidation is believed to be responsible for transferring electrons into the first portion of the electron transport chain (Elthon & Stewart 1981, 1982). The ability of rotenone to inhibit the oxidation of both proline and P5C in maize mitochondria (Elthon & Stewart 1981) strongly suggests that electrons from these substrates enter the respiratory chain prior to at least one of the rotenone sensitive iron-sulphur proteins of the NADH dehydrogenase complex (Lambers 1997; Figure 5.3). Possibly, an elevated rate of mitochondrial electron transport in plants grown in the presence of exogenous proline may cause ubisemiquinone to be regenerated at a rate in excess of the rate at which it is re-oxidised by cytochrome *b*. The increased availability of ubisemiquinone might increase the rates of one electron reduction of O_2 to $O_2^{\cdot-}$, thereby increasing the "leakiness" of the mitochondrial electron transport chain. The induction of *AtPDH* transcript accumulation following exposure to exogenous proline (Kiyosue et al. 1996; Verbruggen et al. 1996a; Peng et al. 1996) further supports the possibility that a high level of exogenous proline might increase rates of mitochondrial electron transport and thus cause elevated levels of production of ROIs capable of causing subcellular damage. Although the effects of an artificial elevation of endogenous proline levels on mitochondrial ultrastructure in roots was not investigated, there seems no reason why an elevation in the rate of mitochondrial electron flow to a level in excess of the normal rate of electron transfer should not also apply for the mitochondria in the roots of seedlings grown in the presence of proline. A negative effect on proline on mitochondrial integrity might contribute to the decreased rate of root growth in the presence of exogenous proline (Figure 4.19).

Surprisingly, although several studies have presented convincing evidence for a protective role for molar concentrations of proline on protein structure and enzyme activity (Schobert & Tshesche 1978; Arakawa & Timasheff 1985; Wang & Bolen 1996; Samuel et al. 1997) and membrane integrity (Rudolph et al. 1986; Alia et al. 1991; Pardha Saradhi et al. 1995), this appears to be the first study to investigate the validity of the compatible solute paradigm *in vivo*, at least in terms of cellular ultrastructure. The limitations of the use of purely biochemical approaches towards assessment of the biophysical significance of proline accumulation during hyperosmotic stress have been reviewed in Section 2.1.1. The observed effects of prolonged exposure to proline on cellular ultrastructure are not consistent with the view of free proline as an inert, "compatible" solute. Gibon et al. (1997) have recently questioned the "compatibility" of another commonly studied osmolyte. These workers observed that rape leaf discs treated with glycine betaine displayed reduced viability, an attenuated capacity to synthesise proline but increased accumulation of soluble sugars upon osmotic upshock, inhibition of synthesis of the large subunit of RUBISCO and the induction or upregulation of polypeptides which were not responsive to

stress (Gibon et al. 1997). Together with evidence that exogenous proline inhibits growth and promotes chlorophyll loss in rice seedlings (Garcia et al. 1997), these data cast doubt on proposals that elevations in the levels of compatible solutes are without effects on cellular metabolism. It should nonetheless be noted that these negative consequences of exogenously applied osmolytes may arise from inappropriate compartmentation in the experimental systems used in these studies. These results do not disprove the compatible solute paradigm.

The concept of "compatibility" of osmolytes was first used by Brown and Simpson (1972) to highlight their observation that polyhydric alcohols were non-perturbing of metabolism at concentrations where Na^+ and Cl^- were disruptive. The fundamental tenet of the compatible solute paradigm is that osmolytes must stabilise macromolecular cell components during stress without affecting the functional activity of these molecules. The accumulating evidence which draws into question the frequently adopted generalisation that proline and glycine betaine are truly "compatible" introduces the important question of suitable nomenclature for these compounds. The use of transgenic strategies to increase levels of either proline or glycine betaine has indicated that protective effects associated with elevated rates of synthesis of these compounds are unlikely to be related to their roles as osmotica, since the levels accumulated are too small to enable osmotic adjustment (Hare et al. 1998). Thus, besides "compatible solute", the term "osmolyte" also appears to be unsuitable. Over the years, the ability to stabilise macromolecules not only under dehydrative stresses but also to confer thermal stability has given rise to the synonym of "osmoprotectant". Not only has the indiscriminate use of this term and the poorly defined concept of "osmoprotection" been criticised (Blum et al. 1996), but it not consistent with the assertion (Hare et al. 1998) that many of the protective functions associated with the accumulation of various osmolytes may be associated with their synthesis and/or degradation and not with their absolute levels *per se*. Although perhaps somewhat clumsy, the more neutral term "osmoresponsive solute" (defined as a small organic molecule which increases in concentration in response to a change in the external osmotic pressure) may be an adequate and descriptive name. It would be consistent with both of the views that (i) the molecule itself has limited intrinsic protective value at concentrations to which it normally accumulates and (ii) that protective effects might be mediated by metabolic effects associated with their accumulation (Hare et al. 1998). While it is still premature to completely dismiss the suitability of the terms "osmolyte" and "compatible solute", a better understanding of the mechanisms by which these compounds act may warrant such a change in the terms currently used to describe them.

5.3.5 The induction of peroxidase activity by exogenous proline

Peroxidases form a family of isoenzymes found in all higher plants. These heme-containing glycoproteins have been implicated in physiological processes as diverse as the removal of toxic hydroperoxides, the biosynthesis and degradation of lignin (Figure 2.3; Lagrimini et al. 1997a, 1997b; Ogawa et al. 1997), the cross-linking of cell wall structural proteins and polysaccharides (Fry 1986), defence against microbial or insect attack (Smith & Hammerschmidt 1988; Ye et al. 1990) and the catabolism of auxin (Hinnman & Lang 1965; Grambow & Langenbeck-Schwich 1983). However, owing to the low degree of specificity of these isoenzyme forms when assayed *in vitro*, it has been difficult to unequivocally establish the specific functions of individual peroxidases *in planta*. Ironically, despite the widespread use of peroxidases in genetic, physiological and pathological studies, they thus remain one of the least understood classes of plant enzymes (Lagrimini et al. 1997b).

The diversity of peroxidase isoforms in plants (up to 35 have been identified in certain species) arises by various post-translational modifications of a smaller number of gene products (Gaspar et al. 1982). Thus far, eleven cDNAs which encode distinct peroxidase isoforms in *Arabidopsis* have been cloned (Capelli et al. 1996). Besides the considerable overlap of activities between peroxidase isoenzymes and the lack of direct evidence of the natural products of individual peroxidase isoforms, studies of peroxidases have been further confounded by a poor understanding of the expression of different peroxidases both the subcellular level and in different tissues, as well as developmentally-regulated expression patterns of different peroxidases.

While only an approximately 50 -75% increase in peroxidase activity in response to proline treatment of *Arabidopsis* seedlings was observed when activity was determined using a spectrophotometric assay (Figure 4.25A), the increase in activity was apparently much greater on polyacrylamide gels (Figure 4.25B). This difference could be due to the variation in the ability of different peroxidase isoforms to hydrolyse particular substrates, or to the presence of inhibitors of peroxidase activity in plant extracts. Such putative inhibitors might be separated from the peroxidase isozymes upon electrophoresis. A proline-mediated induction of peroxidase activity in *Arabidopsis* is consistent with the demonstration by Chen and Kao (1995) that exogenous proline induced total peroxidase activity in rice seedlings. As will be discussed further in Section 5.3.6, it also provides some evidence for the proposal that proline itself might be a stress related signal that affects gene expression (Section 2.5.3; Hare & Cress 1997).

This section concerns how the ability of exogenous proline to increase peroxidase activity may also relate to the inhibitory effects of proline on germination as well as hypocotyl and root growth

in *Arabidopsis* seedlings, and both the stimulatory and inhibitory effects of different concentrations of proline on shoot organogenesis. Central to this discussion is the widely accepted view that peroxidases rigidify cell walls through the cross-linking of phenolic groups in the wall, such as those on structural proteins, lignin and pectins (Cosgrove 1997). In simple terms, the rate of cell expansion is a function of turgor pressure, yield threshold and wall extensibility. However, the biochemical processes that affect these parameters are not yet fully elucidated. When hormones modulate the rate of cell expansion, they usually do so by influencing cell wall properties (Cosgrove 1997). Schopfer (1996) has demonstrated that treatment of maize coleoptile walls with H_2O_2 inhibited elongation growth (*in vivo*) and decreased wall extensibility (*in vitro*). Inhibitors of peroxidase suppressed the wall-stiffening effects of H_2O_2 *in vitro* (Schopfer 1996). The view that proline-mediated induction of peroxidase activity might influence radicle emergence, seedling growth or the organisation of undifferentiated cells into shoot buds does not invalidate the proposals outlined above that implicate a primarily metabolic explanation for the effects of exogenous proline on their processes. The effects of proline on these processes may be multifaceted.

5.3.5.1 Peroxidase action may inhibit germination

Bewley (1997) has suggested radicle cell wall loosening may represent the ultimate block to the germination of the seeds of many species. The observation that ABA can frequently prevent radicle extension when it is applied even an hour or so before germination would be expected to occur supports the view that an ABA-mediated block of radicle cell wall loosening may be a critical event in radicle extension though the structures surrounding the embryo (Bewley 1997). Accordingly, Schopfer and Plachy (1985) observed that ABA inhibits radicle extension in *Brassica napus* embryos through an inhibition of cell wall loosening rather than by a disruption of the osmotic potential of the cells or their ability to take up water.

Thus far in this document, the regulation of seed germination has been considered primarily in terms of the metabolic changes which occur in the embryo. Nonetheless, there may be more than one cause for the dormancy of a given seed. Only in certain instances are the embryos themselves dormant. In the seeds of many species, a second category of dormancy, termed coat-imposed dormancy, arises because growth of the embryo is constrained by its capacity to penetrate the tissues which surround the embryo. The extent to which the germination of *Arabidopsis* seeds is regulated by a primarily metabolic restriction is not clear. However, there is some indication that the testa may normally restrict radicle emergence from a nondormant *Arabidopsis* embryo. Firstly, characterisation of the *ats* (aberrant testa shape) and *ttg* (transparent

testa glabrous) seed shape mutants has demonstrated that the testa contributes to the degree of dormancy exhibited by the seed (Léon-Kloosterziel et al. 1994). It was suggested that mutations which affect the structure of the testa enable easier penetration of the radicle through the testa because this structure is a mechanical barrier that normally limits radicle emergence (Léon-Kloosterziel et al. 1994). Furthermore, pricking *Arabidopsis* seeds improves their germination (Koornneeef & Karssen 1994).

In many species, the initial stages of germination involve cell elongation rather than cell division (Roberts 1973). The relative importances of cell division and cell elongation in radicle growth in *Arabidopsis* do not appear to have been investigated. Since the extensibility of the radicle cell walls is likely to be an important determinant of the ability of the radicle to elongate (Bewley 1997), it is tempting to speculate that a stimulative effect of exogenous proline on peroxidase activity may render these cells less capable of elongation, and might thus account at least partly for the effect of proline on delayed radicle emergence in *Arabidopsis* (Table 4.7). In this regard, it is of interest to note that although Lagrimini et al. (1997b) noted no gross differences in the growth rates and gross morphologies of mature tobacco plants which expressed an antisense RNA directed against an anionic peroxidase, transgenic seeds which overexpressed the same peroxidase displayed a substantially reduced rate of germination. Seeds of the antisense peroxidase lines germinated at a similar rate to WT tobacco seeds, although the cotyledons were more developed (Lagrimini et al. 1997b).

5.3.5.2 Peroxidases may account partly for the inhibition of vegetative growth under adverse environmental conditions

During vegetative growth, peroxidase-mediated stiffening of the cell wall is believed to result in a cessation of cell elongation (MacAdam et al. 1992a, 1992b; Sánchez et al. 1996). While the functional significance of proline accumulation during hyperosmotic stress has traditionally been viewed as a means of ensuring turgor maintenance, it has been emphasised (Section 2.5.2.2; Hare & Cress 1997) that the any beneficial effects of proline on stress tolerance may extend beyond a mere maintenance of osmotic potential, which would not be adequate to ensure continued growth during stress if metabolism is severely disrupted. Besides cell turgor, cell wall extensibility is an important determinant of the rate at which any plant cell enlarges. Moisture stress has been shown to inhibit growth by affecting cell wall plasticity, even when turgor is maintained at a high level (Michelena & Boyer 1982). Increases in lignification following the imposition of either biotic or abiotic stresses have already been discussed (Section 2.2.2.2; Section 2.5.2.2). It is believed that the deposition of lignin and suberin in cell walls may protect

plants from dehydration-induced damage both through maintenance of water status by acting as a water barrier and by preventing membrane collapse (Solecka1997). Changes in phenolic acid levels in the cell wall, particularly dehydrodiferulic acid, can modify wall extensibility (Sánchez et al. 1996) and may account at least partially for growth retardation under adverse conditions (Solecka 1997). Lignin monomers need to be methylated before polymerisation and it is believed that *S*-adenosyl-*L*-methionine (SAM) is the donor of methyl groups. Accordingly, SAM synthetase is preferentially expressed in tissues undergoing lignification under non-stressful conditions (Peleman et al. 1989) and transcripts encoding three isoforms of SAM synthetase accumulate in salt-stressed tomato (Espartero et al. 1994). The coordinate induction by fungal elicitors of SAM synthetase, *S*-adenosylmethionine: caffeic acid 3-*O*-methyltransferase and *S*-adenosyl-*L*-homocysteine hydrolase mRNAs strongly support the hypothesis of enhanced lignification in response to pathogen infection (Gowri et al. 1991; Kawalleck et al. 1992). The cross-linking of cell wall structural proteins following pathogen infection is driven by the accumulation of H_2O_2 produced by an oxidative burst and is mediated by peroxidase action (Brisson et al. 1994). An increase in the abundance of neutral and basic peroxidase isoforms in salt-adapted tomato suspension cell cultures correlated with a higher content of lignin-like compounds in the cell walls than in unadapted cells (Sancho et al. 1996). Levels of a transcript which encodes a peroxidase increased in the roots of salt-stressed tomato plants (Botella et al. 1994).

It thus seems feasible to suggest that the inhibition of growth associated with stressful conditions may arise, at least in part, from an induction of peroxidase activity associated with increased rates of proline biosynthesis. Chen and Kao (1995) have presented this argument for proline in mediating the inhibitory effects of cadmium on root growth of rice seedlings. It remains to be seen whether the selective induction of peroxidase activity by proline (Figure 4.25) can be mimicked by the imposition of osmotic stress, temperature extremes or other stresses capable of inducing proline accumulation in *Arabidopsis*.

5.3.5.3 The involvement of peroxidases in the regulation of morphogenesis

Since cell size and shape frequently underpins tissue form, the regulation of cell expansion is essential for all morphogenic processes in plants (Cosgrove 1997). While shoot regeneration from tissue cultures is not well understood at the biochemical level, alterations of the cell wall composition and structure may be important aspects of cellular differentiation and morphogenesis (Fry 1990; Lozovaya et al. 1996). Changes in the cell wall are critically involved in cytokinesis and cell plate formation, and thus affect the sizes and shapes of plant cells. *In vitro* regeneration provides an attractive experimental system for investigations regarding the early events in plant cell differentiation.

Organ initiation and development in tissue explants are believed to involve the promotion of meristematic activity and its maintenance in certain regions, with a concomitant suppression of meristematic activity in regions of "maturation" (Kay & Basile 1987). The lack of knowledge concerning the importance of changes in cell wall structure in contributing to these events has been addressed by fairly recent advances in the characterisation of a family of cell wall proteins named expansins. Eleven distinct expansin cDNAs have been characterised from *Arabidopsis* (Cosgrove 1997). The ability of expansins to modulate cell wall extension is believed to arise from their capacity to weaken the noncovalent bonds between cellulose and hemicellulose polymers, with the result that the polysaccharide network can move in response to the wall stress generated by cell turgor (Cosgrove 1997).

The recent demonstration that beads loaded with purified expansin protein can induce the ectopic formation of leaf primordia when placed onto the apical meristem of tomato plants (Fleming et al. 1997) is consistent with the view that localised cell wall expansion plays a crucial role in organ development. *In situ* hybridisation analysis indicated that expansin genes are expressed in the apical meristem and their transcripts are particularly abundant in cells which form a primordium bulge (Fleming et al. 1997). This confirms a role for endogenous expansin abundance in leaf initiation. Interestingly, Downes and Crowell (1998) have characterised a CK-inducible expansin gene from soybean. The induction of expansin activity by CK possibly represents the first molecular explanation for the effects of this poorly understood class of growth regulators on cell size and shape. It suggests that an increase in cell wall extensibility might govern the effects of CKs on various aspects of growth and development, including the stimulation of cell division and the promotion of shoot organogenesis (Downes & Crowell 1998).

Although these recent advances strongly indicate that cell wall components are important determinants of the capacity for morphogenesis, the extent to which cell wall stiffening by peroxidases may affect the capacity for *in vitro* shoot regeneration does not appear to have been investigated in detail. However, through analysis of isoperoxidase patterns and their correlation with histochemical changes in tobacco explants induced to form either callus, shoot buds or floral buds, Kay and Basile (1987) were able to correlate the abundances of 25 different isoperoxidases with specific developmental events (e.g. sustained cell division, lignification, suppression of growth and leaf development) that accompany organogenesis. Lozovaya et al. (1996) have presented evidence which suggests that alterations in phenolic compounds in the cell walls of callus tissues of several species correlate with the capability of the tissues to regenerate shoots (Lozovaya et al. 1996). Peroxidase-catalysed oxidative cross-linking of the phenolic side chains of cell wall polymers was suggested to reduce cell wall extensibility in callus lines with a poor capacity for regeneration. These reactions resemble those of lignin synthesis, although they occur

in non-lignified primary cell walls (Lozovaya et al. 1996). Accordingly, in petunia, an anionic peroxidase is induced upon lowering cytokinin to a level that prevents regeneration (Tournaire et al. 1996) and auxin strongly suppresses expression of a tobacco anionic peroxidase (Klotz & Lagrimini 1996). An anti-auxin, *p*-chlorophenoxyisobutyric acid, enhanced activity of the promoter of an anionic peroxidase above that of untreated controls or restored activity when used in combination with IAA (Klotz & Lagrimini 1996). This provides confirmation of a direct effect of auxin on the suppression of expression of this gene. The CK kinetin was shown to inhibit the ABA-mediated induction of a cell wall-localised basic peroxidase which was associated with both the ABA-induced formation of dormant bud structures (turions) as well as growth inhibition in the duckweed *Spirodela polyrrhiza* (Chaloupková & Smart 1994).

Given the well-documented involvement of exogenous proline on the stimulation of somatic embryogenesis in several species (Section 5.3.3), it is also worth noting that Joersbo et al. (1989) identified a peroxidase isoform that was present only in crude extracts of embryogenic cell suspension lines of carrot, and was virtually absent in a non-embryogenic cell line. The inhibition of somatic embryo development in carrot cell cultures by tunicamycin, an inhibitor of protein glycosylation, was shown to be partially overcome by the simultaneous addition of a cationic peroxidase to the culture medium (Cordewener et al. 1991). In this instance, a peroxidase-mediated *restriction* of cell size was proposed to be an important prerequisite for plant cell differentiation. Nonetheless, a concentration-dependent optimum of peroxidase activity capable of rescuing tunicamycin-mediated inhibition of somatic embryogenesis was noted (Cordewener et al. 1991). This parallels the inhibition of shoot organogenesis in *Arabidopsis* by supraoptimal concentrations of proline (Table 4.15).

5.3.5.4 Conclusion

Clearly, the demonstration that peroxidase activity is induced by proline offers several potential explanations for the observed effects of exogenous proline on the rates of radicle emergence, seedling growth and *in vitro* shoot organogenesis in *Arabidopsis*. Since peroxidases use H_2O_2 to create highly reactive free radicals, both substrates and products of peroxidase-catalysed reactions are phytotoxic. It is likely that the expression of total peroxidase activity is highly regulated, since the use and production of ROIs must be tightly controlled to prevent oxidative damage. Given the emerging view that regulated changes in levels of ROIs contribute to an efficient signalling mechanism which employs ROIs as small, diffusible messengers (Section 2.2.2; Section 2.4.5.4; Foyer et al. 1997; Alvarez et al. 1998; Hare et al. 1998), it is tempting to speculate that the effect of exogenous proline on the selective induction of peroxidase activity

may extend into regulation of ROI levels and thereby contribute to cellular signalling mechanisms that regulate gene expression patterns. Assessment of the validity of this proposal requires a better understanding of the signalling events that link increases in proline levels with the changes in plant gene expression.

5.3.6 The signalling capacity of the proline metabolic system

As was discussed in Section 2.4.5, a key factor in the genetic capacity for adaptation to environmental stresses is an appropriately responsive and coordinated signal transduction system. Following stress perception, the primary signal (e.g. turgor loss or a disruption in redox potential) must be amplified and processed to enable integration with other environmental cues and endogenous programs to enable a coordinated response reflected at the level of altered gene expression. Environmental cues are in all likelihood translated into physiological effects through a signalling network that operates against a background of other controlling influences including the plant's developmental state, nutrient status and the overall hormonal balance. Of particular importance in modification of the overall context of this signalling network is likely to be the ability of different signals to modify throughput of parallel cascades upstream of stress-inducible genes. A series of "checks" provided by feedback loops conceivably provides a means by which plants can ensure that the genetic response to stress is appropriate to the prevailing environmental conditions.

Evidence in support of a signalling role for the proline metabolic system in contributing to this network which controls stress-related gene expression has been reviewed in Section 2.5.3. Taken together, these findings are in full accordance with the proposal (Hare & Cress 1997; Hare et al. 1998) that a signal derived from the proline biosynthetic and catabolic pathways, possibly the redox potential of the pyridine nucleotide pools, may control selective gene expression in response to osmotic stress. Owing to the relative lack of understanding of how cellular redox potential may be perceived and how changes in the levels of reduction of the pyridine nucleotide pools might be translated into altered gene expression, it is not yet possible to predict with certainty the mechanism by which proline or proline analogues might affect redox-controlled gene expression. Models which implicate the thioredoxin system in the transcriptional or post-transcriptional regulation of gene expression (e.g. Figure 2.5; Section 2.2.2.3) seem attractive in this regard.

The demonstration that exogenous proline increased peroxidase activity in *Arabidopsis* (Figure 4.25) extends the findings of others (Chen & Kao 1995; Garcia et al. 1997; Iyer & Caplan 1998)

that proline or P5C either directly or indirectly affect plant gene expression. All previous studies which have investigated the effects of proline or proline analogues on gene expression appear to have been conducted using rice plants. Given the involvement of peroxidase activity in affecting the cellular redox state, the effects of proline on peroxidase expression seem consistent with the likely importance of regulated changes in proline metabolism in the buffering of cellular redox potential. Furthermore, this finding underscores the emerging trend that the regulatory capacity of metabolites at the level of metabolic interconversions is often parallelled by their signalling capacities (Section 2.4.5.2; Jang & Sheen 1997). The view that metabolic regulation and signalling are on a continuum offers an ideal mechanism for the coordination of cellular energy status with the regulation of gene expression. Elucidation of the mechanism(s) which underlies proline-mediated changes in gene expression is likely to provide considerable insight into the physiological importance of the sensitivity of proline metabolism to environmental changes.

While the signalling events responsible for a proline-mediated increase in peroxidase activity require further investigation, future studies in this regard may draw on what is known regarding the regulation of at least two plant genes shown to be induced by P5C or 3,4-dehydroproline (Iyer & Caplan 1998). The hormonal regulation of the *salT* and *RAB16* genes has been characterised in some detail and may offer some insight into how a signalling pathway which is triggered by flux through the proline metabolic system might affect plant gene expression. Owing to the diversity of peroxidase isoforms found in higher plants, it is impossible to make generalisations regarding the hormonal regulation of proline-responsive peroxidase isoforms. Induction of peroxidase isogene expression by ABA (Kwak et al. 1996; Lee & Lin 1996; Tsai et al. 1997), jasmonic acid (JA) or methyl jasmonate (Yeh et al. 1995; Lee & Lin 1996; Curtis et al. 1997; Moons et al. 1997; Tsai et al. 1997) and salicylic acid (Thulke & Conrath 1998) has been reported in various species. The hormonal regulation of peroxidase isoforms from *Arabidopsis* does not appear to have been investigated.

Levels of rice *salT* transcript increase not only after exposure to NaCl, drought, proline and P5C, but also after treatment with either JA or ABA. Since proline, P5C or 3,4-dehydroproline (DHP) do not appear to affect ABA levels in the sheaths of rice plants (Iyer & Caplan 1998), gene induction by a proline-related mechanism appears to be independent of an increase in the endogenous ABA concentration. Nonetheless, an effect of carbon flux through the proline biosynthetic pathway on tissue sensitivity to ABA may warrant further investigation. It was suggested in Section 2.5.3 that an increased level of $NADP^+$ associated with elevated rates of flux through the proline biosynthetic pathway may stimulate the synthesis of $NAADP^+$, which appears to be an intermediate in ABA signal transmission (Wu et al. 1997; Y Wu, Rockefeller University,

NY, personal communication). Irrespective of this speculation, Xin and Li (1993) found that none of the ABA-regulated proteins in maize suspension-cultured cells displayed detectable changes in cells treated with 100 mM proline for 12 h.

Interestingly, whereas ABA induces *salT* transcript accumulation in roots and most extensively in the sheath of rice seedlings through activation of the *salT* promoter, salT protein does not accumulate in root or shoot tissues of ABA-treated seedlings (Moons et al. 1997). In contrast to this ABA-mediated negative effect on *salT* expression at a post-transcriptional level, JA-induced *salT* transcript accumulation in shoots is associated with a dose-dependent increase in salT protein abundance (Moons et al. 1997). However, JA is not essential for NaCl-mediated induction of *salT* transcript accumulation, since salinity stress induced the *salT* transcript in roots even in the presence of inhibitors of JA synthesis (Moons et al. 1997). Exactly how a P5C-related signal might interfere with the effects of ABA and JA on *salT* transcript abundance is uncertain. Nonetheless, it is worth noting that induction of *salT* transcript by DHP or P5C in sheaths of rice plants requires at least 24 h exposure (Iyer & Caplan 1998), whereas in roots of rice seedlings, ABA- and JA-induced *salT* transcript accumulation can be detected within 4 h and reaches high levels after 8 h (Moons et al. 1997). Given the ABA-independence of post-transcriptional regulation of *salT* expression, the effects of P5C on salT protein levels warrants investigation in the future.

It may be of some significance to note that treatment of rice plants with 1 mM P5C and DHP increased levels of salicylic acid (SA) in blades of rice plants by almost six- and approximately 12-fold respectively (Iyer & Caplan 1998). An involvement of SA in redox-regulated gene responses to adverse biotic and abiotic stresses is well documented (Section 2.4.5.4). Induction by SA of an anionic peroxidase gene from parsley has been reported (Thulke & Conrath 1998). However, Moons et al. (1997) found that *salT* expression is not responsive to treatment with either SA or ethylene. Therefore, if a common mechanism mediates the induction of gene expression by proline or proline analogues, this does not appear to operate via SA action.

Besides *salT*, another P5C-induced gene which has been studied in some detail is *RAB16*. The responsiveness of rice *RAB16* to ABA application is well-documented (Ono et al. 1996). The involvement of mitogen-activated protein kinase (MAPK) cascades in plant stress responses has been discussed elsewhere (Section 2.4.5.4; Hare et al. 1997; Meskiene et al. 1998). In particular, it was emphasised that MAPK cascades have considerable capacity for signal amplification and that they are known to integrate multiple signals transmitted by various second messengers. In barley aleurone protoplasts, the tyrosine phosphatase inhibitor phenylarsine oxide (PAO) can completely block the activation of both a MAPK as well as *RAB16* expression by ABA (Knetsch

et al. 1996). This led to the conclusion that ABA-mediated induction of *RAB16* transcript accumulation requires activation of a MAPK *via* tyrosine phosphatase action. Future studies might therefore consider whether tyrosine phosphatase and MAPK activities may act downstream of the signal proposed to arise from proline synthesis and degradation (Figure 5.4A). An involvement of proline or P5C in stimulating protein tyrosine phosphatase action is confounded by the demonstration that all mammalian protein tyrosine phosphatases possess a reactive cysteine in their active site, which must be in the reduced form for catalytic activity (Staal et al. 1994). The involvement of NADPH oxidase action in increasing protein tyrosine phosphorylation in human neutrophils has been reported (Fialkow et al. 1993). This increase might arise either from activation of protein tyrosine kinases, the inhibition of protein tyrosine phosphatases or a combination of both processes. Redox regulation of plant tyrosine phosphatases does not appear to have been investigated. However, PAO inhibits mammalian tyrosine phosphatases through its reaction with the conserved cysteine residue found in the active site of these enzymes (Staal et al. 1994). Based on the simple models presented in Figure 5.4, one would anticipate that if P5C increases carbon flux through the proline biosynthetic pathway through the stimulation of a cycle involving proline synthesis and degradation, then the associated decrease in the level of reduction of the NADP pool would inhibit, rather than stimulate, tyrosine dephosphorylation in target proteins.

Obviously, the possibility that the P5C-mediated induction of *RAB16* transcript accumulation in rice plants (Iyer & Caplan 1998) may arise through a mechanism distinct from events downstream of ABA perception cannot be excluded. It should also be noted that although the time course and concentration dependence of MAPK activation by ABA are consistent with a role for MAPK activation in the induction of barley *RAB16* accumulation, an absolute requirement for MAPK action in this process has not been established (Figure 5.4B). Sensitivity of P5C-responsive rice *RAB16* transcript accumulation to PAO has not been investigated. Despite the evidence that ABA regulates proline synthesis (Section 2.4.3), the rapidity of MAPK activation argues strongly against a model in which ABA-elicited accumulation of *RAB16* transcript is mediated through an effect related to proline synthesis. Activation of an ABA-inducible MAPK occurred within less than 1 minute after ABA treatment (Knetsch et al. 1996), whereas strong induction of *AtP5CS1* gene expression by spraying *Arabidopsis* plants with 1 mM ABA was reached only after 2 h, with a maximum level of induction being reached only after 5 h (Yoshiba et al. 1995). Induction of *AtP5CS2* transcript accumulation by ABA appears to be even less rapid (Strizhov et al. 1997). The demonstration that sugars which can be phosphorylated by hexokinase have no effect on *RAB16* expression in barley embryos (Perata et al. 1997) suggests that the P5C- and/or ABA-stimulated signalling pathway(s) that control the expression of the gene are unlikely to interact with signals downstream of the cellular sugar sensing apparatus in barley embryos. The

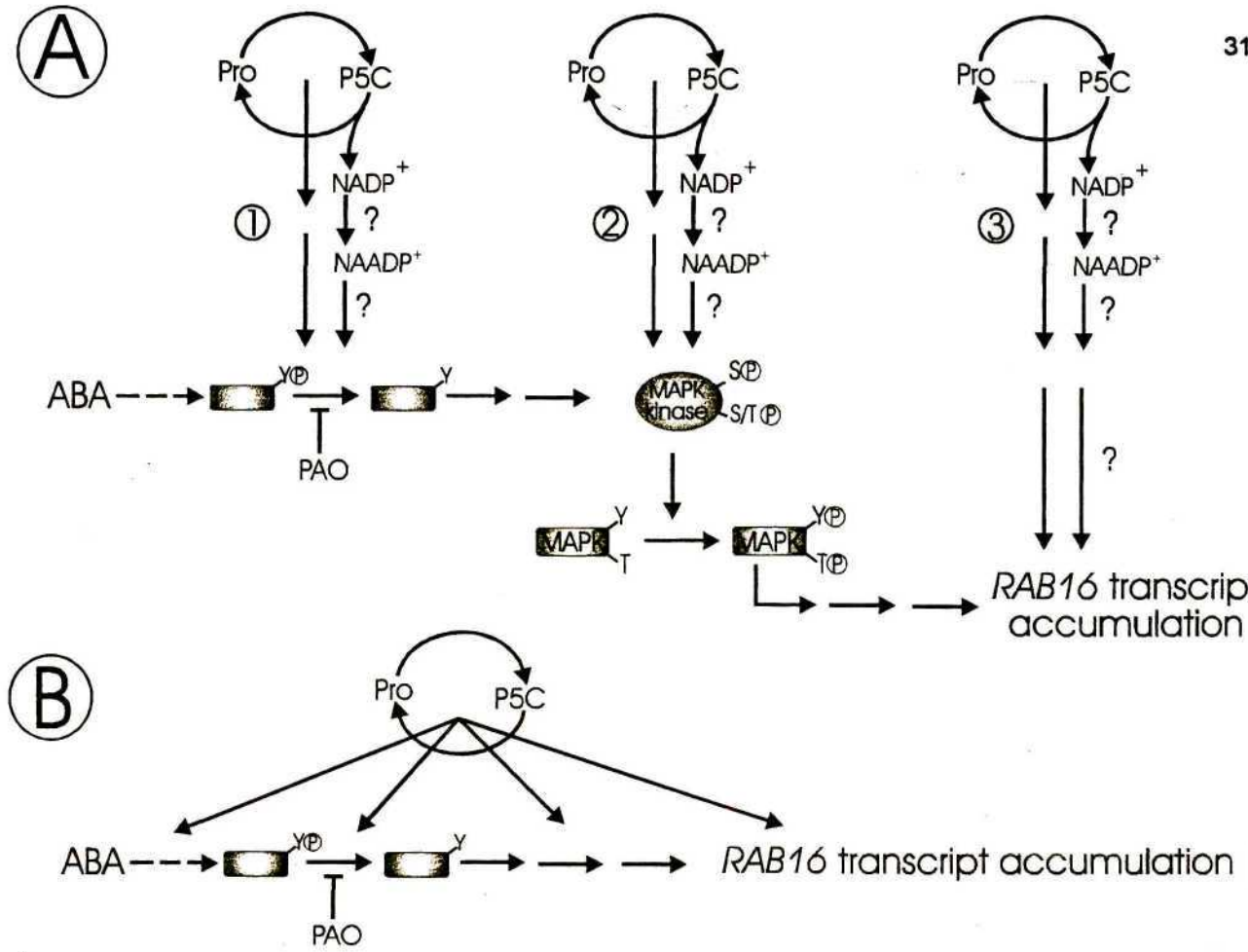

Figure 5.4. Possible interactions between P5C- and ABA-mediated induction of *RAB16* transcript accumulation. (A) Abscisic acid and a signal derived from elevated P5C levels (Iyer & Caplan 1998) may both act at a point upstream of MAPK induction (①, ②), or both signalling routes may be independent (③). **(B)** The actions of ABA or a P5C-related signal on *RAB16* transcript levels may be independent of MAPK activation, although the P5C-related signal may or may not affect ABA-mediated activation of a phenylarsine oxide (PAO)-sensitive protein tyrosine phosphatase upstream of *RAB16* transcript accumulation (Knetsch et al. 1996). It is proposed that P5C-induced increases in gene expression may arise from an increase in $NAADP^+$ levels generated from increased $NADP^+$ concentrations resulting from activation of flux through the proline biosynthetic pathway (Section 2.5.3). Not indicated is the potential involvement of phosphatidic acid in propagating the ABA signal that leads to RAB16 protein accumulation in barley aleurone cells (Ritchie & Gilroy 1998).

cycloheximide-independence of *RAB16* induction by P5C was not investigated by Iyer and Caplan (1998). Controversy surrounds whether ABA-mediated induction of *RAB16* expression in rice is sensitive to inhibition of protein synthesis (Nakagawa et al. 1996). However, the identification of an ABA-, NaCl- and dehydration-inducible rice bZIP protein, OSBZ8, which binds to the ABRE in the rice *RAB16* promoter (Nakagawa et al. 1996) may open the way for further characterisation of the intriguing involvement of proline metabolism in the regulation of stress-inducible genes. Owing to the multitude of peroxidase isoforms in plants, the use of *salT* and *RAB16* as model genes to elucidate the signalling events triggered by an increase in the levels of free proline, P5C or proline analogues may be useful in understanding the selective induction of peroxidase activity following incubation in exogenous proline (Figure 4.25; Chen & Kao 1995).

6. CONCLUSION

Within the limits of this study, it has not been possible to provide conclusive answers to many of the questions posed at the outset. In attempting to present a synthesis of the most significant findings of this study in the context of recent advances made by others working in this field, at least three overall trends can be identified.

Firstly, considerable circumstantial evidence suggests that changes in proline biosynthetic and degradative capacity, but not necessarily increases in absolute proline levels, may constitute an early and efficient stress response in *Arabidopsis*. The identification of several putative stress-regulated promoter elements in the 5'-UTRs of genes which encode enzymes involved in proline synthesis corroborates the findings of others (Table 2.1) that these genes are specifically induced under conditions of hyperosmotic stress. The possibility that some increase in the activities of the constitutively present proline biosynthetic enzymes may arise from increased cofactor availability under stress cannot be dismissed. However, the specific induction of their cognate genes by adverse environmental conditions discredits the notion that increased proline synthesis may arise merely from a stress-induced derangement of metabolism. The quantitative significance of the proposed cycle between proline and its precursors in the absence of stress, and the likely uncoupling of proline synthesis and degradation during stress, clearly requires further critical examination. The statement by Kacser (1987) which has been reproduced in the Preface of this document seems particularly appropriate to resolution of the full extent to which proline synthesis may act as a redox sink under conditions when the energy assimilating capacity of the plant is limited. An advance in the technology to measure flux through metabolic cycles may be necessary before this point can be truly resolved. Nonetheless, it may be comforting to note that despite the intensive efforts of many workers over the past four decades, conclusive evidence that proline accumulation mediates osmotic adjustment in higher plants has never been obtained (Munns 1993; Hare et al. 1998). Nor has any intrinsic biophysical value of proline been demonstrated, at least at the levels to which it is normally accumulated in stressed plants. The evidence obtained in this study, together with other recent advances made using both plant (Iyer & Caplan 1998; Kwok & Shetty 1998) and animal (Downs et al. 1998) systems, is consistent with the original proposal (Hare & Cress 1997) that the metabolic implications of stress-induced proline accumulation may be more important than the absolute levels of the imino acid *per se*.

Secondly, examination of the effects of exogenous proline on various aspects of growth and development in *Arabidopsis* are inconsistent with the classical view of proline as a metabolically inert "compatible" solute. The view that carbon flux through the proline biosynthetic and

degradative pathways is an important determinant of the adaptive capacity of plants to stress provides a promising solution to the long-standing controversy surrounding whether or not the free proline level is a reliable indicator of stress tolerance in breeding programs (Table 2.3). It may also account for the observation that substantial levels of free proline usually accumulate only long after symptoms of stress-induced damage are evident (Moftah & Michel 1987). When stress is severe and/or imposed rapidly, the redox regulating capacity of the proline metabolic cycle may be overworked. Proline accumulation might then merely be a symptom of uncoupling between the biosynthetic and degradative components of the cycle proposed in Figure 2.14. Since substrate cycles constitute extremely sensitive flux control systems, the modification of pathways which are capable of accommodating large fluxes in the absence of stress appears to be a recurrent theme in the evolution of stress tolerance mechanisms (Hare et al. 1997).

Thirdly, the proline metabolic system apparently has considerable signalling potential. Increasing evidence now indicates that ubiquitous metabolites, the regulatory capacities of which were long perceived to be limited to their roles as modulators of enzyme activities, also affect gene expression. Metabolic flux, and not the level of the metabolite *per se*, appears to be the primary trigger of these signalling pathways (Jang & Sheen 1997). The signalling capacities of metabolic fluxes enable integration of developmental programs and environmental cues with the availability of energy and its allocation to processes needed for growth and development. The selective induction of peroxidase activity by proline mirrors the induction of a number of stress-responsive genes by either proline or P5C (Iyer & Caplan 1998). Although the precise nature of the signal(s) triggered by proline and/or P5C awaits resolution, cellular redox status, most probably the level of reduction of the NADP pool, seems a likely candidate (Hare et al. 1998). The role of peroxidases in redox-related cellular functions substantiates this view. If any of the putative redox-responsive promoter elements identified in the three proline biosynthetic genes examined in this study are functional, this would suggest that proline synthesis from glutamate may constitute an autoregulatory system. In other words, under conditions of an excess availability of reductant, proline synthesis would be upregulated at the level of gene expression. Once redox homeostasis has been regained through the oxidation of NADPH that accompanies proline synthesis, the expression of genes which encode proline biosynthetic enzymes would once again return to a basal level required for "housekeeping" functions. It seems possible that a signalling cascade activated by stress-regulated changes in proline synthesis and/or degradation may modify the throughput of parallel cascades upstream of at least certain stress-inducible genes. If so, this would provide a "internal" regulatory mechanism of continuously ensuring that the genetic response to stress is appropriate to the prevailing environmental conditions.

It is hoped that the ideas and findings presented in this document will contribute a platform of information that can progressively be built upon in order to either confirm or discount the primary hypotheses which have been outlined here. Besides the urgent need for an effective approach to quantify metabolic fluxes in extended networks, further assessment of the regulatory roles played by proline metabolism is likely to require a better understanding of proline degradation, the relative contributions of glutamate and ornithine to the P5C pool, as well as the inter- and intracellular transport of proline. The apparent involvement of chloroplasts in proline synthesis (Rayapati et al. 1989; Szoke et al. 1992) remains to be clarified. Regarding the central role played by proline and/or P5C transport in mediating the regulatory effects of shifts in proline metabolism, it is worth emphasising that amino acid transporters may hold the greatest potential for tight control of the interconversions between proline and its precursors in separate subcellular compartments. The classical view that the first enzymatic step in a pathway (P5CS in the case of proline biosynthesis from glutamate) is rate-limiting and has the highest flux control coefficient disregards the fact that the first and last steps involved in the synthetic half of the proposed proline cycle in a single cell (Figure 2.14) involve transport into or out of the cytoplasm, while proline transport into the mitochondrion is a requisite for the degradative portion of the cycle. Unless plant cells have a constitutive capacity to compartmentalise proline, it's overproduction in the cytosol alone may be inadequate to confer maximal benefits to the stressed cell. The overexpression of a subcellular transporter protein(s) as well as the enzymes responsible for P5C production may be necessary. Transgenic plants will no doubt be invaluable in identification of which steps in the biosynthesis, transport and degradation of proline become limiting under adverse conditions. This would enable the bottleneck reactions(s) to be targeted in agriculturally important crops. The characterisation of the antisense plant lines generated in this study has opened the way for alternative approaches towards assessment of how stress tolerance may be affected in transgenic lines in which proline synthesis has been silenced.

Notwithstanding the difficulties associated with finding direct evidence in favour of a primarily metabolic role for the synthesis of proline, or of other osmolytes (Hare et al. 1998), a better understanding of the functional significance of osmolyte accumulation is likely to pay substantial rewards in the form of increased agricultural productivity. This may be of particular importance in developing countries where conditions are frequently suboptimal (Hare et al. 1996). The use of the proline metabolic system as a paradigm for elucidating stress-related signal transduction promises to offer a significant contribution to the ultimate goal of increasing plant stress tolerance under field conditions. The identification in this study of several promoter elements in the genes which encode enzymes involved in proline synthesis from glutamate may provide molecular tools for probing in reverse the signalling events that regulate proline accumulation. Yeo (1998) has argued that despite the enormous insight we have gained regarding the molecular biology of

solute synthesis and compartmentation, the potential of this knowledge to increase coordinated responses at the whole plant level is likely to be overestimated. Tolerance of hyperosmotic stresses most probably resides at a higher level of organisation. The manipulation of genes which encode "master switches" responsible for coordinating the coordinated induction and repression of entire groups of genes represents one approach to tackling this issue (Hare et al. 1996; Hare et al. 1998).

Irrespective of whether the ultimate target of substantial mitigation of stress effects will be realised by efforts to manipulate proline metabolism, there can be little doubt that continued work in this area will not only be mandatory for the identification of sensible strategies to improve crop productivity, but also enable appreciation of how plants coordinate growth and development in a constantly changing environment. A central thrust of this study has been to attempt an integrated approach to resolving the enigmatic accumulation of free proline during stress. With the increasing realisation that merely having all of the genes that regulate a process in hand will not necessarily explain their function *in vivo*, it is hoped that future workers in this area of study will increasingly employ a complementary range of biochemical, physiological and genetic approaches in their efforts.

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APPENDIX

Nucleotide sequences of the 5'-untranslated regions of *Arabidopsis* genes involved in proline synthesis from glutamate

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-2567  CTCCACGTAA ACAAAAAAAC TACGCAAAAA ATCGAAAAAG TAGTTTTTTC
-2517  TCTCTCTCTT TTTCACACTC ATTATATCTA TAACCCACAT ATTCTAACAA
-2467  ATACTAGTAG CATTTACGAA TCAAGATTCT CCAACATAAA ATGTATTTCT
-2417  CACTTACTTC TCCATTATCG TTTTATGTAC CCTTTTTTTT ACGTACCAAT
-2367  CAAAATCGCA TCTTTCTTTT CAAATATAAT TATGTATATA TACGCATGTG
-2317  CAGCTGCTGC GTGTCTATGC ATACTTGAGT CTAAATAACT ATCCCTGACT
-2267  ATGATATGTC GTTGGTTACG AATGTGATCT TTAGTTAAAA TAACAAGAAT
-2217  AATATCACAC AGACAAAAAC AAATAAGTGC ATATTATTTC AACAAGATTG
-2167  GAGTGGGTGG GCCTAAAGGC TTAAAAAAAT AGAGCACATG CACAGAGGAC
-2117  CATTGATTCC CCAGAGACAA TCAGACATCT GAGACCCTAA TCGCATCAAG
-2067  CCGCGTGCCC TTCTTCCATT AACTAATTTT GTGTGTTTGT TTTGGCTTAA
-2017  ACCTGAGAAT TACATTGATT ACTTTATTTG TTTCATTTTC TCCATGAAGG
-1967  AGATAAAAAG AGTAAAAATT AGAGATTGAT GAAAACTGAA AAAGAAACTG
-1916  AAGTCGGTAA GATAGGTGTT GGAACTTGGA ATAATGGCTT GGCTTTGAAC
-1867  AAAACGCATG CACCATTCAT TGCCTTCAAG TTTTTTGCAA TTAGCTTTGT
-1817  TTTTGTTTTT GTTTTTGTTT TTGGGAGAGG TTCTAATGAC CAAGAATCAA
-1767  GAGCGTTGTC TAAAATCTAA ACCATATGAT ACGGTTTTTA ATATTCTCAT
-1717  GCATTAAAAT GTACTATTTC TATATATGAT CTTATATACC CAACATCTTG
-1667  GAATTAATAG TTTGATTCGT TATCATTTGA AGAAGCTCTC AACAGCTTCA
-1617  AAAAGCGAAA TGTAGCATCA TGAAGCGGTA TCCAATTTCA AGAAGCTACC
-1567  AGTAGCTTGT GGAAGTTTTC AAGAAGTTTT CCGGAAGATC CAACGATTGT
-1517  GGAAGCCCCT CATAGTTTTT GGAAGTTTTC AATGATATTA GCAGCGTTGA
-1467  GCGTGGCATG GCTAGACAAT GTAAGAGATT TGATTTGCAA CACATTTGAT
-1417  GTATTTTTTT ACTTTTGAGT TACAATTGTA ATGTATTATT GATTTTGCCC
-1367  AGTTATGATT TATAAACCCT ACAATTTAGT ATCAAAGTTT TTATTTAAAA
-1317  TTCTGAATCT GACATTAATG ATATCTGGCT CATTTACAGA GCCAATGAGA
-1267  TGGATGATGT TCGAAACTGG ATTGGCCATT ATTTATCTTT TTTTTATCTG
-1217  GAGAATCTCG AGGTTGGCAC AAACATTATC ATATTAGCCT TTAGAAATTG
-1167  GATTGGCTAA TCACACATTT ATATATATTC TTACCAAAAT AAATCACCTC
-1117  TCCCGTAATT GAAAAATATC TAAATACTGT AAGTCTGAAA AAATTCACAA
-1067  GGGTTCGAAG AAAGAAGGAA ATATCTAAGC ATCATTAATA AACTATCTGT
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continued...

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-1017   AACCTGAGGG AAAATCATTT CATGTTGAAA TATGTGGATT TGGAAGTTTT
-967    ATAATCTATC TGAATTTGTG AAATTTGATA ACAAGTAAGA TTTGTTTCTT
-917    AACACAAATC TAAAATTTGT TTTCTAATTA GGTTTGAGAG AGAGAGAGAA
-867    AGAAACGCTT TGTATGATAC ACATCTAGGC TATGAATGAA GGCAGCGGAC
-817    AAAGCGGTCT AATTTGTCTG CGGTTTAGTC CATCTCATTT TTGGGGTGGA
-767    CAATAAACCG CTGCGGACCA AGTTTATTTG TATGTAAAAA CGGTCCGCAG
-717    ATGGTCCGCA ACGATTTTCT TCTATTTTTT TAAGTCCAGA CCACTGCGGA
-667    CTATAATTGA TGAATGATAA ATAAAAAACG GTCTGATCCG TTGACGGTTT
-617    TGTCCGCCCC AACCGCCATA ACCATTCAAA CCCCTAATTA TTTCATCAGA
-567    TAACATTATA CACTAATAAT CATTGCACTC AAATATGTCA CACAATCATA
-517    TAATAAAATA ATAACAATGA TTAAAATGAA AAAATTGTTG TGGCGCCGCA
-467    TAAAATAGAA ATCGTGAGAG ACGACGTCAT CTAAAAATTG CCTTGCTGTC
-417    CACTTTTCAC TTTGTCCTCT CTTCTCATCT CCGTTCACTT CCACGGCGTT
-367    TCCTCAGCCG CCGATTTTAT TTATTTCCCA AAATACCCAT CACCTATAGC
-317    GCCACAATCC TCTACATCAC ACCCTAATCT CATTACCATA CACCACCCAA
-267    CGAACACGCG CCACTTCATT TGTTAGTATC TAAAATACCA AACCTACCCT
-217    TAGTTCCACA CGTGGCGTTT CCTGGTTTGA TAACAGAGCC TGAGTCTCTG
-167    GTGTCGCTGG TGTTTATAAA CCCCTTCATA TCTTCCTTGG TGATCTCCAC
-117    CTTTCCCTCA CCTGATATTT ATTTTCTTAC CTTAAATACG ACGGTGCTTC
-67     ACTGAGTCCG ACTCAGTTAA CTCGTTCCTC TCTCTGTGTG TGGTTTTGGT
-17     AGACGACGAC GACGATA

1       ATG
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The 5'-UTR of the *AtP5CS1* gene (Genbank Accession No. AC003000). The sequence shown is the complement of the region between nucleotides 45470 and 48040 in the sequence of the *A. thaliana* (ecotype Columbia) genomic DNA (chromosome 2) in BAC T5I7 (submitted to Genbank by S.D. Rounsley, X. Lin, K.A. Ketchum, M.L. Crosby, R.C. Brandon, S.M. Sykes, S. Kaul, T.M. Mason, A.R. Kerlavage , M.D. Adams, C.R. Somerville and J.C. Venter, The Institute for Genomic Research, Rockville, MD). The numbers indicated denote nucleotide position upstream from the start codon. The transcription initiation site has not yet been reported. Nucleotides at positions -72, -98 and -106 highlighted in bold indicate the respective first nucleotides in the mRNAs characterised by Strizhov et al. (1997) [Genbank Accession No. X86777], Savouré et al. (1995) [Genbank Accession No. X87330] and Yoshiba et al. (1995) [Genbank accession No. D32138]. Using these nucleotides as a guide, a putative TATA box is shown in bold and underlined. The start codon is shown in italics.

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-1344   TTTAAATGGA CCCTTGGACC TTTAAAAAGA TTTATAAATA AAAAACTTTG
-1294   GGAGGCAACT GACCTTTTAG TGATTACCGT GGGATCTACC AATGACTATA
-1244   GAAAATTAAA ATTAAGAATA AACTGATGAA AACAAAAGAC AAAACACAAG
-1194   TGCTCGTCTC TAAATACACT GAAATTCTCC ATATCTATAA AATAAACATT
-1144   TCCTTGTAAA TATATGGACG ATTGTTATTA TTACTACATA GAGAAAATGT
-1094   CACATATTGT GTCAAAATCA TATGAAATTT GGACTTAACG ATAACAAATT
-1044   AAAAACTAAT GATGATAAAG AAATGTTGTG GCGGCGCGTA TGAATTAGGA
-994    ATATATTGAT AAGTTCCATG GAGATATTGC CAAGTAAACT TGCTTTGTTG
-944    TCCAAAAACC TTTGTGTTGT TCACGTTCAC GAAGTTCTCT CAGCCGCCAA
-894    TTTTTAAATT ACCCAAAATA CCCCACCACC ATAGAGTTAA TCCACTGCGC
-844    CACACCCTGA TTCTTTTAGC CTGCACCACC ACACGTCATC ACGTGGCGCC
-794    ATGTGGTTGG AAATATTTAG TTAAAAACAT GAATAATAAT GTAAAGTAAA
-744    CCTAAGTATT TTAAAAAAGG TTCCTACCCA AAAAAAAAAG TATTTTATAA
-694    AAGGTTCAGA AAAAATACAA AATGAATCTT AAATTATAAC TATTTTACAA
-644    TTCGGCCATA TATTTGTTAA AACTTCTAAC CTTAGCAAAT TCTACTGAGT
-594    AAAGTGCAAA TGATTTCACA AAGAATTAAA AACACTGTGG GGCACAAATT
-544    TTAGTGTTGA AATTTGTGCT ATTGCGATGA GTTTGGGAGT TTGGGATACT
-494    AATAATAATT AAGGTATATA ATAAACACTT TACAGACAAA AAGTATCAAA
-444    TAAATACCCA CAACAGATTT CTGATACATT TCTATATAGG CACAATACCA
-394    TATACATTAT AATCTGTGTA TTTTAGTCAT AAGTAACAAA AAATAAATTG
-344    ATTACATGGT AGTTAGCAAT CATTTGCTAA GAACAACCGA CAACAAAATA
-294    ATCACACAAG ACAAAATGCA GACAATTCTC AAATCCACAA AAAAAAAAAC
-244    TATAGACGTT ATCTATTGAT AATTGGAATC TTTAACATAA CCCACTATGA
-194    TTAGGACTTA AATGTTAGTT TTAACAACTT GGGTATTATT ACTTGATGTT
-144    TACAAAGATT AAGGACTTAT TTGACTATTT TGACAAGTTG TCCTACGCTC
                           ▼
-94     TTAAAAACCC GTTCGCTTCC TCACAGGAGA ATCTCTCCAC ACCGGATTAT
-44     CCAATATTCT CCACGTCCGC TTCTTCCCTT CAACTGAGTC CGAC

+1      TAAGTTGACT CGTTCTCTCG TGTTTTCGAT AGACATAAAA TATG
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The 5'-UTR of the *AtP5CS2* gene (Zhang et al. 1997). The numbers indicated denote nucleotide position upstream from the second (downstream) transcription initiation site (Zhang et al. 1997). The upstream transcription start site (nucleotide position -76) is indicated by a filled triangle (▾). Nucleotides at positions -34 and -60 highlighted in bold indicate the respective first nucleotides in the mRNAs characterised by Strizhov et al. (1997) [Genbank Accession No. Y09355] and Zhang et al. (1997). A putative TATA box (Zhang et al. 1997) is shown in bold and underlined. The start codon is shown in italics.

-922 GATCCGATTC AATCGCTTCT CGATGAGCTC CGCCATCTTA TCAACGGTCG
-872 ACATACTCCA ACCGACGCTC CCGAAGCTCT TTTCTCCCTG AAAAAGCACG
-822 TTCAACTAAG TTATCATACT TTCCACTCAT CTTCTCAACC TCGGCTCAAG
-772 ATTTCGTCTA GATCGGGCAC CGCTTGAGCA ATCTTGCCTC TCAAACAGCG
-722 AGCTCAGCAC AAACACCAGA GTTCAAAGCA AAAGGAACGC CTCCTTCAAA
-672 CGAAACGATA ACTCCAATAA TGATCATTGG CACGCAAAAC GCATCGGACG
-622 AGTTCGGAAC GCTAGCTCGT TGTTCATCAC GATTAACGAC CCTCGTCTCT
-572 TCCCTCTCCC CCTCAAAGAA CCACTTTCGA ATGACTCGAA CATCCCAACT
-522 CGAACACCAG ACGACCACTT TGATCCAGGA GGCTCTTCAG AAATGCGACG
-472 GTCACGCAAC AGCCGAGGCG ACCCTTTTAA CCTTCCTTGT CGTCTCGCTT
-422 GACTATCACC GAGACAGCAC ATTGCCGTAA TAGCAGCCTA TACTTTGATA
-372 GCCACGCGAC GTTTCGATCC TCGAACCTTG AGAATCATGA AAGACAAACC
-322 AAACATTGCG TCTTCCCCAT GCGCGACTTT TCACTTCCTT CGATTGAGTT
-272 TCGTGCACCT GAAATCCTGA TCCACGTCAT CAACGATGCA TCAGGATTGG
-222 GGGGCAACTG TTAGGGGTCG GATTGGGCCT ACTTGGCCCA ATGCCCAAAC
-172 CCAAGTCAAA AGCCCAAAAT AAGTTCCAAA CCAAGGAGTA TTAAGGCGAA
-122 GGGTAGTTTC GTCAAATCCC TCGATGAATG CCCTACACGC GCAGAAGGCT
-72 **ATAAATA**CGG AATAGCATGT CTTGTCAAAG GGGGAGGCCG CGAGGACACT
-22 TAGAGACAAA GAGCAGCTCG TC

+1 AGCTCACCCG TCTGAAGTAT CTCGCCAGCC GGCCCGTCTC AAGCAGCTCG
+51 TCAGCTCACC CGTCTGAAGC ATCTCGTCAG CCGGTCCGTC TCAAGTAGCT
+101 CATCAGCTCG CCCGTCTCAA GCAGCTCGTC AGCTCACCCG TCCGAAGTAT
+151 CTCGCCAGCC GGCCCGTTAA ATTTTCCCGA *TG*

The 5'-UTR of the *AtP5CR* gene (Verbruggen et al. 1993) [Genbank Accession No. M76538]. The numbers indicated denote nucleotide position upstream from the transcription initiation site (Verbruggen et al. 1993). Sequencing of an *AtP5CR* mRNA confirmed the primer extension experiment used to identify this transcriptional start site (Verbruggen et al. 1993). A 69 bp region implicated in the tissue-specific expression of *AtP5CR* in *Arabidopsis* seedlings (between -212 and -143 bp; Hua et al. 1997) is underlined. A putative TATA box (Genbank Accession No. M76538) is shown in bold and underlined. The start codon is shown in italics.