THE EFFECT OF ELEVATED GLUTATHIONE REDUCTASE AND SUPEROXIDE DISMUTASE ACTIVITIES IN STRESSED TRANSGENIC TOBACCO

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, from February 1994 to November 1996, under the supervision of Professor Alan M. Amory.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

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ABSTRACT

Life as we know it would be impossible in the absence of oxygen. However, too much oxygen can be toxic to the aerobic organisms which depend on it for their very existence. This apparent paradox arises as a result of oxygen's ability to accept electrons, forming highly reactive (reduced) oxygen species such as superoxide, hydrogen peroxide and the hydroxyl radical. The toxicity of oxygen is greatly enhanced in illuminated plants, due to the photosynthetic reactions which produce both oxygen and highly energetic electrons in close proximity to one another. These problems are further exacerbated when plants are exposed to a variety of stress conditions, since these conditions reduce the ability of plants to utilise excess electrons. As a result of the danger posed by these reactive oxygen species, plants have evolved a complex antioxidant system for their scavenging.

Research has shown that plants with naturally elevated levels of the components of the antioxidant system are better equipped to deal with stress conditions which enhance the production of reactive oxygen species. A considerable amount of research has thus been dedicated to the elucidation of the antioxidant system. Almost as much research has been dedicated to enhancing the antioxidant system, with the aim of improving plant productivity under stress conditions.

This study sought to evaluate plants carrying elevated levels of two of the enzymes of the antioxidant system. For these purposes, tobacco was transformed with the gene for *E. coli* glutathione reductase (GR), an enzyme believed to catalyse the rate limiting reaction in the scavenging of hydrogen peroxide. This gene was fused to the gene for the RUBISCO small subunit transit peptide - a peptide capable of targeting proteins to the chloroplast. Due to the presence of this peptide the transformed plants exhibited high chloroplastic levels of GR activity. These plants were crossed with a second tobacco transformant carrying high levels of chloroplastic tomato superoxide dismutase (SOD) - an enzyme responsible for the scavenging of superoxide. These hybrid plants were shown to exhibit high GR and SOD activities in the chloroplast - the subcellular compartment most susceptible to damage caused by reactive oxygen species.

The transgenic hybrids were evaluated for their ability to tolerate oxidative stress by treating them with paraquat - a herbicide whose mode of action involves the production of large quantities of activated oxygen. Under stress conditions, plants carrying just *E. coli* GR showed a slight improvement in their ability to deal with oxidative stress. In contrast to this, the SOD transformants showed more cellular damage than untransformed control plants. This was attributed to the inability of other enzymes in the antioxidant pathway to deal with the increased flow of metabolites through the pathway. The hybrid transformants showed enhanced stress tolerance in the initial stages of oxidative stress, but this declined with ongoing exposure to stress conditions. As with the SOD transformants, this decline in protection was ascribed to the relatively low activities of the other enzymes in the antioxidant

pathway. It was concluded that elevated levels of the two enzymes conferred greater stress tolerance than just one of the enzymes, but for true stress tolerance it will be necessary to evaluate the antioxidant system and enhance the activity of further enzymes in the pathway. It may also be necessary to improve the regulation of transgene expression, ensuring that none of the enzymes are overwhelmed by the increased flow of metabolites through the system.

TABLE OF CONTENTS

	Page
PREFACE	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	iii
TABLE OF CONTENTS	v
LIST OF FIGURES	x
LIST OF TABLES	xii
ABBREVIATIONS	xiii
PROLOGUE	1

1

THE PRODUCTION AND CHARACTERISATION OF TRANSGENIC *NICOTIANA TABACUM* CARRYING THE *E. COLI* GLUTATHIONE REDUCTASE GENE

1.1 INTRODUCTION	4
1.1.1 Transformation systems	5
1.1.1.1 *Agrobacterium tumefaciens* mediated gene transfer	5
Agrobacterium tumefaciens	5
Ti Plasmid	5
T-DNA	6
Virulence region	6
The mechanism of gene transfer	6
Signal perception and induction	6
Production of the T-strand	7
Production and transfer of the T-complex	7
Integration of the T-strand into the plant genome	8
Vectors for *Agrobacterium* mediated gene transfer	9
Cointegrative vectors	9
Binary vector systems	9
Selection marker genes	10
Gene expression	10
Promoters	10
Termination sequences	11
1.1.2 Physical transformation of plant cells	11
1.1.2.1 Direct gene transfer	11
Chemically mediated gene transfer	11
Liposome fusion	12
Electroporation	12
1.1.2.2 DNA injection techniques	12

1.1.2.3 Biolistics 12

1.1.3 Applications for recombinant DNA technology 13

1.2 MATERIALS AND METHODS 14

1.2.1 *Agrobacterium tumefaciens* 14

1.2.1.1 Bacterial strain and the plasmid 14

1.2.1.2 Growth and maintenance of *Agrobacterium tumefaciens* 14

1.2.1.3 Determination of the growth kinetics of the bacterial population 15

1.2.2 Plant material for transformation 16

1.2.2.1 Germination of seedlings 16

1.2.2.2 Plant tissue culture 16

1.2.3 Transformation of tobacco with *E. coli* glutathione reductase 16

1.2.3.1 Explants 16

1.2.3.2 Infection and co-culture 16

1.2.3.3 Curing and selection of transgenic lines 17

1.2.3.4 Hardening off 17

1.2.4 GR activity in transformed shoots 17

1.2.5 Preparation of acetone powders 18

1.2.6 Western Blotting 18

1.2.6.1 Sample preparation 18

1.2.6.2 Determination of protein concentrations 18

1.2.6.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) 18

1.2.6.4 Electrophoretic blotting 19

1.2.6.5 Immunological detection of proteins 19

1.2.7 Production of F1 plants 20

1.2.8 Characterisation of *E. coli* GR 20

1.2.8.1 Isolation and purification of tobacco chloroplasts 20

1.2.8.2 Preparation of chloroplast samples for electrophoresis 20

1.2.8.3 Preparation of whole leaf samples for electrophoresis 20

1.2.8.4 Western blots 21

1.2.8.5 SOD activity gels 21

1.2.8.6 GR activity gels 21
Casting and running starch gels 21
Staining starch gels for GR activity 22
Running and staining non-denaturing polyacrylamide gels 22

1.2.8.7 TEM localisation of *E. coli* GR using immunogold labelling 22
Plant material 22
TEM using epoxy resin 23
Fixation of tissues 23
Dehydration and embedding 23
Immunolabelling 23
Two step labelling protocol 23
One step labelling protocol 24
TEM using acrylic resin 24
Fixation of tissues 24
Dehydration and embedding 24
Immunolabelling 25
Viewing 25

1.3 RESULTS AND DISCUSSION 26

1.3.1 Bacterial growth and cell counts 26

1.3.2 Transformation 27

1.3.2.1 Shoot formation on selection plates 27

1.3.2.2 Screening for transformed shoots 31

1.3.2.3 Hardening off of shoots 34

1.3.3 Identification and characterisation of *gor* gene transformants 34

1.3.3.1 Glutathione reductase activity in possible transformants and F1 plants 35
Possible transformants 35
F1 plants 35
Type of gene introduced 37
Positional effects 37
DNA methylation 37
Tissue-specific factors 38

1.3.3.2 Confirmation of transformation using western blots 38

1.3.3.3 Distribution of GRA between plant and bacterial GR 42

1.3.3.4 Subcellular localisation of *E. coli* GR 43
Western blots of isolated chloroplasts 43
TEM localisation of *E. coli* GR 45

2

THE RESPONSE OF TRANSGENIC TOBACCO TO OXIDATIVE STRESS

2.1 INTRODUCTION 52

2.1.1 The reactivity of oxygen 53

2.1.1.1 Reactive oxygen intermediates 54

2.1.2 Increased production of activated oxygen under stress conditions 56

2.1.3 Antioxidant defences 58

2.1.3.1 Antioxidant molecules 58
Carotenoids 58
Vitamin E (α-tocopherol) 58
Ascorbate and glutathione 59

2.1.3.2 Antioxidant enzymes 59
The Mehler-peroxidase cycle 59
The ascorbate-glutathione cycle 61

2.1.4 A new role for the superoxide radical 62

2.1.5 The engineering of increased oxidative stress tolerance 63

2.2 MATERIALS AND METHODS 66

2.2.1 Plant material 66

2.2.1.1 Plant types 66

2.2.1.2 Source of transgenic plants 66

2.2.1.3 Germination and maintenance of plants 66

2.2.2 Selection and characterisation of plant types 67

2.2.2.1 *E. coli* GR transformants 67
GR activity 67
Western blotting 67

2.2.2.2 Tomato Cu,Zn SOD transformants 67

2.2.3 Oxidative stress 67

2.2.4 Enzyme assays 67

2.2.4.1 Sample preparation 67

2.2.4.2 GR assays 68

2.2.4.3 SOD assays 68

2.2.5 Stress indicators 69

2.2.5.1 Solute leakage 69

2.2.5.2 Chlorophyll assays 69

2.3 RESULTS AND DISCUSSION 70

2.3.1 Characterisation of transgenic plants 70

2.3.1.1 Chloroplastic *E. coli* GR transformants 70

2.3.1.2 Plants carrying tomato Cu,Zn SOD 70

2.3.1.3 Tomato SOD / *E. coli* GR hybrids 73

2.3.1.4 Cytoplasmic *E. coli* GR transformants 74

2.3.2 Response of transgenic tobacco to oxidative stress 77

2.3.2.1 The induction of oxidative stress in experimental plants 78

2.3.2.2 The response of tobacco plants to oxidative stress 79
Untransformed plants 79
E. coli GR transformants 83
Chloroplastic tomato Cu,Zn SOD transformants 88
Chloroplastic tomato Cu,Zn SOD / *E. coli* GR hybrids 91

CONCLUDING REMARKS 96

REFERENCES 98

LIST OF FIGURES

Figure number		Page
1.1	The Structure of plasmid pBIN19*gor*/T2	15
1.2	Growth kinetics of *Agrobacterium tumefaciens* strain LBA4404	26
1.3	Callus and shoot formation on selection plates containing MS medium supplemented with either 0, 50, 100 or 300 $\mu g\ ml^{-1}$ kanamycin.	29
1.4	Selection of transformed shoots on rooting medium (½ MS) containing 100 $\mu g\ ml^{-1}$ kanamycin	33
1.5	Preparation of transformed shoots for hardening off	34
1.6	Variation in the GRA of F1 transgenic plants	36
1.7	Immunodetection of *E. coli* GR in transgenic tobacco plants	39
1.8	Optimisation of the western blotting procedure	40
1.9	Optimised western blots for F1 transgenics	41
1.10	Relative GR activities of the bacterial and plant forms of GR	42
1.11	SOD activity gel for the determination of the purity of isolated chloroplasts	43
1.12	Immunodetection of *E. coli* GR protein in isolated chloroplasts using western blotting	44
1.13	Localisation of *E. coli* GR in the chloroplasts of transformed tobacco	45
1.14	Enhancement of TEM resolution with a post-labelling OsO_4 treatment in an immunogold labelling protocol	48
1.15	Gold labelling in control sections of tobacco leaves	50
2.1	The electronic structure of oxygen and its derivatives	53
2.2	The antioxidant system of higher plant chloroplasts	60
2.3A	SOD activity gels for leaf extracts of tomato Cu,Zn SOD transformants	71
2.3B	SOD activity gels for extracts from isolated chloroplasts of tobacco plants carrying tomato Cu,Zn SOD in the cytoplasm	72
2.4	SOD activity gels for leaf extracts from hybrid transformants	73
2.5	SOD activity gel for extracts of chloroplasts isolated from a cytosolic *E. coli* GR transformant	75
2.6	Optimisation of the western blotting procedure	76

2.7	Western blots showing the presence of *E. coli* GR in transformed tobacco	77
2.8	The response of GR activity in control plants following paraquat treatment	79
2.9	SOD activity in paraquat-treated control plants	80
2.10	Chlorophyll levels in control plants subjected to paraquat treatment	81
2.11	Electrolyte leakage from leaf discs of control plants treated with paraquat	82
2.12	The response of GR to paraquat in cytoplasmic and chloroplastic *E. coli* GR transformants	84
2.13	SOD activity in cytoplasmic and chloroplastic *E. coli* GR transformants	85
2.14	Chlorophyll levels in *E. coli* GR transformants following paraquat treatment	86
2.15	Leakage rates for *E. coli* GR transformants	87
2.16	SOD activities in the tomato chloroplastic SOD transformants relative to control plants	88
2.17	GR activities in chloroplastic SOD transformants	89
2.18	Chlorophyll decline in the chloroplastic SOD transformants treated with paraquat	90
2.19	Leakage rates for chloroplastic SOD transformants relative to control plants	90
2.20	GR activity in the hybrid transformants	92
2.21	SOD activity in the transgenic hybrids carrying SOD and *E. coli* GR transgenes	93
2.22	The decline of chlorophyll in the SOD / *E. coli* GR transformants	93
2.23	Electrolyte leakage in the hybrid transformants relative to the transformants carrying only one of the transgenes	94

LIST OF TABLES

Table number		Page
1.1	The response of leaf discs to varying kanamycin concentrations in the selection medium	31
1.2	Glutathione reductase activity in three transformed plants and their corresponding F1 lines	35
1.3	Labelling density in leaf sections incubated with anti-*E. coli* GR antibodies and in control sections	46
2.1	Factors enhancing the production of activated oxygen under stress conditions	57
2.2	Transgenic plants used to study oxidative stress	64

ABBREVIATIONS

APx	ascorbate peroxidase
bp	base pairs
BAP	benzylaminopurine
bya	billions of years ago
BSA	bovine serum albumin
°C	degrees Celsius (centigrade)
CaMV	cauliflower mosaic virus
cm	centimetre
cm^3	cubic centimetre
Da	Dalton
DHA	dehydroascorbate
DHAr	dehydroascorbate reductase
DNA	deoxyribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DTNB	5,5'-dithiobis(2-nitrobenzoic) acid
DTT	dithiothreitol
U	enzyme unit
EDTA	ethylenediaminetetraacetic acid
Fd	ferredoxin
Fig.	figure
FAD	flavin adenine dinucleotide
fwt	fresh weight
g	gravitational acceleration
g	grams
GUS	β-glucuronidase
gor	*E. coli* glutathione reductase gene
GSSG	glutathione (oxidised)
GSH	glutathione (reduced)
GR	glutathione reductase
GRA	glutathione reductase activity
ICC	immunocytochemical
IgG	immunoglobulin G
kb	kilobases
kbp	kilobase pairs
l	litre
mRNA	messenger ribonucleic acid
m	metre
m	milli- (x 10^{-3})
mA	milliampere
mg	milligram
ml	millilitre
mM	millimolar
μ	micro- (x 10^{-6})
μg	microgram
μl	microlitre
μmol	micromole

min	minute
M	molar (concentration)
MDHA	monodehydroascorbate radical
MDHAr	monodehydroascorbate reductase
MS	Murashige and Skoog nutrient formulation
n	nano- (x 10^{-9})
nm	nanometre
NAA	naphthaleneacetic acid
NPTII	neomycin phosphotransferase type II enzyme
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NBT	nitrobluetetrazolium
nos	nopaline synthase gene
NLS	nuclear localisation signal
OD	optical density
PBS	phosphate buffered saline
PS I	photosystem I
PEG	polyethylene glycol
pAg	protein A-gold
pH	hydrogen ion concentration, negative logarithm
hv	radiant energy
ROS	reactive oxygen species
RUBISCO	ribulose 1,5-bisphosphate carboxylase/oxygenase
s	second (time)
ssDNA	single stranded DNA
SSB	ssDNA binding protein
SDS	sodium dodecyl sulphate (sodium lauryl sulphate)
SEV	split end vector
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SOD	superoxide dismutase
T-DNA	transferred DNA
TEM	transmission elecron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TP	transit peptide
TP-GOR	fusion protein comprising a transit peptide and GOR
Ti	tumor inducing
UV	ultra violet
vir	virulence gene
Vir	protein product of a virulence gene
v/v	concentration on a volume basis
%	percent

PROLOGUE

"Like Janus, oxygen has two faces, one benign and the other malignant."
(Irwin Fridovich, 1979b)

A survey of the planets in our solar system reveals that the majority of them have substantial atmospheres, and that with one exception all of these atmospheres are strongly reducing. This preponderance of reducing atmospheres is hardly surprising. Shklovskii and Sagan (1977) point out that hydrogen is the most common element in the universe and reduced molecules like methane, ammonia and water are thus widespread. In this regard, the earth is unique in that it has an oxidising atmosphere.

What makes the situation more surprising is that the earth teems with life and all of these living organisms are comprised of compounds which are characteristically reduced. Under oxidising conditions such compounds should be readily oxidised to carbon dioxide, water and dinitrogen. As Dickerson (1978) puts it: "One tends to forget that oxygen is a dangerously corrosive and poisonous gas ....". Despite the inherent dangers of an existence in the presence of oxygen, most earthly organisms possess an aerobic metabolism which makes life in the absence of oxygen impossible. Winston (1990) comments that this may well represent one of the great paradoxes of the history of our planet.

In fact, the existence of chemically reduced life-forms in an oxidising atmosphere is not paradoxical when one examines their evolution. Evidence acquired from the fossil record, biochemistry, geology and mineralogy all indicates that life originated at a time when the Earth's atmosphere was predominantly reducing. This topic is extensively reviewed by Lemmon (1969), Schopf (1978) and Cloud (1983). The evidence suggests that the early atmosphere was similar to the current atmospheres of the Jovian planets - rich in the reduced forms of nitrogen, carbon and oxygen. In the absence of oxygen and an ozone layer, these molecules were exposed to high levels of ultraviolet (UV) radiation. This radiation provided sufficient energy for the synthesis of the organic compounds on which life is based. A tentative sequence of events compiled by Schopf (1978) suggests that the first life forms arose 3.5 billion years ago (bya) - some 1 billion years after the formation of the earth. By 3 bya these anaerobic prokaryotes had given rise to the first organisms capable of aerobic photosynthesis. These organisms were able to utilise light energy to remove electrons from water and channel them for biosynthesis. One of the products of this process is oxygen and this resulted in the gradual accumulation of atmospheric oxygen. The resulting transition from a reducing to an oxidising atmosphere conferred a distinct advantage on many organisms, in that it allowed the evolution of respiration. Respiratory metabolism allows 18 times more energy to be extracted from glucose than does the fermentation process utilised by anaerobic organisms, and is one of the reasons why aerobic organisms now dominate the biological community (Schopf, 1978).

Despite the advantages acquired from oxidative metabolism, there are distinct disadvantages associated with a life in an oxidising atmosphere. The dangers of living with oygen were recognised as early as 1775 by Joseph Priestly (cited in Bensasson *et al.*, 1993), who stated: "[Oxygen] might not be so proper for us in the usual state of the body: for as a candle burns out much faster in dephlogisticated than in common air, so we might, as may be said, live out too fast, and the animal powers be too soon exhausted in this pure kind air".

Due to its electron configuration molecular oxygen itself is relatively inert (Cadenas, 1989) but under certain conditions it will undergo a series of single electron transfers. This results in the production of several reactive intermediates and it is these oxygen species which are so dangerous to living organisms (Monk, 1989). Anaerobes have either developed barriers against atmospheric oxygen or retreated to environments that are free of oxygen. However, aerobic organisms must live with oxygen if they are to benefit from it and have developed defenses against oxygen - defenses which probably evolved in parallel with their respiratory metabolism (Winston, 1990). These defences include water- and lipid-soluble antioxidants, as well as numerous enzymes which either scavenge reactive oxygen species directly or maintain the reduced status of antioxidant pools (Larson, 1988, Winston, 1990). These defences offer sufficient protection under normal conditions but are easily overwhelmed, leading to free radical damage (Fridovich, 1979a). Halliwell and Gutteridge (1989) review a number of human ailments which may be attributed in part to insufficient protection against oxygen radicals. Recent research suggests that even under normal conditions oxygen is deleterious and that ageing in organisms may be due to the gradual accumulation of damage caused by radicals which escape various defence mechanisms. Orr and Sohal (1994) have extended the lifespan of *Drosophila* by as much as 34% by producing transgenic flies carrying elevated levels of enzymes which scavenge reactive oxygen species (ROS).

That defence mechanisms are so readily overwhelmed is of particular significance in plants. Illuminated chloroplasts utilise light energy for the production of high-energy electrons. These electrons are obtained from water, with oxygen as a by-product. As a result, chloroplasts develop high oxygen concentrations in close proximity to an extremely efficient electron producing system. To quote Asada and Takahashi (1987): "... plants are potentially exposed to the most severe environmental conditions in respect of the production of active oxygen and resulting oxidative damage". Since plants face the risk of oxidative oxidative damage even under optimal conditions, it seems likely that stress conditions will further exacerbate such damage. An increase in the levels of ROSs have been shown in response to drought, chilling, ozone, herbicide exposure, UV and a number of other stresses (Scandalios, 1993; Bartels and Nelson, 1994). One of the effects of stress in plants is a reduction in productivity. Boyer (1982) has shown that in the US, unfavourable physicochemical environments reduce crop yields by as much as 71%. For this reason, the improvement of stress tolerance in crop species is an urgent requirement in the field of agricultural research.

Numerous studies have shown that the activity of several antioxidant enzymes increases during oxidative stress and that plants which show the greatest increase in activity generally show increased stress tolerance. Furthermore, plants which have been selected for resistance to oxidative stress have increased activities of these enzymes (Allen, 1995). This evidence suggests that one way to improve stress tolerance is to produce plants having increased levels of antioxidant enzymes. In recent years the isolation and transfer of plant genes to other plants has become a standard technique, and as such genetic engineering presents an opportunity to test this hypothesis. In fact several such studies have been conducted in the last 7 - 8 years, with plants being engineered to express elevated levels of various antioxidant enzymes. Some of these studies have resulted in increased stress tolerance, but the majority have conferred no advantages to the transformed plants. There are two suggestions regarding the lack of success in these studies. One is that the transformations may have targeted the enzymes to the wrong subcellular compartment - the chloroplast is the site of most active oxygen production, whereas many of the studies have involved an increase in the cytoplasmic levels of the enzymes. The second suggestion for the lack of success is that the enzymes in the antioxidant pathway act to maintain a balance between several metabolite pools in the pathway, and that an increase in just one enzyme upsets this balance - having a detrimental effect on the plant. Based on this hypothesis, it has been suggested that plants may benefit from an enhancement of two or more of the enzymes in the antioxidant pathway.

Taking into account the above suggestions, it was decided to produce plants having elevated chloroplastic levels of two of the antioxidant enzymes, and to examine their response to oxidative stress. As such, this study involved the transformation and characterisation of tobacco plants carrying elevated chloroplastic levels of one of the enzymes in the plant antioxidant system (glutathione reductase). These plants were then cross-pollinated with a tobacco variety having enhanced chloroplastic levels of superoxide dismutase - a second antioxidant enzyme. Finally, the response of these hybrids under conditions of oxidative stress was examined and compared to the response of untransformed plants, as well as plants having elevated levels of just one of the enzymes.

1

THE PRODUCTION AND CHARACTERISATION OF TRANSGENIC *NICOTIANA TABACUM* CARRYING THE *E. COLI* GLUTATHIONE REDUCTASE GENE

1.1 INTRODUCTION

Until recently, the production of new genotypes for crop improvement or research in the plant sciences relied either on the traditional method of sexual hybridisation or on mutagenesis. Sexual hybridisation is limited by several factors, the most obvious being the finite range of genotypes present within the species of interest. Furthermore, hybridisation can be a time consuming process. Mutagenesis, although providing unique genotypes, is limited by the fact that the mutations are random and very few produce plants of interest. Moreover, the selection of plants carrying desirable traits is normally only achieved after an exhaustive screening process - once again a time consuming exercise.

In the early 1980s the first transgenic plants carrying engineered genes were produced, bringing about a revolution in the field of plant science. With the advent of reproducible transformation protocols it became possible to generate plants carrying genes for a specific trait, eliminating the uncertainty (and much of the time) which had previously been the limiting factor in plant breeding and research. Since the first successful transformation many plant species have been subjected to genetic alteration. Many of the genes introduced into these plants have originated from organisms belonging to entirely different kingdoms - an event which is rare in nature and was previously impossible to achieve in a laboratory.

Since the first transformation, transgenic plants have been used to develop crop species carrying genes for specific traits, one good example being resistance to certain herbicides. Transgenic plants have also been used to study gene expression and the functioning of biochemical pathways. In recent years several studies of the plant antioxidant pathway have been carried out using transgenic plants, with most of the emphasis being placed on plants carrying genes for the chloroplastic expression of foreign forms of the enzyme superoxide dismutase (SOD). A number of studies have also examined plants carrying foreign forms of the enzyme glutathione reductase (GR) in the cytoplasm. Very few workers in this field have produced plants carrying foreign GR in the chloroplast - a compartment which requires an active antioxidant pathway. Furthermore, only one study has examined the effect of engineering plants carrying both of these enzymes at elevated levels. The aim of this study was to produce tobacco plants carrying elevated levels of both SOD and GR, and to examine the response of these hybrids to oxidative stress. This chapter deals with the *Agrobacterium*-mediated transformation of tobacco plants with *E. coli* GR, targeted to the chloroplast. It also describes

the methods used to confirm the transformation and show that the gene product is localised in the chloroplasts of the transformed plants.

1.1.1 Transformation systems

The aim of any transformation is the stable insertion of DNA into the plant genome (either nuclear or organellar) and the subsequent regeneration of whole fertile plants capable of transferring the gene to their progeny (Draper and Scott, 1991; Gruber and Crosby, 1993). Since the first transformations were carried out, intense research in the field has resulted in a variety of transformation protocols which exhibit varying degrees of success. These protocols are extensively reviewed by Potrykus (1990), Potrykus (1991) and Draper and Scott (1991) and only the more commonly used methods will be mentioned in this review of the field.

1.1.1.1 *Agrobacterium tumefaciens*-mediated gene transfer

In any review of plant transformations, the most obvious starting point is the *Agrobacterium*-mediated system of DNA transfer. These bacteria have evolved a natural mechanism for gene transfer and the first plants expressing engineered foreign genes were transformed using *Agrobacterium tumefaciens* (Horsch *et al.*, 1984). Since *Agrobacterium* was used in the current transformation, this system warrants more detailed description than the other methods, which will only be mentioned briefly.

Agrobacterium tumefaciens

Agrobacterium tumefaciens, a gram-negative soil bacterium, is an obligate anaerobe capable of saprophytic or parasitic growth (De Cleene and De Ley, 1976 cited in Winans, 1992). It infects mostly dicotyledenous plants and produces tumerous crown galls on infected species. It may also produce a teratoma containing stunted shoots (Winans, 1992). However, not all strains of *Agrobacterium* are virulent. It has been shown that those strains which cause infection contain a large plasmid responsible for DNA transfer to host plants, and the subsequent development of disease symptoms (Gasser and Fraley, 1989). This *Agrobacterium*-plant interaction represents the only known case of a natural interkingdom transfer of DNA (Zambryski, 1992).

Ti Plasmid

As mentioned, the DNA which is transferred during infection by *Agrobacterium* originates on a specific plasmid. This is a 200 - 500 kbp circular plasmid referred to as the Ti (Tumour inducing) plasmid (Gruber and Crosby, 1993). It contains two separate components which are necessary for the transfer of DNA to a plant cell. The first is the T-DNA (Transferred DNA) - a single stranded (ss) copy of this DNA (the T-strand) is transferred to the plant cell. The second component is the virulence (*vir*)

region. This region contains the genes which encode most of the components necessary for the production and transfer of the T-DNA copy.

T-DNA

The T-DNA is situated between two 25-bp direct repeats - these are the T-DNA borders (Zupan and Zambryski, 1995). Any DNA situated between these borders is transferred to the plant cell. The T-DNA in wild type *Agrobacterium* has genes which produce plant growth regulators (cytokinins and auxins), resulting in the formation of the tumours observed on infected plants. It also carries the genes for the synthesis of opines - an amino acid derivative utilised exclusively by *Agrobacterium* as a carbon and nitrogen source (Zupan and Zambryski, 1995).

Virulence region

The virulence region forms a 30 kbp section of the Ti plasmid and has approximately 25 genes arranged in seven operons (Winans, 1992). These are termed *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G and *vir*H respectively (Zambryski, 1992). The *vir* genes are tightly controlled and are transcriptionally induced by compounds produced by plants in response to wounding (Stachel *et al.*, 1986a). These compounds include plant phenolics as well as certain sugars. Stachel *et al.* (1985) have isolated two molecules - acetosyringone and α-hydroxyacetosyringone - which have proven to be potent inducers of the *vir* genes. Once induced, these genes produce the components necessary for the production of the T-strand and its transfer to the plant cell.

The mechanism of gene transfer

The mechanism whereby *Agrobacterium* transfers DNA to plant cells is fairly complex and occurs in several stages, under the control of the genes situated on the Ti plasmid. This mechanism will dealt with in some detail.

Signal perception and induction

Agrobacterium infection occurs at wounds in plant tissues. *Agrobacterium* spp. are peritrichously flagellated (Schlegel, 1985) and their motility plays a role in the early stages of infection - a positive chemotactic response to the exudates at wound sites results in direct contact between *Agrobacterium* and the wounded plant cells (Winans, 1992). The proteins involved in signal perception and induction are encoded by *vir*A and *vir*G. The *vir*A gene product is an inner membrane protein which recognises compounds released by plant wounds and transduces this information to an inactive form of the *vir*G gene product (Zambryski, 1992). Signal transduction involves the activation of VirG by means of a VirA-mediated phosphorylation. VirG then activates transcription of its own, as well as other, *vir* loci (Zambryski, 1992; Winans, 1992).

Production of the T-strand

Following induction of the *vir* loci, a single-stranded copy of the lower strand of the T-DNA is produced. This single stranded DNA (ssDNA) is termed the T-strand. Stachel *et al.* (1986b) have proposed that the generation of a T-strand involves the displacement of the lower T-DNA strand, followed by the synthesis of a new lower strand. T-strand production requires the formation of a nick in the left and right borders of the lower T-DNA strand. This is accomplished by the *vir*D protein products (VirD1 and VirD2). It seems that VirD1 relaxes the DNA in the region of the T-DNA borders, while VirD2 is involved in site-specific nicking (Zambryski, 1992). The nicking of the T-DNA is enhanced by the presence of VirC1 and VirC2, although these proteins are not essential for the process (Toro *et al.*, 1988).

Production and transfer of the T-complex

Once the T-strand has been produced it must leave the bacterial cell, enter the plant cell and integrate with the plant genome. During this process it must cross several membranes and at the same time avoid degradation by nucleases. The products of several of the *vir* loci are involved in this process. Stachel *et al.* (1986b) proposed that the T-strand is transferred as part of an ssDNA-protein complex - the proteins bind the DNA, protecting it and ensuring that it is transferred across membranes and into the plant nucleus. Two proteins which form part of this complex - VirD2 and VirE2. When the T-strand is generated VirD2 (which also nicks the DNA) binds strongly to the 5' end (Ward and Barnes, 1988). This gives the T-strand a polar nature which may facilitate transport of the ssDNA complex (T-complex), by targeting it to specific bacterial and plant cell components (Zambryski, 1992). It may also protect the T-strand from the action of exonucleases (Ward and Barnes, 1988). VirE2 comprises the bulk of the T-complex - about 350 - 700 VirE2 molecules are required to cover all of the nucleotides in a 20 kb T-strand (Citovsky *et al.*, 1988). VirE2 is an ssDNA binding protein (SSB) which associates strongly with the T-strand (Christie *et al.*, 1988; Citovsky *et al.*, 1988) and protects it from endonucleases as well as 3' and 5' exonucleases (Citovsky *et al.*, 1989; Sen *et al.*, 1989). The VirE2 molecules extend and unfold the T-strand, giving a linear complex with a diameter of less than 2 nm (Citovsky *et al.*, 1989). This may well aid in its transfer through membrane channels.

In addition to VirD2 and VirE2 there are several other proteins, encoded by the *vir*B and *vir*D loci, which may play a role in the T-strand transfer. These are the two largest *vir* loci and they each encode a number of proteins. Analysis of the amino acid sequences and subcellular locations of these proteins indicates that several of the *vir*B products may form membrane channels through which the T-complex may be exported (Zambryski, 1992). Christie *et al.* (1989) have shown that one of these proteins, VirB11, has an ATPase activity. These authors propose that

VirB11 induces a conformational change in a *vir*B encoded membrane complex, thus opening a pore which allows T-complex transport. This is the only product of these two loci for which a definite role has been proposed. The functions of the rest of these proteins in T-complex transport have yet to be defined.

Integration of the T-strand into the plant genome

Once the T-complex has been transferred to the plant cell, nuclear uptake and integration of the T-strand into the plant genome must occur. Targeting to the nucleus appears to be carried out by both the VirD2 and VirE2 proteins in the T-complex. It has been shown that VirD2 has a highly conserved region of 29 - 31 amino acids at the C-terminus (Zambryski, 1992). Within this region there are two sequences which are homologous to a known nuclear localisation signal (NLS) (Howard *et al.*, 1992). Furthermore, Howard *et al.* (1992) have shown that chimeric proteins comprising one of these sequences fused to GUS is localised in the nucleus, although nuclear localisation of the chimeric protein is more efficient if both sequences are present. As pointed out by Zupan and Zambryski (1995), the T-complex has a mass of 50 x 10^6 Da and a single protein (VirD2) may not be sufficient to mediate the nuclear uptake of a complex of this size. This suggests that VirE2, the largest component of the T-complex, may also play a role in the nuclear uptake. Indeed, it has been shown that each VirE2 molecule also has two NLSs capable of targeting proteins to the nucleus (Citovsky *et al.*, 1992). Thus the T-complex has nuclear localisation signals along its entire length. The fact that NLSs occur along the length of the T-complex raises the question as to whether the single VirD2 NLS is necessary - does it have any other functions? Citovsky *et al.* (1992) point out that the VirD2 NLSs are stronger than those of VirE2. It has thus been suggested that the VirD2 protein targets the T-complex to the nuclear pore in a polar fashion, ensuring that the uptake of the complex occurs in a 5' to 3' direction (Citovsky *et al.*, 1992; Zupan and Zambryski, 1992). This vectorial transfer across the nuclear membrane may well be a common feature of ss nucleic acids (Zupan and Zambryski, 1992).

The integration of the T-DNA into the host chromosome is the final, and least characterised, step in the transformation process. It is assumed that transcriptionally active areas are more accessible to T-DNA due to unravelling of nucleosomes and other chromatin structures (Zambryski, 1992). From evidence gathered in the studies of 15 insertion events, it would seem that the integration occurs as an illegitimate recombination event. The following mechanism was proposed by Gheysen *et al.* (1991) (cited in Zupan and Zambryski, 1992). They suggest that the T-DNA inserts at a nick in the plant DNA. The evidence shows that the 5' end of the T-DNA, protected by the VirD2, joins to the plant DNA. The plant DNA then unwinds to form a gap and the 3' end of the T-strand joins to a nearby region on the plant DNA. Once the T-strand has joined to one

strand of the plant DNA a nick forms in the opposite strand and DNA synthesis fills the gap, using the T-strand as a template.

Vectors for *Agrobacterium*-mediated gene transfer

For successful gene transfer, the transforming vector must first of all have restriction sites which allow easy insertion of the gene(s) of interest. In addition, it must carry a gene which allows selection of the transformed cells either by selection or screening. Furthermore, it must carry regulatory sequences for successful expression of both the gene of interest and the selection genes.

Since normal plants cannot be regenerated from oncogenic cells, the first step in producing vectors based on the Ti plasmid is the formation of a disarmed plasmid (Draper and Scott, 1991). This is accomplished either by removing the tumour inducing gene or by removing the entire T-DNA - as is the case in *Agrobacterium* strain LBA4404 (Ooms *et al.*, 1981). The next step is to insert the gene of interest into the correct site on the plasmid. The large size of the Ti plasmid makes this difficult. This has resulted in the development of two different strategies when working with *Agrobacterium* - one uses a *cis*-acting vector system and the other a *trans*-acting vector system (Draper and Scott, 1991).

Cointegrative vectors

Cointegrative vectors are *cis*-acting, with the T-DNA and the virulence genes being situated on a single engineered plasmid. This plasmid is generated by the fusion of two independent plasmids which carry a homologous sequence. One of these, a disarmed Ti plasmid with T-DNA borders situated on either side of the homologous sequence, is carried in *Agrobacterium*. The second (called the intermediate vector), is an *E. coli* plasmid. It is much smaller and easier to manipulate (Gruber and Crosby, 1993). The gene of interest is inserted into this plasmid and the two bacteria are co-incubated to allow conjugation. Once conjugation has occurred the plasmids undergo homologous recombination, giving a single large co-integrated vector carrying the gene of interest (and the rest of the smaller plasmid) between the T-DNA borders. The DNA from the intermediate vector is transferred with the gene of interest to the plant host and may limit the usefulness of this vector system (Draper and Scott, 1991). For this reason the split end vector (SEV) system was developed. In this system the two plasmids each carry one of the T-DNA borders, and are constructed such that the only superfluous DNA between the borders of the co-integrated vector is the homologous sequence (Draper and Scott, 1991).

Binary vector systems

These systems are made possible by the fact that the *vir* genes act in *trans* to the T-DNA, and will process the T-strand even if situated on a

separate plasmid to the T-DNA. The concept of a binary vector system was first introduced by Hoekema *et al.* (1983). This transformation system involves two plasmids - a disarmed Ti plasmid and a binary vector (also called a shuttle plasmid). The *vir* genes are carried on the Ti plasmid, which is situated in *Agrobacterium*. The binary vector is a plasmid capable of replicating in both *E. coli* and *Agrobacterium*. It carries the T-DNA borders, selectable markers, expression signals and polylinkers for the insertion of genes within the borders (Gruber and Crosby, 1993). This plasmid is maintained and manipulated in *E. coli*. Once the gene of interest has been inserted between the borders, the binary vector is transferred into *Agrobacterium*, where the two plasmids exist autonomously. Since cointegration of the two plasmids is not required, the introduction into *Agrobacterium* occurs much more readily than the introduction of a cointegrating vector (Klee *et al.*, 1987).

Selection marker genes

Following the transformation protocol, it becomes necessary to select cells which have undergone transformation. This is accomplished by including in the vector a gene which will readily allow transformed cells to be distinguished from untransformed ones. This marker gene may be one of two types. The first type is a biochemical reporter gene which produces a readily assayed enzyme. The most extensively used biochemical marker is the *E. coli* gene encoding β-glucuronidase (GUS) (Gruber and Crosby, 1993). This enzyme produces an intense blue stain in transformed cells when they are grown on a medium containing the appropriate substrates for the enzyme.

Alternatively, the vector can carry a selectable marker gene whose product confers resistance to a plant growth inhibitor (for example, kanamycin). This inhibitor is included in the plant regeneration medium. During the regeneration process transformed cells will grow normally, while untransformed cells will either die or grow at a much reduced rate. The most widely used selectable marker is the neomycin phosphotransferase type II enzyme (NPTII), which detoxifies kanamycin by phosphorylation (Klee *et al.*, 1987).

Gene expression

For successful expression of introduced genes, there is a need for transcriptionally active 5'-regulatory regions, a 5'-untranslated leader sequence, translational start sequences and a transcription termination sequence (Gruber and Crosby, 1993). Both the gene of interest and the marker gene require all of these components.

Promoters

The CaMV virus carries a strong promoter and a number of studies have attempted to utilise the CaMV virus as a transformation vector by

replacing dispensable viral genes with genes of interest (Fütterer *et al.*, 1990). However, due to the size of the virus, there is a limit to the amount of DNA that can be inserted into the genome. This restricts the use of the virus as a vector. However, the CaMV promoter is widely used in bacterial vectors - such as the Ti plasmid. An added advantage to using the CaMV promoter is that it's activity is greatly enhanced if it is duplicated (Kay *et al.*, 1987). The use of two 35S promoters in tandem increases the expression of marker genes by as much as 4 times (Gruber and Crosby, 1993). Another promoter commonly utilised in vectors is that of the nopaline synthase gene (*nos*). Both the *nos* and CaMV promoters are constitutive promoters. An alternative to these constitutive promoters is an inducible promoter. These include heat shock promoters, a nitrate-inducible promoter, hormone-inducible promoters and light-inducible promoters (Gruber and Crosby, 1993).

Termination sequences

In addition to the promoter, it is also common for vectors to include sequences for the termination of transcription and for polyadenylation of the mRNA. These sequences help to stabilise the mRNAs resulting from transcription of the foreign gene (Gruber and Crosby, 1993).

1.1.2 Physical transformation of plant cells

Agrobacterium-mediated transformation is the most widely used system for the transformation of plant cells, the most obvious reasons being the simplicity of the transformation and selection protocols. However, many hosts are either inefficiently transformed or not transformed at all - the most commonly cited cases being the inability of *Agrobacterium* to transform the majority of monocotyledonous plants (Binns, 1990). It seems that the low transformation efficiency in these plants is due to a lack of wound response on the part of these plants. This is unfortunate, since the plants in this group include most of the cereals which form the backbone of modern agriculture. For this reason, a considerable amount of research has been devoted to alternative transformation protocols.

1.1.2.1 Direct gene transfer

Chemically mediated gene transfer

The direct transfer of genes to plant cells is accomplished by using plant protoplasts, and the process may be carried out in a number of ways. A number of chemical compounds are known to stimulate the uptake of naked DNA into protoplasts. One of the most commonly used is the polymer polyethylene glycol (PEG). At concentrations of 15 - 25%, PEG precipitates DNA and stimulates its uptake by endocytosis (Draper and Scott, 1991).

Liposome fusion

A second method involves the fusion of DNA-carrying liposomes with protoplasts (Caboche, 1990). This process is stimulated by PEG but is no more efficient than the uptake of naked DNA in the presence of PEG (Draper and Scott, 1991).

Electroporation

Electroporation involves the application of electrical impulses to a solution containing protoplasts. The impulses induce pores in the plasma membrane which allow the uptake of DNA. The pores have a diameter in excess of 30 nm and last for several minutes (Okada *et al.*, 1986). The efficiency of electroporation techniques is improved in the presence of PEG.

The main disadvantage to the above techniques is that the regeneration of plants from protoplasts is not easily accomplished (Potrykus, 1990).

1.1.2.2 DNA injection techniques

This technique involves the injection of DNA into protoplasts or single cells, using a microcapillary (Neuhaus and Spangenberg, 1990). The DNA may be injected into the cytoplasm or directly into the nucleus, and regeneration of plants from walled single cells is easier to accomplish than the regeneration of protoplasts (Draper and Scott, 1991). The main disadvantage to this technique is the time and skill required to inject individual cells.

1.1.2.3 Biolistics

A technique which has lately received considerable attention is that of biolistics (transformation via a particle gun). This method was invented by J.C. Sanford, T.M. Klein, E.D. Wolf and N. Allen in 1984 (Miki *et al.*, 1993). It involves the acceleration of DNA-coated particles to speeds sufficient to penetrate the cells of the target tissue (Potrykus, 1990). The method uses either gold or tungsten particles which are accelerated using a compressed gas, gun powder or electrical discharges (Miki *et al.*, 1993). The advantage to this method is that it allows the introduction of DNA into a wide range of plant materials. Despite this advantage, it has been pointed out by Potrykus (1991) that relatively few successful transformations have been achieved in the plant species which are recalcitrant to *Agrobacterium*-mediated transfer. This author suggests that, once again, it is the plant material rather than the DNA delivery protocol which is the limiting factor - these plants probably have very few of the competent cells which are required for successful transformation.

1.1.3 Applications for recombinant DNA technology

Genetic engineering has greatly expanded the horizons of the plant sciences. It has contributed considerably to the development of new genotypes in important agricultural crops. Following the first transformations in the early 1980s, researchers have produced crop species with enhanced herbicide, insect and disease resistance; improved seed quality; altered fruit ripening and improved flower pigmentation - to mention but a few cases (Gasser and Fraley, 1989; Draper and Scott, 1991; Greenberg and Glick, 1993). With recent advances in this field, even more exotic applications for transformed plants have been developed. Molecular farming allows the large scale production of valuable biomolecules in plants - this process has been used to produce mammalian antibodies in tobacco (Hiatt and Mostov, 1993), human serum albumin in potato tubers and bacterial α-amylase in tobacco (Pen *et al.*, 1993). Furthermore, transformations have greatly advanced the understanding of plant genetics, providing information on promoters, enhancers, silencers, terminators, *cis*- and *trans*-acting elements and a host of other gene regulatory systems (Potrykus, 1990).

Recombinant DNA technology is also a powerful tool for studies in the fields of plant pathology, physiology and biochemistry. By enhancing or reducing the activities of enzymes in various biochemical pathways, it has become possible to improve our understanding of how plants respond to their environment. As mentioned in the prologue environmental stress greatly reduces plant productivity, and an understanding of how plants respond to non-lethal stress events may lead to a reduction in these losses.

One general response in plants under several types of stress is an increase in the components and turnover of the antioxidant cycle - a topic which will dealt with in some detail in Chapter 2. This response resulted in a large amount of work being carried out to examine the role of the antioxidant cycle in dealing with stress. It has also raised the question of whether plants carrying elevated levels of the enzymes in this cycle are better equipped to tolerate certain stress conditions. With this in mind, *Agrobacterium* was used to transform tobacco with the *E. coli* GR gene, as discussed in the introduction. This chapter deals with the transformation process and the characterisation of the transformants. The response of these plants to oxidative stress, as well as the response of other transformants carrying foreign antioxidant enzymes, will be dealt with in Chapter 2.

1.2 MATERIALS AND METHODS

1.2.1 *Agrobacterium tumefaciens*

1.2.1.1 Bacterial strain and the plasmid

Agrobacterium tumefaciens strain LBA4404 (pBIN19*gor*/T2) was obtained from Dr Karl Kunert (AECI Research and Development Department, Modderfontein). The organisation of the plasmid is shown in Fig. 1.1. The plasmid was produced by Foyer *et al.* (1995) as follows: The *E. coli* glutathione reductase gene (*gor*) was obtained from the plasmid pGR (Greer and Perham, 1986) and inserted into the *Hinc*II site of plasmid pUC18 as described by Kunert *et al.* (1990). The *gor* gene was then subcloned as a *Bam*HI fragment into pJIT62 (Guerineau *et al.*, 1990), an expression vector carrying the CaMV 35S promoter, a pUC9 polylinker and the CaMV polyadenylation sequence. This produced the vector pKG1 (Foyer *et al.*, 1991). The *gor* expression cassette (CaMV 35S-*gor*-poly(A)) was removed from pKG1 as a *Hinc*II/*Eco*RI fragment and cloned into the *Sma*I/*Eco*RI site of pJIT117 (Guerineau *et al.*, 1990), to create a translational fusion with the pea *rbcS* gene encoding a chloroplastic transit peptide. The chimeric gene (*rbcS gor*), along with the CaMV promoter and poly(A) was excised as a *Kpn*I fragment and inserted into the binary vector pBIN19 (Bevan, 1984), creating pBIN19*gor*/T2. The vector pBIN19 carries the neomycin phosphotransferase gene under the control of the nopaline synthetase gene promoter and terminator. This gene is carried between the T-DNA borders and confers kanamycin resistance on transformed cells. The binary vector also carries a prokaryotic kanamycin resistance gene (Aph 1) outside the T-DNA borders, kanamycin resistance in bacteria carrying the vector.

1.2.1.2 Growth and maintenance of *Agrobacterium tumefaciens*

The bacterium was grown and maintained on GT medium (10 g l^{-1} tryptone, 3 g l^{-1} yeast extract, 2 g l^{-1} glucose, 1 g l^{-1} Tris, 10 g l^{-1} sodium glycerophosphate, 0.4 g l^{-1} $CaCl_2$, 15 g l^{-1} agar, pH 7.4). The medium was supplemented with 100 μg ml^{-1} rifampicin, 100 μg ml^{-1} kanamycin sulphate and 200 μg ml^{-1} streptomycin sulphate to select for the vector. Stock cultures of the bacteria were stored in 40% glycerol at -80°C. For long term storage of working stocks, bacterial stabs were prepared using solid GT medium. These were kept at room temperature in the dark. For short term work GT plates were streaked, incubated at 28°C for 24 hours and then stored at 4°C. For the transformation, bacteria were grown in liquid GT medium at 28°C with shaking.

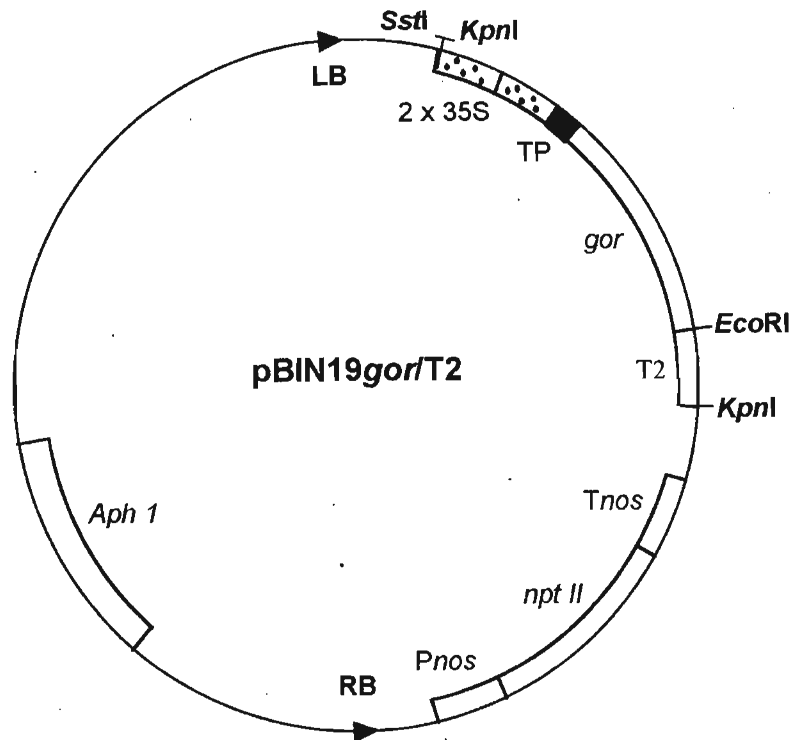


Figure 1.1: The Structure of plasmid pBIN19*gor*/T2. The plasmid carries the *gor* coding sequence fused to the coding sequence for pea *rbcS*. This chimeric gene is controlled by a CaMV promoter and terminator. Abbreviations: LB, left T-DNA border; 35S, Cauliflower mosaic virus (CaMV) 35S promoter; TP, coding sequence for pea *rbcS* (RUBISCO small subunit) transit peptide; *gor*, coding sequence for *E. coli* glutathione reductase; T2, CaMV terminator; T*nos*, terminator of nopaline synthetase gene; *npt II*, coding sequence of neomycin phosphotransferase gene; P*nos*, promoter of nopaline synthetase gene; RB, right T-DNA border; *Aph 1*, prokaryote kanamycin resistance gene. (Information regarding the structure of the plasmid was supplied by Dr. K. Kunert, pers comm.)

1.2.1.3 Determination of the growth kinetics of the bacterial population

A. tumefaciens was grown in liquid GT (supplemented with antibiotics as described above) at 28°C over 28 hours with shaking. The optical density (OD) (600 nm) was monitored over this period, to determine the time required for the culture to enter the log phase of growth. Further cultures were then initiated, and during the log phase OD was measured and serial dilutions of the culture were plated on solid GT medium. These plates were maintained at 28°C for 2 days and the resulting bacterial colonies were counted, allowing a relationship between OD and bacterial concentration to be established.

1.2.2 Plant material for transformation

1.2.2.1 Germination of seedlings

Tobacco seeds (*Nicotiana tabacum* var. samsun) were germinated in seed trays in a growth room adjusted to 27°C and with a 14 hour photoperiod (120 μmol m^{-2} s^{-1}). The seedlings were watered with Long Ashton nutrients containing 0.14 g l^{-1} nitrates (Hewitt, 1952). Once the first 2 leaves were fully expanded, these seedlings were potted individually in 250 cm^3 pots containing a commercial potting soil.

1.2.2.2 Plant tissue culture

Plants were propagated in culture via direct somatic embryogenesis, using a slight modification of the method described by Stolarz *et al.* (1991). Fully expanded leaves were taken from plants in the growth cabinet and surface sterilised for 20 minutes in 1% sodium hypochlorite containing 0.1% tween-20. These leaves were then rinsed three times in sterile water and cut into squares of approximately 1 cm x 1 cm, which were plated on MS induction medium (1 x MS salts (Murashige and Skoog, 1962), 30 g l^{-1} sucrose, 10 g l^{-1} agar, 1 mg l^{-1} benzylaminopurine (BAP), 0.1 mg l^{-1} napthylacetic acid (NAA), pH 5.7) in sealed Petri dishes. The petri dishes were maintained in a growth room at 25°C on a 16 hour photoperiod (200 μmol m^{-2} s^{-1}) until embryos formed (2 - 3 weeks).

Once embryos had formed, the explants were plated on MS germination medium containing 0.5 x MS salts (Murashige and Skoog, 1962), 30 g l^{-1} sucrose and 10 g l^{-1} agar (pH 5.7) under the same conditions described above. The embryos were allowed to germinate and form shoots, which were excised and placed on the same germination medium in culture bottles. The shoots were allowed to root and form 2 - 3 fully expanded leaves, which were then used as a source of sterile plant material for the transformation.

1.2.3 Transformation of tobacco with *E. coli* glutathione reductase

1.2.3.1 Explants

The method of Horsch *et al.* (1988) was followed for the transformation process. The plant material used was sterile leaf material grown in culture as described above. The leaves were cut into 1 cm x 1 cm squares under sterile conditions to provide the explants for the transformation.

1.2.3.2 Infection and co-culture

Liquid GT, supplemented with antibiotics as described above, was inoculated with *A. tumefaciens* and incubated at 28°C overnight with shaking. The OD of the culture was measured and the culture diluted with

liquid MS medium (without plant growth regulators) to 5 x 10^6 cells ml^{-1}. The explants were dipped in the diluted cell cultures for 2 minutes and then briefly blotted to remove excess liquid. They were then placed on co-culture plates (solid MS medium) in the light (200 μmol m^{-2} s^{-1}) at 25°C for 3 days.

1.2.3.3 Curing and selection of transgenic lines

Following the co-culture period, the explants were washed in liquid MS (without plant growth regulators) supplemented with 1 mg ml^{-1} Cefotaxime for 1 hour. This wash was carried out in the dark, as Cefotaxime is light sensitive. Subsequently, the explants were transferred to selection plates (solid MS + 500 μg ml^{-1} Cefotaxime + kanamycin sulphate) and placed in the dark. Because kanamycin inhibits plant growth the explants were divided into four groups, each receiving a different kanamycin concentration (0, 50, 100 or 300 μg ml^{-1}) in the selection medium. These plates were examined twice weekly and any explants showing signs of fungal contamination were discarded. Explants that showed bacterial contamination were subjected to a further 1 hour wash in liquid MS medium containing 1 mg ml^{-1} Cefotaxime. The healthy explants were transferred to fresh medium every three weeks until callus and shoot formation occurred at the edges of the leaf discs.

Shoots were removed from the callus and transferred to culture tubes containing ½ strength MS medium supplemented with 100 μg ml^{-1} kanamycin sulphate. These tubes were placed in the light with a 16 hour photoperiod (200 μmol m^{-2} s^{-1}) at 25°C, and were subcultured every three weeks. Shoots that developed fungal contamination were discarded, as were shoots that showed signs of necrosis.

Once the shoots had started to root in this medium, they were transferred to ½ strength MS medium (without kanamycin sulphate) in culture bottles and allowed to develop leaves and root systems.

1.2.3.4 Hardening off

Hardening off was accomplished by transferring the fully developed shoots to potting soil in 250 cm^3 pots, which were covered with plastic bags. The pots were placed in a growth cabinet with a 12 hour photoperiod (120 μmol m^{-2} s^{-1}) at 27°C. The bags were removed from the pots daily for increasing lengths of time, until the plants acclimatised to the growth cabinet humidity.

1.2.4 GR activity in transformed shoots

Glutathione reductase (GR) assays were carried out to determine the activity of the enzyme in the hardened off plants. These assays were performed as described in section 2.2.4.2.

1.2.5 Preparation of acetone powders

Samples for western blotting were prepared as described by Ibrahim and Cavia (1975). Leaf material was ground to a fine powder in liquid nitrogen using a mortar and pestle. This powder was further ground with five volumes of cold (-25°C) 80% acetone. The resulting slurry was filtered under vacuum through Whatman No. 2 filter paper and the precipitate was washed with 100% acetone until a pale green to white colour was achieved. This powder was allowed to dry by passing air through it for several minutes. The acetone powder was then stored at -25°C until needed.

1.2.6 Western Blotting

1.2.6.1 Sample preparation

Acetone powder samples were prepared for western blotting by suspending 20 - 25 mg of the relevant acetone powder in 500 μl cold (4°C) 0.2 M Tris buffer (pH 7.5). The suspension was incubated on ice for 45 - 60 minutes with frequent agitation. The suspension was then centrifuged at 10 500 g and 6°C for 15 minutes, using a Biofuge B microfuge (Heraeus Sepatech).

Purified *E. coli* GR was supplied by R. Ponquett as a suspension in 3.6 M ammonium sulphate. In order to remove the ammonium sulphate from the solution, the sample was dialysed against 50 mM Tris buffer (pH 7.5) for 72 hours. The buffer was changed every 24 hours. To concentrate the final *E. coli* GR solution, the dialysis tubing containing the sample was placed on granular PEG 6000 until most of the liquid in the tubing had been removed.

1.2.6.2 Determination of protein concentrations

The protein concentration in the supernatants were assayed using the protein-dye binding method of Bradford (1976). Bovine serum albumin (BSA) fraction V was used to construct a standard curve over the range 0 - 1000 $\mu g\ ml^{-1}$ protein.

1.2.6.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were denatured by mixing with one volume of gel-loading buffer (50 mM Tris.HCl (pH 6.8), 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulphate (SDS), 0.1% bromophenol blue, 10% glycerol) and boiling for 2 minutes. The bromophenol blue served as a marker dye during electrophoresis.

SDS-PAGE was performed as described by Sambrook *et al.* (1989). Proteins were separated on 10% denaturing gels (10% acrylamide, 0.3% N,N'-methylenebisacrylamide, 0.375 M Tris (pH 8.8), 0.1% SDS, 0.4 $ml\ l^{-1}$ N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.1% ammonium

persulphate) overlaid with a 5% polyacrylamide stacking gel (5% acrylamide, 0.15% bisacrylamide, 0.125 M Tris buffer (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate and 1 ml l^{-1} TEMED). The electrophoresis buffer used contained 25 mM Tris (pH 8.3), 250 mM glycine and 0.1% SDS. Electrophoresis was performed at 4°C and 60 mA per gel in a vertical SE 600 electrophoresis apparatus with a PS 500X power pack (Hoeffer Scientific Instruments) until the marker dye was within 1 cm of the end of the gel. Rainbow molecular weight markers (Amersham) in the range 4300 - 200 000 Da were run in each gel.

1.2.6.4 Electrophoretic blotting

Following electrophoresis, proteins were blotted from the polyacrylamide gels onto nitrocellulose sheets using a modification of the method described by Towbin *et al.* (1979). The procedure was carried out in a TE 77 Semiphor semi-dry transfer unit (Hoeffer Scientific Instruments), with the membrane and gel arranged as per the manufacturer's instructions. The transfer buffer comprised 48 mM Tris (pH 8.3), 39 mM glycine and 20% (v/v) methanol. The transfer was carried out at 114 mA for 1.5 hours and the nitrocellulose membrane was then air dried for 30 minutes.

1.2.6.5 Immunological detection of proteins

E. coli glutathione reductase was detected using a protocol described by P. Badenhorst (1993). The antibody used to detect the *E. coli* GR was raised in rabbits and was provided by R. Ponquett.

The air-dried nitrocellulose membranes were incubated in blocking solution (0.2 M Tris (pH 7.5), 0.5 M sodium chloride, 0.05% tween-20 and 5% fat free milk powder) containing the antibody. This was carried out overnight at 4°C with shaking. This step allowed primary antibody binding and the blocking of protein binding sites on the membrane to be carried out simultaneously. The membrane was then washed three times, 10 minutes each, in blocking solution. This wash was followed by a three hour incubation with an anti-rabbit IgG antibody (Sigma) conjugated to alkaline phosphatase. This antibody was diluted 1:8500 with blocking solution. The membrane then received four 10 minute washes in a wash solution (50 mM Tris.Cl (pH 7.5) with 150 mM sodium chloride). Following this wash, the binding sites of the secondary antibody were determined by incubating the membrane in alkaline phosphatase buffer (100 mM Tris.Cl (pH 9.5), 100 mM sodium chloride, 5 mM magnesium chloride) containing 330 mg l^{-1} nitrobluetetrazolium (NBT) (dissolved in 70% dimethylformamide) and 165 mg l^{-1} 5-bromo-4-chloro-3-indolyl-phosphate (dissolved in 100% dimethylformamide). The alkaline phosphatase reaction was allowed to continue until the blue bands were clearly visible. The reaction was terminated by placing the membrane in phosphate buffered saline (pH 7.2) containing 2 mM EDTA for 10 minutes. Finally, the nitrocellulose membrane was washed in distilled water for 5 minutes and air dried.

1.2.7 Production of F1 plants

Transformed plants were selected on the basis of high GR activity (GRA) as well as a positive immunological response in the western blots. In order to show the stable transfer of the *E. coli* glutathione reductase gene to the F1 generation, these selected plants were allowed to flower and self pollinate. The resulting seeds were germinated and maintained as described in section 1.2.2.1 above. The F1 plants were tested for high GRA (see section 1.2.4) and were subjected to western blotting (as described in 1.2.6), to confirm the presence of *E. coli* GR.

1.2.8 Characterisation of *E. coli* GR

1.2.8.1 Isolation and purification of tobacco chloroplasts

The plants used were maintained in a growth chamber at 27°C with a 12 hour photoperiod at 120 μmol m^{-2} s^{-1}, and were watered with Long Ashton nutrients (Hewitt, 1952). In order to reduce starch levels in the leaves, the photoperiod was reduced to 4 hours daily, three days before the extraction and the lights were switched off for the final 24 hours.

Chloroplasts were isolated mechanically from tobacco leaves according to Mills and Joy (1980). Briefly, leaf material was homogenised at 4°C with extraction buffer (50 mM N-tris-(hydroxymethyl)methylglycine(Tricine)-KOH, pH 7.9, 2 mM EDTA, 1 mM magnesium chloride, 330 mM sorbitol, 0.1% BSA) in the ratio 1 g leaf tissue : 6 ml buffer. The homogenisation was carried out at high speed in a Wareing blender for 3 seconds. The homogenate was filtered through four layers of muslin and placed in ice cold centrifuge tubes. This filtrate was then underlayered with 10 ml (3 cm) of Percoll medium (50 mM Tricine-KOH, pH 7.9, 330 mM sorbitol, 0.1% BSA, 40% (v/v) Percoll) and centrifuged at 2 300 g for 2.5 minutes in a Beckman GP centrifuge. The supernatant and Percoll layer were removed by aspiration and the pellet was resuspended in 500 μl of 0.2 M Tris buffer (pH 7.5).

1.2.8.2 Preparation of chloroplast samples for electrophoresis

Chloroplast proteins were released by sonicating the resuspended chloroplasts for 5 minutes. These samples were then centrifuged at 10 500 g for 15 minutes at 6°C. The resulting supernatant was used for superoxide dismutase (SOD) activity gels and western blotting.

1.2.8.3 Preparation of whole leaf samples for electrophoresis

These samples were prepared according to Perl *et al.* (1993). Leaf samples (0.5 g) were ground to a fine powder in liquid nitrogen and then further ground with 1 ml of extraction buffer (0.05 M potassium phosphate buffer (pH 7.8), 0.5 mM EDTA, 14 mg ml^{-1} isoascorbic acid). The

homogenate was centrifuged at 10 500 g for 15 minutes at 6°C and the supernatant was utilised for running gels. Protein levels in the supernatant were assayed as in section 1.2.6.2.

1.2.8.4 Western blots

Western blots of the isolated chloroplasts were performed as described in 1.2.6 above.

1.2.8.5 SOD activity gels

Samples were mixed with an equal volume of the gel loading buffer described in 1.2.6.3, but the DTT was omitted and the samples were not boiled.

These non-denaturing gels were performed as described in 1.2.6.3, the only difference being the omission of SDS from all steps. Once the gel had run, it was stained for SOD activity as described by Beauchamp and Fridovich (1971). Briefly, the gel was immersed in the stain solution (0.05 M sodium phosphate buffer (pH 7.8), 1 mM EDTA, 0.2 mg ml^{-1} NBT, 31 μg ml^{-1} riboflavin and 200 μl TEMED) for 40 minutes in the dark. It was then transferred to a tank of water on a light box for 20 minutes allowing the background stain to develop, with clear bands showing the sites of SOD activity.

1.2.8.6 GR activity gels

Both chloroplast and acetone powders were used for GR activity gels. Chloroplast samples were prepared as described in section 1.2.7.2, while the preparation of acetone powder samples is described in 1.2.6.1. Protein determinations were once again carried out as per Bradford (1976). As with the SOD gels, the gel loading buffer did not contain DTT and samples were prepared for electrophoresis without boiling. Both starch and polyacrylamide activity gels were run.

Casting and running starch gels

The starch gels were cast and run as described by Badenhorst (1993). Briefly, 12% starch gels were prepared by boiling hydrolysed starch in TVB buffer (50 mM Tris, 2 mM EDTA, 65 mM boric acid, pH 8.0). Horizontal gels were cast (150 x 200 x 10 mm) and allowed to set for 2 hours. A slit was cut approximately 5 cm from one end of the gel and the samples, spotted onto 3 MM paper wicks, were inserted into this slit. Paper wicks containing bromophenol blue were also inserted into the slit, to provide a marker dye. The anode was set up at the end of the gel closest to the slit and a current of 60 mA was applied across the gel, using 10 x TVB as the electrode buffer. After 30 minutes the current was

switched off and the paper wicks were removed. Electrophoresis was then continued until the marker dye was 1 cm from the cathode.

Staining starch gels for GR activity

GR activity was localised on starch gels using two different staining protocols. The first protocol was described by Amory *et al.* (1992). The starch gel was overlaid with stain solution (10 mM phosphate buffer (pH 7.5), 0.05 mM EDTA, 10 mM magnesium chloride, 250 mg l^{-1} oxidised nicotinamide adenine dinucleotide phosphate (NADP), 250 mg l^{-1} glucose-6-phosphate, 7 U glucose-6-phosphate dehydrogenase, 2 g l^{-1} oxidised glutathione (GSSG), 0.75 mM 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), and 1% agar) and maintained at 37°C until yellow spots developed at the sites of GR activity.

The second staining protocol was that of Murphy *et al.* (1990). Briefly, the gel was overlaid with the stain (0.1 M Tris.HCl (pH 8.0), 85 μg ml^{-1} 2,3-dichlorophenolindophenol, 85 μg ml^{-1} flavin adenine dinucleotide (FAD), 0.85 mg ml^{-1} GSSG, 0.4 mg ml^{-1} reduced nicotinamide adenine dinucleotide (NADH), 0.2 mg ml^{-1} MTT and 0.7% agar) and incubated at 37°C until blue bands corresponding to GR activity appeared.

Running and staining non-denaturing polyacrylamide gels

The gel was run as described for SOD activity gels in 1.2.7.5. Staining for GR activity was carried out according to Foyer *et al.* (1991). Briefly, the gel was immersed in the stain (0.25 M Tris.HCl buffer (pH 8.4), 4 mM GSSG, 1.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 2 mM DTNB) until yellow bands coinciding with GR activity appeared. The gel was immediately photographed as the yellow reaction product readily leaches from the gel.

1.2.8.7 TEM localisation of *E. coli* GR using immunogold labelling

Plant material

Tobacco plants were maintained in a growth chamber at 27°C with a 12 hour light, 12 hour dark photoperiod at 120 μmol m^{-2} s^{-1}. The plants were watered with Long Ashton nutrient medium (Hewitt, 1952). The growth cabinet lights were switched off 3 days prior to leaf harvesting, in order to reduce starch levels in the chloroplasts. Only mature leaves were harvested for investigation. The leaves were prepared for transmission electron microscopy (TEM) using an epoxy resin as well as an acrylic resin.

TEM using epoxy resin

Fixation of tissues

Leaves were cut into blocks of 2 - 5 mm^3 and fixed (0.1 M potassium phosphate (pH 7.2), 2.5% gluteraldehyde, 0.5% caffeine) for 20 hours at 4°C. The leaf material was then washed three times for 5 minutes each in 0.1 M phosphate buffer (pH 7.2). Half of the material was then taken directly through the dehydration and embedding protocols. The remaining tissue was postfixed in 0.5% osmium tetroxide for 1 hour and then washed three times for 5 minutes each in 0.1 M phosphate buffer (pH 7.2), before being dehydrated and embedded.

Dehydration and embedding

Tissue blocks were dehydrated in an acetone series, starting with 30% acetone through 50%, 75% and finishing in 100% acetone. The dehydrated tissue blocks were first infiltrated with 50% resin in acetone for 4 hours and then in 100% resin for 20 hours. They were then embedded in epoxy resin (Spurr, 1969) which was polymerised at 70°C for 10 hours.

Immunolabelling

Leaf sections were cut (Reichert-Jung Ultracut E microtome) at a gold interference colour and initially collected on copper grids. These grids proved unsuitable for immunolabelling and subsequent sections were collected on nickel grids. Labelling was carried out using a rabbit antiserum containing antibodies against purified *E. coli* GR. This antiserum was provided by R. Ponquett. The sections were labelled in one of two ways:

Two step labelling protocol

The sections were first etched with 9% H_2O_2 for 1 minute. Following etching, the grids were washed twice with water for 2 minutes each wash. The grids were then incubated in phosphate buffered saline (PBS) (pH 7.2) containing 2% glycine for two 5 minute periods. The grids were then jet-washed (washed in a stream of liquid) with PBS (pH 7.2). Following this wash, the grids were pre-incubated in PBS (pH 7.2) containing 1% ovalbumin and 0.1% gelatine for four 5 minute periods. After the pre-incubation step the grids were incubated with antiserum diluted to either 1:200 or 1:100 with the pre-incubation medium. This step was carried out over 3 hours and was followed by two PBS washes of 2 minutes each and a PBS jet-wash. The grids were again placed on the pre-incubation medium for three 5 minute periods and then transferred to a solution of protein A-gold (pAg) (Sigma) diluted 1:50 in PBS (pH 7.2) containing 1% ovalbumin, 0.1% gelatine, 0.1% tween 20 and 0.1% triton X-100. The incubation with pAg was carried

out over 1 hour and was followed by two 2 minute washes and a jet-wash in the pre-incubation medium, two 2 minute washes and a jet wash in PBS (pH 7.2) and two 2 minute washes and a jet wash in distilled water. After washing, the grids were stained for 4 minutes in lead citrate (Reynolds, 1963) and washed again with water.

Controls were labelled as above but the rabbit antiserum was replaced with either plain blocking solution or a 1:50 dilution of pre-immune IgG (Sigma).

One step labelling protocol

Prior to the labelling of the grids, 50 μl of antiserum and 6 μl of pAg were mixed with 1000 μl of PBS (pH 7.2) containing 1% ovalbumin. This mixture was incubated at room temperature for half an hour to allow the formation of pAg-IgG aggregates. These aggregates were collected by centrifugation at 2 000 g (MC 200 nanofuge, Hoefer Scientific instruments) and the pellets were resuspended in 300 μl PBS (pH 7.2) containing 1% ovalbumin. The aggregates were redispersed by sonicating the suspension for 3 minutes, giving a solution containing pre-coupled immunogold complexes.

The grids were etched, washed and pre-incubated as for the two step protocol above. They were then incubated with freshly dispersed pAg-IgG complexes. The incubation time ranged from 1 to 2.5 hours. Following incubation, the grids received three 2 minute washes in PBS (pH 7.2) with 1% ovalbumin, two 2 minute washes and a jet-wash in PBS (pH 7.2) and two 2 minute washes and a jet-wash in distilled water. The washed grids were then stained for 4 minutes in lead citrate (Reynolds, 1963) and jet-washed with water.

TEM using acrylic resin

Fixation of tissues

The leaf tissue was fixed as for the epoxy resin but the blocks that were postfixed only received a 6 minute treatment in 0.5% osmium tetroxide.

Dehydration and embedding

Dehydration was carried out in an ethanol series starting with 25%, through 50% and finishing in 70% ethanol. The leaf material was then infiltrated for 1 hour in 2 parts LR white (London Resin Company): 1 part 70% ethanol. This was followed by two 30 minute infiltrations in pure LR white and an overnight infiltration at room temperature, again in pure LR white. The material was infiltrated for a further 24 hours in LR white at 4°C and then transferred to fresh LR white in gelatine capsules from

which all air bubbles had been excluded. Polymerisation was carried out in the gelatine capsules over 24 hours at 50°C.

Immunolabelling

The following labelling protocol was initially carried out using sections cut to a gold interference colour. The resulting contrast was extremely poor and sections were subsequently cut thicker - at a copper to blue-copper interference colour.

The grids were pre-incubated for three 5 minute periods in a blocking solution containing 0.5 M Tris buffer (pH 7.4) containing 0.1% gelatine, 1% ovalbumin, 1% tween 20 and 0.02% sodium azide. Following pre-incubation, the grids were incubated for 1 hour in antiserum diluted 1:10 with the blocking solution. The grids then received two 2 minute washes and a jet wash in 0.5 M Tris buffer (pH 7.4) and were again incubated for three 2 minute periods in blocking solution. This was followed by a 1 hour incubation in pAg diluted 1:50 with blocking solution. The grids were then taken through a series of washes (two 2 minutes washes in blocking solution, two 2 minutes washes and a jet wash in 0.5 M Tris and two 2 minute washes and a jet wash in distilled water). After washing grids were immersed for 10 minutes in 0.5% osmium tetroxide, followed by three 5 minute washes in water and 5 minutes of staining in lead citrate (Reynolds, 1963). For some of the grids the osmium treatment was omitted in order to determine the improvement in contrast that this step provided.

Controls were labelled as above but the rabbit antiserum was replaced with either plain blocking solution or a 1:50 dilution of pre-immune IgG (Sigma).

Viewing

All sections were viewed using a JEOL JEM-1010 transmission electron microscope.

1.3 RESULTS AND DISCUSSION

The primary aim of this work was to produce transgenic tobacco carrying the *E. coli* GR gene, a goal which was successfully achieved. Furthermore, the study examined the effect of kanamycin as a selection agent during the tranformation process. The transformants were characterised with respect to the activity and concentration of the transgene product, and the subcellular distribution of the *E. coli* GR was examined in some detail.

1.3.1 Bacterial growth and cell counts

Liquid GT medium supplemented with antibiotics was maintained at 28 °C for 28 hours. The OD of the bacterial population was monitored at 600 nm and showed an initial lag phase lasting approximately 15 hours. The bacterial population then underwent a logarithmic growth phase, with the most rapid increase occurring between 19 and 27 hours after the inoculation of the medium (Fig. 1.2). After 27 hours of growth there was a decline in the growth rate.

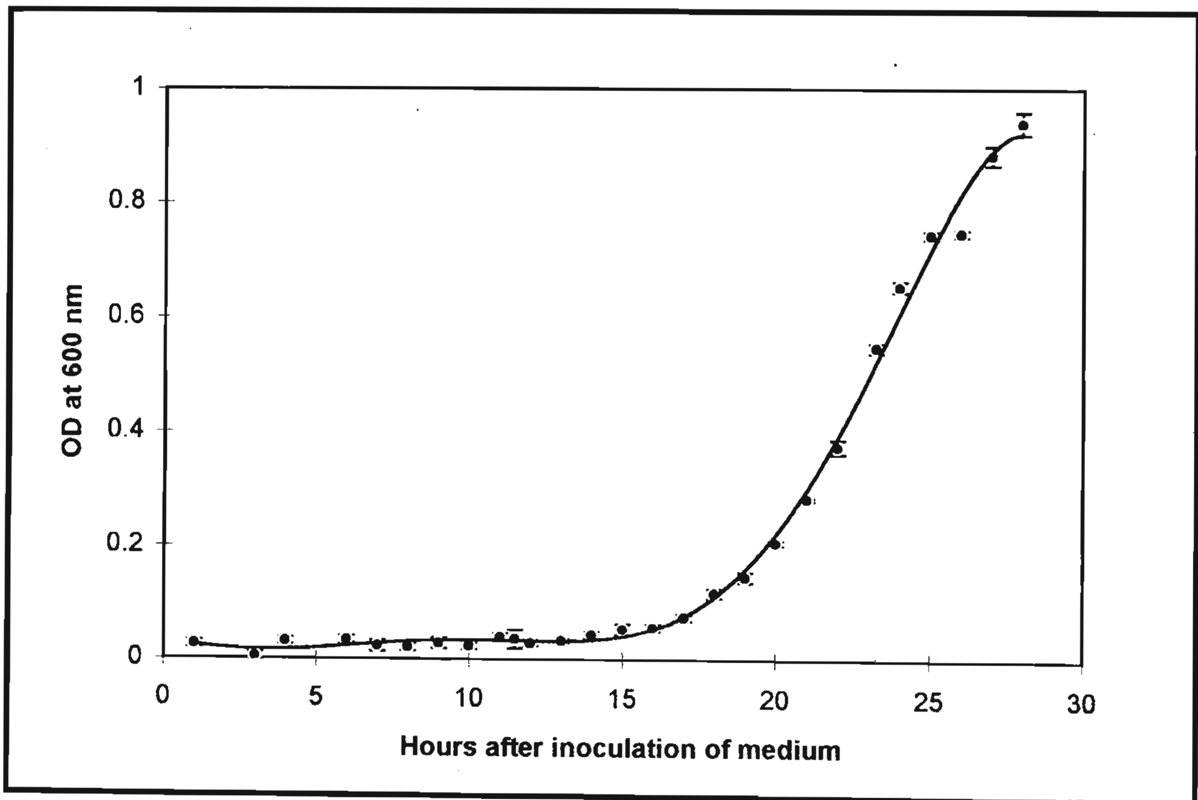


Figure 1.2: Growth kinetics of *Agrobacterium tumefaciens* strain LBA4404. The optical density (600 nm) of the *A. tumefaciens* culture was monitored over 28 hours to determine the growth of the bacterial population. Data represents the mean and standard deviation of three readings.

Subculturing of bacteria and the removal of aliquots for the transformation took place 23 - 25 hours after inoculation of the medium, when the growth was most vigorous.

Before performing the transformation, aliquots of the bacterial culture were removed between 23 and 25 hours and a relationship between OD and cell number was determined as described in section 1.2.1.3. The aliquots were found to have an OD_{600} of 0.706, corresponding to a cell count of 5 x 10^8 cells ml^{-1}. These cultures were diluted with liquid MS medium to provide a cell density of 5 x10^6 cells ml^{-1} for the transformation.

1.3.2 Transformation

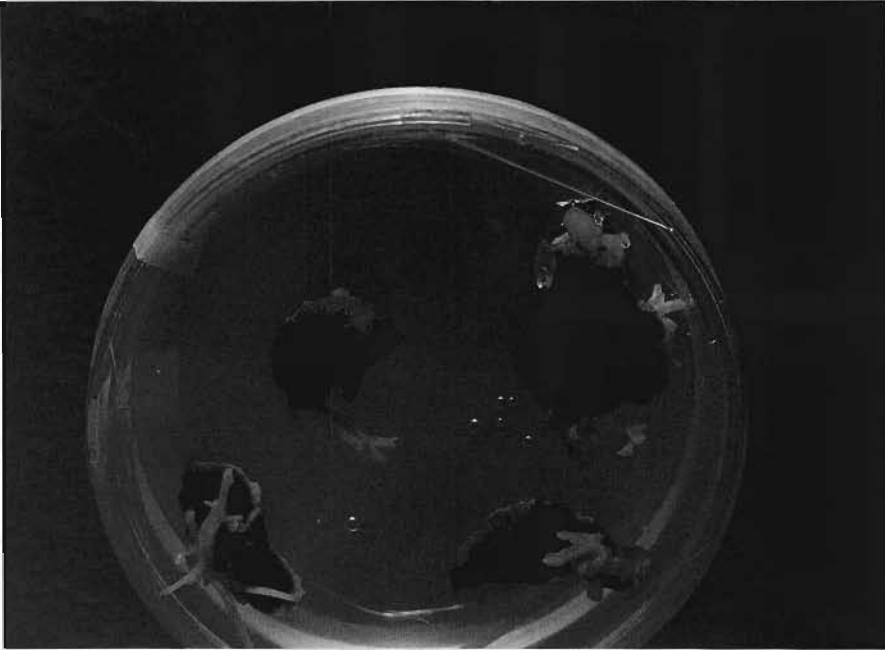
The infection and curing of the leaf discs with *A. tumefaciens* was carried out as described in section 1.2.3. Following this, the leaf discs were put through selection protocols for the production of transformed plants.

1.3.2.1 Shoot formation on selection plates

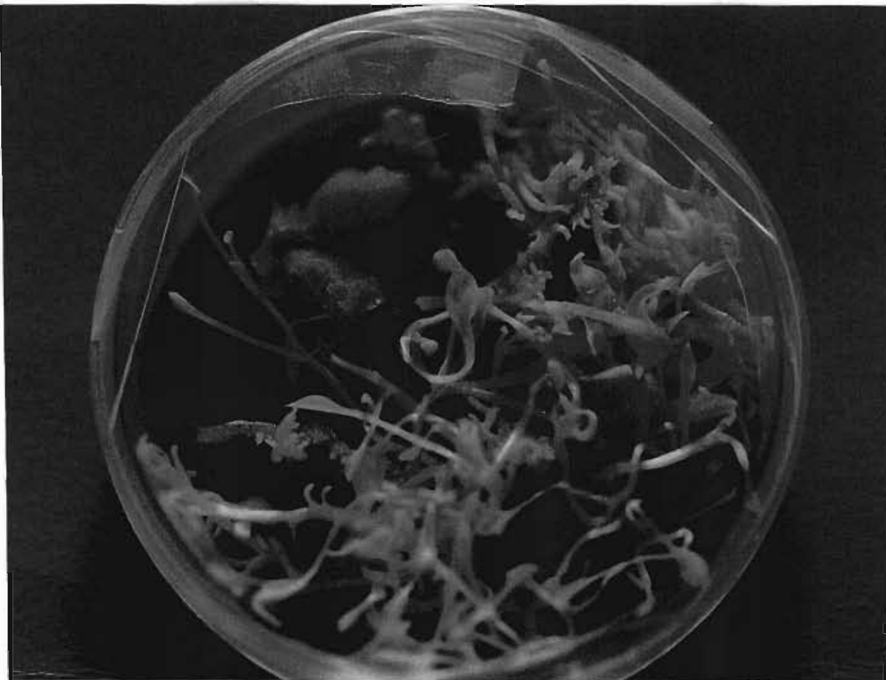
Kanamycin is capable of inhibiting plant growth, even in plant cells carrying the gene for kanamycin resistance. Sriskandarajah *et al.* (1994), working on apple transformations, showed that selection media with kanamycin concentrations between 40 and 100 $\mu g\ ml^{-1}$ cause a reduction of callus, shoot and root production relative to media with no kanamycin. These authors demonstrated that for callus production and rooting the reductions in growth were independent of kanamycin concentration. However, shoot production showed a sharp decline as kanamycin concentrations increased. Working with flax, Dong and McHughen (1993) also showed a decline in shoot production with increasing concentrations of kanamycin. In choosing a kanamycin-based selection protocol it was thus necessary to determine a kanamycin concentration which would allow shoot production, and still inhibit growth in untransformed cells. For this reason, the inoculated explants were divided randomly into four groups, each group being incubated on selection plates with a different kanamycin concentration (0, 50, 100 or 300 $\mu g\ ml^{-1}$ kanamycin).

Figs. 1.3A - 1.3E show the effect of various kanamycin concentrations on the formation of shoots and callus on selection plates. As seen in Figs. 1.3A and 1.3B, leaf discs incubated without kanamycin showed shoot production within just three weeks. After six weeks these leaf discs showed a substantial amount of callus along all of the leaf disc margins, and a proliferation of shoots had occurred. The major drawback to this treatment was the large number of shoots which required screening in the later stages of the selection protocol. Leaf discs incubated on 50 or 100 $\mu g\ ml^{-1}$ kanamycin showed the start of callus production after only 4 weeks (Fig. 1.3C). This callus formed at isolated points, presumably where cell transformations had taken place. Another two weeks were required for further callus development and the start of shoot production (Fig. 1.3D). The leaf discs incubated on plates containing 300 $\mu g\ ml^{-1}$ kanamycin showed no signs of either callus or shoot production. After six weeks many of these leaf discs were necrotic, turning brown as shown in Fig. 1.3E.

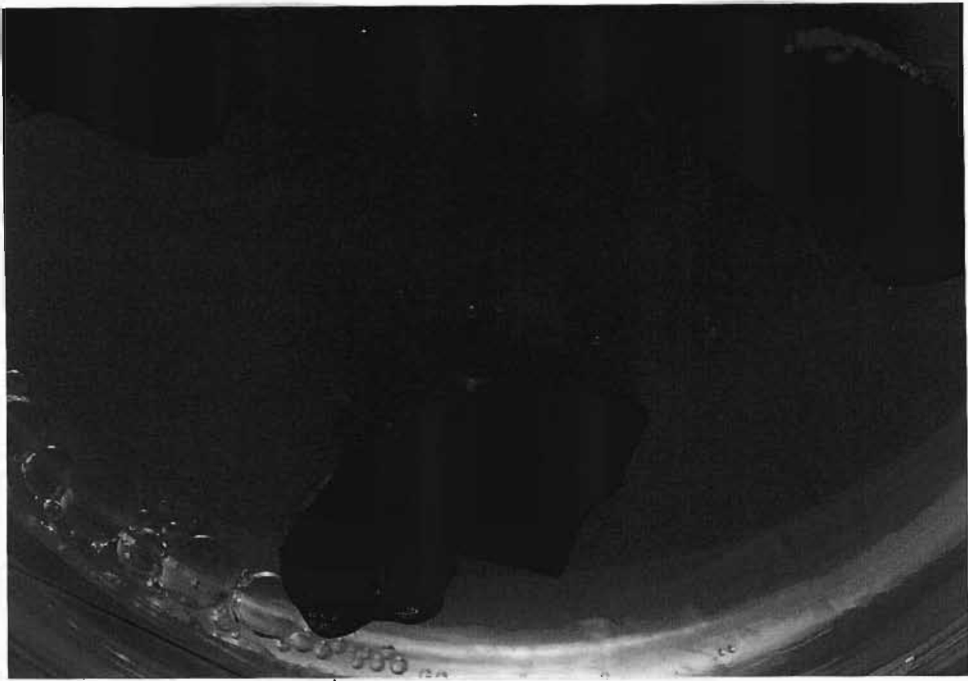
A



B

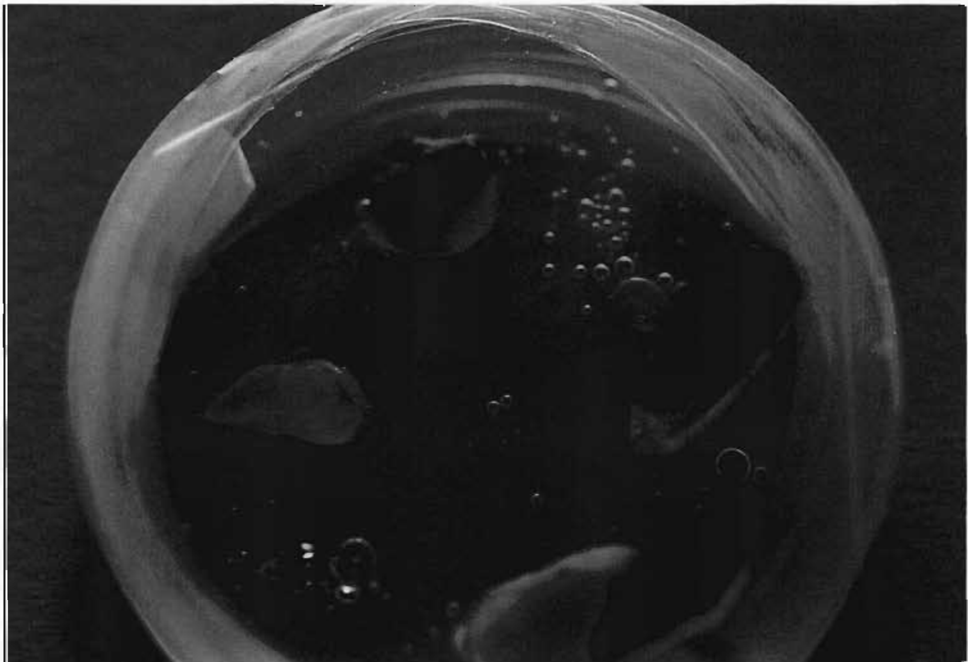


C



D

E



After sixteen weeks in culture the effects of the kanamycin concentration on callus and shoot production were evaluated (Table 1.1).

Table 1.1: The response of leaf discs to varying kanamycin concentrations in the selection medium. The table shows the percent of explants forming callus and shoots compared to the percent showing necrosis after 16 weeks of incubation with varying concentrations of kanamycin.

	Kanamycin concentration (μg ml^{-1})			
	0	50	100	300
% leaf discs forming callus	100	11	22	0
% leaf discs forming shoots	100	6	22	0
% leaf discs necrotic	0	17	17	45

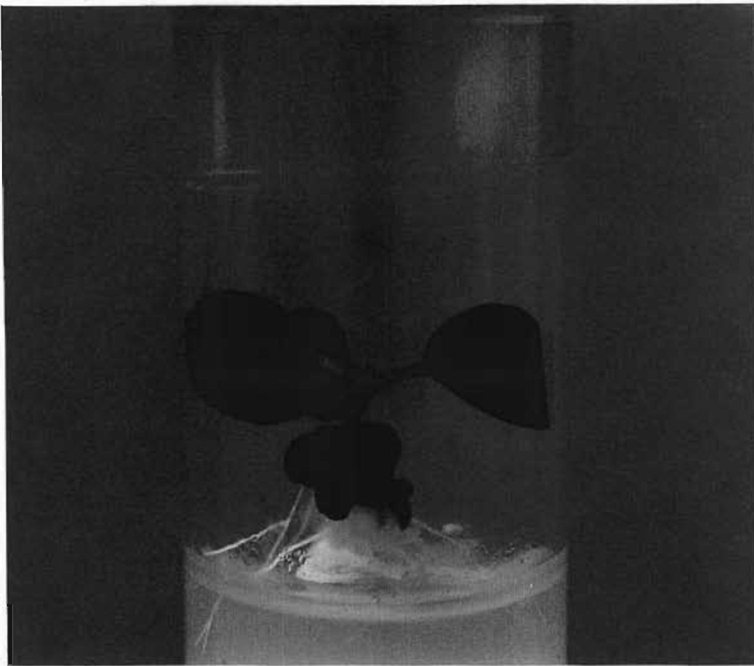
n = 18 leaf discs per kanamycin treatment

The table shows the percentage leaf discs on each concentration that formed callus and/or shoots, as well as the percentage which showed necrosis. The explants incubated without kanamycin showed no necrosis, while all of them had produced callus and shoots. In contrast, nearly half of the explants incubated with 300 μg ml^{-1} kanamycin were necrotic and none showed signs of callus or shoot development. The explants on intermediate kanamycin concentrations showed some necrosis, but several leaf discs also formed callus and shoots. These results are in agreement with those of Dong and McHughen (1993) and Sriskandarajah *et al.* (1994).

1.3.2.2 Screening for transformed shoots

Dong and McHughen (1993) showed that although kanamycin severely inhibits the growth of plant cells, a large percentage of the shoots selected on lower kanamycin concentrations are not transformed. These plants are commonly referred to as 'escapes' (Ying *et al.*, 1992). The number of untransformed shoots escaping the selection protocol declines as kanamycin concentrations increase. The escapes probably arise due to protection of untransformed cells by neighbouring transformed cells. In order to detect these escapes, shoots from the leaf discs were placed on a rooting medium containing 100 μg ml^{-1} kanamycin. Sriskandarajah *et al.* (1994) showed that this concentration of kanamycin is sufficient to prevent rooting in a large percentage of untransformed shoots. Fig. 1.4 shows the effects of including 100 μg ml^{-1} kanamycin in the rooting medium. Transformed plants showed rapid chlorophyll development and formed aerial roots within four days (Fig. 1.4A). Maintenance on the rooting medium resulted in the formation of roots within the medium (Fig. 1.4B). This rooting started within 10 days of transfer to the rooting medium, and was accompanied by the growth of new leaves. In contrast, the untransformed shoots failed to develop on the rooting medium. They remained a pale green colour, indicting a lack of chlorophyll production

A



B

C

(Fig. 1.4C). They also failed to form new leaves, and after several weeks in culture necrosis was evident in most of these shoots.

1.3.2.3 Hardening off of shoots

Shoots which rooted on the rooting medium supplemented with kanamycin were subsequently transferred to the same rooting medium without kanamycin. The removal of kanamycin from the medium resulted in a rapid increase in growth, and within three weeks the plants developed sufficient roots and leaves to be hardened off (Fig. 1.5).

Figure 1.5: Preparation of transformed shoots for hardening off. Plants were prepared for hardening off on rooting medium (½ MS) without any kanamycin. Three weeks after transfer to this medium, the shoots had developed sufficient roots and leaves to be hardened off.

The hardening off process required was relatively fast, with plants adapting to growth room humidities within 7 - 10 days. These plants were then analysed for the presence of the *gor* gene product.

1.3.3 Identification and characterisation of *gor* gene transformants

The plants resulting from the transformation were analysed for the presence of the *gor* gene in several ways, all involving the detection of the gene product - *E. coli* GR. The first assay was for an increase in GR activity. Although this would not differentiate between the bacterial and plant enzymes, a substantial increase in GRA would strongly suggest a positive transformation. To further characterise these plants, soluble protein was extracted and subjected to western blots using an antibody raised against *E. coli* GR. Three plants showing high GR activity and which were positive for *E. coli* GR in the western blots were self fertilised to produce the F1

generation. The resulting F1 plants were again checked for high GR activity and the presence of *E. coli* GR, to confirm the stable transfer of the gene to the progeny. One of these F1 lines was selected for furhter analysis. These F1 plants were used for GR activity gels to provide an idea of the ratios of native to bacterial GR. They were then used to determine the subcellular localisation of *E. coli* GR. This was accomplished in two ways. Firstly, isolated chloroplasts were subjected to western blotting, to confirm the presence of *E. coli* GR in the chloroplasts. The second method involved an immunocytochemical (ICC) stain, with the anti-*E. coli* GR antibody being used for the detection of *E. coli* GR in TEM sections. Protein A gold (pAg) was used for the visualisation of antibody binding sites on TEM sections.

1.3.3.1 Glutathione reductase activity in possible transformants and F1 plants

Possible transformants.

Glutathione reductase assays showed a wide range of activities in the plants which resulted from the transformation process. Of the 15 plant lines chosen from the selection process 47% showed a GR activity equal to, or lower than, that of the control plants. These are probably escapes (discussed in section 1.3.2.2). In the remaining 53% of possible transgenics GRA ranged from 0.72 μmol NADPH oxidised min^{-1} g fwt^{-1} to 32.39 μmol NADPH oxidised min^{-1} g fwt^{-1}. Three of the high expressing lines were self-fertilised for the production of F1 plants.

F1 plants

Seeds from the three selected lines were grown as described in the materials and methods. Twelve plants from each of these lines were randomly selected for GR assays. In all three lines, there were plants exhibiting GRA equal to those of untransformed plants. Table 1.2 shows the average GRA of the transformed plants in each line relative to the parent plants. The percentage of untransformed plants in each F1 line is also shown (assuming that a GR activity equal to that of the control plants indicates an untransformed plant).

Table 1.2: Glutathione reductase activity in three transformed plants and their correspondig F1 lines. The table also shows the percentage plants in each line that were not transformed. GRA for F1 plants is given as the mean and standard deviation of the readings for transformed plants in each line.

	GRA in parent plants (μmol NADPH min^{-1} g fwt^{-1})	GRA in F1 plants (μmol NADPH min^{-1} g fwt^{-1})*	% F1 plants untransformed•
Line 1	32.39	25.13 ± 7.01	58
Line 2	25.85	32.63 ± 6.46	8
Line 3	20.21	19.39 ± 12.07	33

* n = 5 for line 1, n = 11 for line 2, n = 8 for line 3

• n = 12 for each plant line

From Table 1.2 it can be seen that a number of plants in each line show no extra GRA, although all of the parent plants had high GRA. If the *gor* gene were inserted at a single site in the parent plants, only 25% of the F1 plants would be expected not to carry the gene (consistent with the Mendelian laws of inheritance). As this was not the case, two conclusions are possible. One is that the number of plants sampled was too low to show population trends. The second possibility is that multiple insertions of the *gor* gene occurred. In an attempt to rule out one of these possibilities, line 2 was selected for further analysis and GR assays were carried out on 63 more plants, giving a total of 75 plants (including the 12 already analysed) (Fig. 1.6). Of the 75 plants, 39% showed no increase in GRA. Based on this GRA data, it seems that this line does not carry just a single copy of the *gor* gene.

The GR assays for the plants with increased GRA show an activity (mean $\pm$ std deviation) of 25.41 $\pm$ 11.47 μmol NADPH min^{-1} g fwt^{-1}. As shown in Fig. 1.6, there was considerable variation in the GRA of these plants.

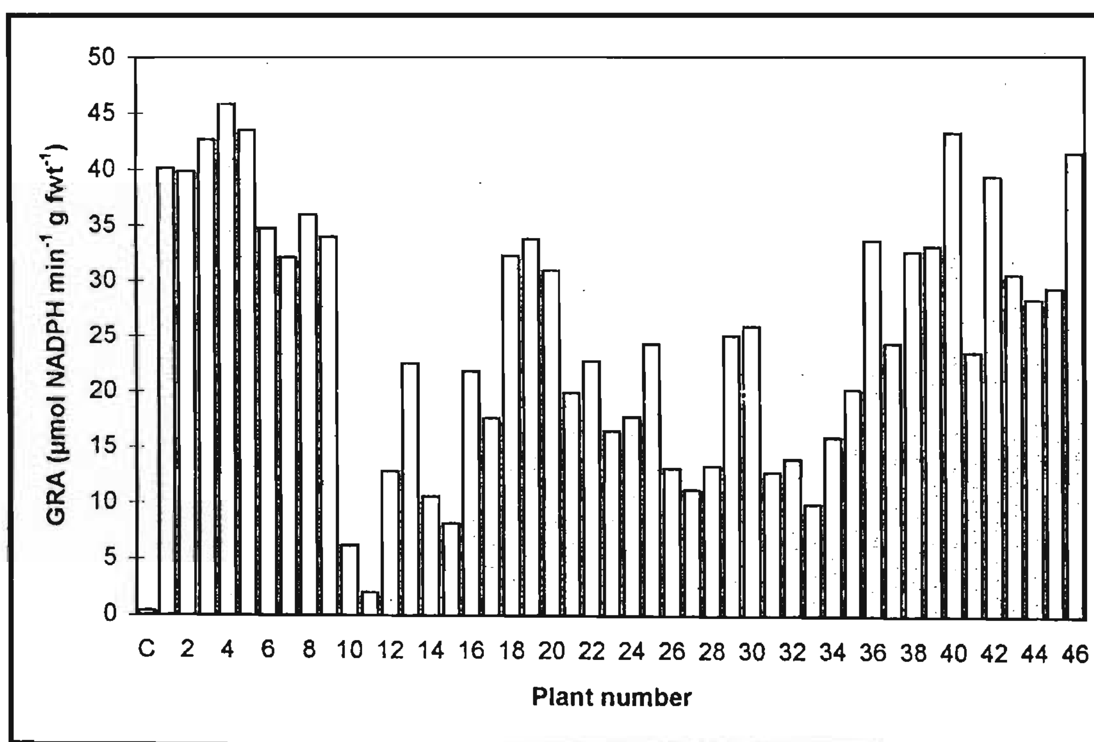


Figure 1.6: Variation in the GRA of F1 transgenic plants. Only the plants showing higher activities then the control (labelled 'C' in the figure) are shown.

The GRA of these F1 plants varies from 5 - 120 times that of the control plants. Similar variations have been reported in other transformation studies (Lagrimini *et al.*, 1990; Shirsat *et al.*, 1989). One explanation for the variation is that the parent plants had two or more copies of the gene inserted on different chromosomes, resulting in the F1 plants having a variable number of gene copies. However, such a scenario would be

difficult to prove simply by examining the gene product levels. Shirsat *et al.* (1989) carried out a detailed analysis of tobacco plants carrying different copy numbers of the pea *legA* gene and showed that gene copy number is not directly related to the variation in levels of the gene product. These authors proposed alternative reasons for the differences in gene expression, including the effect of the insertion location (positional effects) and methylation of the introduced DNA. Other factors which may affect gene expression include developmental stage of the tissue, the type of tissue and the type of gene examined. Each of these will be considered briefly.

Type of gene introduced

Dunsmuir *et al.* (1988) have reported that the expression of foreign genes is partly dependent on the type of gene introduced. They report a 2 - 5-fold variability in the expression of introduced seed protein genes, compared to a variability of several hundred fold for the maize *Adh*1 gene. Dunsmuir *et al.* (1988) point out that the cause of the variability is not well understood, but may well be due to positional effects (discussed in the next section).

Positional effects

The expression of T-DNA genes in different transformants is affected by the surrounding DNA or chromatin structure, the extreme example of this being the total loss of expression of the introduced gene (Horsch *et al.*, 1988). According to these authors, such a loss occurs in one quarter to one third of transformants during differentiation of the plantlet. Dunsmuir *et al.* (1988) have attempted to buffer inserted genes against effects induced by local DNA sequences, by surrounding the inserted DNA with long regions of flanking DNA. They found that this strategy afforded no change in the variability of expression, and concluded that the positional factors affecting gene expression operate over long distances - e.g. chromatin folding.

DNA methylation

DNA methylation has been proposed to play a significant role in the control of gene expression in plants. Up to 30% of cytosine residues in plant DNA are methylated compared to 5% in animal cells (Sinkar *et al.*, 1988). These methylated regions are transcriptionally inactive. Since these regions are so large in the plant genome, there is a good possibility that a foreign gene will insert at a methylated site and remain untranscribed. Furthermore, plants may possess the ability to actively silence foreign DNA (Sinkar *et al.*, 1988). Genes which have been silenced by means of cytosine methylation may be activated by treatment with a hypomethylating agent, and several studies (Peerbolte *et al.*, 1987; Sinkar *et al.*, 1988) have used such agents to show that the

lack of expression of many introduced genes is indeed due to methylation of cytosine residues.

Tissue-specific factors

The expression of foreign genes in plant tissues depends to a certain extent on the age and the type of tissue examined. This is particularly relevant for genes under the control of the CaMV 35s promoter. Although this promoter has been classified as constitutive, Williamson *et al.* (1989) have shown that it is more active in young, actively dividing tissues than in older tissues. These authors have also shown differences in promoter activity for different types of tissue within the same plant. One of the proposals forwarded by Williamson *et al.* (1989) is that the activity of the CaMV 35s promoter is dependent on changes in the conformation of DNA that occur in actively dividing cells. An alternate explanation for the age-specific differences is that cells of different ages have different methylation levels. This is supported by the findings of Grierson and Covey (1988), who have shown that dividing cells have lower methylation levels than older less active cells.

There are thus a number of possible reasons for the variation observed in the GRA levels for the plants used in this study. An attempt was made to rule out age and tissue specific factors by utilising leaf material from plants of the same age and by using leaves at the same stage of development. However, Williamson *et al.* (1989) have shown that gene expression levels vary even within a single leaf, depending on which part of the leaf is sampled. It is therefore not unlikely that the variation observed in this study is due, in part, to variations in the activity of the CaMV 35s promoter. A second possibility is that variation was caused by different degrees of DNA methylation from plant to plant. Confirmation of this would require a study of methylation levels in the different plants used. Finally, differences in gene copy number may still have played a role in the variation of gene expression levels. This is particularly relevant when one considers the large differences between the plants with the highest GRA and those with the lowest GRA.

1.3.3.2 Confirmation of transformation using western blots

As mentioned in section 1.3.3, GR assays do not differentiate between plant GR and bacterial GR. It was therefore necessary to confirm the transformation by showing that the plants with high GRA did in fact carry the bacterial form of the enzyme. This was accomplished by means of western blots, using an antibody raised against *E. coli* GR. The initial western blot was carried out using soluble protein extracted from several plants selected for kanamycin resistance. Controls included soluble protein extracted from a control plant and from a plant known to carry *E. coli* GR in the cytoplasm, as well as purified *E. coli* GR (supplied by R. Ponquett). The results of this blot are shown in Fig. 1.7. The control plant (lane 1) shows several protein bands having very low cross-reactivity with the antibody, all

of which are much less intense than the band obtained with purified *E. coli* GR (lane 9). This protein shows extremely strong cross-reactivity with the antibody, giving a single dark band. The plant carrying cytoplasmic *E. coli* GR shows two proteins which react with the antibody - neither of which is seen in the control plant. Five of the six possible transgenic plants also show these two bands, although they are much more intense than those in the cytoplasmic transformant. The darker of the two bands in these lanes migrates the same distance as the purified *E. coli* GR, confirming the successful transformation of these plants. This band has a molecular weight of 47 kDa, which corresponds well to the 48.717 kDa predicted by Greer and Perham (1986) for *E. coli* GR.

The second band in the transgenic plants has a molecular weight of around 97 kDa - slightly more than double the weight of the protein identified as *E. coli* GR. This band is not present in the control plants, indicating a protein associated exclusively with the transformants. It is known that the active form of *E. coli* GR is a dimer of two identical subunits and that the enzyme maintains an equilibrium between the monomeric and dimeric forms, with the equilibrium being affected by NADPH and glutathione (Scrutton *et al.*, 1992; Arscott *et al.*, 1989). The molecular weight of the heavier band suggests that it is the dimeric form of GR. A possible explanation is that the dimeric GR was not completely denatured prior to electrophoresis, hence the additional band.

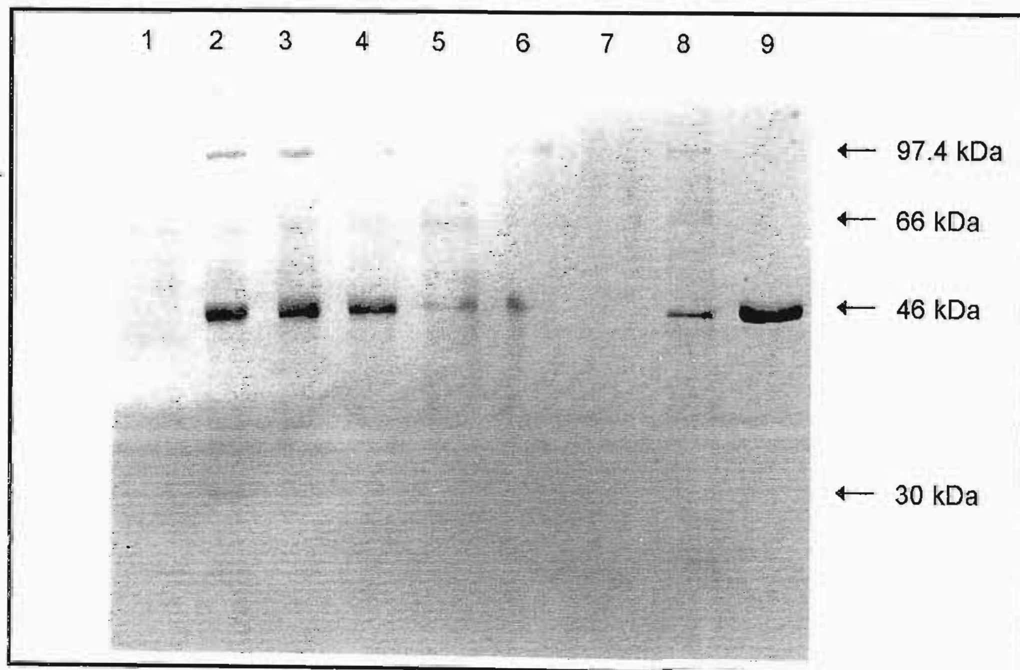


Figure 1.7: Immunodetection of *E. coli* GR in transgenic tobacco plants. Soluble protein was extracted from seven plants selected for kanamycin resistance and subjected to western blotting. Lane 1 carries 60 µg of soluble protein from an untransformed control plant, while lanes 2 - 7 each carry 60 µg of protein from a possible transformant. Lane 8 was loaded with 60 µg of protein from a tobacco line carrying *E. coli* GR in the cytoplasm and lane 9 received purified *E. coli* GR, supplied by R. Ponquett.

As mentioned, the antibody shows very little cross-reactivity with proteins other than *E. coli* GR. Whittaker (1990) obtained a band at 60 kDa using an anti-*E. coli* GR antibody. This 60 kDa band was hypothesised to be the plant GR, since this protein has a molecular weight of about 60 kDa (Tanaka *et al.*, 1988a). Such cross reactivity between the antibody used in this study and the plant GR was not observed. Due to the low cross reactivity observed in Fig. 1.7, it was decided to use the same antibody concentration in all further blots.

Having identified plants carrying *E. coli* GR, and decided on an antibody concentration, the protein loading levels for the western blotting procedure were optimised. This was accomplished by carrying out another blot utilising a series of protein loadings from one of the plants identified as an *E. coli* GR transformant. The optimisation blot is shown in Fig. 1.8.

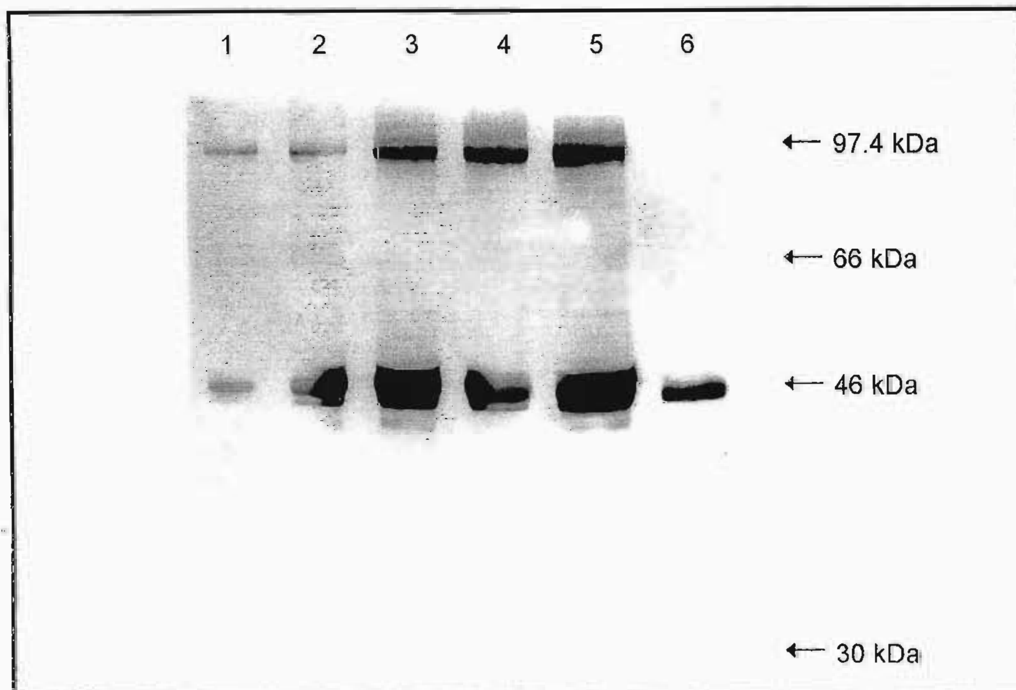


Figure 1.8: Optimisation of the western blotting procedure. A series of protein levels were loaded into lanes 1 - 5, with the lanes receiving 50, 75, 100, 125 and 150 µg of soluble protein respectively. Lane 6 was loaded with purified *E. coli* GR.

All of the lanes for the transgenic plants show the two bands discussed for Fig. 1.7. The band with the lower molecular weight again co-migrates with the band seen for purified *E. coli* GR in lane 6. The intensity of this band is very similar for lanes 3 - 5, indicating that protein levels above 100 µg are saturating. A protein loading of 60 µg per lane was selected as optimal, since this would allow the detection of increases in *E. coli* GR. A feature of this blot not seen in the first one is the presence of a second band very close to the 47 kDa band. This second band is more readily seen in lanes 1 and 2 where protein levels are lower - the two bands tend to overlap where the protein concentrations are saturating. The difference in molecular weights of these two bands is 4 000 - 5 000 kDa. This is the molecular weight of the transit peptide (TP) used to target *E. coli* GR to the

chloroplast (Van den Broeck *et al.*, 1985). This transit peptide is the peptide responsible for the chloroplastic insertion of the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO). Van den Broeck *et al.* (1985) have shown that this transit peptide will readily transport foreign proteins into the chloroplast, after which it is cleaved from the foreign protein. It would thus seem that the darker of these two bands is the unprocessed TP-*E. coli* GR protein in the cytoplasm, while the paler band (of lower molecular weight) is the processed *E. coli* GR in the chloroplast. From the relative intensities of the two bands, it seems that the unprocessed TP-*E. coli* GR protein is present in much larger quantities than the processed *E. coli* GR. Aono *et al.* (1993) carried out a similar transformation using the transit peptide for ferredoxin and only found the processed form of *E. coli* GR in extracts from whole leaves, indicating a rapid uptake and processing of the TP-*E. coli* GR protein. However, the plants produced by these authors only showed a GRA increase three times that of control plants, as opposed to the 50 times increase in GRA shown here. It thus seems that the plants produced in this study generate the TP-*E. coli* GR protein more rapidly than it can be incorporated into the chloroplasts.

Having optimised the western blotting protocol, ten of the high expressing plants from the selected F1 line (see section 1.3.3.1) were subjected to western blotting. The western blot is shown in Fig. 1.9.

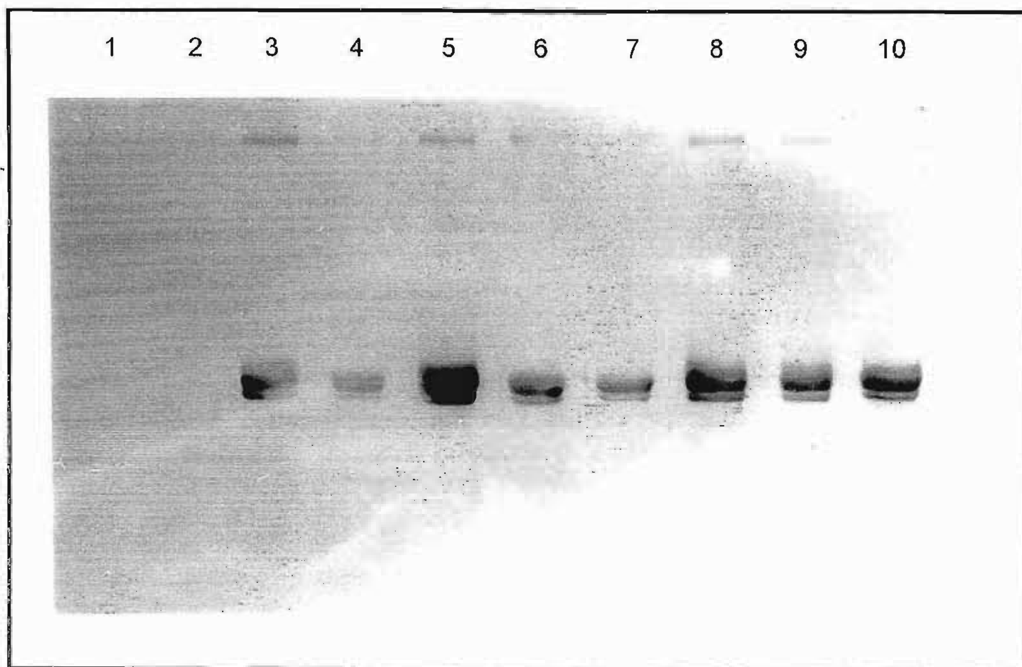


Figure 1.9: Optimised western blots for F1 transgenics. Western blots were run for 10 of the plants tested for GR activity in section 1.3.3.1. Eight of the plants were selected at random from the group showing high GRA, while the other two were chosen from the group showing GRA equivalent to the control plants. Each lane was loaded with 60 µg soluble protein from the relevant plant.

Two of the plants, selected for having GRA similar to the control plants, show no *E. coli* GR protein. This indicates that the GR assays are a reliable means of selecting the transformants. The remaining eight plants, selected at random from those having high GRA, all show the same banding patterns observed in the optimisation blot. That is, they show bands representing the monomeric and dimeric forms of processed *E. coli* GR, as well as a band representing *E. coli* GR attached to the chloroplastic transfer peptide from the small subunit of RUBISCO. The blot also shows variations in the levels of the *E. coli* GR protein, confirming that the observed variations in GRA are due to variations in gene expression.

1.3.3.3 Distribution of GRA between plant and bacterial GR

Before confirming the chloroplastic localisation of the *E. coli* GR, the relative contributions of *E. coli* GR and plant GR to the total GRA were examined. This was accomplished by separating the two forms of GR on non-denaturing gels and then staining the gels with a stain for GR activity. This experiment was performed using both starch and polyacrylamide gels. The starch gels were unable to provide much information, as the stain used was too faint to analyse. The polyacrylamide gels proved more effective and allowed localisation of the isozymes to be visualised (Fig. 1.10).

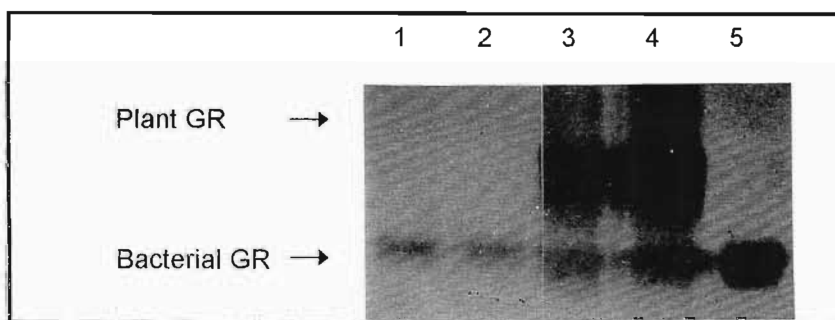


Figure 1.10: Relative GR activities of the bacterial and plant forms of GR. Transformed and control plants were electrophoresed on a 10% non-denaturing polyacrylamide gel, which was then stained for GR activity. Extracts from control plants were run in lanes 1 and 2, which received 60 and 80 µg of soluble protein respectively. Lanes 3 and 4 received 60 and 80 µg of soluble protein from a transformed plant, while lane 5 carried purified *E. coli* GR.

The gel shows only a single faint band of GR activity in the control plants. In contrast, the transformed plant shows several bands of activity. The band of lowest molecular weight in the transformants co-migrates with the band of activity in the lane carrying purified *E. coli* GR. Due to the dark smear in the lanes for the transformants, the plant isozyme in these plants cannot be seen. However, a third band is observed between the *E. coli* GR band and the point where the plant GR should occur. This same GRA banding pattern was observed by Foyer *et al.* (1995) in poplar trees transformed with foreign GR targeted to the chloroplast, but these authors did not speculate as to the origin of the intermediate band in the transformants. It is suggested here that since the active forms of GR are homodimers (Scrutton *et al.*, 1992; Arscott *et al.*, 1989), the intermediate band may represent a heterodimer comprised of one bacterial and one

plant subunit. If this is the case, the weight of the intermediate band is not readily explained, since the plant form of the enzyme is present in very low quantities.

1.3.3.4 Subcellular localisation of *E. coli* GR

Up to this point in the discussion, it has been assumed that the transformed plants carry the *E. coli* GR in the chloroplast. This has been supported by circumstantial evidence, such as the presence of both *E. coli* GR and TP-*E. coli* GR. The subcellular location of the *E. coli* GR was investigated in two ways. The first involved the analysis of the protein content of isolated chloroplasts using the western blotting technique. The second method involved visualisation of the *E. coli* GR location on TEM sections prepared from transformed plants. The anti-*E. coli* GR antibody used in the western blots was used as the primary antibody in an immunogold staining protocol.

Western blots of isolated chloroplasts

Before performing the western blots, the purity of the isolated chloroplasts was confirmed by examining the distribution of a marker enzyme in the cellular fractions. The marker enzyme used was superoxide dismutase (SOD), which has several isozymes specific to certain subcellular compartments. SOD is readily assayed using an activity gel (Fig. 1.11).

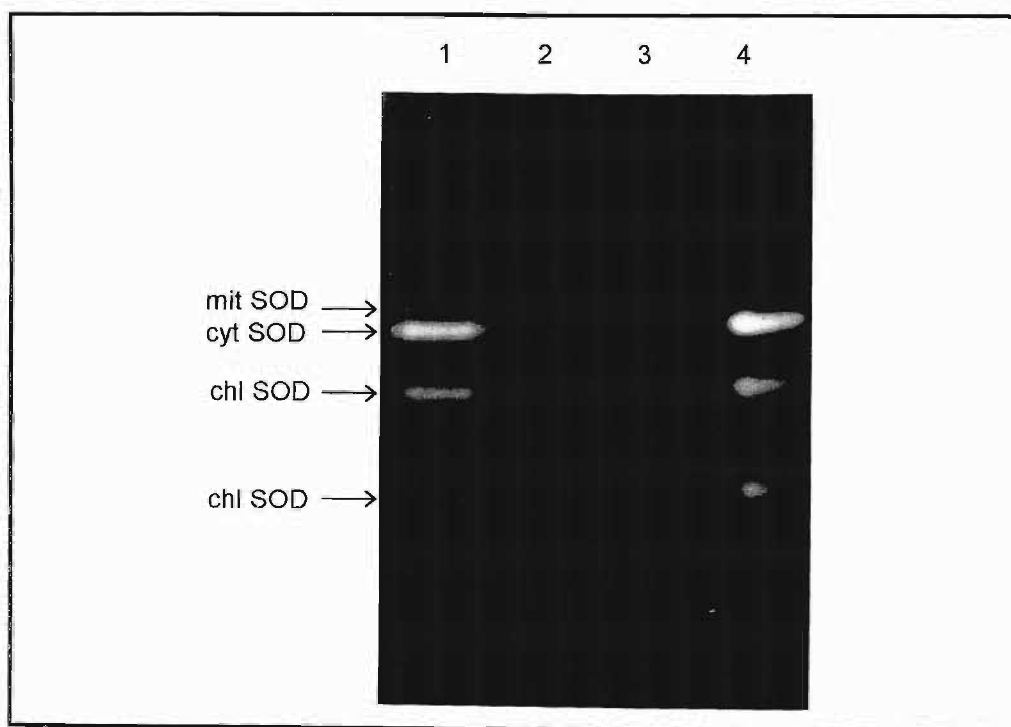


Figure 1.11: SOD activity gel for the determination of the purity of isolated chloroplasts. Soluble protein from isolated chloroplasts was electrophoresed on 10% polyacrylamide gels. A SOD stain was used to visualise the SOD isoforms present. Lane 1 is loaded with soluble protein from the leaves of transformants (80 µg). Lanes 2 and 3 carry 40 and 60 µg respectively of protein from isolated chloroplasts. Lane 4 is a control lane loaded with 80 µg soluble protein from an untransformed plant. Abbreviations: chl = chloroplastic, mit = mitochondrial, cyt = cytoplasmic.

It can be seen that the control plants and the whole leaves of transformed plants carry four SOD isozymes. One of these is a mitochondrial enzyme, two are chloroplastic and the fourth is cytoplasmic. These SOD isozymes are discussed in greater detail in Chapter 2. Fig. 1.11 shows that the cytoplasmic form of SOD is not present in the isolated chloroplasts, indicating that the chloroplasts are not contaminated by cytoplasmic elements. There is, however, some mitochondrial contamination. Having determined the levels of contamination in the isolated chloroplasts, they were subjected to western blotting, the results of which are shown in Fig. 1.12.

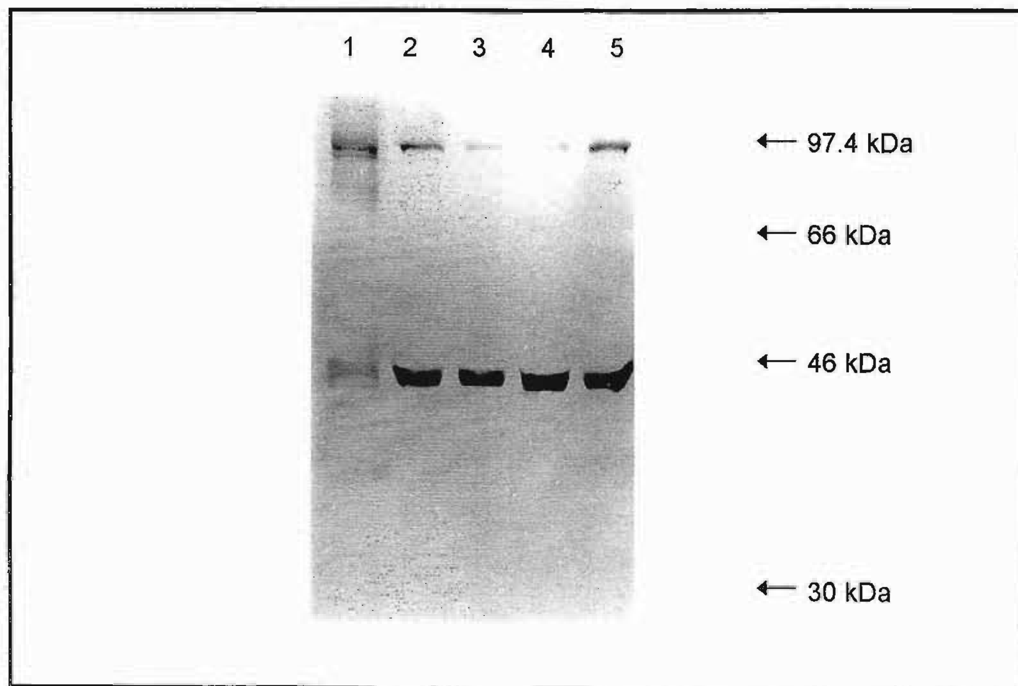


Figure 1.12: Immunodetection of *E. coli* GR protein in isolated chloroplasts using western blotting. Lane 1 is loaded with 80 μg soluble protein from entire leaves of transformants, while lanes 2 - 5 carry soluble protein from two batches of isolated chloroplasts. Lanes 2 and 3 have 60 and 80 μg of protein respectively from the first batch of chloroplasts and lanes 4 and 5 carry 80 and 100 μg of protein respectively from the second batch.

The first lane in Fig. 1.12 shows the same banding patterns obtained for the previous western blots of whole leaf material from transgenics. Lanes 2 - 4 show the proteins present in the chloroplasts of the transformants. These lanes contain the band of lowest molecular weight seen in the whole leaf extracts - a band previously interpreted as *E. coli* GR which has been processed during chloroplast insertion. The isolated chloroplasts also carry the band previously interpreted as the dimeric form of the processed *E. coli* GR protein. The band originally interpreted as the TP-*E. coli* GR protein is absent from the chloroplasts. This is consistent with the earlier hypothesis that this fusion protein is only present in the cytoplasm, with the TP being cleaved from the TP-*E. coli* GR protein as it enters the chloroplast.

TEM localisation of *E. coli* GR

In order to confirm the subcellular localisation of *E. coli* GR, leaf tissue from the transformed plants was prepared for TEM viewing. The embedding of the sections was carried out in LR white resin as well as an epxoy resin. The epxoy resin proved unsuitable for labelling, giving very little labelling even at high antibody concentrations. The LR white, a hydrophilic resin specifically manufactured for immunostaining procedures (London Resin Company data sheet), was therefore used for the labelling procedue. As with most immunocytochemical approaches osmium tetroxide was not used for a post-fixation treatment of the tissue, as it tends to decrease the antigenicity of the sections (London Resin Company data sheet). The TEM sections were first incubated with the same antibody utilised in the western blots, following which they were incubated with protein A that had been conjugated with colloidal gold. Protein A is a cell wall protein from *Staphylococcus aureus* which has a high affinity for the F_c region of immunoglobulins - particularly IgG (Robards and Wilson, 1993). This interaction with immunoglobulins is a pseudoimmune reaction and, as such, occurs very rapidly. For this reason the protein A-gold reagent is now widely used in immuno-electron microscopy (Varndell and Polak, 1987). This reagent was used to visualise the binding sites of the anti-*E. coli* GR antibody (an IgG) in the current study (Fig. 1.13).

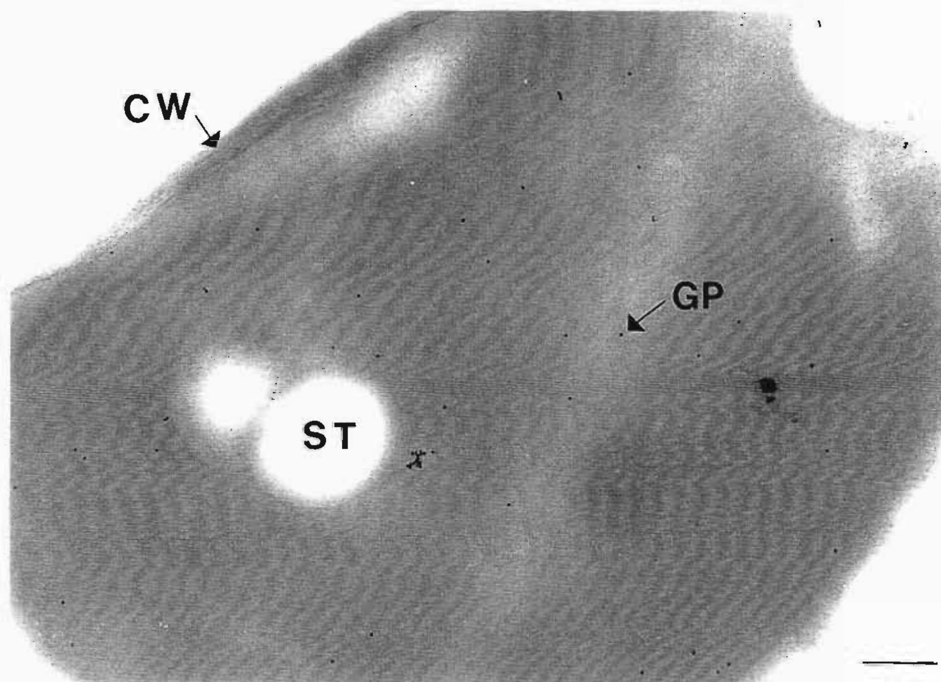


Figure 1.13: Localisation of *E. coli* GR in the chloroplasts of transformed tobacco. The section was not treated with osmium tetroxide, resulting in low resolution of membrane structures. Magnification: 57 270x. Bar represents 0.175 µm. Abbreviations: GP = gold particle, ST = starch grain, CW = cell wall.

Fig. 1.13 clearly shows gold labelling in the chloroplast. The resolution in the image is very low, due to the lack of an osmium tetroxide treatment. The resin manufacturers state that the resin is hydrophilic but has a low lipid solvency, resulting in the retention of membrane lipids during the embedding process. For this reason, it was decided to subject all further TEM sections to an osmium tetroxide treatment after the gold labelling step. This seemed a reasonable proposition, since antigenic sites would already have been bound and the reasons for not using osmium tetroxide prior to labelling (a decline in antigenicity) would no longer be valid. Figs. 1.14 A - C clearly show the enhancement in resolution afforded by this step.

The osmium tetroxide treatment allows visualisation of membranes - particularly the thylakoids, which were not seen before the incorporation of this step. No references were found for a similar treatment in other studies, so it is not known if this treatment would enhance resolution in other tissues or in tissues embedded in other resins. To determine the labelling density on these sections, micrographs of 15 chloroplasts were analysed to determine the number particles per unit area (Table 1.3).

Table 1.3: Labelling density in leaf sections incubated with anti-*E. coli* GR antibodies and in control sections. Controls included sections incubated without antibodies as well as sections treated with pre-immune antibodies.

	antibody (type and dilution)		
	1:5 rabbit antiserum against *E. coli* GR	no antibody	1:50 pre-immune rabbit IgG
gold particles μm^{-2}	7.42 ± 1.28 (n = 15)	4.17 ± 0.61 (n = 6)	3.89 ± 0.71 (n = 9)

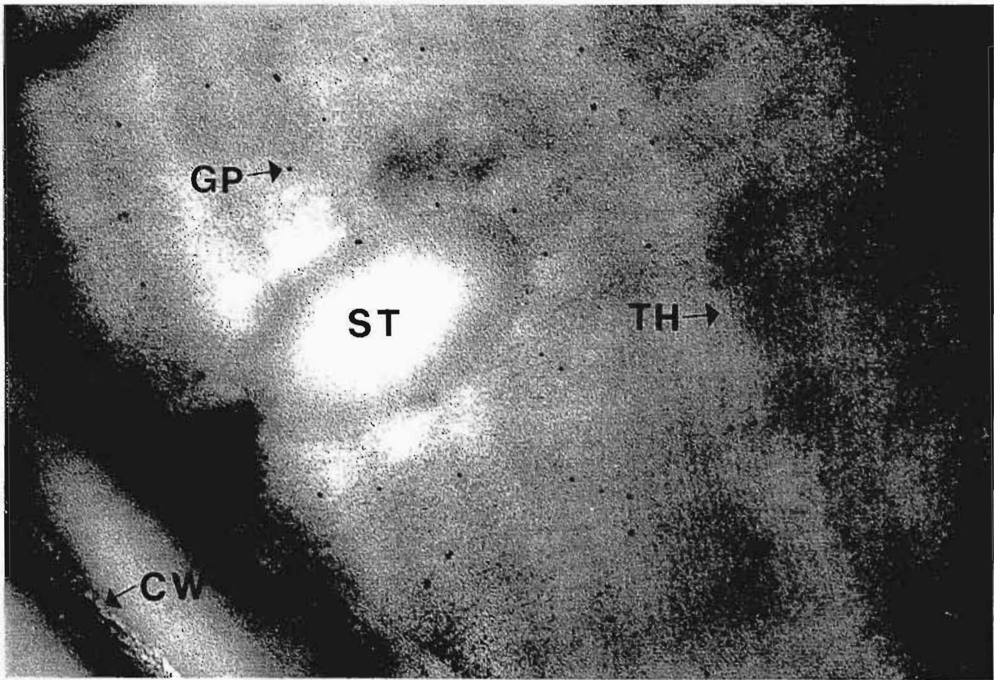
n = number of micrographs used to determine particle densities

In order to determine whether the gold labelling was significant, two controls were used in this study. In the first, sections were labelled as before, with the exception that no incubation with an antibody took place. This allowed the extent of non-specific binding between protein A-gold and the sections to be determined (Fig. 1.15A, Table 1.3). The second control utilised a pre-immune IgG instead of the anti-*E. coli* GR IgG, to determine the amount of labelling due to non-specific binding of IgG to sections (Fig. 1.15B, Table 1.3).

From Figs. 1.15A and 1.15B, it appears that the labelling density in the controls was considerably lower than in sections incubated with the anti-*E. coli* GR antibody. This is in agreement with the particle counts registered in Table 1.3. It is thus clear that the particle binding in Figs. 1.14A - 1.14C is significant and represents specific binding at points of *E. coli* GR localisation. This confirms the chloroplastic location of *E. coli* GR in the transformed plants.

The two methods used to determine the subcellular localisation of *E. coli* GR (western blots and immunogold labelling of TEM sections) are thus in agreement. They provide conclusive evidence that the transformation was

A



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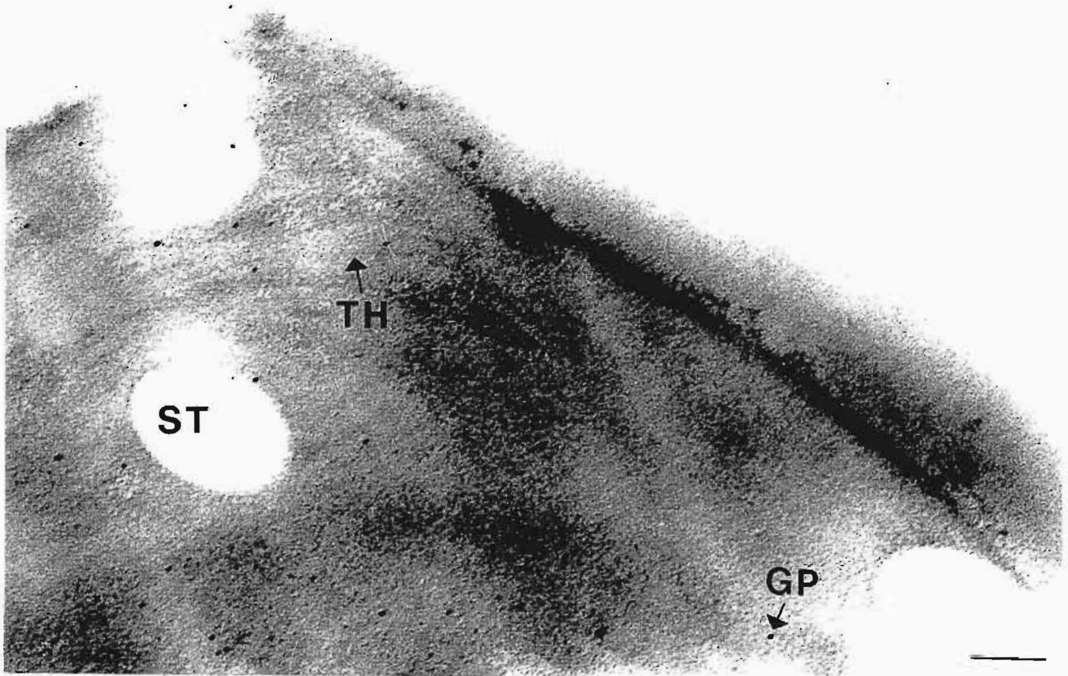

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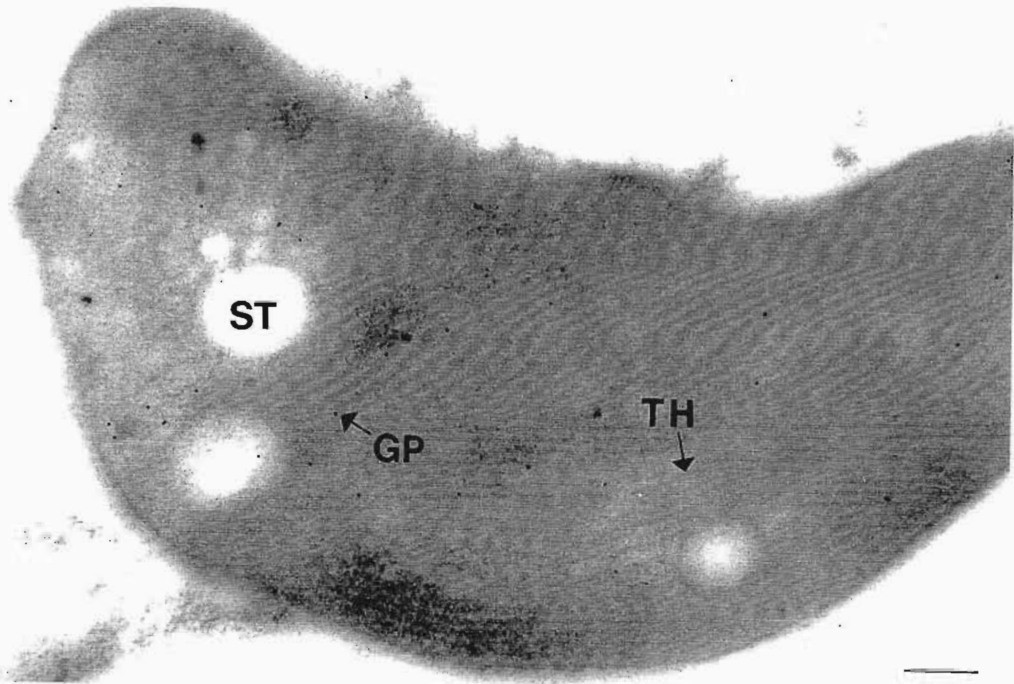

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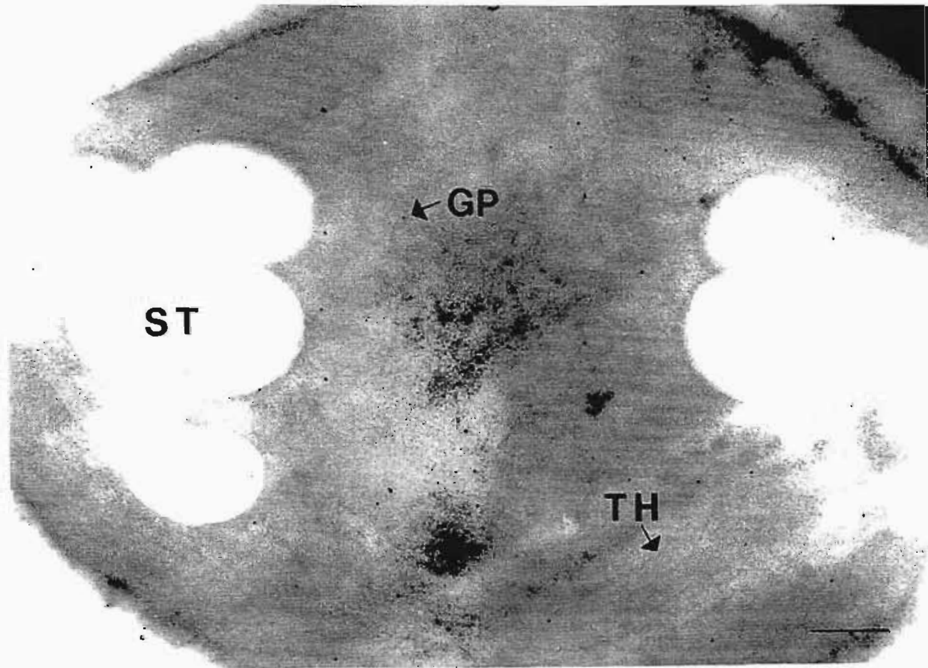

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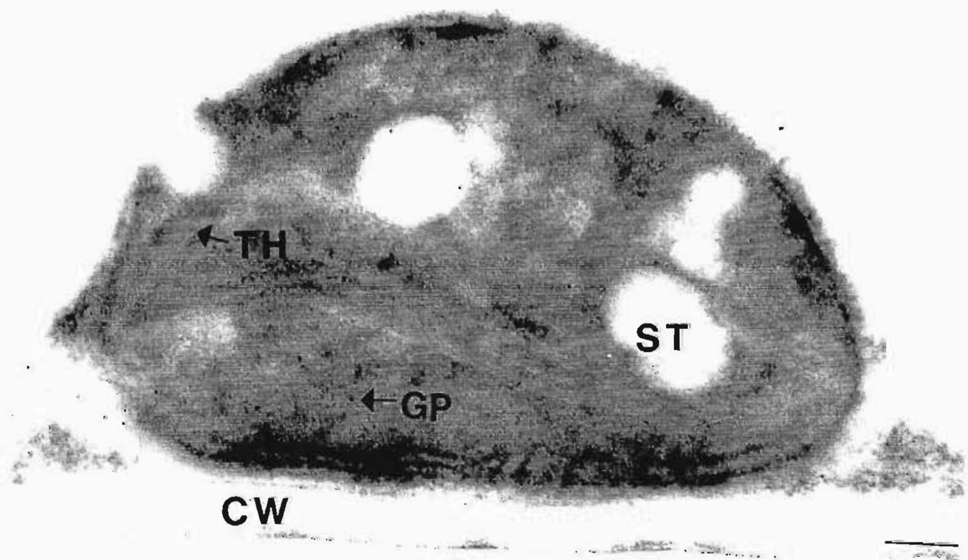


successful, and that the transit peptide from the small subunit of RUBISCO functioned to target the *E. coli* GR to the chloroplast.

In summary, this chapter has shown that *Agrobacterium*-mediated gene transfer is a successful means of introducing foreign genes into plant cells. The findings of other authors regarding the use of kanamycin as a selection agent were confirmed: namely, kanamycin inhibits the growth of plant cells and the optimal concentration should be determined for the plant material being transformed. It was also shown that despite this inhibition considerable numbers of untransformed plants are produced on the selection plates, and that further screening is necessary for selection of transformants. From the analysis of the transformants it was shown that fusing the gene for the RUBISCO transit peptide to a gene of interest is an efficient means of inserting the gene product into the chloroplast. It was also shown that this transit peptide is cleaved from the protein as chloroplastic insertion occurs. Finally, it was shown that the *E. coli* GR is stable in plant chloroplasts and that the transformed plants exhibit a high GR activity. The transformation was thus a success, producing tobacco plants which allow for an analysis of the antioxidant system under oxidative stress.

2

THE RESPONSE OF TRANSGENIC TOBACCO TO OXIDATIVE STRESS

2.1 INTRODUCTION

As mentioned in the prologue, the earth's atmosphere is characterised by an abundance of oxygen. However, this has not always been the case. Life on earth evolved in an essentially reducing atmosphere, where the only oxygen present was that produced as a result of the photolysis of water by UV radiation. With the advent of photosynthetic organisms some 3 billion years ago, the oxygen content of the atmosphere steadily rose to the current level of 21%. This steady rise in the oxygen concentration allowed a significant change in metabolism - namely the development of aerobic respiration. Prior to the development of an oxidising atmosphere, most organisms relied on fermentation as an energy source - a process which extracts far less energy from organic molecules than respiration. Thus the appearance of an oxidising atmosphere made possible the efficient use of energy sources and permitted the evolution of aerobic organisms. In addition to being the terminal electron acceptor in respiration, oxygen plays a role in more than 200 other enzyme reactions and as such the dependence of plant life on oxygen is absolute (Hendry, 1994).

However, life in the presence of oxygen has also resulted in the possibility of the unwanted oxidation of cell components. Plant chloroplasts are particularly sensitive to damage mediated by oxygen for several reasons, as outlined by Halliwell and Gutteridge (1989):

1. Photosystem II, situated in the thylakoid membranes of the chloroplast, is the site of the water-splitting apparatus. One of the by-products of the water-splitting reactions is oxygen. Illuminated chloroplasts thus experience oxygen concentrations which are higher than those present in the surrounding atmosphere.
2. The thylakoid membranes contain a high percentage of unsaturated lipids which are very susceptible to peroxidation.
3. Chlorophyll molecules in the thylakoid membranes absorb light energy and enter an excited singlet state. If this energy is not dissipated the chlorophyll may enter the triplet state. In this state it may transfer the energy to oxygen, producing singlet oxygen - a highly reactive oxygen species which causes carotenoid destruction, chlorophyll bleaching, lipid peroxidation and membrane deterioration.
4. Chloroplasts contain an electron generating system and an electron transport chain. It is possible for some of these electrons to be leaked to oxygen, producing superoxide - a reaction first described by Mehler (1951) and referred to as the Mehler reaction.

Plants have evolved numerous defences against reactive oxygen species, which allows them to function efficiently under most conditions. However, the

damage associated with reactive oxygen species is exacerbated under certain stress conditions and may overwhelm the plant defence systems under these conditions. The aim of this investigation was to study the stress responses of transgenic plants expressing high levels of some of the enzymes involved in the scavenging of reactive oxygen species. Before examining the plants produced for this study, a review of the relevant antioxidant enzymes and the pathways mediated by them is necessary. In addition, a brief overview of oxygen and its reactivity is pertinent.

2.1.1 The reactivity of oxygen

The electronic configuration of dioxygen is unusual in that it has two unpaired electrons, each occupying one of the two antibonding molecular orbitals ($2P\pi_x^*$ and $2P\pi_y^*$) (see Fig. 2.1).

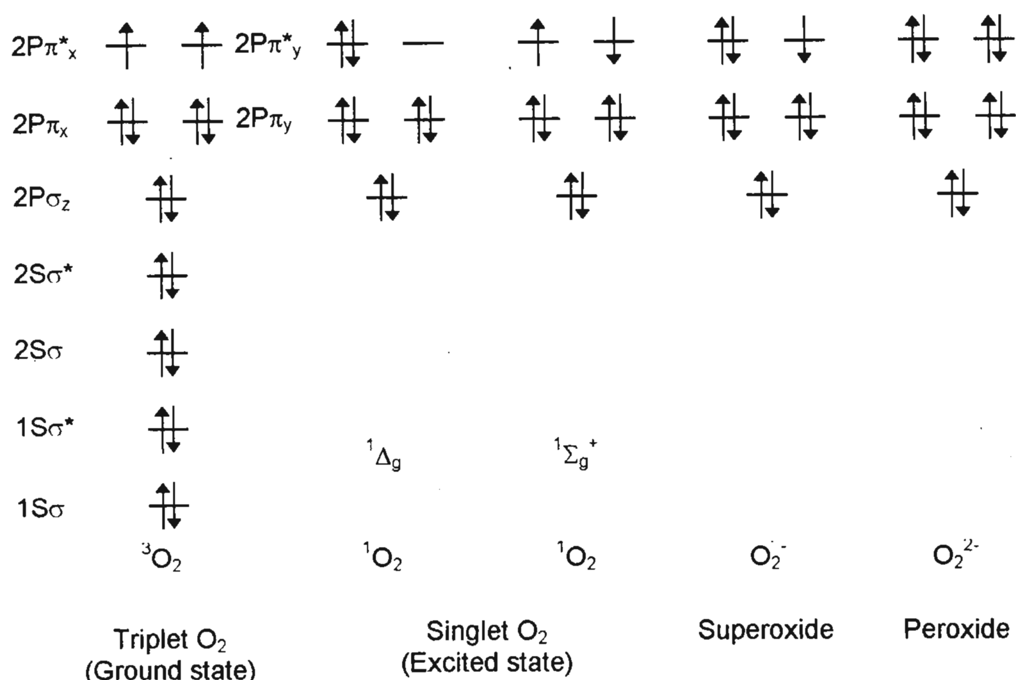


Figure 2.1: The electronic structure of oxygen and its derivatives. Note that the 2Sσ, 2Sσ*, 1Sσ and 1Sσ* molecular orbitals of singlet oxygen, superoxide and peroxide are omitted in the diagram. In the ground state, dioxygen has two unpaired electrons with parallel spin in the antibonding orbitals. One-electron additions fill these orbitals producing first superoxide and then peroxide. The restrictions imposed by the parallel spin of the unpaired electrons are lost when singlet oxygen forms as a result of energy input (see text). (Figure from Asada and Takahashi, 1987).

In keeping with Hund's rule, the two unpaired electrons have parallel spin. Dioxygen therefore has a triplet ground state, and takes the form of a biradical (Asada and Takahashi, 1987). Theoretically this should make oxygen extremely reactive - one would expect it to react spontaneously with the components of living organisms. However, everyday observations show that this is not the case. The reason for the lower than expected reactivity displayed by oxygen is the spin restriction imposed by the unusual electronic configuration. For dioxygen to oxidise another atom or molecule by accepting two electrons from it, both new electrons must be of parallel

spin to fill the vacant spaces in the π^* orbitals (Cadenas, 1989). However, a pair of electrons from another orbital would have opposite spin, in accordance with Pauli's exclusion principle (Halliwell and Gutteridge, 1989). The spin restriction placed on dioxygen can be overcome by an input of energy, resulting in the production of singlet oxygen. In singlet oxygen the two electrons have opposite spin and can accept two electrons form another molecule.

Dioxygen requires a four-electron reduction to form water. As a result of the spin restriction these electrons are acquired in a series of univalent reductions, mainly via reactions with radical species and unpaired electrons (Salin, 1987). This results in the sequential production of a series of intermediate oxygen species, namely the hydroperoxyl radical, hydrogen peroxide and the hydroxyl radical (reaction 1).

$$O_2 \longrightarrow HO_2^- \longrightarrow H_2O_2 \longrightarrow 2\ OH^\bullet \longrightarrow 2\ H_2O \qquad (1)$$

Along with singlet oxygen, these intermediate species are all far more reactive than triplet dioxygen (Asada and Takahashi, 1987).

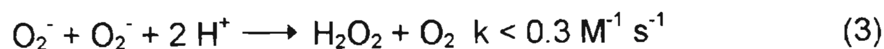
2.1.1.1 Reactive oxygen intermediates

Superoxide is the first reactive oxygen species produced by the reduction of dioxygen (reaction 1). In addition to various biological sources of superoxide, a number of systems have been devised for the experimental generation of this reactive intermediate - a topic reviewed by Bensasson *et al.*, 1993; Fridovich, 1978; Hassan and Scandalios, 1990. In illuminated chloroplasts there are two possible mechanisms of superoxide production. The first, proposed by Mehler (1951), is the direct transfer of an electron from a component of the electron transport chain to oxygen. The most likely site of such an electron transfer is the reducing side of photosystem I (PS I) (Foyer *et al.*, 1994b). Superoxide also forms via reduced ferredoxin (Fd) (reaction 2).

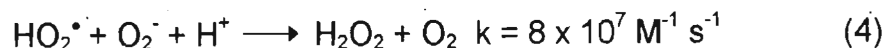
$$2\ O_2 + 2\ Fd_{red} \longrightarrow 2\ O_2^- + 2\ Fd_{ox} \qquad (2)$$

Badger (1985) suggests that this is the primary source of superoxide in chloroplasts. However, the Fe-S centres (X and A/B) at the reducing side of PS I have reduce oxygen at a much higher rate (250 s^{-1}) than ferredoxin (0.081 s^{-1}) (Asada, 1994 cited in Vácha, 1995). Although superoxide is more reactive than dioxygen, it is relatively inert in aqueous solutions. However, in the presence of protons it gives rise to the hydroperoxyl radical - a much more reactive species (Cadenas, 1989). More importantly, it also reacts with peroxide to form the highly reactive hydroxyl radical. This may account for much of the toxicity originally ascribed to superoxide (Salin, 1987; Thompson *et al.*, 1987).

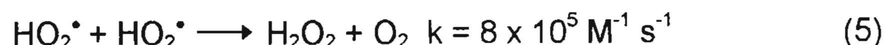
The toxicity of superoxide and its tendency to form more reactive species is partly ameliorated by dismutation reactions (Halliwell and Gutteridge, 1989):



$$O_2^- + O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2 \quad k < 0.3\ M^{-1}\ s^{-1} \qquad (3)$$



$$HO_2^\bullet + O_2^- + H^+ \longrightarrow H_2O_2 + O_2 \quad k = 8 \times 10^7\ M^{-1}\ s^{-1} \qquad (4)$$

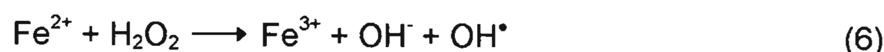


$$HO_2^\bullet + HO_2^\bullet \longrightarrow H_2O_2 + O_2 \quad k = 8 \times 10^5\ M^{-1}\ s^{-1} \qquad (5)$$

As shown in reaction 3, this reaction proceeds very slowly for superoxide on its own. However, in the presence of the more reactive hydroperoxyl radical the dismutation is extremely rapid (reactions 4 and 5). In addition, dismutation is catalysed by superoxide dismutase (SOD). Under physiological conditions, the rate constant for the enzyme-catalysed reaction is $2 \times 10^9\ M^{-1}\ s^{-1}$ (Elstner, 1982) and the steady-state concentration of superoxide is kept below 10^{-8} M (Badger, 1985).

As shown in reactions 3, 4 and 5, the dismutation of superoxide gives rise to hydrogen peroxide. This is the major source of chloroplastic hydrogen peroxide. In 1975 Egneus *et al.* demonstrated that intact chloroplasts take up oxygen and that the oxygen is used to produce hydrogen peroxide, thus inhibiting CO_2 assimilation. Many experiments have confirmed these findings - Kaiser (1976) showed that CO_2 fixation is decreased 50% by peroxide concentrations as low as 10^{-5} M. Peroxide exerts its effect on photosynthesis by oxidising thioredoxin, which is necessary for the reductive activation of enzymes in the reductive pentose phosphate pathway (Charles and Halliwell, 1980; Foyer, 1993). Kaiser (1979) has shown that peroxide affects fructose bisphosphatase, sedoheptulose bisphosphatase, phosphoribulokinase as well as glucose-6-phosphate dehydrogenase. This oxidation of sulfhydryl groups is the primary mode of peroxide toxicity. In general, however, H_2O_2 is relatively unreactive and is the most stable of the reactive oxygen intermediates (Salin, 1987). Like superoxide, it contributes to cellular damage by participating in the production of the more reactive hydroxyl radical.

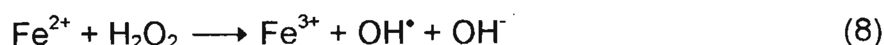
As mentioned, both superoxide and peroxide may give rise to the hydroxyl radical. The production of the hydroxyl radical from peroxide was noticed as early as 1894 by Fenton (cited in Bensasson *et al.*, 1993). This is a metal-catalysed reaction and proceeds as follows:



$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^\bullet \qquad (6)$$

The hydroxyl radical may also be formed via the Haber-Weiss reaction. Once again, this is catalysed by a transition metal ion:

$$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2 \quad (7)$$



$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^\bullet + OH^- \quad (8)$$

$$O_2^- + H_2O_2 \longrightarrow OH^\bullet + OH^- + O_2 \quad (9)$$

The net reaction (9) is also referred to as a superoxide-driven Fenton reaction (Benasson *et al.*, 1993). The hydroxyl radical will undergo electron transfer, hydrogen abstraction and double bond addition reactions, all with rate constants in the range 10^9 - 10^{10} M^{-1} s^{-1} (Bensasson *et al.*, 1993). As a result of this high reactivity, this radical is indiscriminate and will react with the first substrate it encounters. This makes the specific enzymatic scavenging of the hydroxyl radical impossible. The only way to prevent damage by this radical is to scavenge superoxide and peroxide, preventing its formation (Fridovich, 1978).

2.1.2 Increased production of activated oxygen under stress conditions

Plant stress may be defined as "... a state in which increasing demands made upon a plant lead to an initial destabilisation of functions, followed by normalisation and improved resistance" but "If the limits of tolerance are exceeded and the adaptive capacity is overworked, the result may be permanent damage or even death" (Larcher, 1987 cited in Lichtenthaler, 1996). Plants are exposed to a wide range of stresses, both natural (such as drought, chilling and high light intensities) and man-made (air pollution, for example). As already mentioned, activated oxygen is produced as a result of normal plant metabolism. However, studies in recent years have shown that many of the stress factors encountered by plants increase the production of activated oxygen (Table 2.1).

Schlee (1992) - as cited in Elstner and Osswald (1994) - has stated that all stress events in plants may be attributed to one or more of seven stress factors - namely light, radiation, biological influences (*e.g.* insects), mechanical factors, chemical factors, hydration and temperature. Furthermore, he has pointed out that these factors cannot be differentiated from each other in terms of their mode of impact or symptoms, since all of the resulting stress reactions are very closely interconnected in plant metabolism. Based on these observations, Elstner *et al.* (1988) have attempted to identify a "stress point" in plant metabolism - a point beyond which plant metabolism is sufficiently impaired to result in loss of vital functions and cellular death. A characteristic feature of the stress point is an increase in light sensitivity (De Luca d'Oro and Trippi, 1987). As Elstner *et al.* (1988) put it: "Plant stress is governed by photodynamic effects more or less clearly expressed by oxygen activation, concomitant to membrane degradation and finally fortified by hormonal responses such as ethylene formation". These authors propose that the stress point may be defined as the transition from heterolytic to homolytic biochemistry - a point where an

Table 2.1: Factors enhancing the production of activated oxygen under stress conditions.

Factor causing oxygen activation	Plant species	Activated oxygen species implicated	Reference
greening	*Pisum sativum*	O_2^-	Gillham and Dodge (1985)
high light	*Spinacia oleracea*	O_2^-, H_2O_2	Schöner and Krause (1990)
high light	*Conyza bonariensis*		Jansen *et al.* (1989)
high light	*Euglena gracilis*	O_2^-, H_2O_2, $OH^•$	Tschiersch and Ohmann (1993)
high light	**review**	$^1O_2$, O_2^-	Demmig-Adams and Adams (1992)
high light + chilling	**review**	$^1O_2$, O_2^-, H_2O_2, $OH^•$	Wise (1995)
high light + chilling	*Triticum aestivum*	O_2^-, H_2O_2	Mishra *et al.* (1993)
chilling	*Arabidopsis thaliana*	H_2O_2	O'Kane *et al.* (1996)
chilling	*Zea mays*		Massacci *et al.* (1995)
chilling	*Zea mays*	H_2O_2	Jahnke *et al.* (1991)
freezing + thawing	*Malus pumila*	H_2O_2	Kuroda *et al.* (1992)
Mg deficiency	*Phaseolus vulgaris*	O_2^-, H_2O_2	Cakmak and Marschner (1992)
Mg deficiency K deficiency	*Phaseolus vulgaris* *Phaseolus vulgaris*	H_2O_2 H_2O_2	Cakmak (1994)
Fe	*Pisum sativum*	$OH^•$	Iturbe-Ormaetxe *et al.* (1995)
Fe	*Nicotiana plumbaginifolia*	H_2O_2, $OH^•$	Kampfenkel *et al.* (1995)
Fe + drought	*Triticum aestivum*	O_2^-	Price and Hendry (1991)
drought	*Pisum sativum*	$OH^•$, H_2O_2	Moran *et al.* (1994)
drought	*Triticum aestivum*	O_2^-, $OH^•$	Price *et al.* (1989)
drought	*Zea mays*	O_2^-, H_2O_2	Leprince *et al.* (1994)
hyperoxia	*Glycine max*	H_2O_2	Dalton *et al.*(1991)
post anoxia	*Triticum aestivum*		Albrecht and Wiedenroth (1994)
wounding	**review**	O_2^-, H_2O_2	Thompson *et al.* (1987)
NaCl	*Vigna unguiculata*	H_2O_2, $OH^•$	Swamy and Reddy (1991)
NaCl	*Pisum sativum*	O_2^- H_2O_2	Hernández *et al.* (1995)
UV-B + O_3	*Arabidopsis thaliana*	H_2O_2	Rao *et al.* (1996)
O_3	*Spinacia oleracea*	O_2^-	Sakaki *et al.* (1983)
SO_2	*Nicotiana tabacum*		Tanaka *et al.* (1988a)
SO_2	*Hordeum vulgare*		Navari-Izzo and Izzo (1994)
SO_2 + NO_2	*Pinus sylvestris*	H_2O_2	Wingsle and Hällgren (1993)
herbicide (paraquat)	*Lemna minor* *Lolium perenne*	$OH^•$, O_2^-	Babbs *et al.* (1989)
herbicide (paraquat)	*Phaseolus vulgaris*	O_2^-	Chia *et al.* (1982)

increase in the production of cellular radicals (including active oxygen) occurs.

As pointed out by Polle and Rennenberg (1993), plants have limited mechanisms for the avoidance of stress. As such, they have evolved a wide range of defences against activated oxygen.

2.1.3 Antioxidant defences

The plant antioxidant system includes a host of small molecules as well as a number of enzymes. With a few exceptions, these enzymes function for the regeneration of the reduced forms of the small molecules once they have been oxidised.

2.1.3.1 Antioxidant molecules

The chloroplast contains both lipid and water soluble molecules for the scavenging of oxidants. The lipid soluble species include the carotenoids (primarily β-carotene and the xanthophylls) and vitamin E (α-tocopherol), while the primary water soluble scavengers are ascorbate and glutathione. A further scavenger, ferredoxin, is closely associated with photosystem I at the stromal face of the thylakoid membrane.

In addition, a wide variety of flavenoids, phenolic compounds, alkaloids, amino acids and amines have been shown to have antioxidant properties (see Larson (1988) for review). Many of these are specific to certain plant species, while the *in vivo* effectiveness of others has yet to be ascertained. These antioxidants will not be dealt with in this review.

Carotenoids

The carotenoids are situated in the thylakoid membranes. They function to accept excess energy from triplet chlorophyll ($^3chl^•$), reducing the possibility of singlet oxygen ($^1O_2$) formation. They have a dual role, in that they can also scavenge $^1O_2$ once it has formed (Halliwell and Gutteridge, 1989). The energy transfer from $^1O_2$ and $^3chl^•$ to the carotenoids occurs at diffusion-controlled rates, making them highly effective scavengers (Asada and Takahashi, 1987).

Vitamin E (α-tocopherol)

The thylakoid membranes are rich in vitamin E, which protects them from oxidation (Halliwell and Gutteridge, 1989). This antioxidant is also capable of accepting energy from $^1O_2$ and $^3chl^•$ but this reaction is two orders of magnitude lower than the energy transfer to carotenoids, and as such vitamin E probably plays a minor role in the quenching of these high energy species (Asada and Takahashi, 1987). The major function of vitamin E is the scavenging of chain-propagating peroxy radicals ($ROO^•$) formed by the peroxidation of unsaturated membrane lipids (Larson,

1988; Winston, 1990). This produces the α-tocopheryl radical which can be re-reduced to α-tocopherol by ascorbate (Fig. 2.2).

Ascorbate and glutathione

Green leaves contain as much ascorbate as chlorophyll, and this is not unexpected when one considers the fundamental role played by ascorbate in the defence against oxygen radicals (Foyer, 1992). The main function of ascorbate is to reduce peroxide in the chlroplast stroma, but it also reduces the α-tocopheryl radical. It thus has an antioxidant role in both the hydrophilic and hydrophobic environments of the chloroplast (Foyer and Lelandais, 1993). Furthermore, ascorbate may react directly with superoxide and the hydroxyl radical (Foyer *et al.*, 1994b). The oxidation of ascorbate results firstly in the formation of the monodehydroascorbate radical (MDHA) (Fig. 2.2). If this radical is not reduced back to ascorbate, it spontaneously disproportionates to form ascorbate and dehydroascorbate (DHA) (Foyer *et al.*, 1994a).

Dehydroascorbate is unstable above pH 6.0 and is either cleaved to form compounds like tartrate and oxalate, or decomposes to produce toxic derivatives (Foyer, 1992). Besides the damage caused by these derivatives, they represent a loss to the ascorbate pool. The chloroplast therefore has a highly efficient system for the recycling of dehydroascorbate. This involves the enzymatic and non-enzymatic reduction of dehydroascorbate by reduced glutathione (GSH) - the major role of glutathione in the plant antioxidant system.

2.1.3.2 Antioxidant enzymes

A number of enzymes in plants have been shown to play a role in the defence against active oxygen. The evidence for the role played by these enzymes is threefold, as reviewed by Allen (1995):

1. The activity of these enzymes increases under conditions causing oxidative stress, and the increase in activity generally correlates with an enhanced stress tolerance,
2. Plants with an induced tolerance caused by exposure to one oxidative stress factor are generally more tolerant to other stress factors,
3. Plant lines selected for natural tolerance to a stress factor have high levels of one or more of these enzymes and display the cross tolerance mentioned in (2).

These enzymes occur in several of the subcellular compartments in the plant cell, but this discussion will be confined to the chloroplast. The chloroplastic enzymes participate in two cycles - the Mehler-peroxidase cycle and the ascorbate-glutathione cycle.

The Mehler-peroxidase cycle

The first step in this cycle is the reduction of oxygen to superoxide by ferredoxin (Fd). This reaction generally occurs when there is a lack of

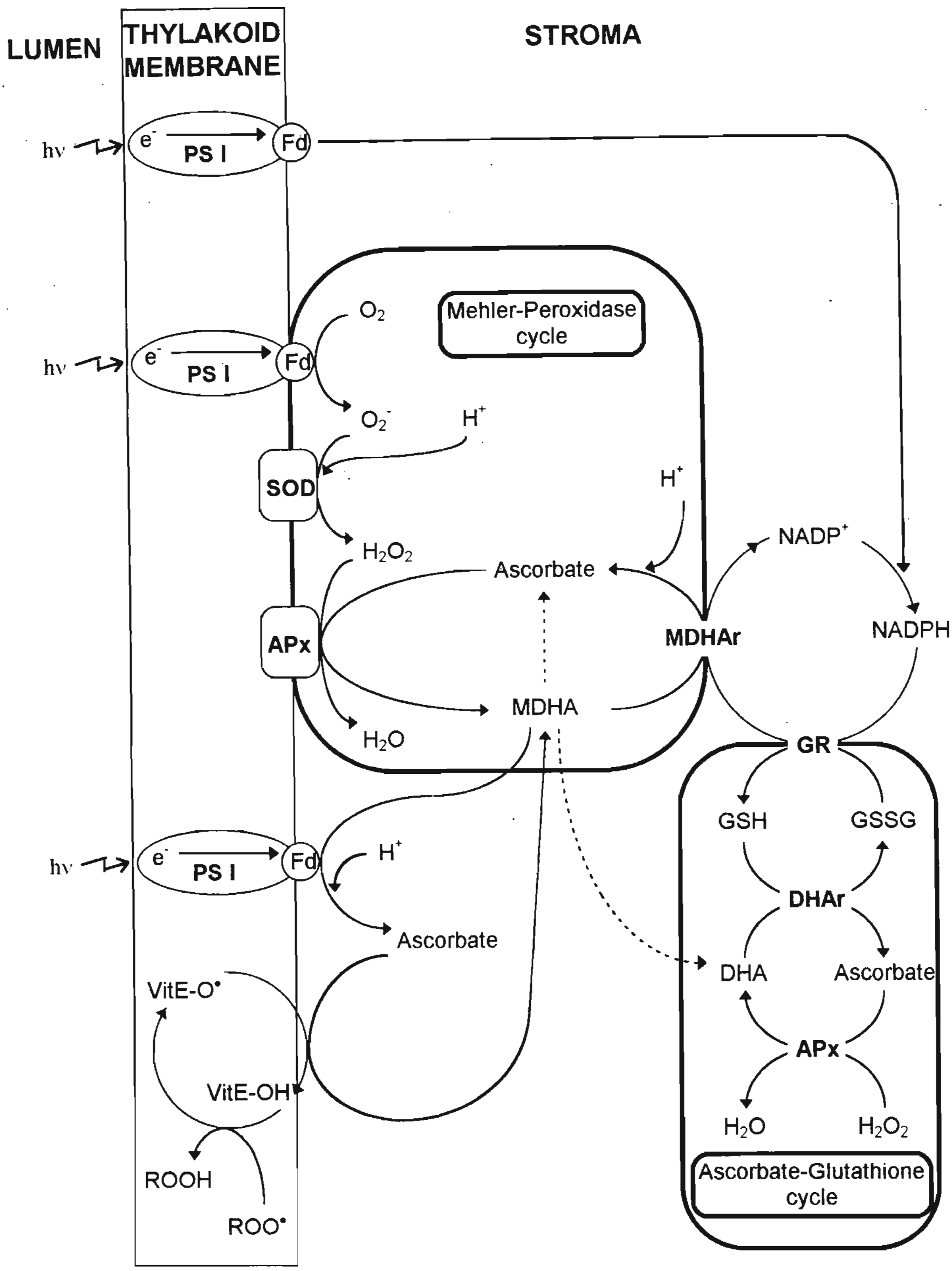


Figure 2.2: The antioxidant system of higher plant chloroplasts. Plant chloroplasts rely on two interconnnected cycles for the scavenging of superoxide and peroxide. The Mehler-peroxidase cycle traps superoxide, resulting in the oxidation of ascorbate. This ascorbate is then reduced by the ascorbate-glutathione cycle, utilising reduced glutathione. This glutathione is re-reduced in this cycle, consuming NADPH. These cycles scavenge radicals and utilise some of the excess reducing power which would otherwise produce more radicals. Abbreviations are as in the text. With the exception of PS I, bold type indicates enzymes. Broken arrows show the disproportionation of MDHA.

carbon dioxide, resulting in an excess of reducing power. Superoxide may also be generated within the thylakoid membrane by the primary electron acceptor of photosystem I (PS I) (Asada, 1992). This superoxide may be scavenged within the membrane itself or may enter the stroma, where it is scavenged along with the superoxide produced by ferredoxin.

The initial reaction in the scavenging of photoreduced oxygen is the dismutation of superoxide by SOD, a process which consumes protons and produces peroxide (Fig. 2.2). SOD is a ubiquitous enzyme in aerobic organisms and its evolution has resulted in a number of types of SOD. These are classified according to the metal co-factor(s) associated with the enzyme (Mn, Fe or Cu + Zn). The Mn and Fe SODs are very similar and are found in prokaryotes and eukaryotes, while the Cu,Zn SOD is structurally different and found only in eukaryotes (Bowler *et al.*, 1992). This indicates that the Cu,Zn SOD has evolved independently of the other forms and at a later stage than them. In plants the different types of SOD are specific to particular compartments. Cu,Zn SOD has different isoforms in the chloroplast and the cytosol, while Mn SOD is found in the matrix of the mitochondrion (Hassan and Scandalios, 1990). Fe SOD is not found in all plants (Hassan and Scandalios, 1990), but where it does occur it is found in the chloroplast.

Chloroplastic Cu,Zn SOD has been shown to be localised at the stromal face of the thylakoid membrane, and the evidence suggests that it is probably in close proximity to PS I (Ogawa *et al.*, 1995). This indicates that superoxide is scavenged before it can leave its site of production and participate in deleterious reactions. The peroxide produced by the dismutation of superoxide is reduced to water by ascorbate peroxidase (APx) (Groden and Beck, 1979; Nakano and Asada, 1981). This reaction generates the monodehydroascorbate radical. Miyake and Asada (1992) have shown that the chloroplastic form of APx exists in both soluble and thylakoid-bound forms, and that the thylakoid isozyme is also closely associated with PS I. These authors suggest that this thylakoid-bound isozyme consumes most, if not all, of the peroxide generated by the photoreduction of oxygen. The monodehydroascorbate produced by APx may be reduced back to ascorbate by accepting electrons directly from the Fd associated with PS I. Alternatively, it may be reduced enzymatically by monodehydroascorbate reductase, utilising NADPH as an electron donor (Fig. 2.2) (Hossain *et al.*, 1984). Miyake and Asada (1992) have shown that most of the monodehydroascorbate is reduced by PS I although their evidence suggests that it may be reduced by one of the electron acceptors of PS I rather than the Fd associated with this photosystem. As discussed previously, the monodehydroascorbate may also disproportionate to form ascorbate and dehydroascorbate.

The ascorbate-glutathione cycle

As mentioned above, Miyake and Asada (1992) have shown the presence of a soluble (stromal) form of APx. They have proposed that this

isozyme is part of a secondary scavenging system which traps peroxide that is formed in the stroma, or which escapes the primary scavenging system of the thylakoids. The scavenging of peroxide in the stroma results in the production of dehydroascorbate, adding to the dehydroascorbate which results from disproportionation of the monodehydroascorbate produced by the Mehler-peroxidase reactions. As dehydroascorbate is potentially toxic, the stromal scavenging system must have components for the efficient reduction of this unwanted molecule.

In 1976 Foyer and Halliwell showed that reduced glutathione (GSH) is capable of the non-enzymatic reduction of dehydroascorbate, but could not show the presence of an enzyme catalysing this reaction in chloroplasts. In 1981, Jablonski and Anderson showed the presence of an enzyme (DHAr) capable of the glutathione-dependent reduction of dehydroascorbate in chloroplasts. This reaction constitutes the major function of glutathione in the protection of cells against free radicals (Hausladen and Alscher, 1992). As a result of this reaction, a system is required for the reduction of oxidised glutathione (GSSG). Even before the discovery of dehydroascorbate reductase, it had already been shown that plant chloroplasts contain glutathione reductase (GR) (Foyer and Halliwell, 1976; Jablonski and Anderson, 1978) for the NADPH-dependent reduction of (GSSG). This series of stromal reactions involving soluble APx, DHAr and GR constitutes the ascorbate-glutathione cycle. This cycle was first proposed in 1976 by Foyer and Halliwell, and is also referred to as the Foyer-Halliwell cycle. Some authors may also refer to it as the Halliwell-Asada cycle.

2.1.4 A new role for the superoxide radical

Until recently the photoreduction of oxygen by PS I (the Mehler reaction) was considered by many to be a unavoidable consequence of the generation of excess reducing power - a process responsible for the generation of oxygen radicals, resulting in cellular damage. New evidence, and new interpretations of existing data, suggests that photoreduction has in fact evolved as a valve for the dissipation of excess excitation energy and is, in fact, an important mechanism in the protection of the photosynthetic apparatus.

Polle (1996) recently reviewed the role of the Mehler reaction in photosynthesis and showed that the photosynthetic reduction of oxygen plays a significant role in the protection of the photosynthetic apparatus. As pointed out in the review this takes place by decreasing the amount of reducing power in a number of ways:

1. As shown in Fig. 2.2, the Mehler peroxidase reaction not only consumes excess electrons from PS I, but also generates the MDHA radical. This radical in turn readily accepts electrons from PS I. MDHA is also reduced enzymatically, utilising reducing power in the form of NADPH and

generating $NADP^+$. The reduction of $NADP^+$ uses yet more electrons from PS I.

2. DHA produced as a result of the Mehler-peroxidase reactions also requires reducing power in the form of NADPH, via its coupling with ascorbate and glutathione (Fig. 2.2).
3. The Mehler-peroxidase cycle consumes protons, contributing to the generation of a trans-thylakoid pH gradient. This gradient energises the thylakoid membranes, a process which regulates electron flow between the photosystems.
4. The development of the pH gradient mediates the formation of zeaxanthin, which in turn enables the dissipation of energy from the light harvesting pigments.

This role played by superoxide becomes even more significant when one considers the hypothesis of Winterbourn (1993). This author has proposed that oxygen will accept electrons from a wide range of cellular radicals to form superoxide, thus acting as an intracellular sink for radicals which would otherwise cause cellular damage. As a result of this radical chain reaction it becomes possible for the highly efficient SOD to scavenge not only superoxide but also many other radicals (via superoxide), increasing the turnover of the Mehler-peroxidase cycle.

With these findings, consumption of O_2 in the photoreduction process begins to resemble the protection afforded by the oxygenase reaction of RUBISCO (ribulose bisphosphate carboxylase/oxygenase) in the photorespiratory cycle (in fact, the photoreduction of oxygen is described by Osmond and Grace (1995) as 'Mehler-ascorbate peroxidase photorespiration'). A number of studies have been carried out to compare the effectiveness of the Mehler reaction and photorespiration in protecting the photosynthetic apparatus against photoinhibition. Wu *et al.* (1991) show that under high light photorespiration offers more protection than the Mehler reaction. This finding is supported by Guy *et al.* (1993), who have estimated that at the CO_2 compensation point one sixth of O_2 uptake is due to the Mehler reaction. However, Biehler and Fock (1996) have shown that in drought stressed wheat 29.1% of photosynthetic electrons are consumed by the Mehler-peroxidase cycle, while only 18.4% are used in maintaining photorespiration. This indicates that under stress conditions the dissipation of energy via photoreduction plays an important role in protecting the photosynthetic apparatus.

2.1.5 The engineering of increased oxidative stress tolerance

In recent years the genes for many of the enzymes involved in the antioxidant response, including glutathione reductase (GR), Mn-, Fe-, and Cu,Zn-superoxide dismutases (SODs), ascorbate peroxidase (APx) and catalase have been cloned, and transgenic plants having both reduced and enhanced levels of some of these enzymes have been generated (Table 2.2). These plants have given unique insights into the functioning of these

enzymes and the role they play in the protection against both environmental and man-made stress (Foyer *et al.*, 1994a).

Table 2.2: Transgenic plants used to study oxidative stress

Transformed plant	Transgene product	Origin of transgene	Target organelle	Reference
alfalfa	Mn SOD	tobacco	chloroplast mitochondrion	McKersie *et al.* (1993, 1996)
tobacco	Cu,Zn SOD	petunia	chloroplast	Tepperman and Dunsmuir (1990)
	Cu,Zn SOD	petunia	chloroplast	Pitcher *et al.* (1991)
	Mn SOD	tobacco	chloroplast mitochondrion	Bowler *et al.* (1991)
	GR	*E. coli*	cytosol	Foyer *et al.* (1991)
	GR	*E. coli*	cytosol	Aono *et al.* (1991)
	Cu,Zn SOD	pea	chloroplast	Sen Gupta *et al.* (1993a,b)
	GR	*E. coli*	chloroplast	Aono *et al.* (1993)
	GR	pea	chloroplast, mitochondrion, cytosol	Creissen *et al.* (1994)
	antisense GR mRNA	spinach		Aono *et al.* (1995a)
	GR and Cu,Zn SOD	*E. coli* (GR) rice (SOD)	cytosol	Aono *et al.* (1995b)
	Mn SOD	tobacco	chloroplast	Slooten *et al.* (1995)
tomato	Cu,Zn SOD	petunia	chloroplast	Tepperman and Dunsmuir (1990)
poplar	GR	*E. coli*	cytosol chloroplast	Foyer *et al.* (1995)
potato	Cu,Zn SOD	tomato	cytosol chloroplast	Perl *et al.* (1993)

The study of these transgenic species has greatly advanced the understanding of the regulation of the antioxidant genes and the role of cellular oxidants in signal transduction. They have also provided insights into the response of the antioxidant system to oxidative stress. Work carried out using these transgenic plants has shown that it is possible to obtain increased stress tolerance under certain conditions, and has raised hopes that it may be possible to engineer increased stress tolerance in economically important crop species (Allen, 1995). However, not all of the transformants listed above exhibit increased stress tolerance. This can be partly explained in terms of the genes which have been used. Foyer *et al.* (1994a) point out that SOD merely converts one type of reactive oxygen species to another, and unless this second species is also effectively dealt with no real benefits will be derived from the transformation. Furthermore, many of these transformations fail to target the transgene products to subcellular compartments where they would allow the greatest advantage in terms of radical scavenging. Lastly, most of these studies only examine the effects of an increase in a single component of the antioxidant system. In their review of this topic, Foyer *et al.* (1994a) conclude that an increase in

several components of the antioxidant system will be necessary in order to obtain substantial increases in stress tolerance.

In view of the above findings, this study sought to examine oxidative stress tolerance in transgenic tobacco carrying enhanced levels of more than one antioxidant. Furthermore, it was decided that the transformants should carry these foreign enzymes in the chloroplast, since this organelle is the subcellular site most likely to produce reactive oxygen species under conditions of oxidative stress. To this effect, plants carrying chloroplastic *E. coli* GR (described in Chapter 1) were crossed with plants carrying chloroplastic tomato Cu,Zn SOD. It was hoped that a study of these hybrids, together with the plants carrying enhanced levels of the individual enzymes, would give an insight into the functioning of the antioxidant system under stress conditions. It was also hoped that the hybrid plants would show enhanced tolerance to oxidative stress.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

2.2.1.1 Plant types

The control plants were *N. tabacum* var. samsun. The transformants included *N. tabacum* var. samsun carrying cytoplasmic *E. coli* GR, *N. tabacum* var. samsun carrying chloroplastic *E. coli* GR, *N. tabacum* var. xanthi carrying chloroplastic tomato SOD and a xanthi x samsun hybrid carrying both chloroplastic *E. coli* GR and chloroplastic tomato Cu,Zn SOD.

2.2.1.2 Source of transgenic plants

The plants carrying cytoplasmic GR were transformed by M. Roberts (unpublished work) and grown from seed provided by P. Badenhorst. The tomato SOD transformants were grown from seed provided by D. Aviv (Weizmann Institute of Science, Rehovot, Israel). The chloroplastic GR transformants were generated as described in Chapter 1. The hybrids carrying both chloroplastic GR and SOD transgenes were produced by cross pollinating the plants carrying the individual transgenes.

In order to generate the hybrids, both plant types carrying the individual transgenes were used as pollen donors as well as pollen recipients. The anthers were dissected out of the recipient flowers 1 - 2 days before the flower bud opened, and the bud was then covered with a paper bag to prevent accidental pollination. Pollen from the donor plants was used to fertilise the recipients on the day that the bud opened. Seeds were removed from the plants 4 - 5 weeks after pollination.

2.2.1.3 Germination and maintenance of plants

Seeds of the five plant types were placed on soil in seedling trays, and germinated in a growth room with a 12 hour photoperiod (300 μmol m^{-2} s^{-1}) and a 20°C night / 25°C day temperature at 70% humidity. After 6 - 7 weeks the seedlings had developed 2 - 3 fully expanded leaves, and were transferred to individual pots and maintained under these same conditions. They were watered with Long Ashton nutrients containing 0.14 g l^{-1} nitrates (Hewitt, 1952). The plants were utilised 4 weeks after transfer to individual pots.

2.2.2 Selection and characterisation of plant types

2.2.2.1 *E. coli* GR transformants

GR activity

All plants carrying cytoplasmic or chloroplastic *E. coli* GR were subjected to GR assays as described below (2.2.4.2). Those plants having the highest GRA for their genotype were used for the oxidative stress experiment.

Western blotting

Western blots were carried out on whole leaf material and isolated chloroplasts of all *E. coli* GR transformants, in order to confirm the subcellular compartmentalisation of the transgene product. The chloroplast isolations and western blots were performed as outlined in the methods and materials section of Chapter 1.

2.2.2.2 Tomato Cu,Zn SOD transformants

Extracts from whole leaves of all tomato Cu,Zn SOD transformants were subjected to SDS-PAGE and staining for SOD activity in order to confirm the presence of the tomato Cu,Zn SOD. Furthermore, isolated chloroplasts from these plants were also subjected to SDS-PAGE and SOD staining to confirm the localisation of the tomato SOD in the chloroplast. The chloroplast isolations and SOD activity gels were carried in the manner described in Chapter 1.

2.2.3 Oxidative stress

Four fully expanded leaves from each of the five plant types were removed from the plants (each leaf was taken from a separate plant). The petioles of these leaves were cut a second time under water to remove air bubbles in the xylem vessels. The petioles were then placed in a 75 μM paraquat (methyl viologen) solution and the leaves were placed in a dark growth cabinet at 25°C for 6 hours. After 6 hours the growth cabinet lights (200 μmol m^{-2} s^{-1}) were switched on and the first batch of plant material was harvested (0 hour readings). Leaf material was subsequently harvested at 8, 16 and 24 hours.

2.2.4 Enzyme assays

2.2.4.1 Sample preparation

At each sampling time, leaf discs were cut from the 4 leafs of each plant type. These leaf discs were randomly mixed and divided into 3 batches to provide three samples for each plant type. The samples for assays were

prepared as described by Smith *et al.* (1988). They were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The samples were then further ground at 4°C with 10 volumes of extraction buffer (0.1 M potassium phosphate (pH 7.5), 0.5 mM EDTA), and the resulting homogenate was centrifuged at 10 500 g (Heraeus Sepatech Biofuge B) for 15 minutes at 6°C. The supernatant was then utilised for enzyme assays both GR and SOD assays.

2.2.4.2 GR assays

The supernatant was assayed for GR activity as described by Carlberg and Mannervik (1985). The assay mixture comprised 0.1 M potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 0.2 mM NADPH, 2 mM GSSG and extract in a final volume of 1 ml. The reaction was initiated by the addition of the NADPH, and the decrease in NADPH concentration was monitored at 340 nm using a Beckman DU 7500 spectrophotometer. The reaction rate was calculated using the kinetics software package provided with the spectrophotometer. GR activity was calculated on a protein basis by determining supernatant protein levels, using the method of Bradford (1976). A protein standard curve was constructed using BSA as a protein standard.

2.2.4.3 SOD assays

SOD activity was assayed using the method of Beauchamp and Fridovich (1971), modified in that the buffer used was that of van Rensburg and Kruger (1994). The assay mixture had a final volume of 3 ml and contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.5 $\mu g\ ml^{-1}$ riboflavin, 2 $mg\ ml^{-1}$ methionine, 0.05 $mg\ ml^{-1}$ NBT and extract. This mixture was incubated in the light (100 $\mu mol\ m^{-2}\ s^{-1}$) for eight minutes and the reaction was then terminated by placing the assay mixture in the dark. The absorbance was read at 560 nm against a blank comprising the above mixture (minus the extract) kept in the dark until used.

This assay depends on the ability of the SOD to prevent a colour change due to superoxide production - that is, it measures the inhibition of the reaction by SOD. Beauchamp and Fridovich (1971) defined one enzyme unit as the amount of SOD necessary to inhibit the reaction by 50%. To determine the amount of inhibition due to SOD, 3 assays were set up and run without any extract, thus allowing the maximum possible colour development in the assay mixture. According to Giannopolitis and Ries (1977), the SOD units ml^{-1} = [(V/v) - 1] x (extract dilution factor), where V and v are the reaction rate in the absence and presence of SOD respectively. This equation was used to calculate the amount of SOD present.

2.2.5 Stress indicators

In order to determine the effect of paraquat on the leaf material, the increase in solute leakage and the decline in chlorophyll levels were monitored.

2.2.5.1 Solute leakage

At each harvesting time 4 leaf discs (1 leaf disc/leaf) from each plant type were cut using a cork borer 6.8 mm in diameter. Each leaf disc was floated on 1 ml distilled water in a well of a 100-well sample tray from a CM 100 conductivity meter (Reid and Associates). After a 24 hour incubation period, the conductivity of each leaf disc was read 5 times. The leaf discs were then frozen in liquid nitrogen and returned to the sample tray for a further 1 hour. The conductivity was again read to determine the maximum leakage for each leaf disc. Ten samples of plain distilled water were also read to determine baseline conductivity. The average baseline value was subtracted from each reading, and leakage for each leaf disc was expressed as a percentage of the total possible leakage for that leaf disc.

2.2.5.2 Chlorophyll assays

Chlorophyll was assayed according to Arnon (1949). Briefly, 50 μl samples were removed from the homogenate prepared for enzyme assays (2.2.5.1) prior to centrifugation. These samples were mixed with 500 μl of ice cold 80% acetone and incubated on ice for 15 minutes. The samples were then centrifuged at 100 g for 5 minutes (Sigma 201M centrifuge), and the absorbance of the supernatant was measured at 652 nm (Beckman DU 7500 spectrophotometer) using 80% acetone as the blank. Chlorophyll concentration was calculated using an extinction coefficient of 36.1 mg^{-1} ml cm^{-1}.

2.3 RESULTS AND DISCUSSION

As mentioned in the introduction, plants carrying high levels of more than one antioxidant enzyme have not been widely studied to date. To determine whether plants would derive any benefit from having enhanced activities of more than one antioxidant enzyme, transformants carrying both *E. coli* GR and tomato SOD were produced. The hybrids expressing high chloroplastic activities of both enzymes were obtained by cross-pollinating the plants described in Chapter 1 with plants having high levels of chloroplastic tomato SOD activity. This chapter describes the characterisation of these hybrids and goes on to examine their response to paraquat-induced oxidative stress.

2.3.1 Characterisation of transgenic plants

Four types of transgenic tobacco were used in this study. These included plants carrying chloroplastic *E. coli* GR, plants with cytoplasmic *E. coli* GR, plants with chloroplastic tomato SOD and a hybrid having both chloroplastic *E. coli* GR and chloroplastic tomato SOD. Prior to studying the oxidative response of these plants, it was necessary to confirm the presence of the relevant transgene product in the appropriate subcellular compartment.

2.3.1.1 Chloroplastic *E. coli* GR transformants

The analysis of the tobacco plants carrying chloroplastic *E. coli* GR is described in Chapter 1 and these plants will only be mentioned here where they were used as controls in gels.

2.3.1.2 Plants carrying tomato Cu,Zn SOD

Extracts from leaves as well as isolated chloroplasts of Cu,Zn SOD transformants were analysed for SOD activity, using non-denaturing polyacrylamide gels which were subjected to a SOD activity stain. This stain incorporates NBT (yellow in colour) and a superoxide generating system. On exposure to light superoxide is produced and reacts with the NBT, converting it to a blue formazan (Halliwell and Gutteridge, 1989). Where SOD is localised the superoxide is scavenged and the gel remains clear, resulting in clear bands at the points where SOD is situated.

Figure 2.3A shows the results of SOD activity gels for extracts from entire leaves. The control plant (lane 4) shows four bands of SOD activity, although the two bands of lowest molecular weight are very faint. These bands are shown to better effect in lane 1 of Fig. 2.3B (also a control plant extract). An analysis of the types of SOD present in these four bands was not possible, but the banding patterns are in agreement with those observed by Bowler *et al.* (1991) for tobacco. From the results of these authors, the isozymes in the tobacco control plants were identified as shown to the left of Fig. 2.3A. The tomato isozymes were identical to those observed by Perl *et al.* (1993), and are identified to the right of the figure.

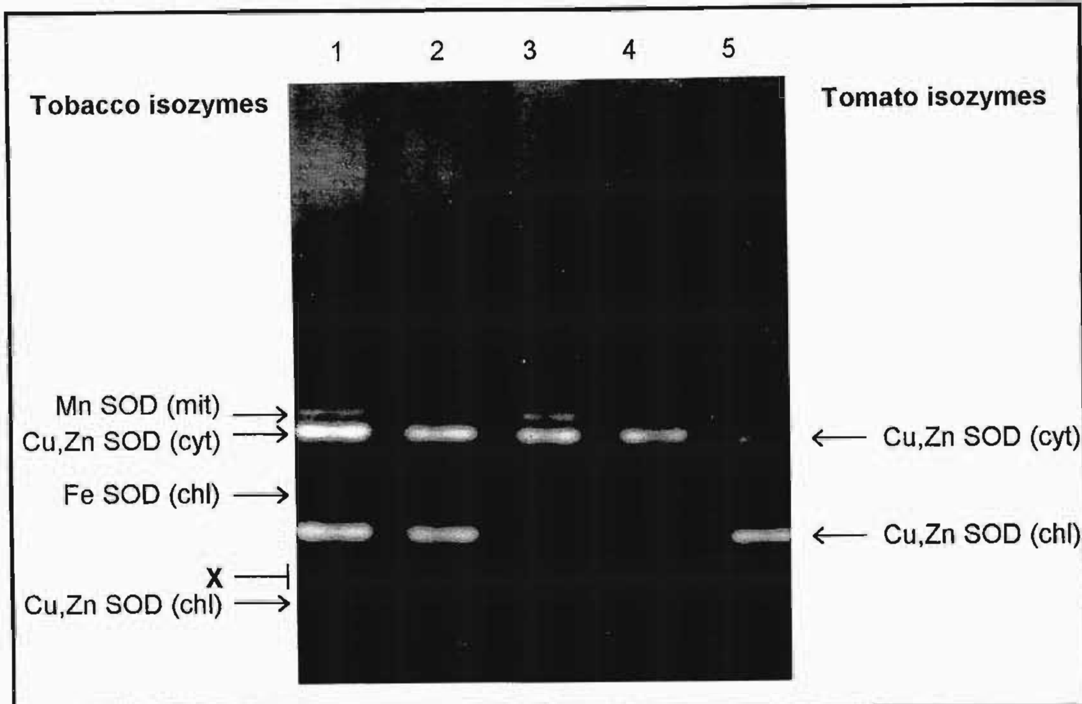


Figure 2.3A: SOD activity gels for leaf extracts of tomato Cu,Zn SOD transformants.
Samples (60 µg of total soluble protein) from leaf extracts were electrophoresed on 10% polyacrylamide gels. Lanes 1 and 2 have extracts from Cu,Zn SOD transformants, while lane 3 contains an extract from a chloroplastic *E. coli* GR transformant. Lane 4 represents an untransformed control plant and lane 5 contains leaf extract from a tomato plant. Abbreviations: mit = mitochondrial, cyt = cytoplasmic, chl = chloroplastic.

From Fig. 2.3A it can be seen that the tomato cytoplasmic isozyme migrates identically to the cytoplasmic isozyme of tobacco. However, the chloroplastic Cu,Zn SOD isozymes for the two plants migrate differently, allowing easy identification of the tomato SOD in the transgenic tobacco (see lanes 1 and 2 of Fig 2.3A). Although these gels only allow a semi-quantitative analysis of SOD activity it is apparent that the activity of the tomato enzyme is relatively high, indicating stable expression of the transgene in tobacco. Furthermore, the intensity of the band indicates that the transgene confers a significant increase in the overall SOD activity of the chloroplast.

A point worth mentioning is that there is no discernible Fe SOD activity in any of the transgenic plants. There are two possible explanations for this. Firstly, the increase in SOD activity in the chloroplast may result in an increase in the peroxide levels. Since Fe SOD is irreversibly inhibited in the presence of peroxide (Scandalios, 1993) such an increase may account for the disappearance of the Fe SOD band. However, Cu,Zn SODs are also irreversibly inhibited by peroxide (Scandalios, 1993). Since the chloroplastic SOD band was not affected in the transformants this explanation is unlikely to account for the loss of the Fe SOD band.

The alternative explanation is that the expression of the Fe SOD gene may require a signal in response to cellular oxidation levels. Bowler *et al.* (1992) have shown that Fe SOD activity in higher plants does increase under photoinhibitory conditions (such as chilling mediated photoinhibiton and greening of etiolated plants), indicating that the expression of the SOD gene does require an oxidising signal. It thus seems likely that the higher levels of chloroplastic SOD conferred by the tomato transgene inhibit Fe SOD production at the transcriptional level - possibly by preventing an oxidation signal responsible for the induction of Fe SOD transcription.

One further point worth noting is the appearance of two additional isozymes in the transgenic tobacco plants in lanes 1 and 2 (these isozymes are marked X in Fig 2.3A). These isozymes have a similar level of activity to the chloroplastic Cu,Zn SOD of tobacco and do not appear in either the tomato or the control tobacco. Perl *et al.* (1993) also observed the appearance of an additional band midway between the endogenous and transgene Cu,Zn SODs when they transformed potato plants with tomato chloroplastic Cu,Zn SOD. They attributed this band to a heterodimer composed of subunits from the homodimeric potato and tomato chloroplastic Cu,Zn SODs. Such a dimer composed of one tomato and one tobacco monomer would account for the upper of the two new bands observed in Fig. 2.3A, but at present the second of the two new bands cannot be accounted for.

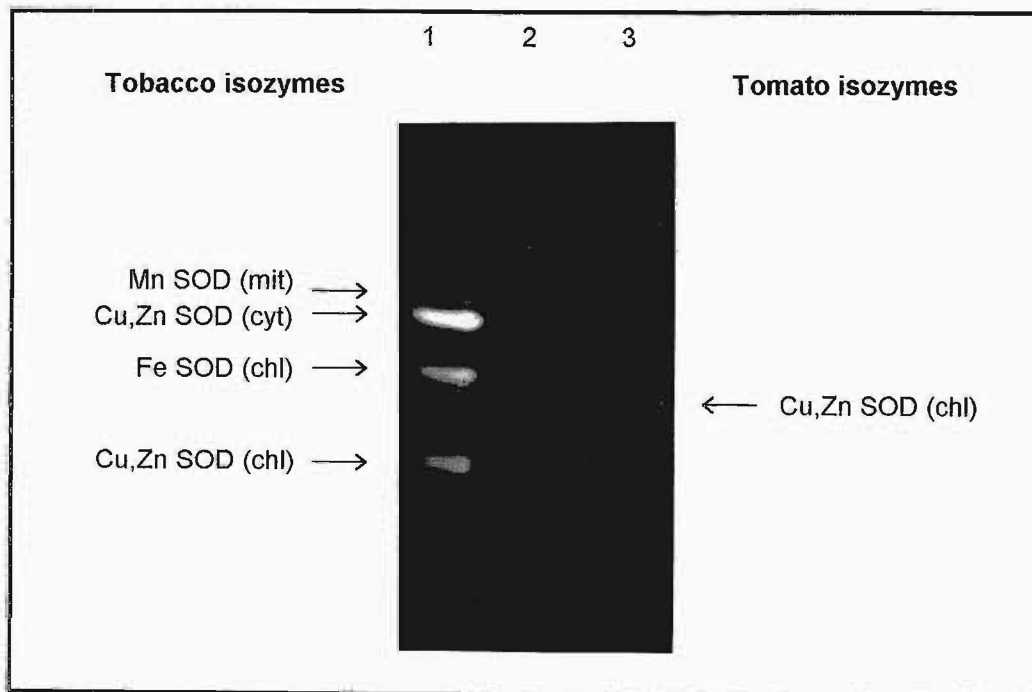


Figure 2.3B: SOD activity gels for extracts from isolated chloroplasts of tobacco plants carrying tomato Cu,Zn SOD.
Lane 1 was a control lane loaded with 80 μg soluble protein extracted from leaves of untransformed tobacco, while the samples in lanes 2 and 3 were chloroplastic extracts isolated from Cu,Zn SOD transformants (40 μg and 60 μg of soluble protein respectively). Abbreviations as for Fig. 2.3A.

Fig. 2.3B shows SOD activity in extracts of chloroplasts isolated from two SOD transformants. This gel was run in order to determine the subcellular location of the transgene product. It was initially assumed that the product (the chloroplastic isozyme of tomato Cu,Zn SOD) would be situated in the chloroplastic compartment, since all of the SODs are nucleus encoded and contain the necessary NH_2-terminal targeting sequences for transport to the relevant organellar locations (Bowler *et al.*, 1992). Fig. 2.3B shows the banding pattern for isolated chloroplasts (lanes 2 and 3) at different protein loading levels. The only band present in these lanes is the tomato Cu,Zn SOD, although this band is very faint - indicative of a very low protein loading. However, it is sufficient to show that the enzyme is localised in the chloroplast (in whole leaf extracts, the tomato isozyme band and the cytoplasmic Cu,Zn SOD band of tobacco are of equal intensity - if this band in the chloroplast fraction was due to cytoplasmic contamination, then the tobacco isozyme should also contaminate at a similar level). Further evidence for the chloroplastic insertion of the transgene product is the formation of heterodimeric SOD. These heterodimers can only form in the presence of the tobacco monomer, which is situated in the chloroplast.

2.3.1.3 Tomato SOD / *E. coli* GR hybrids

All of the hybrids used in the stress experiment were first analysed for the presence of both tomato SOD and *E. coli* GR. Fig. 2.4 shows a SOD activity gel run for four of the hybrid plants.

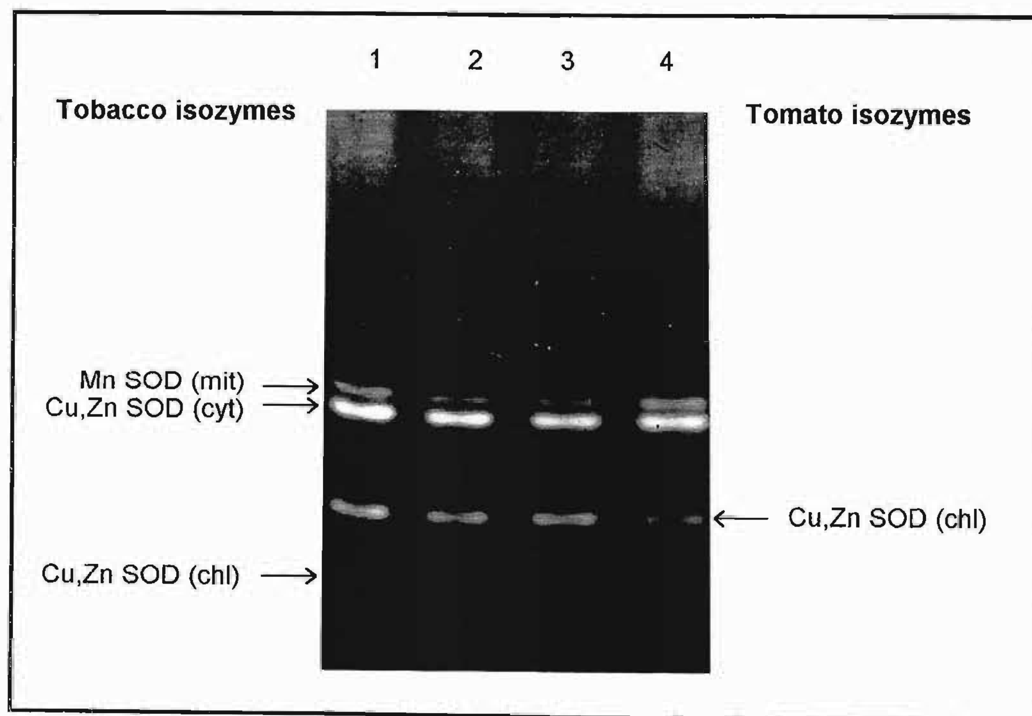


Figure 2.4: SOD activity gels for leaf extracts from hybrid transformants. All four lanes contain samples from hybrid tobacco plants carrying both *E. coli* GR and tomato Cu,Zn SOD. Each lane contained 60 μg of soluble protein. Abbreviations as for Fig. 2.3A.

The lanes shown in Fig. 2.4 were run in the same gel and at the same protein loading as the lanes shown in Fig. 2.3A. The chloroplastic tomato Cu,Zn SOD isozyme is clearly present in all four lanes, indicating the stable transfer of the gene during the cross pollination of the SOD and *E. coli* GR transformants. As with the SOD transformants, the tobacco Fe SOD is not noticeable.

Another feature of these plants is the lower intensity of the tomato isozyme bands, compared to the bands observed from plants transformed with only the tomato Cu,Zn SOD (compare with Fig. 2.3A). Furthermore, the two new bands observed in Fig. 2.3A are also less intense in these plants - probably as a result of the lower levels of tomato Cu,Zn SOD subunits. This decrease in the activity of the tomato isozyme is not due to protein loading levels - protein loading levels were identical for the hybrids and for plants carrying only the tomato SOD. This is confirmed by the observation that the tobacco isozymes from the two plant types showed bands of equal intensity. This decrease in tomato SOD cannot be attributed to a DNA transcription signal as discussed for endogenous Fe SOD, since the tomato isozyme is under the control of the constitutive CaMV promoter.

To confirm the presence of *E. coli* GR in the hybrids, these plants were tested for GR activity. The hybrids showed a GR activity (average $\pm$ std deviation) of 17.7 ± 4.8 μmol NADPH oxidised min^{-1} g fwt^{-1} (n = 25), compared to the control plant activity of 0.39 ± 0.26 μmol NADPH oxidised min^{-1} g fwt^{-1} (n = 4). As established in Chapter 1, such high rates of NADPH utilisation confirms the presence of large quantities of *E. coli* GR.

2.3.1.4 Cytoplasmic *E. coli* GR transformants

The cytoplasmic *E. coli* GR transformants used in this study were produced by M. Roberts (unpublished work). Western blots were performed on leaf material to confirm the presence of *E. coli* GR. Western blots were also performed on isolated chloroplasts, to confirm that none of the transgene product was situated in the chloroplast. Prior to running the western blots, isolated chloroplast extracts were subjected to a SOD activity stain to test that no there was no contamination by cytoplasmic components - *i.e.* SOD was used as a marker enzyme for chloroplast purity.

Fig. 2.5 shows the results of the SOD activity gel. Lane 1 contains soluble protein extracted from entire leaves and shows an identical banding pattern to that observed for control plants in Figs. 2.3A and 2.3B. Lanes 2 and 3 contain soluble protein extracted from isolated chloroplasts. These extracts lack the band attributed to the cytoplasmic Cu,Zn SOD of tobacco, indicating that the isolated chloroplasts are not contaminated by cytoplasmic elements.

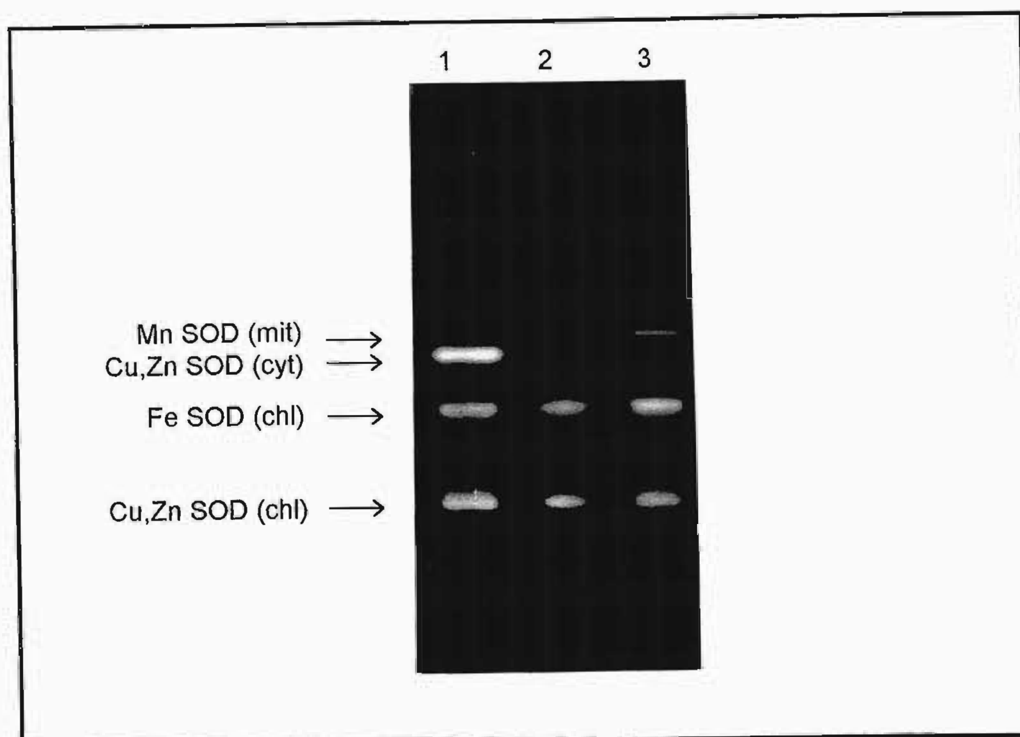


Figure 2.5: SOD activity gel for extracts of chloroplasts isolated from a cytosolic *E. coli* GR transformant.
Lane 1 received 80 μg soluble protein from a whole leaf extract, while lanes 2 and 3 were loaded with extracts from chloroplasts isolated from the same plant (60 μg and 80 μg of protein respectively). Abbreviations as for Fig. 2.3A.

Having confirmed the purity of the isolated chloroplasts, western blots were carried out to determine location of the *E. coli* GR in the transformed plants. Before running the western blots on leaf and chloroplast extracts, however, the western blotting procedure needed optimisation. Extracts from entire leaves were analysed for protein levels and western blotting was carried out using a series of protein loadings.

The results of the optimisation blot are shown in Fig 2.6. The lanes loaded with extract from the transgenic plants show two bands - one at 48 kDa and the second at 97 kDa. The 48 kDa band reacts strongly with the anti-GR antibody and corresponds well with the 48.717 kDa predicted by Greer and Perham (1986) for *E. coli* GR. The second band also reacts strongly with the anti-GR antibody and is not present in the lane for the untransformed control, indicating another protein associated exclusively with the transformed plants. It is known that the active form of *E. coli* GR is a dimer of two identical subunits and that the enzyme maintains an equilibrium between the monomeric and dimeric forms, with the equilibrium being affected by NADPH and glutathione (Scrutton *et al.*, 1992; Arscott *et al.*, 1989). The molecular weight of the heavier band suggests that it is the dimeric form of GR. It is possible that insufficient DTT was added to the gel loading buffer to separate all of the *E. coli* GR into two subunits prior to electrophoresis, hence the additional band. Previous studies involving the antibody used here have indicated a strong cross reactivity with tobacco

GR, as well as other proteins (Whittaker, 1990; Badenhorst, 1993). The blots performed in this study show no cross reactivity with plant GR which has a molecular weight of around 60 kDa (Tanaka *et al.*, 1988a). Cross reactivity with other proteins in this study was also minimal, indicating a suitable dilution of the primary antibody.

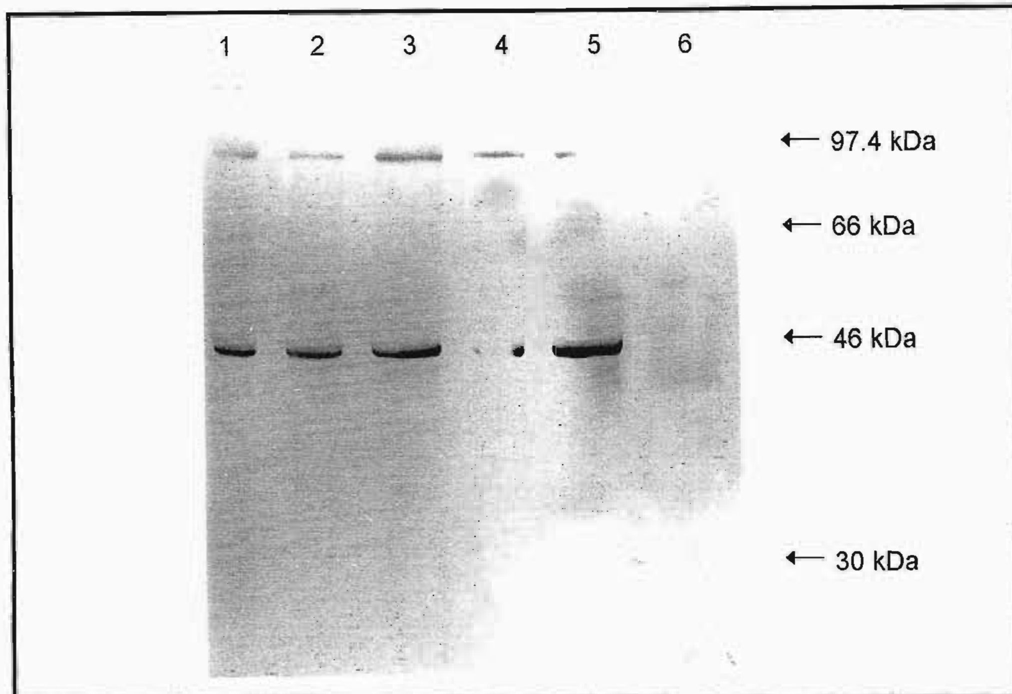


Figure 2.6: Optimisation of the western blotting procedure. Lanes 1 - 5 were loaded with soluble protein extracted from leaves of plants transformed with cytoplasmic *E. coli* GR. These lanes were loaded with 50, 75, 100, 125 and 150 µg of protein respectively. Lane 6 received 150 µg of protein extracted from leaves of untransformed control plants.

The band in lane 4 was not clearly resolved, possibly due to the blotting procedure. The bands in the other lanes were easily distinguished, indicating sufficient protein levels for the blotting procedure. Lanes 3 and 5 show bands of similar intensity, indicating that the procedure is not readily able to distinguish between samples loaded at these protein levels. The greatest difference in banding intensity occurs between lanes 1 (50 µg protein) and 2 (75 µg protein). It was decided to use a loading level of 60 µg protein per lane for future blots, since this loading was not saturating and would allow the detection of increases in *E. coli* GR levels.

Fig. 2.7 shows the western blot for isolated chloroplasts as well as leaf material. The extracts from leaf material (lanes 2 and 4) show the 48 kDa band attributed to *E. coli* GR. The purified *E. coli* GR loaded in lane 6 has the same mobility as the band identified as *E. coli* GR in the transgenic plants. The extracts from isolated chloroplasts (lanes 3 and 5) show no bands which react with the anti-GR antibody, indicating that *E. coli* GR is not associated with the chloroplasts of the transgenic plants.

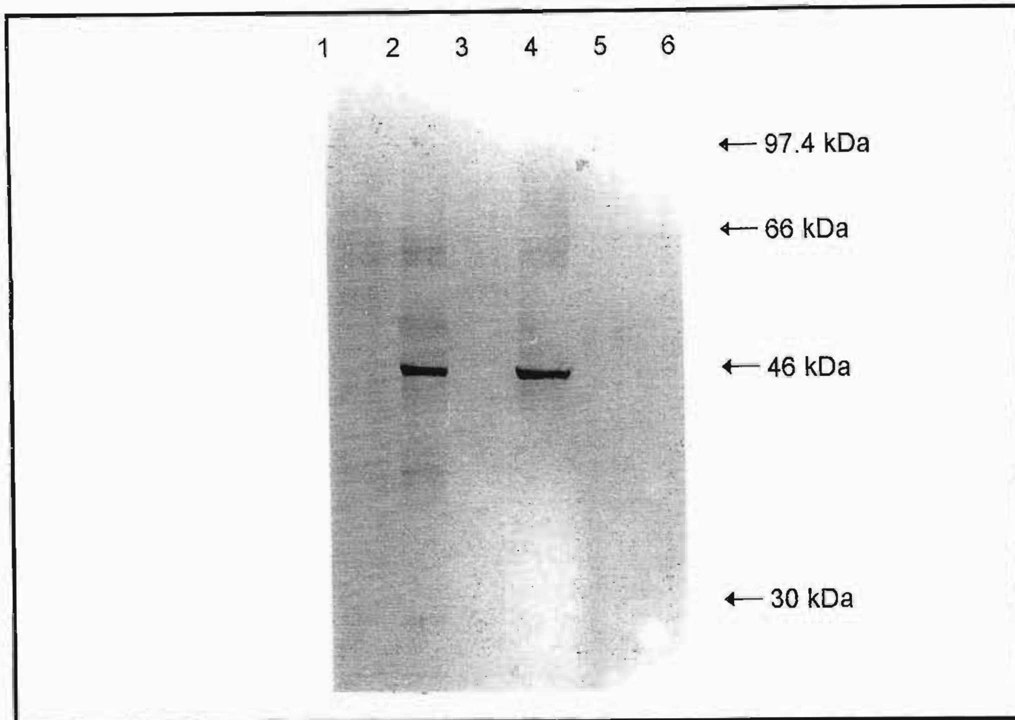


Figure 2.7: Western blots showing the presence of *E. coli* GR in transformed tobacco. This gel was loaded with extracts from leaves and isolated chloroplasts of transformed plants with *E. coli* GR targeted to the cytoplasm. Lane 1 contained soluble protein (60 μg) extracted from an untransformed control plant, while lanes 2 and 4 were loaded with soluble protein from entire leaves of transformed plants (60 μg and 80 μg respectively). Lanes 3 and 5 show banding patterns for extracts of chloroplasts isolated from transformed plants (40 μg and 60 μg respectively), while lane 6 received purified *E. coli* GR supplied by R. Ponquett.

The cytoplasmic *E. coli* GR transformants were also tested for the levels of GR activity. These plants showed a GR activity (average ± std deviation) of 1.06 ± 0.49 μmol NADPH oxidised min^{-1} g fwt^{-1} (n = 10), compared to control plant activity of 0.39 ± 0.26 μmol NADPH oxidised min^{-1} g fwt^{-1} (n = 4).

2.3.2 Response of transgenic tobacco to oxidative stress

In this study, tobacco plants having increased levels of two antioxidant enzymes (SOD and GR) were studied under conditions of elevated oxidative stress. GR has been proposed as the rate limiting enzyme in the ascorbate-glutathione antioxidant cycle (Jablonski and Anderson, 1981) and this cycle has been shown to operate in both the chloroplast and the cytoplasm (Smith *et al.*, 1989). Although the chloroplasts are theoretically exposed to higher levels of oxygen radicals than other subcellular compartments, previous studies have shown that an enhancement of either chloroplastic or cytoplasmic GR may protect plants against oxidative stress (Aono *et al.*, 1991). Experimental material thus included plants carrying *E. coli* GR in either the chloroplast or the cytoplasm. The enzyme SOD catalyses the initial reaction in the scavenging of photoreduced oxygen and a number of

studies have already indicated that elevated levels of SOD may protect plants against the effects of excess oxygen activation arising under various stress conditions (reviewed by Hérouart *et al.*, 1993; Allen, 1995). The experiment thus incorporated plants carrying chloroplastic Cu,Zn tomato SOD. As reviewed in the introduction (section 2.1.5), Foyer *et al.* (1994a) have suggested that an increase in a single enzyme in the radical-scavenging system may not be as beneficial as a balanced increase in two or more of these enzymes. For this reason it was decided to examine plants transformed with both SOD and GR in the chloroplast, to determine whether these transformants would offer more protection against oxidative stress than just one of these enzymes alone.

2.3.2.1 The induction of oxidative stress in experimental plants

As discussed in section 2.1.2, a number of stress conditions may lead to an increase in the production of oxygen radicals in plants. In choosing a protocol for inducing oxidative stress in the experimental plants, it was necessary to select a method that would have few stress effects other than the production of oxygen radicals. In this regard, it was decided that paraquat (methyl viologen) was a good candidate for the production of oxidative stress. This bipyridinium herbicide is highly toxic and functions to produce oxygen radicals in illuminated chloroplasts. Paraquat is a dicationic molecule capable of accepting a single electron to form the monocationic paraquat radical. The formation of this radical occurs readily at the reducing side of PS I, with paraquat probably accepting the electron from P430 - an Fe-S protein bound to PS I (Asada and Takahashi, 1987). As mentioned in section 2.1.1 oxygen reacts readily with radicals, and the paraquat radical is no exception. It reacts rapidly with molecular oxygen ($k = 7.7 \times 10^8\ M^{-1}\ s^{-1}$), producing the superoxide radical (Winston, 1990). This reaction regenerates the paraquat dication, which can then produce another paraquat radical - thus giving paraquat the effect of a superoxide-generating catalyst. Babbs *et al.* (1989) have also produced evidence that the paraquat radical can react with H_2O_2, producing the hydroxyl radical and regenerating paraquat (reaction 10):

$$\text{paraquat radical} + H_2O_2 \longrightarrow \text{paraquat} + OH^{\bullet} + OH^{-} \qquad (10)$$

In illuminated chloroplasts this reaction produces large quantities of hydroxyl radicals, equivalent at least to the number of hydroxyl radicals produced per gram of fresh tissue on exposure to 10 000 rads of high energy gamma irradiation (Babbs *et al.*, 1989). Paraquat treated plants are thus ideal candidates for a study of the radical scavenging system - any peroxide that is not scavenged will result in the formation of lethal doses of hydroxyl radicals.

2.3.2.2 The response of tobacco plants to oxidative stress

Untransformed plants

Numerous studies involving the response of plants to oxidative stress have shown increases in the activity of antioxidant enzymes, as well as increases in the levels of antioxidant molecules (particularly ascorbic acid and glutathione). These studies have been conducted with a variety of plants with no particular adaptations imparting above average tolerance of oxidative stress. Stress factors giving rise to increases in components of the antioxidant pathway include drought (Gamble and Burke, 1984; Sgherri and Navari-Izzo, 1995; Baisak *et al.*, 1994; Burke *et al.*, 1985), chilling (Schöner and Krause, 1990; Walker and McKersie, 1993; Mishra *et al.*, 1993), elevated oxygen levels (Foster and Hess, 1980; Foster and Hess, 1982) and paraquat exposure (Edwards *et al.*, 1994; Tsang *et al.*, 1991). The most commonly observed change in stressed plants is an increase in GR and SOD activity. A similar trend was observed in this study, with both enzymes showing nearly a 50% an increase in activity in control plants 8 - 16 hours after the imposition of the stress treatment (Figs. 2.8 and 2.9).

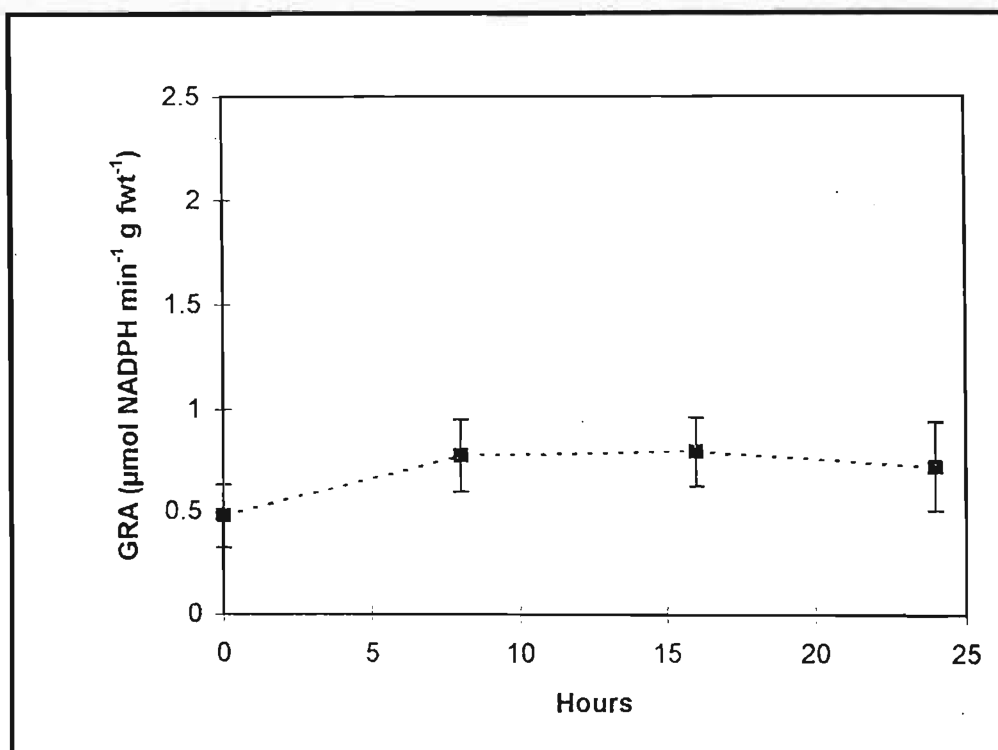


Figure 2.8: The response of GR activity in control plants following paraquat treatment. The readings were taken at the indicated times and each the data at each point is the mean and standard deviation of nine determinations.

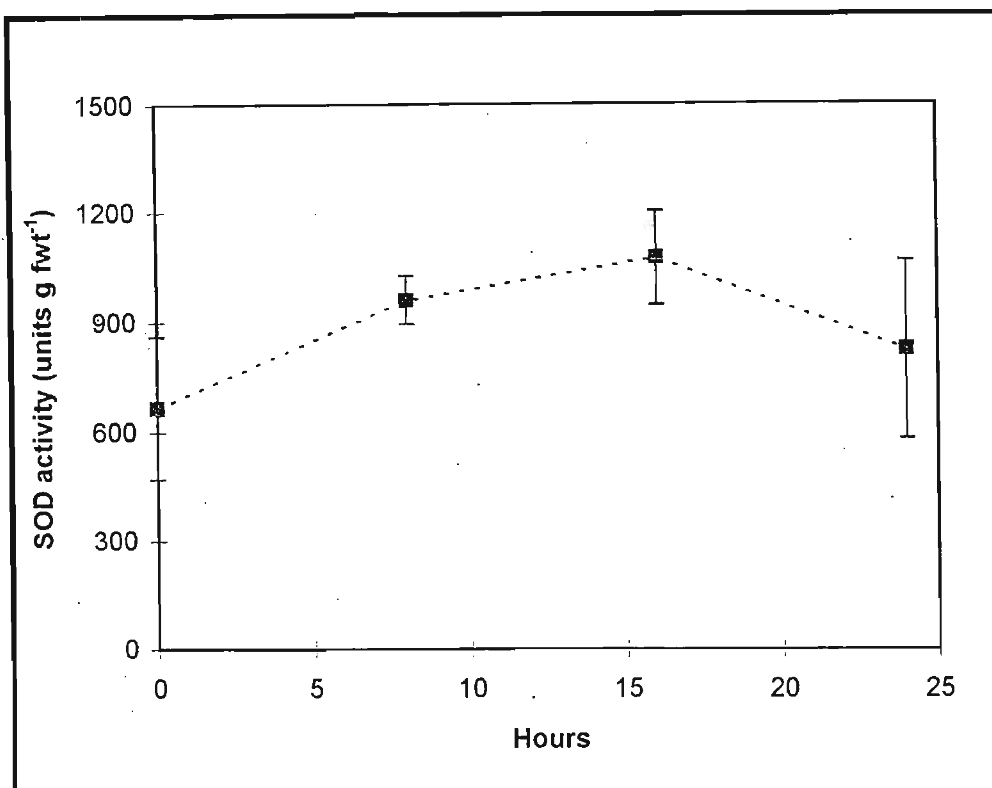


Figure 2.9: SOD activity in paraquat-treated control plants. The readings were obtained at regular intervals following the initiation of the oxidative stress, and the data at each point represents the mean activity and the standard deviation.

The increase in enzyme activities can be attributed to a number of factors, including increased production of enzyme, a decrease in the rate at which enzyme is degraded or an increase in the affinity of the enzyme for its substrates. Studies utilising protein synthesis inhibitors have shown that SOD activity in stressed plants increases partly as a result of increased SOD production (Mishra *et al.*, 1993; Baisak *et al.*, 1994). Tsang *et al.* (1991) have shown that the increase is due to an elevation of SOD mRNA levels, with all of the SOD mRNAs (Fe, Mn and Cu,Zn) exhibiting enhanced levels. This would account for the observed increase in SOD levels observed in this study.

GR polypeptide levels have also been shown to increase under stress conditions (Baisak *et al.*, 1994). Pastori and Trippi (1992) have shown that this increase is not due to an increase in mRNA production - it occurs rather because of an increase in the rate of translation of existing mRNA. Furthermore, Edwards *et al.* (1994) have also found that GR has a number of isoforms and that the composition of the GR isoform population changes under stress conditions, favouring the production of those isoforms with a higher affinity for GSSG. Both the increase in mRNA translation and a change in the isoform population would account for the observed GRA increase in control plants.

The signals for increases in mRNA levels and for increased translation are not clear. However, Pastori and Trippi (1992) have shown that

elevated levels of SOD and GR may be induced by treating leaf discs with either peroxide or with superoxide-producing systems. This indicates that activated oxygen may well play a role in inducing synthesis of SOD and GR.

Although the increases in enzyme activity agree with the findings of the studies cited above, the drop in SOD activity after 16 hours of treatment (Fig. 2.9) is not seen in any of the cited experiments. This decline may be related to the activity of APx - the first enzyme in the peroxide scavenging cycle. This enzyme commonly shows enhanced activity in the early stages of oxidative stress but this response is often a transient one. Baisak *et al.* (1994) attributed drought-induced oxidative damage to a decline in APx activity, suggesting that this lead to an accumulation of peroxide and concomitant cellular damage. Although APx activity was not determined in this study, several factors point to an accumulation of peroxide following paraquat treatment. Firstly, the control plants showed the mentioned decline in SOD activity after 16 hours. This decline may have occurred because the Cu,Zn SOD and Fe SOD isozymes are both irreversibly inhibited by peroxide. A high peroxide-related SOD turnover would explain the results of Karpinski *et al.* (1993), who showed that the SOD mRNA levels are up to 20 times higher than those of GR mRNAs in stressed plants. A second indication of peroxide accumulation in the 16 - 24 hour period was a significant decline in the chlorophyll levels of the controls (Fig. 2.10), accompanied by an increase in electrolyte leakage (Fig 2.11).

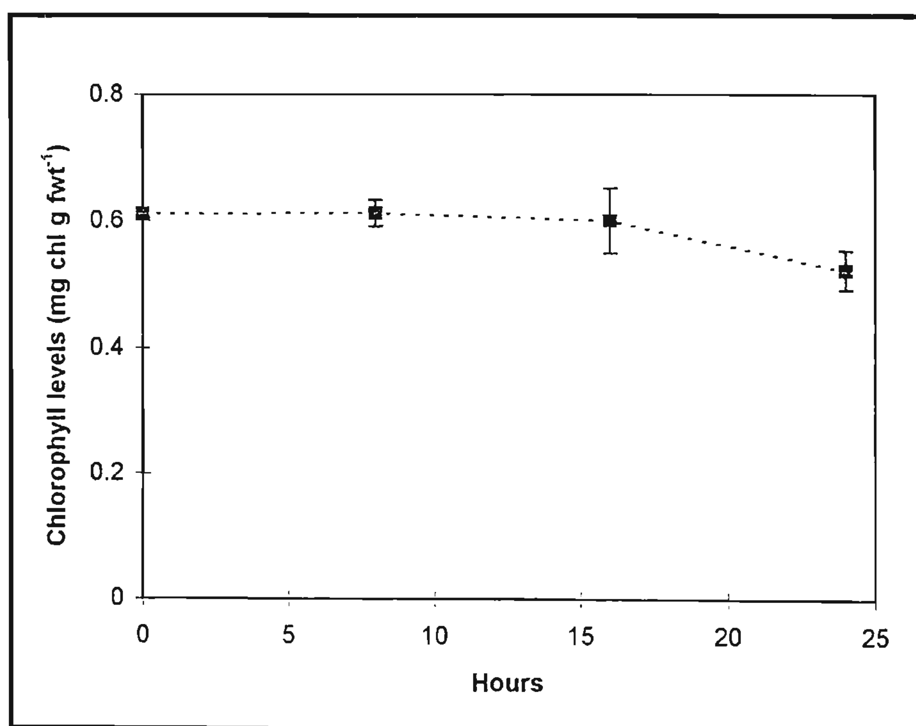


Figure 2.10: Chlorophyll levels in control plants subjected to paraquat treatment. The chlorophyll levels were determined at the indicated times. Each data point represent the mean and standard deviation of nine readings.

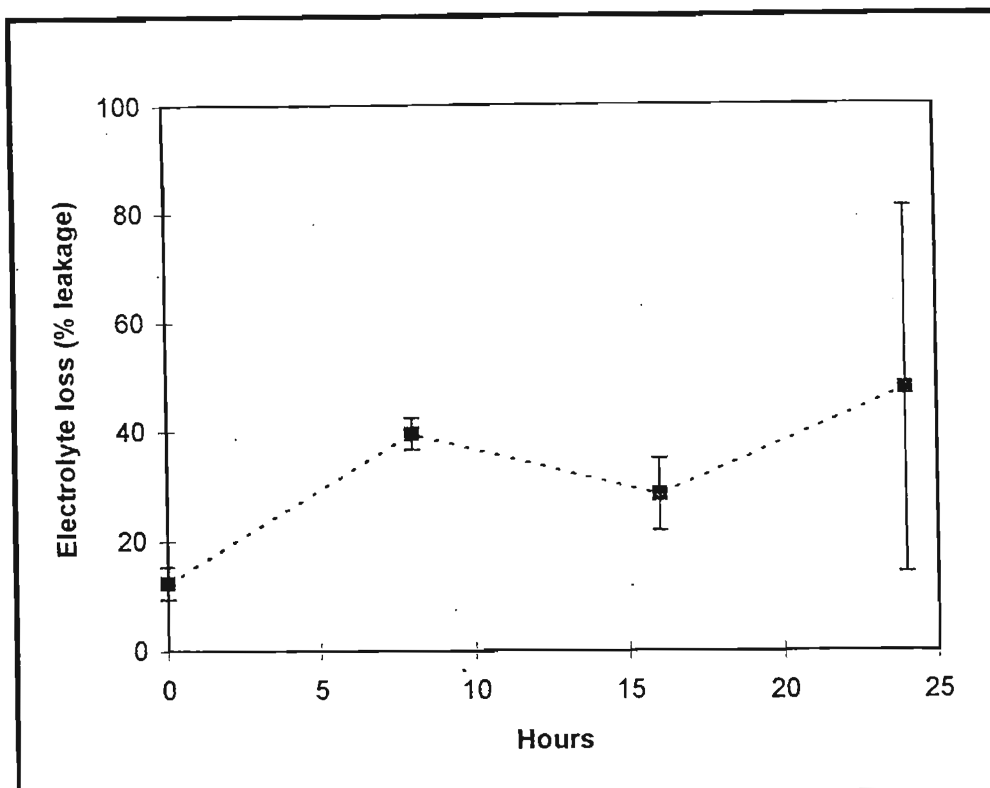


Figure 2.11: Electrolyte leakage from leaf discs of control plants treated with paraquat. Leakage at each time was determined as a percent of the total possible leakage. Each data point represents the mean and standard deviation of leakage from leaf discs cut from the leaves of four treated plants.

Chlorophyll loss and cell leakage are both indicators of cellular damage, and this response to stress reflects an inability to scavenge reactive oxygen species. The decline in SOD activity declined after 16 hours would lead to increases in superoxide and peroxide concentrations. This combination of high superoxide and high peroxide levels would favour the Haber-Weiss reaction, resulting in the formation of the hydroxyl radical (see section 2.1.1.1) - a primary agent in damage mediated by photo-reduced oxygen.

The above responses of the control plants represent a typical plant response to oxidative stress - that is, an increase in cellular oxidants, followed by increases in the components of the scavenging system. If the stress is maintained, the protective systems are overwhelmed and cellular damage follows. However, some plants show a natural resistance to oxidative stress and detailed studies of these plants have shown that it is possible for the antioxidant system to provide comprehensive protection, even under conditions of relatively severe stress. Working with drought tolerant maize (Pastori and Trippi, 1992; Malan *et al.*, 1990) and paraquat resistant *Conyza bonariensis* (Ye and Gressel, 1994; Shaaltiel *et al.*, 1988), it has been shown that these plants all tolerate paraquat treatments due to naturally high levels of antioxidant enzymes. In all of these studies SOD and GR activities were shown to be higher

than in paraquat-sensitive plants, and in several of the studies APx activity was also shown to be elevated. In addition, these paraquat-tolerant plants showed an ability to increase the activity of these enzymes much more rapidly than sensitive plants. These studies, and others like them, have raised the possibility of producing genetically engineered plants which tolerate a variety of oxidative stresses.

***E. coli* GR transformants**

Because GR has been suggested as the rate limiting step in the antioxidant cycle (Jablonski and Anderson, 1981), several transformations have been carried out over the last five years to determine the effect of elevated GR levels under conditions of oxidative stress. These studies have involved plants carrying a gene for either the cytoplasmic expression or the chloroplastic expression of foreign GR. One of the aims of this study was to produce tobacco plants carrying chloroplastic *E. coli* GR and compare the stress response of these plants to that of an existing transformant which expresses a cytoplasmic form of *E. coli* GR. As shown in Figs. 2.12A and 2.12B, there are considerable differences in the GR activity in these two plants.

The plants carrying cytoplasmic *E. coli* GR had a total GRA which is nearly double that of the control plants. This is consistent with the findings of previous studies, where similar transformations have resulted in GRA levels 2 - 10 times higher than that of the GRA of control plants (Foyer *et al.*, 1991; Aono *et al.*, 1991; Creissen *et al.*, 1994; Foyer *et al.*, 1995). The plants carrying chloroplastic *E. coli* GR show a much higher GRA - up to 50 times higher than the control plant GRA. Similar transformations by other workers have resulted in a wide range of GRA in transformed plants. Aono *et al.* (1993) reported activities 3 times higher than control plants, while Foyer *et al.* (1995) obtained transgenic plants with a GRA 100 - 500 times higher than untransformed controls. Foyer *et al.* (1995) showed that chloroplastic transformants also had *E. coli* GR protein levels and *gor* mRNA levels that were higher than those of cytosolic transformants. All of the available evidence suggests that the bacterial GR is more stable in the chloroplast than in the cytoplasm.

Figs. 2.12A and 2.12B also show that the paraquat treatment resulted in a GRA increase in the transformed plants, and that this was greater than in the controls. The GRA in the cytoplasmic transformants tripled over 24 hours, while a 50% increase in GRA was observed in the chloroplastic transformants. This increase cannot be ascribed to an increase in the transcription rate of the *gor* gene, since the gene is under the control of the constitutive CaMV 35s promoter in both the chloroplastic and cytosolic transformants. Foyer *et al.* (1991) observed a similar increase in GRA in tobacco plants carrying cytoplasmic *E. coli* GR, and suggested that this is due to in increase in the translation rate of existing mRNA. This hypothesis is corroborated by the data of Pastori and Trippi (1992),

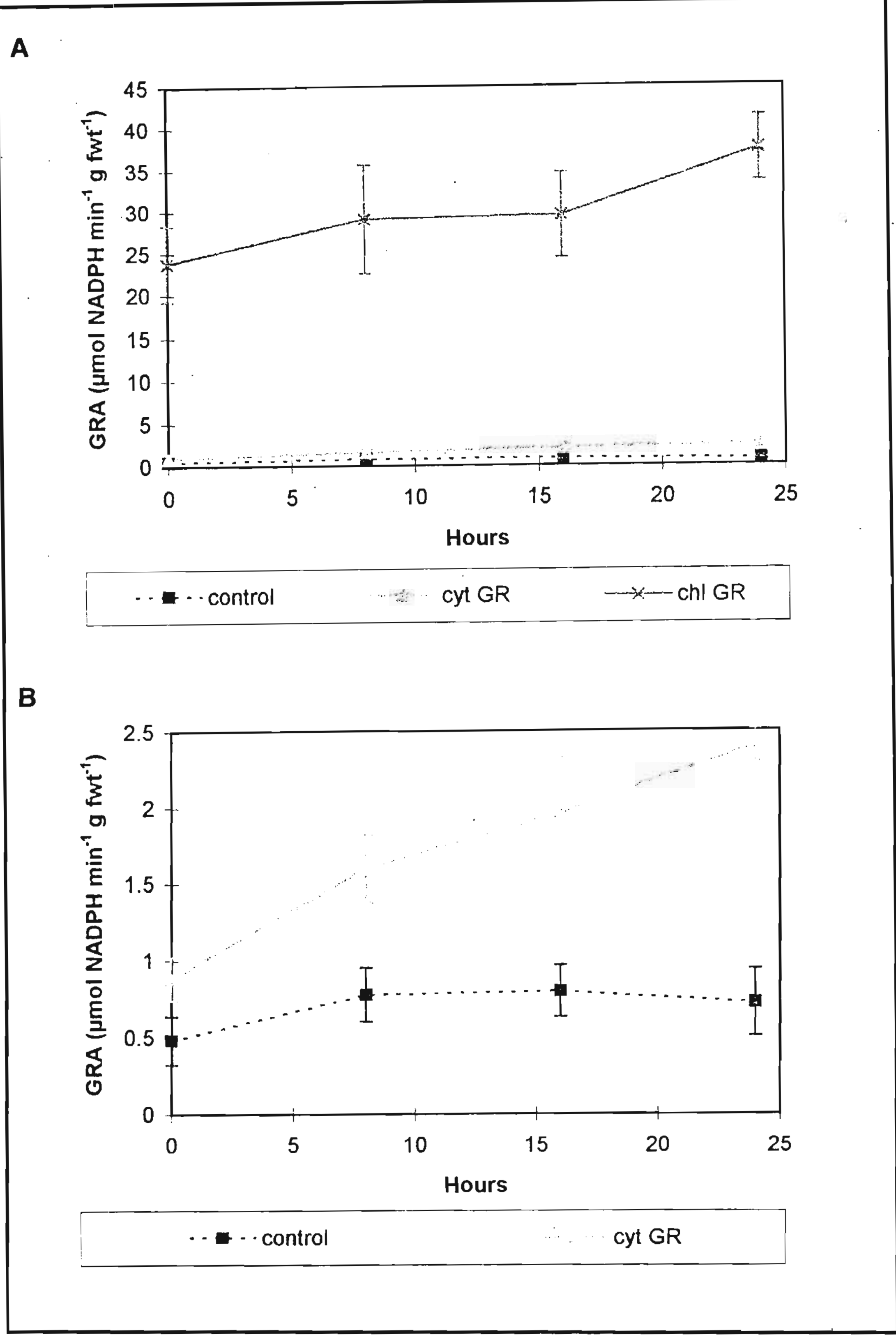


Figure 2.12: The response of GR to paraquat in cytoplasmic and chloroplastic *E. coli* GR transformants. A. The response of chloroplastic *E. coli* GR in relation to the control plants and cytoplasmic transformants. Each data point is the mean and standard deviation of nine readings.

B. This figure shows the control plants and cytoplasmic transformants on an expanded scale, for clarification of the response of this transformant. Abbreviations: cyt GR = cytoplasmic *E. coli* GR transformant, chl GR = chloroplastic *E. coli* GR transformant.

who used protein synthesis inhibitors to analyse similar increases in plant GRA under stress conditions.

The *E. coli* GR transformants were also analysed for SOD activity under stress conditions. The SOD activity in these plants showed an almost identical stress response to that observed in the stressed control plants (Fig. 2.13).

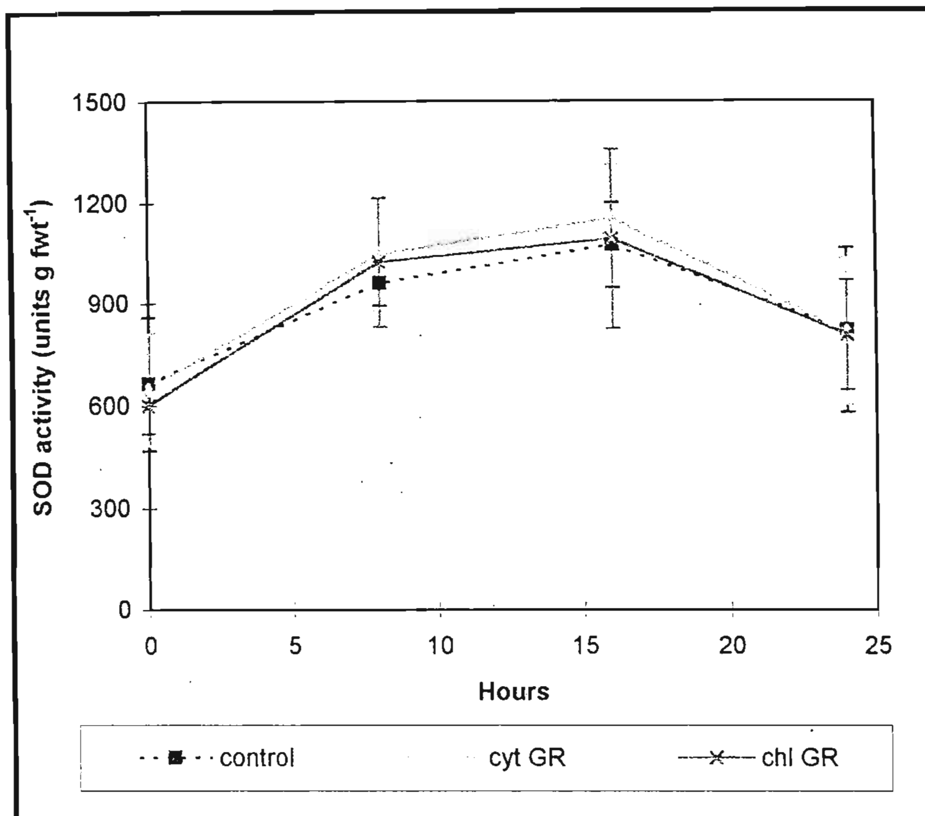


Figure 2.13: SOD activity in cytoplasmic and chloroplastic *E. coli* GR transformants. *E. coli* GR activity was determined at eight hour intervals, with each data point being the mean and standard deviation of nine readings. Abbreviations as for Fig. 2.12.

The transformants showed an initial increase in SOD activity, followed by an abrupt decline, indicating a possible inhibition of SOD due to peroxide accumulation. A second possibility is that the increase in GRA affected SOD activity. Wingsle and Karpinski (1996) suggest that high levels of reduced glutathione may affect the redox status of the cell and down-regulate SOD transcription.

Fig. 2.14 shows that the chlorophyll levels declined steadily in the *E. coli* GR transformants over the 24 period, and that the decline was more rapid than in the controls - further evidence of peroxide-mediated oxidative damage. Only one of the previous studies involving GR transformants evaluated chlorophyll levels in transformants treated with paraquat (Creissen *et al.*, 1994). These authors examined chlorophyll levels in

plants treated with a range of paraquat concentrations and showed that paraquat levels of 15 μM resulted in a chlorophyll decline similar to that seen in controls. It is possible that the paraquat concentration used in this study (75 μM) overwhelmed the antioxidant system, even with enhanced GR levels.

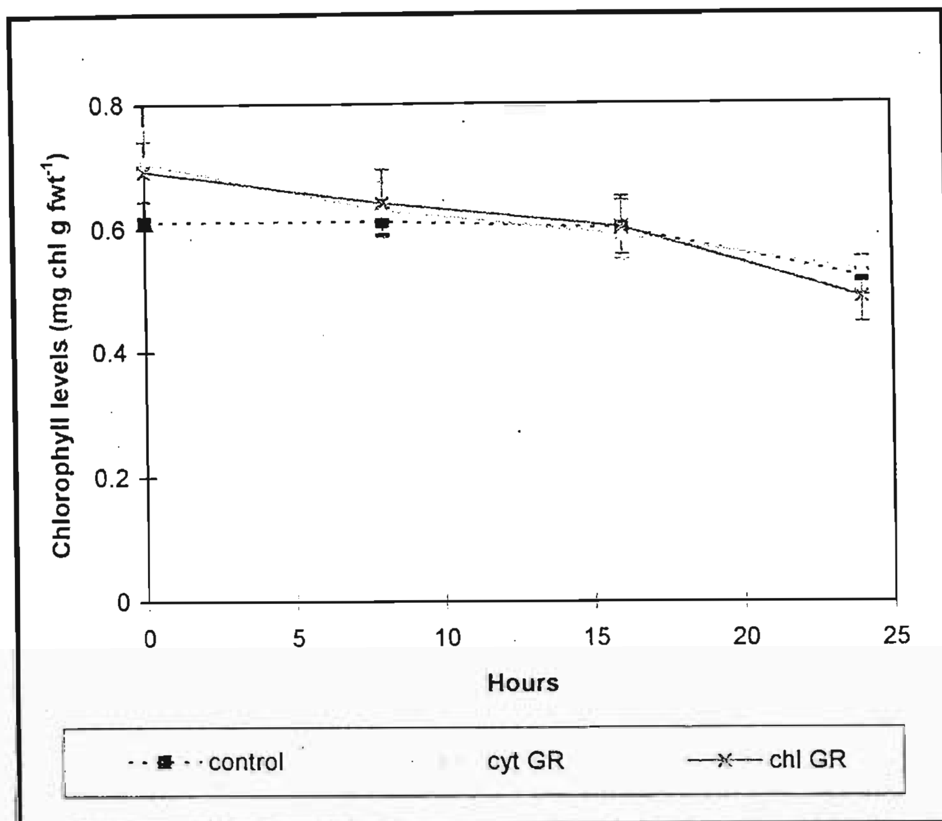


Figure 2.14: Chlorophyll levels in *E. coli* GR transformants following paraquat treatment. The chlorophyll levels for the *E. coli* GR transformants were determined at the indicated intervals, with the mean and standard deviation of nine readings recorded at each point. Abbreviations as for Fig. 2.12.

The leakage rate data is not entirely consistent with the observed loss of chlorophyll. Both the cytoplasmic and the chloroplastic transformants show significantly lower leakage rates after 8 hours than the control plants, indicating that the elevated GR levels may have afforded the transgenic plants some protection in the initial stages of the stress experiment (Fig. 2.15). These low initial leakage rates indicate that the elevated GR levels enhance the peroxide-scavenging capabilities enough to protect the cell membranes despite the destruction of chlorophyll in the thylakoids. A question which arises at this point is how cytoplasmic *E. coli* GR protects the plant cell against oxygen radicals which form in the chloroplast - these oxygen species are likely to react with membrane lipids rather than cross the chloroplast membranes. Aono *et al.* (1991) offer two explanations in this regard. The first is that paraquat may actually result in the production of some oxygen radicals within the cytoplasm, and the increased GRA assists with the scavenging of these

radicals. This is supported by observations that the enzymes of the ascorbate-glutathione cycle are present in the cytoplasm in sufficient quantities to allow the cycle to operate there (Smith *et al.*, 1989). The second possibility proposed by Aono *et al.* (1991) is that the GR in the cytoplasm "co-operates" with the GR in the chloroplasts, and that the small molecules in the antioxidant cycle are transported across the chloroplast envelope.

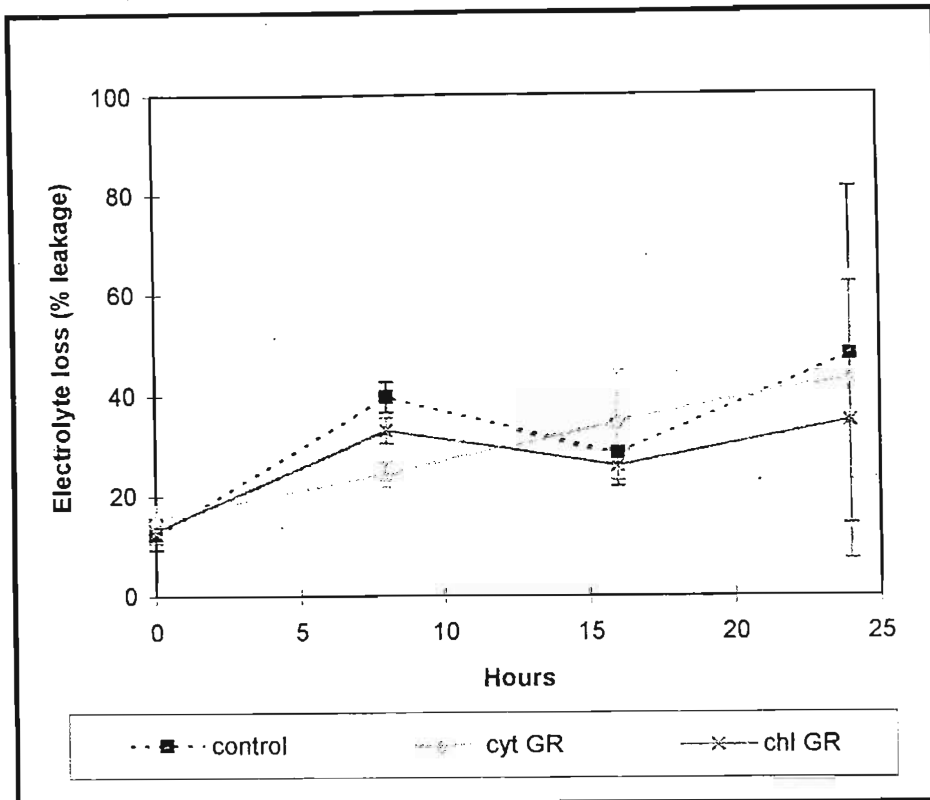


Figure 2.15: Leakage rates for *E. coli* GR transformants. Leakage rates are expressed as a percentage of the maximum possible leakage. For each data point, the mean and standard deviation of the readings from four leaf discs was recorded. Abbreviations as for Fig. 2.12.

As mentioned, the protection afforded by the higher GRA was only temporary - an unexpected result, considering suggestions that GR is the rate limiting step in the scavenging of peroxide (Jablonski and Anderson, 1981). Based on existing evidence, two explanations can be offered for the decline in protection. One is that GR is not the rate limiting step. As mentioned earlier, Baisak *et al.* (1994) have suggested that APx may play a role in limiting the effectiveness of the antioxidant cycle. An alternate suggestion, already discussed, is that the higher GRA results in a larger pool of reduced glutathione, and that this glutathione down-regulates SOD production - possibly by interfering with an oxidising signal which would normally promote the production of SOD and other antioxidant enzymes.

Chloroplastic tomato Cu,Zn SOD transformants

In the last seven years, a number of plant species have been transformed with the genes for Mn SOD and Cu,Zn SOD from bacteria as well as several other plant species (see Table 2.2). The current study utilised tobacco plants transformed with the gene for the chloroplastic isoform of Cu,Zn SOD from tomato. Although the SOD activity gels confirmed the presence of the enzyme in the transformants (Fig. 2.3A), the spectrophotometric assay for SOD did not show an appreciable increase in SOD activity relative to the controls (Fig 2.16).

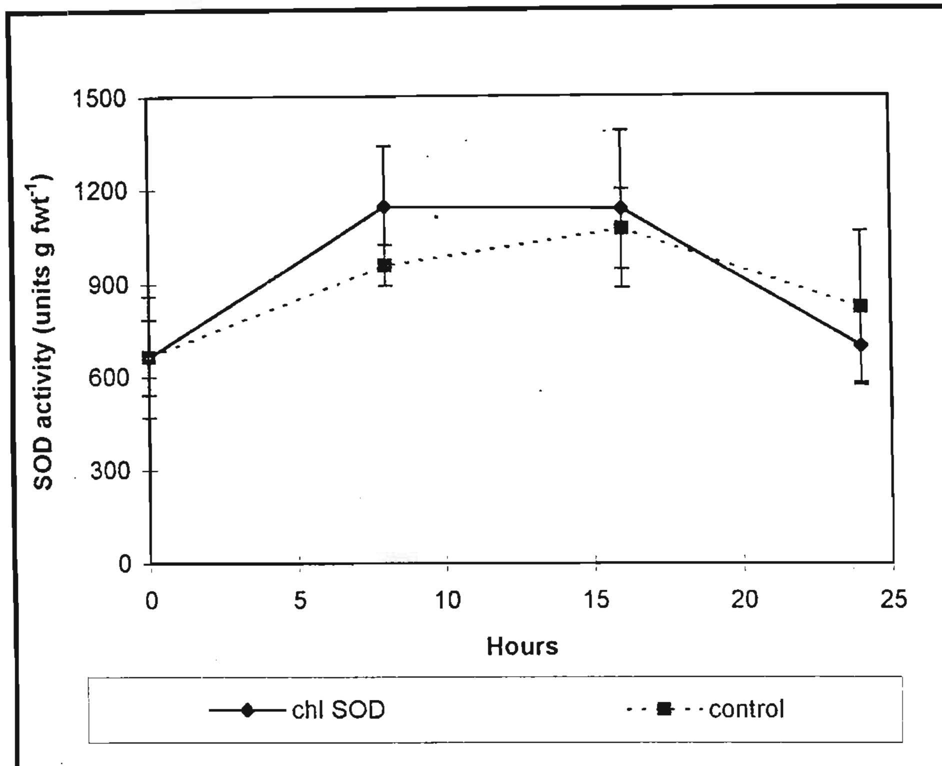


Figure 2.16: SOD activities in the tomato chloroplastic SOD transformants relative to control plants. The SOD activities were measured at eight hour intervals. Each data point is the mean and standard deviation of nine readings. Abbreviations: chl SOD = chloroplastic SOD transformant.

In response to stress, the enzyme in the transformed plants showed similar changes to that of the controls, with the exception of a slightly larger increase in activity at 8 hours. The enzyme activity of the transformed plants declined rapidly after 16 hours of paraquat treatment.

The response of GR in the SOD transformants was not significantly different to the response in the control plants either, showing an initial increase in activity followed by a gradual decline (Fig 2.17).

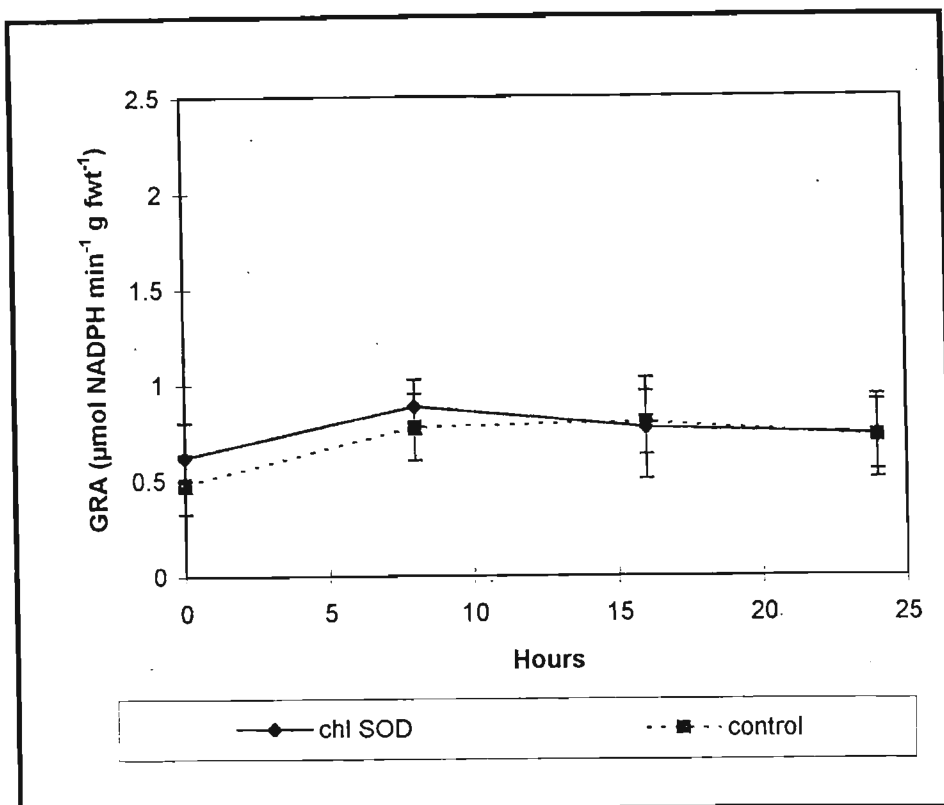


Figure 2.17: GR activities in chloroplastic SOD transformants. GR activity was determined at the intervals indicated. Each data point represents the mean and standard deviation of nine readings. Abbreviations as for Fig. 2.16.

Under stress conditions, these plants showed a decline in chlorophyll levels which was greater than the decline observed in the control plants (Fig 2.18). This may point to an inability of the antioxidant system to scavenge peroxide - a fact supported by the decline in the activity of the peroxide-sensitive SOD (Fig. 2.16).

The oxidative damage to the cells of the transformed plants is highlighted by the rate of electrolyte leakage under stress conditions (Fig 2.19). Of all the transformed plant types used in this study, these SOD transformants show the most rapid increase in electrolyte leakage. This can only be due to a rapid accumulation of reactive oxygen species, and indicates that the excess SOD in these plants affords no protection against the stress conditions used in this study. Tepperman and Dunsmuir (1990) performed a similar transformation using the chloroplastic petunia Cu,Zn SOD and also found that the extra SOD gave no additional protection against paraquat treatment. These authors suggest that the downstream enzymes in the antioxidant pathway (GR and APx) were not capable of scavenging the peroxide produced by the SOD. Similarly, Sen Gupta *et al.* (1993b) also transformed tobacco with a chloroplastic Cu,Zn SOD and found that it only afforded protection against oxidative stress at very low paraquat concentrations (up to 2.4 µM). These studies are further

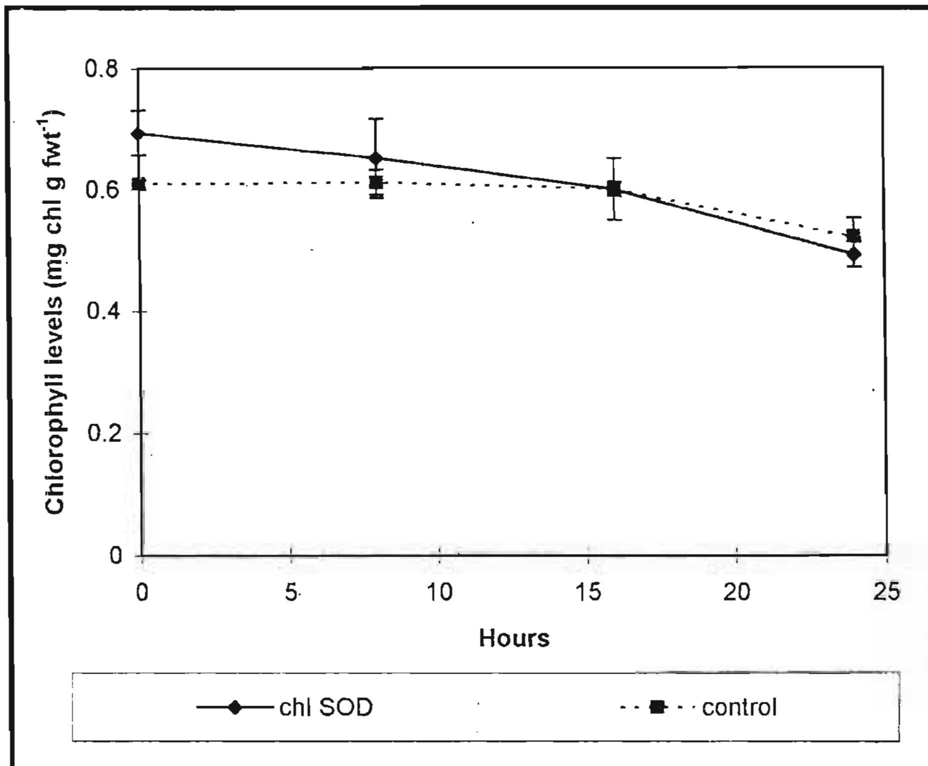


Figure 2.18: Chlorophyll decline in the chloroplastic SOD transformants treated with paraquat. The chlorophyll readings taken at the indicated times are the mean and standard deviation of nine determinations. Abbreviations as for Fig. 2.16.

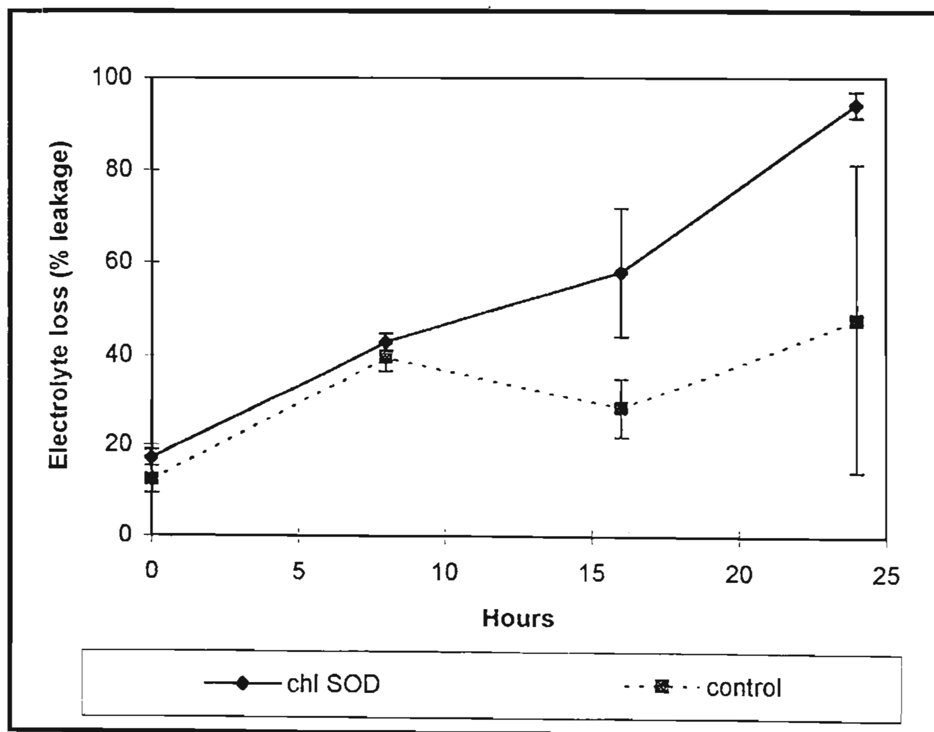


Figure 2.19: Leakage rates for chloroplastic SOD transformants relative to control plants. Leakage rates are expressed as a percentage of total leakage. Each data point represents the mean and standard deviation of readings from four leaf discs.

corroborated by that of Pitcher *et al.* (1991), who showed that tobacco plants carrying chloroplastic petunia Cu,Zn SOD are not tolerant to ozone induced oxidative stress. Perl *et al.* (1993) showed enhanced resistance to paraquat in potato plants carrying tomato Cu,Zn SOD. However, the paraquat concentration in this study was once again relatively low (10 μM). It thus seems that the Cu,Zn SODs are not capable of greatly enhancing the functions of the antioxidant system in plants exposed to paraquat. The most likely reason for the failure to enhance antioxidant capabilities is the irreversible activation of Cu,Zn SOD by its own reaction product, namely hydrogen peroxide. One exception, however, is a study performed by Sen Gupta *et al.* (1993a). These authors showed that transformed plants with elevated chloroplastic Cu,Zn SOD were afforded significant protection against chilling-induced oxidative stress.

In contrast to the above findings, plants transformed with the peroxide-insensitive Mn SOD have been found to be tolerant of paraquat (Bowler *et al.*, 1991), drought (McKersie *et al.*, 1996) and freezing (McKersie *et al.*, 1993). Slooten *et al.* (1995) also showed that Mn SOD transformants are resistant to paraquat, but concluded that the resistance was due, in part, to an observed increase in other components of the antioxidant system (Fe SOD, APx, DHA, MDHA, ascorbic acid and glutathione).

The above studies involving SOD transformants highlight two important features of the antioxidant system. Firstly, successful engineering of oxidative stress resistance may require an increase in more than one component of the antioxidant system. Secondly, where enzymes exist in more than one isoform the choice of isozymes for the transformation is vital. With the first point in mind, it was decided to examine oxidative stress resistance in tobacco plants carrying elevated levels of more than one antioxidant enzyme.

Chloroplastic tomato Cu,Zn SOD / *E. coli* GR hybrids

Only one study carried out to date has examined the effects of elevated SOD and GR in the same plant. Aono *et al.* (1995b) examined the effect of paraquat on tobacco plants with foreign SOD and GR in the cytoplasmic compartment. The plants produced for this study differed in that both of the transgene products were localised in the chloroplast.

Fig. 2.20 shows that the GRA in these plants was almost as high as that of the plants carrying only *E. coli* GR. As with the *E. coli* GR transformants, the GRA increased steadily over the stress period until it was 65% higher than the GRA prior to the oxidative stress.

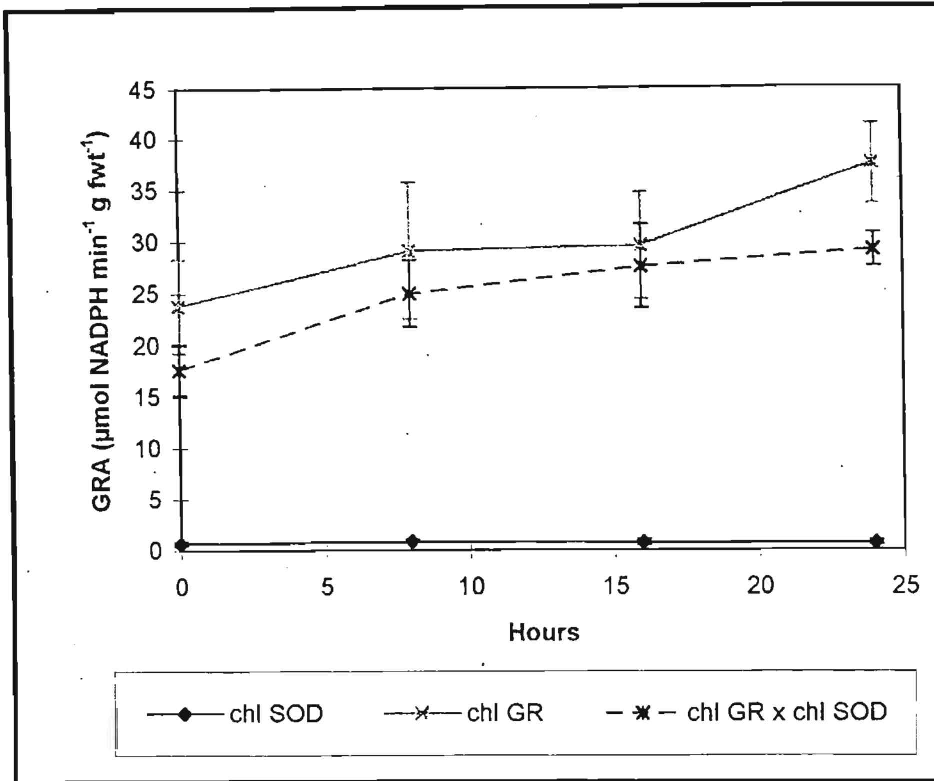


Figure 2.20: GR activity in the hybrid transformants. The GR activity in these plants was determined at eight hour intervals, with each data point representing the mean and standard deviation of nine readings. Abbreviations: chl GR = chloroplastic *E. coli* GR transformants, chl SOD = chloroplastic SOD transformant, chl GR x chl SOD = hybrid plants with chloroplastic GR and SOD.

For the first 16 hours of the experiment, the SOD activity also increased, with the average activity reaching a higher level than in any of the other plant types (Fig. 2.21). The high level of SOD activity attained after 16 hours indicates that these plants were protected against peroxide accumulation during this period. However, after 16 hours the SOD levels declined as for the other plant types. There are two possible reasons for this decline. One is that the increased GRA is insufficient to deal with the peroxide produced by the extra SOD. This is not likely, considering that the increase in GRA is much higher than the increase in SOD activity in these hybrids. The second possibility is that APx cannot deal with the increased flow through the cycle, since it has not been enhanced along with the GR and SOD.

The postulated protection during the first 16 hours is not supported by the chlorophyll levels, which declined as rapidly as the chlorophyll levels in the other transgenic plants (Fig 2.22).

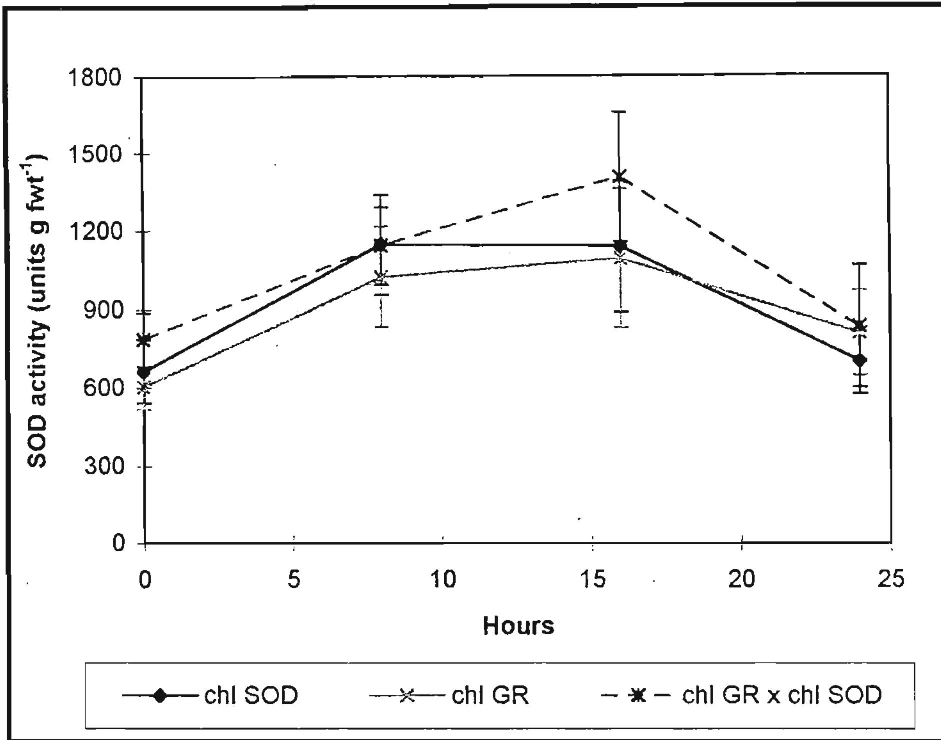


Figure 2.21: SOD activity in the transgenic hybrids carrying SOD and *E. coli* GR transgenes. The figure shows SOD activity relative to the GR and SOD transformants. Each point is the mean and standard deviation for nine readings. Abbreviations as for Fig. 2.20.

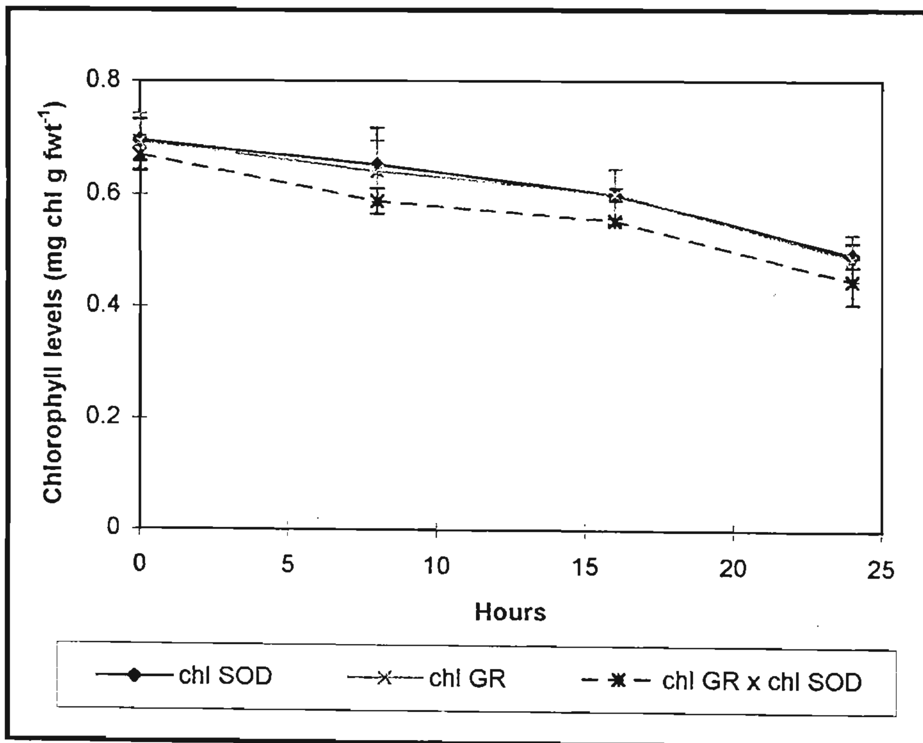


Figure 2.22: The decline of chlorophyll in the SOD / *E. coli* GR transformants. Each point on the graph is the mean and standard deviation of nine chlorophyll readings. Abbreviations as for Fig. 2.20.

The chlorophyll data is in stark contrast to the leakage data. These hybrid transformants show the lowest leakage rates over the first 16 hours - corresponding with the period where an enhanced SOD activity was maintained (Fig. 2.23).

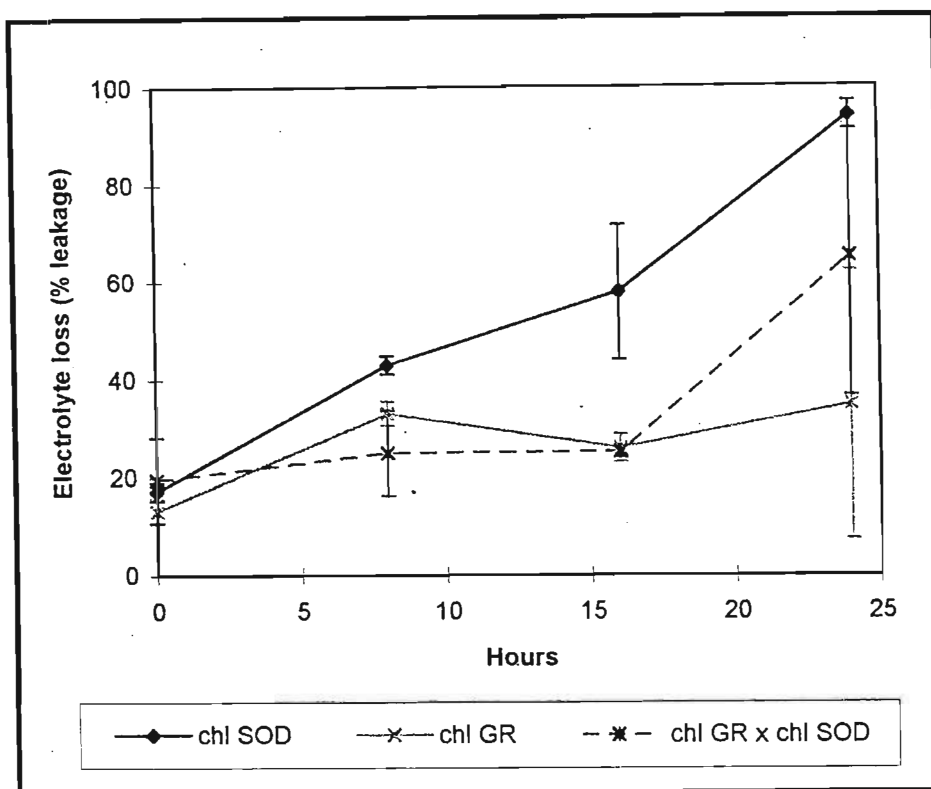


Figure 2.23: Electrolyte leakage in the hybrid transformants relative to the transformants carrying only one of the transgenes. The leakage at the indicated times are reported as a percentage of the total leakage for each leaf disc. Each data point represents the mean and standard deviation of readings from four leaf discs. Abbreviations as for Fig. 2.20.

The low initial levels of leakage are consistent with the results of Aono *et al.* (1995b), who showed a significant reduction in leakage for plants transformed with both GR and SOD. The combined chlorophyll and leakage data suggests that the elevated enzyme levels were unable to protect the photosynthetic apparatus, but were able to prevent membrane damage by efficiently scavenging reactive oxygen species leaving the site of photoreduction in the first 16 hours. However, this protection was rapidly lost following the inhibition of SOD, showing once again that APx may have been overwhelmed by the increase in the turnover of the antioxidant cycle. This shows that although increases in the activity of more than one enzyme may afford some protection against oxidative stress, it is vital to attain a balance in the levels of the various enzymes in the antioxidant cycle. In order to raise the stress tolerance of plants, it will be necessary to increase the levels of enzymes other than just GR and SOD.

In summary, this stress experiment has shown that it is possible to enhance oxidative stress tolerance by increasing the activity of certain enzymes catalysing reactions in the plant antioxidant pathway. However, this depends to a large extent upon which enzymes are manipulated. In the case of the plants carrying tomato SOD, leakage rates indicated greater membrane damage in transformants than in control plants. In contrast, the plants transformed with both cytoplasmic and chloroplastic forms of GR showed a slight increase in stress tolerance. This contrast may well relate to the hypothesis that GR is the rate limiting step in the antioxidant pathway. As such, increases in GR activity would allow the scavenging of the excess activated oxygen which would normally accumulate under stress conditions. An increase in SOD activity without a corresponding increase in GRA would simply enhance the production of activated oxygen under stress conditions - as shown by the rapid increase in membrane leakage experienced by the plants transformed with SOD. The use of the hybrid plants confirms this observation - the plants having increased activities of both GR and SOD showed greater stress tolerance than the plants transformed with the gene for just one of these enzymes. This indicates that transformations with several genes may allow the successful engineering of stress tolerance in plants.

Despite the apparent benefits of transforming the plants with more than one gene, even the hybrid plants eventually showed signs of cellular damage. This gives rise to an important point worth considering when attempting to alter the functioning of a biochemical pathway. All pathways within an organism have evolved over long periods and there is a definite balance between the functioning of the various enzymes within the pathway. Thus some form control is required over the expression of the foreign genes, in order to maintain this balance in the activities of the enzymes. Furthermore, one needs an understanding of the pathway if one is to determine which enzymes should be manipulated in order to derive the optimum benefits from the transformation. In this study, the increase in GR activity seems to have overcome the problems arising from the fact that GR is the rate limiting step in the antioxidant cycle. By increasing SOD activity, more peroxide was produced than could be scavenged by APx, effectively making APx the new rate limiting step in the pathway.

Although the hybrid transformants did not exhibit the hoped for resistance to oxidative stress, they did give an insight into the functioning of the antioxidant pathway. It is hoped that the information derived from this study will contribute to an understanding of oxidative stress tolerance in plants, and perhaps assist in the eventual production of stress-tolerant plants.

CONCLUDING REMARKS

The aim of the current study was to produce transgenic tobacco plants having high levels of chloroplastic GR and SOD activity, with the ultimate goal being to study the response of these plants to oxidative stress. To accomplish this, tobacco leaf discs were transformed with the *E. coli gor* gene using an *Agrobacterium*-mediated transformation system. The resulting transgenic plants were characterised in terms of their GR activity and the subcellular location of the transgene product, and were shown to express extremely high levels of GR activity in the chloroplast. These plants were then cross-pollinated with tobacco plants carrying chloroplastic tomato SOD. The hybrid plants, carrying both GR and SOD, were then subjected to paraquat-induced oxidative stress.

The stress experiment allowed a number of conclusions to be drawn regarding the plant antioxidant system. Firstly, it indicated that GR is the rate limiting step in the antioxidant pathway, since plants having elevated levels of GR were protected against oxidative damage in the first 4 - 8 hours following oxidative stress. The experiment also showed that high levels of both SOD and GR are capable of protecting plants against oxygen radicals under stress conditions. This is exemplified by the low leakage rates in the hybrids over the first 16 hours of the stress experiment. However, it would seem that a large increase in both these enzymes brings about a situation where other antioxidant enzymes are not capable of dealing with the increased flow of metabolites through the cycle. It would seem that APx in particular is detrimentally affected by the imbalances resulting from these transformations.

Another point raised by this study confirms what has already been said by Foyer *et al.* (1994a), namely that increasing just one enzyme is not always sufficient to confer considerable stress resistance. In fact, increasing the activity of just one enzyme in the cycle may actually be detrimental to the plant, causing more oxidative damage than in the controls. This point is highlighted by the rapid increase in cell leakage in plants carrying elevated levels of only SOD. This indicates that it is not sufficient to just increase the levels of the enzymes. It seems that the activities of the individual enzymes must be balanced, such that the redox status of the metabolite pools is maintained. In this regard, it seems that changes in the redox status interferes detrimentally with the endogenous signals responsible for both DNA transcription and mRNA translation.

From the results of this study, it seems that it is possible to enhance stress tolerance by the genetic engineering of plants. However, it will be necessary to include promoters capable of tightly controlling the expression of the relevant transgenes. The control of antioxidant gene expression is an area of active research, but a lot more work in this field is required before the required control of gene expression is obtained. A more practical approach to the enhancement of stress tolerance would be the production of transgenic plants carrying genes for enzymes other than GR and SOD. Previous studies have focused on GR and SOD since they catalyse the first and the last

reactions in the antioxidant pathway. It may be of benefit to examine some of the other enzymes in the pathway, with APx being a good candidate for manipulation. In this regard, Pitcher et al. (1994) have transformed tobacco with the gene encoding APx and have shown increased stress tolerance in these plants. Transgenic plants having elevated GR, SOD and APx activities may well exhibit more stress tolerance than the plants produced in this study. This is not to say that the plants produced here are of no benefit. The oxidative stress induced in this experiment was particularly severe, with the paraquat concentrations applied being greater than those used in similar studies. Further experiments on the GR/SOD hybrids using lower paraquat concentrations or alternative stress inducers (drought and chilling being two good examples) may provide more information about the functioning of the antioxidant system under stress conditions. Such experiments may also reveal a practical use for such transformants should they show greater stress tolerance under such conditions.

There is thus still a lot to be accomplished in this field. However, with advances in plant transformation techniques and a better understanding of the biochemical pathways being manipulated, rapid progress in the development of stress tolerant plants should be possible. In this regard, it is hoped that the current study will be of some benefit.

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