A MOLECULAR STUDY OF γ-AMINOBUTYRIC ACID SYNTHESIS IN *ARABIDOPSIS THALIANA* UNDER ABIOTIC STRESS

MOHOHLO W. MOLATUDI

Submitted in fulfilment of the requirements

for the Master of Science degree

in the Department of Botany,

University of Natal, Pietermaritzburg

PREFACE

The experimental work done for this project was carried out in the Botany Department of Botany, University of Natal, Pietermaritzburg.

This thesis represents work done by the author and has not been submitted to another University for a degree or diploma. Where the work of other authors have been used this has been duly acknowledged.

_____

November 1997

ACKNOWLEDGEMENTS

I would like to thank Prof. J. van Staden my supervisor for his advice and support at all stages of this study.

I am grateful to Dr W.A. Cress my co-supervisor for sharing ideas that helped to shape this study.

I thank my family particularly my mother Moelo Molatudi for her unwavering support over all these years.

I am grateful to Karen van Rooyen for her encouragement and for putting up with a lot of frustration during the course of the study.

I appreciate the help given by all my laboratory colleagues.

A MOLECULAR STUDY OF γ-AMINOBUTYRIC ACID SYNTHESIS IN *ARABIDOPSIS THALIANA* UNDER ABIOTIC STRESS

M.W. Molatudi

ABSTRACT

γ-Aminobutyric acid (GABA) is a ubiquitous non-protein amino acid found in many plants and organisms. GABA accumulation in plants has previously been reported as result of environmental stresses such as water deprivation, high salinity and temperature extremes. It is thought that GABA accumulates as a compatible solute in the cytoplasm where it becomes a major constituent of the free amino acid pool. GABA is synthesised from the decarboxylation of glutamate by glutamate decarboxylase (GDC). In some plants, GDC is activated by the lowering of the cytoplasmic pH and the presence of calmodulin and Ca^{2+}. A calmodulin-induced activation of GDC may be due to these physiological factors and environmental stimuli acting in concert leading to the synthesis and accumulation of GABA.

The GABA content of *Arabidopsis thaliana* var. Columbia (L.) Heynh leaves was found to increase by over 130% due to water deprivation. NaCl concentrations of up to 100 mM seemed to cause GABA accumulation due to a decrease in osmotic potential. Concentrations of NaCl above 100 mM probably caused

GABA accumulation due to combined hyperosmosis and salt toxicity effects. The high levels of GABA in the leaves were maintained throughout a 24 h stress-application period, consistent with its role as compatible solute.

The accumulation of GABA followed by its decline in the dark could be attributed to its rapid metabolism because of an active GABA shunt. This is in contrast to the absence of major variations in the amount of GABA in the light confirming its decreased role as a channel for the glutamate carbon and nitrogen under such conditions. A substantial increase in the GABA content was followed by a dramatic decrease in the last 12 h of incubation. This profile of GABA could support its proposed role as a temporary sink for nitrogen and carbon from glutamate during environmental stress.

Glutamate decarboxylase appears to be encoded by a single gene in the genome of *Arabidopsis*. Sequence analysis reveals that the protein possesses what could be a carboxy-terminal, calmodulin- binding domain, which is consistent with other glutamate decarboxylases. The 30-amino acid peptide contains a TrpLysLys motif found in some calmodulin targets. The secondary structure predictions of this peptide suggest a potential to form an α- helix which is also consistent with proteins known calmodulin- binding domains.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABA:	Abscisic acid
amp^r:	Ampicillin
ATP:	Adenosine triphosphate
BSA:	Bovine serum albumin
CaM:	Calmodulin
cDNA:	copy DNA
CO_2	Carbon dioxide
CTAB:	Cetyltrimethylammonium bromide
dbEST:	Expressed sequence tags database
2,4-D:	Dichlorophenoxyacetic acid
ddNTP:	Dideoxyguanylate triphosphate
dGTP:	Deoxyguanylate triphosphate
dH_2O:	Distilled water
DNA:	Deoxyribonucleic acid
dNTP:	Deoxynucleotide triphosphate
DTT:	Dithiothreotol
EDTA:	Ethylenediamine tetraacetate
EST:	Expressed sequence tag
GABA:	γ-Aminobutyric acid/4-Aminobutyric acid
GABA-T:	GABA:Transaminase
GAD:	Glutamate decarboxylase
GDC:	Glutamate decarboxylase
GDH:	Glutamate dehydrogenase
GOR:	Garnier-Osguthorpe-Robson

kb: Kilobase

IAA: Indoleacetic acid

kDa: Kilodalton

K_m: Michaelis constant

LB: Lauria broth

Mb: Megabase

mRNA: Messenger RNA

NAA: Naphthalene acetic acid

NADH: Nicotinamide adenine dinucleotide

NaOH: Sodium hydroxide

OGDH: 2-Oxoglutarate: glutamate dehydrogenase

OH^-: Hydroxyl ions

PEG: Polyethyl glycol

RNA: Ribonucleic acid

rin: Ripening inhibitor

rss: Reduced salt sensitivity

SDS: Sodium dodecyl sulphate

SSA: Succinic semialdehyde

SSADH: Succinic semialdehyde dehydrogenase

STE: Sodium-Tris-EDTA solution

TAE: Tris-Acetate-EDTA

TBE: Tris-Borate-EDTA

TCA: Tricarboxylic acid

TE: Tris-EDTA

UV: Ultraviolet

Vmax: Maximal velocity

CHAPTER 1

INTRODUCTION

1.1 Distribution and Occurrence of γ-Aminobutyric Acid (GABA)

γ-Aminobutyric acid (GABA) is a ubiquitous, non-protein amino acid and a major constituent of the free amino acid pool in many plants. Such an occurrence is unlike that of other non-protein amino acids which act mainly as intermediates for the biosynthesis of various amino acids (LARSEN, 1980). It was for the first time conclusively shown to occur in potato tubers by STEWARD, THOMPSON AND DENT (1949) using paper chromatography.

GABA is widely distributed, and is found in virtually every plant species. This is unusual because non-protein amino acids are usually restricted to a few species (LEVITT, 1980). It has been detected in various plant organs including tubers (STEWARD, THOMPSON AND DENT, 1949), legume nodules (FRENEY AND GIBSON, 1975) and leaves (MIZUSAKI, NOGUCHI AND TAMAKI, 1964). LÄHDESMÄKI (1968) found GABA in both young and old leaves of *Datura suaveolens*, *Medicago sativa* and *Salvinia natans*. Results obtained by CHEN, BAUM AND FROMM (1994) indicated that GABA is present in flowers, leaves, stems, roots and seeds. It has also been reported in cultured rice (KOIWAI AND NOGUCHI, 1972) and tobacco cells (KOIWAI AND NOGUCHI, 1972).

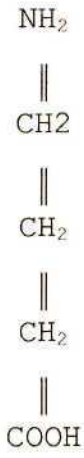

Fig. 1: The chemical structure of GABA with a molecular weight of 103.1 g/mol (BENDER, 1975).

Besides occurring as a free amino acid (Fig. 1) bound forms of GABA have also been reported. LARHER, GOAS, LE RUDULIER, GERARD AND HAMELIN (1983) reported six peaks from the crude extract of *Medicago sativa* which, following hydrolysis, caused an increase of the GABA peak. *N*-Caffeoyl-4-amino-*n*-butyric acid was identified by Gas Chromatography and Mass Spectroscopy as a conjugated form of GABA from extracts of cultured tobacco cells grown on putrescine as a nitrogen source (BALINT, COOPER, STAEBELL AND FILNER, 1987).

Apart from plants, GABA is found in substantial amounts in mammalian brain tissue where it is thought to act as an inhibitory neurotransmitter in the central nervous system (MARAS, SWEENEY, BARRA, BOSSA AND JOHN, 1992). In the vertebrate brain its wide distribution is unparalleled by any known inhibitory neurotransmitter (ERLANDER AND TOBIN, 1991).

Inhibitors that block the synthesis of GABA in experimental animals have induced epileptic seizures (YOUNGS AND TUNNICLIFF, 1991). Its presence has been shown in bacteria while LANE AND STILLER (1970) employed paper chromatography to show its existence in the green alga, *Chlorella pyrenoidosa*.

1.2 Stress and the Free Amino Acid Pool

The accumulation of amino acids seems to be a primary effect of altered protein metabolism (REGGIANI, CANTÚ AND BRAMBILLA, 1988). A lower demand for amino acids during stress due to a net decline in protein synthesis has been suggested as the cause for the accumulation of amino acids during stress. The accumulated amino acids contribute to the maintenance of the osmotic potential and compensate for the depleted hexose sugars degraded during the fermentative process (NICOLETTA, BERTANI AND REGGIANI, 1995). THOMPSON, STEWART AND MORRIS (1966) showed that an increase in the free amino acid pool due to wilting was accompanied by a decrease in sugar and protein content.

Quantitative chromatography has been employed to monitor the mobilization of storage materials in germinating seeds as a contribution to understanding plant growth and development. Changes associated with the soluble nitrogen fraction have

proved to be significant indicators of physiological changes involved in growth. Mature leaves of long day plants showed an abundance of glutamine and asparagine while short-day plants had comparatively more aspartic and glutamic acids (STEWARD AND POLLARD, 1957). Similar results have also been obtained in later studies (MIZUSAKI, NOGUCHI AND TAMAKI, 1964; RHODES, HANDA AND BRESSAN, 1986; CHIANG AND DANDEKAR, 1995). The changes in amino acid composition contained in these reports, could be explained in terms of complex stress effects related to such experimentation and not solely as a result of the photoperiod.

1.3 Environmental Stress and its Impact on Plant Growth

Plants depend on the availability of resources (such as water, energy and minerals) which they obtain from their environment for growth. A limitation of these resources can pose restrictions on plant growth and development. Physical and chemical factors in the environment may also affect plant growth directly. Plants respond to these environmental constraints by altering their rate and patterns of growth by altering affected metabolic processes (FITTER AND KAY, 1987). In this study, physical environmental factors will be considered.

The information, which directs this development, resides in DNA and is transcribed by mRNA and then carried from the nucleus to the cytoplasm where synthesis of the required proteins takes place. Therefore, considerable research has gone into understanding the changes associated with environmental stress and importantly, ways in which plants circumvent stress effects. This study examines the link between GABA and stress in *Arabidopsis thaliana* and how such changes are effected at a molecular level. THOMPSON, STEWARD AND MORRIS (1966) were the first to report the association between environmental stress and the GABA content of tomato leaves. Various studies reviewed by SATYANARAYAN AND NAIR (1990) have since confirmed the observed increase of GABA due to conditions such as water stress, anaerobiosis, temperature extremes and light.

Therefore, as with any stress response, GABA synthesis must be related to the general plant metabolism. Although the study will focus on the effect of abiotic stresses on GABA, the overall impact on plant metabolism is complex. The impact includes changes in the composition of the total amino acid pool as well as protein synthesis. Most stresses also trigger a signal transduction pathway which involves changes in the cellular calcium levels.

In this study the physiological importance of GABA in plant growth and its metabolism from glutamate will be reviewed. The significance and role of GABA in nitrogen metabolism will also be discussed. The study will also consider the presence of GABA in *Arabidopsis* and how its content is determined by environmental stress conditions. The relative amounts of GABA under water stress; temperature extremes; salinity; and different photo periods will be investigated. The genes involved and their possible roles will be investigated by correlating their structure, function and regulation. Gene sequencing and genomic copy number determinations could reveal something of their function. Comparisons of homology of the gene responsible for GABA production with similar genes could give some indications about the regulation of GABA production.

CHAPTER 2

METABOLISM OF γ-AMINOBUTYRIC ACID

2.1 Biosynthesis of γ-Aminobutyric Acid

Decarboxylation of the alpha carbon of glutamate by glutamate decarboxylase (GDC)(EC 4.1.1.15) is the major pathway of GABA synthesis. Another pathway of GABA formation has been reported for extracts of rye, barley and tomato. This involves oxidative decarboxylation of ornithine; firstly to 4-aminobutanamide and then to GABA (SATYANARAYAN AND NAIR, 1990). Studies using labelled arginine showed that ornithine can be converted to GABA (MAZAELIS, 1980) through the intermediates γ-guanidobutyric acid in spruce buds, and ornithine in chestnut fruit. GABA can also be formed from putrescine by bacteria of the genus *Pseudomonas* (SATYANARAYAN AND NAIR, 1990). Spermidine is also broken down to GABA via the intermediate 4-acetamidobutyrate in fungi (BAGNI AND PISTOCCHI, 1992).

The above-mentioned pathways have been studied using radioactive tracer experiments. No enzymes have been isolated. Hence, the relative significance of these pathways has not yet been established. Since arginine, putrescine and ornithine are

derived from glutamate in higher plants it could be argued that glutamate is the only source of GABA.

When STEWARD, THOMPSON AND DENT (1949) found GABA in potato tubers they also detected some glutamate decarboxylase activity, although it was low. Their work cast some doubt on the possibility that GABA was a product of glutamate decarboxylase activity. LÄHDESMÄKI (1968) reported that GABA increased with age in *Salvania natans*, *Medicago sativa* and *Datura suaveolens*. In addition this author also showed a correlation between GABA accumulation and increased enzyme activity. The failure by previous workers to detect the decarboxylase activity could have been due to employment of insensitive methods.

Radio tracer experiments using labelled glutamate and GABA helped shed some light on the metabolism of GABA through decarboxylation of glutamate in plants. Using uniformly labelled L- [^{14}C]glutamate, fed to excised leaves, STREETER AND THOMPSON (1972(a)) showed that glutamate decarboxylation is the major route of GABA formation. Their findings were corroborated by SATYANARAYAN AND NAIR (1986) who found that the label from L-[U- ^{14}C]glutamate was predominantly incorporated into GABA. It was only after 96 h that the label was detected in glutamine, aspartate, asparagine and succinate.

In addition to substrate labelling experiments, crude extracts of glutamate decarboxylase have been prepared (STREETER AND THOMPSON, 1972(a&b); WALLACE, SECOR AND SCHRADER, 1984). These studies have shown a parallel increase in GDC activity with GABA accumulation. This work supported earlier claims by DIXON AND FOWDEN (1961) of a correlation between GABA synthesis and accumulation, and GDC activity. Recent work on purified GDC from various sources has corroborated these findings (SATYANARAYAN AND NAIR, 1985; MATSUMOTO, YAMAURA AND FANATSU, 1996).

STREETER AND THOMPSON (1972(b)) investigated the possibility of GABA production from the amination of succinic semialdehyde (a product of GABA transamination). Less than 10% of GABA was produced in this manner. Therefore, the authors confirmed that glutamate decarboxylation is the main source of GABA production.

In intact pea cotyledons, labelled carbon from glutamate was incorporated mainly into homoserine and glutamine while about 1% was incorporated into GABA (MELCHER, 1986). Such results seem to support the evidence that GABA is not a major component of the free amino acid pool in peas as is found in other species. However, in sliced pea cotyledons radioactivity was predominantly in GABA, almost to the exclusion of other amino acids.

2.2 GABA Metabolism

2.2.1 The γ-Aminobutyric Acid Shunt

Following its formation from glutamate by glutamate decarboxylase, GABA is transaminated to succinyl semialdehyde (SSA). DIXON AND FOWDEN (1961) showed that the initial conversion of labelled GABA fed to the mitochondrial fraction of peanut cotyledons involved a transamination reaction. Extracts of mature radish leaves showed the presence of GABA:transaminase (EC 2.6.19), an enzyme responsible for the transamination reaction. Succinyl semialdehyde, however, could not be extracted in plant cells probably due to its rapid oxidation to succinate (STREETER AND THOMPSON, 1972(b); SATYANARAYAN AND NAIR, 1986).

The oxidation to succinate is catalysed by succinic semialdehyde dehydrogenase (EC 1.2.1.16). STREETER AND THOMPSON (1972(b)) were able to show its presence in radish cotyledon extracts by measuring the formation of NADH in the presence of artificially made succinic semialdehyde. The GABA shunt refers to the pathway of glutamate decarboxylation to GABA, its subsequent transamination to SSA and lastly, oxidation of SSA to succinate which then enters the tricarboxylic acid (TCA) cycle. This route offers alternate entry of glutamate into the TCA cycle as succinate while it

bypasses the glutamate dehydrogenase (GDH) and the 2-oxoglutarate:glutamate dehydrogenase (OGDH) reactions (Fig. 2) (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995).

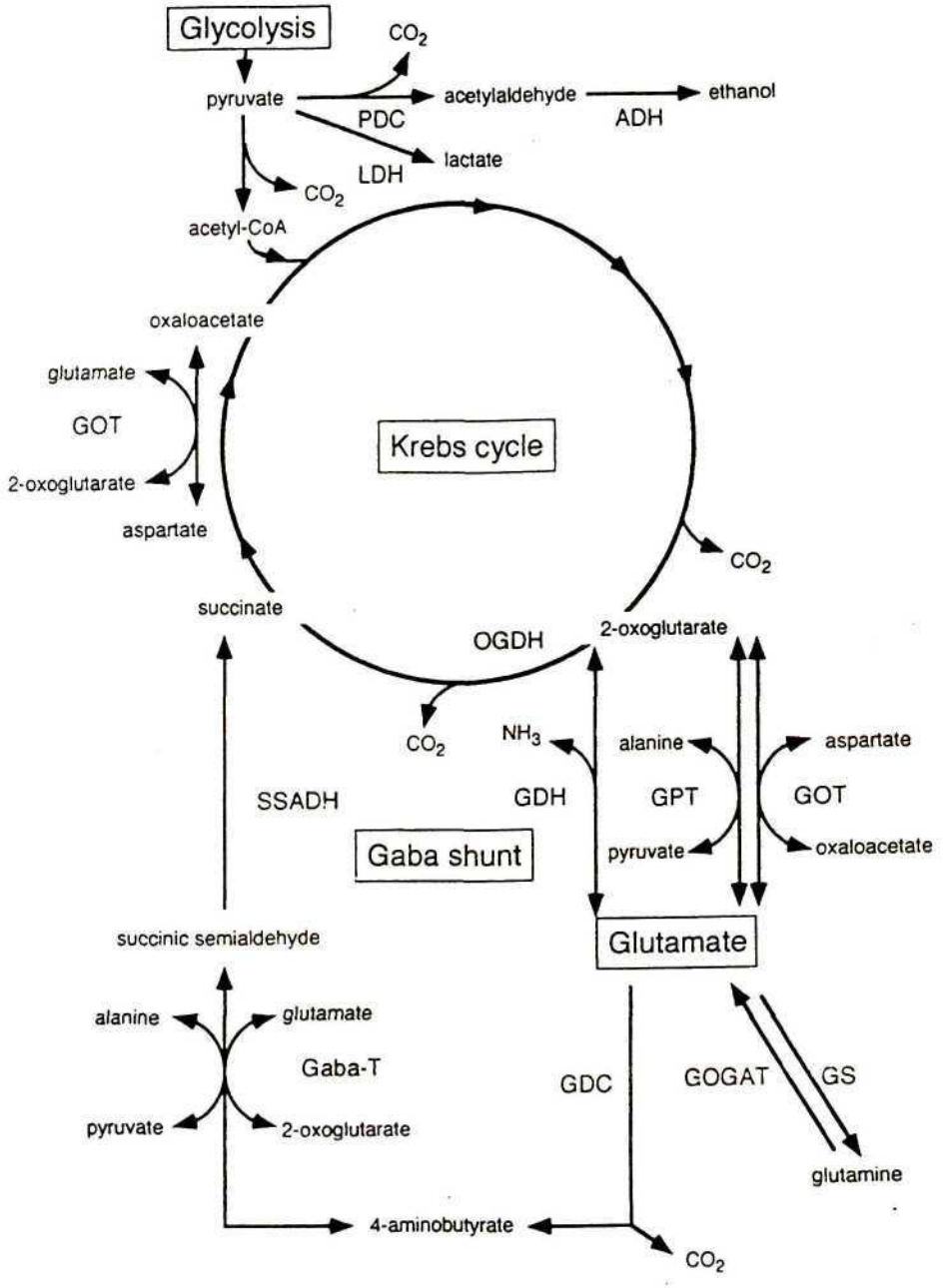

Fig. 2: Diagram of the metabolic pathways associated with the GABA shunt (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995).

The existence of the GABA shunt was demonstrated for the first time in the leaf discs of peas and peanuts using radio tracer experiments (DIXON AND FOWDEN, 1961). These same authors found

that the label from [U-^{14}C]GABA was incorporated into the TCA cycle intermediates and related amino acids (mainly asparagine and glutamine). SATYANARAYAN AND NAIR (1986) recovered 85-90% of radioactivity from respiratory carbon dioxide and the ethanolic extract of potato when [U-^{14}C]GABA was used. VANDEWALLE AND OLSSON (1983) separately fed L-[U-^{14}C]-glutamate and L-[1- ^{14}C]glutamate to germinating *Sinapis alba* seeds, and then subtracted the amount of labelled carbon dioxide ($^{14}CO_2$) evolved due to the latter from $^{14}CO_2$ due to the former. Therefore, a comparison could be made between the degradation of uniformly labelled GABA and that of glutamate via unlabelled GABA or 2- oxoglutarate. Therefore, these authors were able to obtain some evidence for the presence of the shunt. SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL (1995) have convincingly shown the presence and cellular location of the enzymes comprising the shunt in *Glycine max* L. Merr seeds.

2.3 Enzymology of the γ-Aminobutyric Acid Shunt

2.3.1 Glutamate decarboxylase

Crude and purified extracts of GDC reveal that it is strictly compartmentalized in the cytoplasm (DIXON AND FOWDEN, 1961; SATYANARAYAN AND NAIR, 1986; BREITKREUZ AND SHELP, 1995). It has a narrow but acidic pH range of 5.7 to 6.2 (STREETER AND THOMPSON, 1972(b)). SNEDDEN, CHUNG, PAULS AND BOWN (1992) reported optimal enzyme activity at pH 6.0. They also found that activity was inhibited by reagents which reacted with sulfhydryl groups. Using spectral absorption, MATSUMOTO, YAMAURA AND FANATSU (1996) showed that squash GDC had optimal activity at pH 5.8 and was dependent on pyridoxal phosphate. INATOMI AND SLAUGHTER (1975) used extracts of barley embryos to also show that GDC is dependent on pyridoxal-5'-phosphate as a cofactor.

Both mammalian and bacterial glutamate decarboxylases are also pyridoxal phosphate-dependent. These findings prompted suggestions of common ancestral origins of the respective GDC genes, in particular, the common three dimensional core structure of the enzyme (MARAS, SWEENEY, BARRA, BOSSA AND JOHN, 1992). Mammalian and bacterial glutamate decarboxylases also share acidic pH optima although the latter is more acidic.

A glutamate decarboxylase from petunia with a 32-amino acid carboxy end, calmodulin-binding domain was mapped by binding truncated petunia GDC to a recombinant petunia ^{35}S-calmodulin as well as to a biotinylated bovine calmodulin (BAUM, CHEN, ARAZI, TAKATSUJI AND FROMM, 1993). The calmodulin-binding domains were identified by polyclonal antibodies which were raised against recombinant calmodulins which detected similar bands from plant extracts on a nitrocellulose membrane. The truncated calmodulin- binding region led to loss of calmodulin-binding capacity. A 26- amino acid synthetic peptide, corresponding to part of the calmodulin-binding domain of petunia GDC formed a stable complex with calmodulin on a 1:1 stoichiometry in the presence of Ca^{2+} (ARAZI, BAUM, SNEDDEN, SHELP AND FROMM, 1995).

Earlier work on petunia GDC revealed that a cDNA encoding a 58 kDa homodimer was expressed in all petunia organs (CHEN, BAUM AND FROMM, 1994). The differences in mRNA abundance and GDC activity among the various organs suggested a developmental regulation. The same workers observed two proteins in the seeds which cross- reacted with the anti-GDC serum. They had a different expression profile to the 58 kDa GDC. The observation could support earlier reports of a possible existence of three enzyme isoforms in barley embryos (INATOMI AND SLAUGHTER, 1975). A suggestion of a second mammalian GDC

has been made although other authors report the existence of a single enzyme with two subunits (ERLANDER AND TOBIN, 1991)

A calmodulin-activated GDC was also reported from the soybean seed coat, cotyledons, leaves and roots (SNEDDEN, ARAZI, FROMM AND SHELP, 1995). Calmodulin caused a two- to eight-fold increase in GDC activity at pH 7.0. This is consistent with the neutral cytosolic pH and cytoplasmic location of GDC. LING, SNEDDEN, SHELP AND ASSMANN (1994) also identified a 62 kDa calmodulin binding protein from *Vicia fava* roots. A 13-residue sequence was identified from this protein, and found to contain highly conserved regions in the GDC sequences of petunia, *E. coli*, *Drosophila* and the domestic cat. The presence of the protein was correlated with GDC activity except in the cotyledons and leaves. The discrepancy between the presence of the 62 kDa protein and the lack of GDC activity is probably due to the presence of different isoforms as previously proposed. The activity of some of these isoforms may not be modulated by calmodulin (LING, SNEDDEN, SHELP AND ASSMANN, 1994).

Work by MELCHER (1986) showed differences in the amount of GABA produced by intact and sliced pea cotyledons and could indicate the presence of different enzyme forms. Although INATOMI AND SLAUGHTER (1971) found GDC activity in the growing

parts of the barley plant and the embryo, no activity was found in the endosperm.

A tomato ripening mutant (*rin* i.e., ripening inhibitor) was found to possess a GDC amino acid sequence with its active site 98% homologous to the petunia GDC and also appeared to have a similar calmodulin-binding domain (GALLEGO, WHOTTON, PICTON, GRIERSON AND GRAY, 1995). However, neither calmodulin nor Ca^{2+} seem to affect GDC activity in petunia. The difference between prokaryotic and eukaryotic enzymes is that the former does not seem to have a calmodulin binding site.

WALLACE, SECOR AND SCHRADER (1984) observed that glutamate decarboxylase activity was higher relative to GABA:transaminase activity. Their observation confirmed similar results obtained previously (STREETER AND THOMPSON, 1972(b)). Such observations led SATYANARAYAN AND NAIR (1986) to speculate that the rapid production of GABA was followed by a slow turnover rate which resulted in its accumulation.

A substantial decrease in K_m and an increase in V_{max} of GDC due to a decrease in pH suggests an increase in substrate affinity and an increase in catalytic rates at acidic pH values (SNEDDEN, CHUNG, PAULS AND BOWN, 1992). These results are consistent with the acidic pH optimum of glutamate

decarboxylase and the accumulation of GABA under acidic conditions.

2.3.2 γ-Aminobutyric Acid Transaminase and Succinic Semialdehyde Dehydrogenase

In comparison to GDC, less is known about GABA:transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). GABA-T has been associated with the mitochondrial fraction of radish leaf extract (STREETER AND THOMPSON, 1972(b)). Its activity has also been observed in the cytosolic fraction, although in lower amounts. BREITKREUZ AND SHELP (1995) found GABA-T exclusively in the mitochondria of developing soybean cotyledons. It can use either pyruvate or 2-oxoglutarate as an amino receptor during GABA transamination. SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL (1995) confirmed observations by DIXON AND FOWDEN (1961) that GABA:transaminase had a higher activity with pyruvate than with 2-oxoglutarate, contrary to other reports (STREETER AND THOMPSON, 1972(b): SATYANARAYAN AND NAIR, 1986). However, FRENEY AND GIBSON (1975) observed specific activity for pyruvate only in nodules inhabited by *Rhizobium trifolii*.

SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL (1995) observed a pH optimum of 8.2 with both pyruvate and 2-

oxoglutarate while other researchers reported 8.9 for GABA:pyruvate transaminase from radish leaves (STREETER AND THOMPSON, 1972(b)) and 8.3 for GABA:oxoglutarate transaminase from *R. trifolii* (FRENEY AND GIBSON, 1975). No conclusive study of the pH profiles of GABA transaminase with pyruvate and 2-oxoglutarate has yet been made.

When pyruvate is used as an amino receptor, the glutamate carbon skeleton enters the Krebs cycle as succinate and the amino group is used to form alanine. However, when 2-oxoglutarate is used the amino group from glutamate is used to regenerate glutamate while the carbon skeleton enters the Krebs cycle (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995).

$$\text{GABA} + \text{2-oxoglutarate/pyruvate} \leftrightharpoons \text{glutamate}$$

$$\text{SSA} + \text{NAD}^+ \leftrightharpoons \text{succinate} + \text{NADH} + \text{H}^+$$

$$\text{glutamate} + \text{H}^+ \rightarrow \text{GABA} + \text{CO}_2$$

Succinic semialdehyde dehydrogenase is also found in the mitochondrion, has a pH optimum of 9.0, and is dependent on NAD^+. Like GABA transaminase some of its activity has also been associated with the cytosol (STREETER AND THOMPSON, 1972(b); SATYANARAYAN AND NAIR, 1986). However, BREITKREUZ AND SHELP (1995) using protoplasts from developing soybean cotyledons reported that GABA transaminase and SSADH are strictly

mitochondrial enzymes. The authors suggested that activities of GABA:transaminase and SSADH noticed in the cytosolic fraction could be a consequence of contamination following organelle breakage. The enzyme may be membrane-bound or complexed with GABA-T. Both enzymes are strictly mitochondrial in the mammalian systems.

Although present in all the tissues of developing *Glycine max* seeds (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995) and all *Petunia hybrida* organs (CHEN, BAUM AND FROMM, 1994), the enzymes of the GABA shunt exhibit different developmental profiles. Expression and activity of the protein, and the consequent accumulation or decrease of GABA depend on the type of tissue and the stage of development. For example, GDC activity in the soybean seed coat increases during early stages and then declines by up to 80% (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995) while the activity increased in germinating petunia seeds.

RHODES, HANDA AND BRESSAN (1986) compared GABA catabolism of tomato cell cultures adapted to 25% PEG (polyethylene glycol) and control cells. Both cell types utilized GABA with the rate of catabolism higher in adapted cells. The increase could be indicative of the increase in transamination of GABA in cells adapted to water stress.

CHAPTER 3

γ-AMINOBUTYRIC ACID AND THE PLANT NITROGEN BUDGET

3.1 Introduction

Nitrogen is a growth-limiting element and thus very important in plant development. The global production of crop plants depends largely on the supply of inorganic nitrogen in the form of nitrogen fertilizers. As illustrated in Figure 3, following nitrogen fixation, nitrate is reduced to nitrite by nitrate reductase in the cytosol, which is transported to the chloroplast where it is reduced to ammonium by nitrite reductase. Ammonium is then assimilated via glutamine synthetase to glutamine and finally to glutamate from which other amino acids are produced (MENGEL, 1992). The reducing power and ATP required for these processes are supplied by photosynthesis.

The ultimate aim of the above processes is the incorporation of inorganic nitrogen into organic matter such as proteins and nucleic acids which are necessary for growth (MIFLIN AND LEA, 1980). The conversion of ammonia to glutamine and then glutamate, mediated by glutamine synthetase and glutamate synthase, respectively, is generally thought to be the most plausible mode of ammonia entry into organic matter (MIFLIN AND LEA, 1980; BRAY, 1983) while GDH is thought to be

responsible for no more than five percent of this incorporation (LEA, BLACKWELL AND JOY, 1992). Investigations into nitrogen metabolism have mainly employed radio-labelled nitrogen (^{15}N) to track the ^{15}N-ammonium flux through the various pathways.

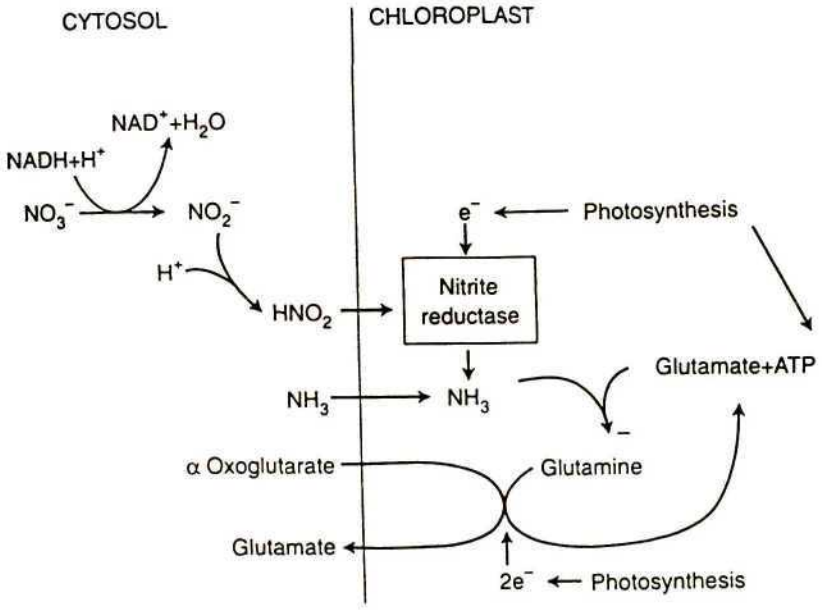

Fig. 3: Nitrogen fixation, reduction and assimilation and the interaction with photosynthetic processes (MENGEL, 1992).

3.2 The Importance of Glutamate in Nitrogen Metabolism

TUIN AND SHELP (1994(a)) showed that exogenous glutamate was rapidly metabolized via deamination or transamination to 2-

oxoglutarate and decarboxylation to GABA. The formation of GABA was not however, a stress response but seemed to be part of normal glutamate metabolism. In the same study the label from [U- ^{14}C]glutamate was found in amino acids such as asparagine and arginine which together with glutamate were incorporated into proteins. The label was also recovered as $^{14}CO_2$ and in various intermediates of the TCA cycle. Therefore, glutamate is involved in the various synthetic reactions which lead to formation of other amino acids.

GDH could be involved in the oxidation of glutamate after protein breakdown to supply the cell with reduced nucleotides and carbon skeletons during carbohydrate shortage (LEA, BLACKWELL AND JOY, 1992). Nuclear magnetic resonance studies show that GDH is capable of oxidizing glutamate to provide the carbon necessary for the functioning of the TCA cycle. Transportation of glutamate needs conversion to asparagine and GDH provides the amino group from glutamate for the manufacture of asparagine (LEA, BLACKWELL AND JOY, 1992).

3.3 The Role of GABA in Nitrogen Metabolism

In the first experiment to establish the GABA shunt DIXON AND FOWDEN (1961) found that radioactivity from GABA was incorporated among other products into glutamine and

asparagine both of which are important forms of transportable nitrogen (PATE, 1989). OJI AND IZAWA (1972) found that there was an increase in the free amino acid and amide pools after one hour of ammonia supply to barley plants. Free amino acids were synthesized rapidly after ammonia assimilation. KISHINAMI AND OJIMA (1980) observed that GABA accumulated in cultured rice cells which were supplied with ammonium and nitrate. GABA accumulation was preceded by a temporary increase in glutamine. When the cells were supplied with glutamine, GABA accumulation was also observed. KISHINAMI AND OJIMA (1980) then suggested that the GABA pool is controlled by glutamine and that it is the first assimilatory product of ammonium.

This observation could be due to the direct increase of the glutamate pool as a result of ammonia. Azaserine inhibited glutamate synthase as well as GABA accumulation under anaerobic conditions. This observation led AURISANO, BERTANI AND REGGIANI (1995) to suggest that the assimilated ammonia served as the source of glutamate which in turn served as substrate for glutamate decarboxylase. Ammonium assimilation was shown to cause a pH decrease in plant cells probably accounting for the increase in GABA synthesis (CARROLL, FOX, LAURIE, PHILLIPS, RATCLIFFE AND PHILLIPS, 1994). Glutamate synthase was responsible for the maintenance of the glutamate pool and GABA accumulation, especially under stress, could act

as a temporary storage for nitrogen due to its low toxicity (AURISANO, BERTANI AND REGGIANI, 1995).

Non-protein amino acids are considered as products with a limited or indirect role in plant growth and development (BEEVERS, 1976). There is evidence however, that GABA could have a more direct function than has been acknowledged previously. Given the importance of nitrogen metabolism in plant growth, GABA unlike other non-protein amino acids, seems to play a more significant role in plant metabolism. Therefore, it could be more than just a metabolite synthesized during stress conditions but part of routine plant metabolism. Some plants when grown in nutrient medium can utilize GABA as the sole nitrogen source (BARNS AND NAYLOR, 1959).

A reduction in the alcohol-soluble nitrogen content relative to the protein-nitrogen content occurs in growing potato tissue as opposed to non-growing tissue. GABA was found to be the only amino acid which increased in content in growing potato cells while glutamine was reduced (STEWARD, THOMPSON AND POLLARD, 1958). LÄHDESMÄKI (1968) suggested that the increase in GABA content of ageing leaves is related to the decline and cessation of growth processes, primarily the formation of new proteins.

In the light, glutamine is the main product from glutamate while compounds associated with the Krebs cycle, and probably produced via the GABA shunt, are predominant in the dark. The ratio of labelled malate to aspartate was high (JORDAN AND GIVAN, 1979). SELMAN AND COOPER (1978) also found the same relationship between GABA accumulation and the light regime in young tomato plants.

GABA was found in nodules of both *Trifolium subterraneum* by INATOMI AND SLAUGHTER (1971) and in clover (FRENEY AND GIBSON, 1975) which are centres of nitrogen fixation. Accumulation of GABA was accompanied by a decrease in shoots and roots of barley. FRENEY AND GIBSON (1975) argued that GABA accumulation depended more on GABA utilization than on glutamate availability and GDC activity.

A study by FORD, RATCLIFFE AND ROBINS (1996) on the phytohormone- induced production of GABA in root cultures of *Datura stramonium* is further proof of the inextricable link between GABA content and the plant's nitrogen status. Kinetin- and NAA-treated roots were found to have small pools of ^{15}N-labelled glutamine and glutamate with a parallel increase in GABA following the assimilation of ^{15}N-ammonium. The assimilation and increase in GABA was shown to be independent of cytoplasmic acidification since alkalinisation did not suppress GABA accumulation. Dedifferentiation caused by the

hormone-treatment seemed to be associated with the redirection of the primary nitrogen metabolism and the synthesis of GABA (FORD, RATCLIFFE AND ROBINS, 1996). Such observations may support an earlier suggestion by KISHINAMI (1988) that a high auxin concentration may shift nitrogen metabolism from asparagine to GABA during ammonium assimilation.

RHODES, HANDA AND BRESSAN (1986) observed an increase in nitrogen flux from glutamate to GABA and alanine. The occurrence was attributed to a possible increase in pyruvate availability and the increase in GDC activity. The utilisation of the amino acids was dependent on the maintenance of the amino acid pool with growth, protein synthesis requirements and rate of catabolism either through decarboxylation or transamination. The study seemed to indicate that GABA synthesis is linked to nitrogen requirements of the cell. The claim by TUIN AND SHELP (1994(a)) that in developing soybean cotyledons, GABA was not solely synthesized as a result of stress seems to support this suggestion. It should be noted however, that intact soybean cotyledons have been considered to be inherently operating under conditions of limited oxygen (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995). TUIN AND SHELP (1994(b)) also mention that co-operation exists between the GABA shunt and the deamination and/or transamination of glutamate leading to the TCA cycle although

the relative magnitudes of these alternate routes are not known.

CHAPTER 4

γ-AMINOBUTYRIC ACID ACCUMULATION

4.1 Introduction

LEVITT (1980) defines biological stress as any environmental factor which is potentially injurious, to plants. Plants due to their sessile nature are more prone to variations in their environment than mobile organisms. Therefore, factors or characters which allow plants to track these fluctuations are important for adaptability (FITTER AND KAY, 1987). Each plant is capable of responding to environmental changes. Therefore, an adaptive response to stress may be regarded as an execution of a plant's inherent mechanism to resist environmental damage. Failure to resist detrimental environmental factors may result in the death of all or part of the plant or reduced growth rate due to physiological malfunctioning. The physical or abiotic environment which influences plant development includes light, temperature, water, atmosphere and soil (TRESHOW, 1970). Therefore, abiotic stresses are extremes of these environmental factors which are likely to cause plant injury or death.

There is a large body of data about the increase in synthesis, the corresponding GDC activity and subsequent accumulation of

GABA due to various abiotic stresses. THOMPSON, STEWARD AND MORRIS (1966) observed an increase in the accumulation of GABA as well as alanine in excised tomato leaves. The observation suggested an increase in pyruvate supply due to decreased oxidation in the Krebs cycle and increased glycolysis. Proline was found to accumulate due to water stress but decreased under anaerobic conditions. Some of the constituents which were also found to decrease after protracted water stress were protein amino acids, sugars and chlorophyll. A net increase in non- protein amino acids was observed.

GABA synthesis seems to be associated with a decrease in the cytoplasmic pH and the activation of a signal transduction pathway due to stress. However, not all treatments which lead to GABA synthesis are necessarily associated with a low pH (CRAWFORD, BOWN, BREITKREUZ AND GUINEL, 1994). A study by KNIGHT, CAMPBELL, SMITH AND TREWAVAS (1991) showed rapid increases in Ca^{2+} levels in transgenic tobacco plants transformed with the calcium-sensitive luminescent protein, aequorin. The transient increase was elicited by mechanical and cold stresses which are known to lead to a GABA increase. In addition, various authors mentioned elsewhere in this review, have shown that the plant GDC has a calmodulin-binding domain and in most cases is activated by calmodulin. CRAWFORD, BOWN, BREITKREUZ AND GUINEL (1994) speculated that Ca^{2+} might be the immediate messenger which stimulates GDC activity.

4.2 pH Regulation

The cytoplasmic pH under normal physiological conditions is generally between 7.1 and 7.6 (FELLE, 1988). Enzymes in a cell operate at pH values different from their optima, thus making them sensitive to minor changes in pH. Rapid synthesis of GABA following stress application could point to the sensitivity of GDC to small pH changes. Work on cultured *Acer pseudoplatanus* using nuclear magnetic resonance pointed to the existence of some pH regulation mechanism after recovery was observed following strong initial acidification of the cytoplasm (GUERN, MATHIEU, PEAN, PASQUIER, BELOEIL AND LALLEMEND, 1986).

FELLE (1988) distinguished between short- and long-term pH regulation features. The former involves the removal of excess hydroxyl (OH^-) ions and expends energy while the latter is a reaction to sudden changes in pH to avoid severe metabolic disturbances. This review will be limited to the short-term control of pH. This regulation of the cytoplasmic acid load could involve passive physico-chemical buffering, ATP-driven sequestration of H^+ to the vacuole or extracellular medium and their consumption in proton-consuming reactions like decarboxylation (BOWN, CHUNG, SNEDDEN AND SHELP, 1989).

Transient decreases in pH due to lactic acid fermentation in hypoxic maize root tips could be a signal by which the plant

triggers ethanol production to ameliorate cytoplasmic acidosis (ROBERTS, CALLIS, WEMMER, WALBOT AND JARDETZKY, 1984). The same authors observed cytoplasmic acidification within 2 minutes and a 0.5 unit decrease in pH within 20 minutes of transfer of maize root tips to anaerobic conditions. CARROLL, FOX, LAURIE, PHILLIPS, RATCLIFFE AND STEWART (1994) also suggested that glutamate decarboxylation is a necessary reaction in short-term pH homeostasis to normalize a pH decline due to ammonium assimilation. A decrease of 0.2 pH units did not result in any measurable GABA levels, prompting suggestions that the pH must be reduced to a threshold value before decarboxylase activity can be stimulated (CRAWFORD, BOWN, BREITKREUZ AND GUINEL, 1994).

Glutamate/H^+ cotransport has been mentioned as another possible source of excess protons. This proton/glutamate symport has been observed in isolated mesophyll cells of *Asparagus sprengeri* (SNEDDEN, CHUNG, PAULS AND BOWN, 1992). The symport was shown to cause an acid load which if not countered by the proton-consuming reactions would cause decreases in pH at a rate of 1.9 pH units per hour. The authors claim that the decarboxylation accounts for about 40% of the protons entering through the symport while other mechanisms such as the ATP-driven H^+ efflux would account for the rest. ATP-driven H^+ efflux was observed to excrete 50% of protons in acid-stressed *A. pseudoplatanus* cells (MATHIEU, GUERN, PASQUIER, BELOEIL AND

LALLEMAND, 1986). Stimulation of both the proton and GABA effluxes occurred with the addition of permeant weak acids leading to a suggestion that both effluxes are sustained by H^+-stimulated GDC activity (SNEDDEN, CHUNG, PAULS AND BOWN, 1992).

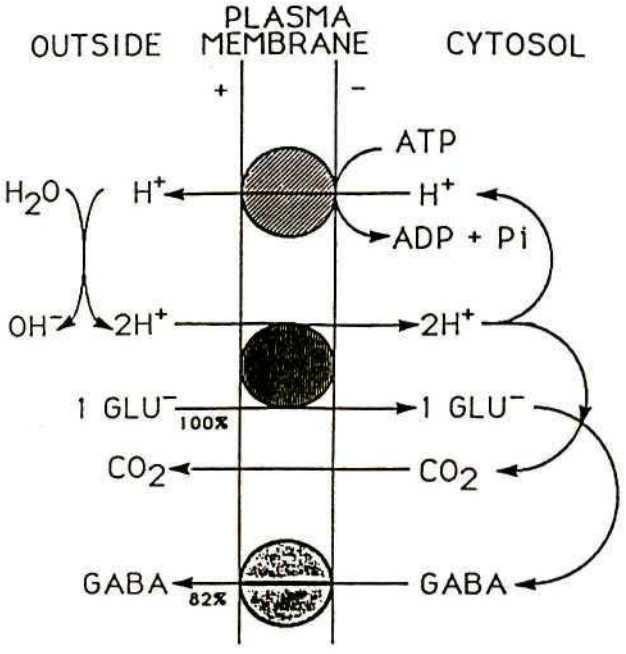

Fig. 4: Diagram of the suggested model for the metabolic pH-stat involving GABA synthesis and efflux (SNEDDEN CHUNG, PAULS AND BOWN, 1992).

In the model proposed in Figure 4 above, SNEDDEN, CHUNG, PAULS AND BOWN (1992) demonstrate how increased ATPase activity in the plasma membrane and glutamate decarboxylation to GABA may be involved in regulating pH. The model is consistent with the alkalinisation of the extracellular medium, glutamate uptake and GABA uptake the authors observed. It should be noted

however, that glutamate decarboxylation to GABA is not the only proton- consuming reaction. There is also the decarboxylation of malate to alanine catalyzed by the malic enzyme and the conversion of glutamine to glutamate which are stimulated by cytoplasmic acidosis (ROBERTS, HOOKS, MAULLIS, EDWARDS AND WEBSTER, 1992).

Contrary to reports by SNEDDEN CHUNG, PAULS AND BOWN (1992) that GABA synthesis in response to cytoplasmic acidosis is a rapid process and that GDC was very sensitive to minor pH changes. ROBERTS, HOOKS, MAULLIS, EDWARDS AND WEBSTER (1992) found that decarboxylation of GABA was only a major factor in pH regulation after acid-producing reactions had ceased. The same authors considered GABA accumulation to be a metabolic marker for dead or dying cells. The observation that GABA synthesis once stimulated by acidification, countered this decrease in pH made GABA accumulation a poor marker for dying cells (CARROLL, FOX, LAURIE, PHILLIPS, RATCLIFFE AND STEWART, 1994). Although the addition of glutamate to asparagus cells did not cause any decrease in pH it increased GDC activity. A decrease in cytosolic pH seems to be a sufficient, but not necessarily the only, stimulus of GDC activity. In such a case the activation of GDC may be due to increases in calcium ions or glutamate concentrations (CRAWFORD, BOWN, BREITKREUZ AND GUINEL, 1994).

4.3 Accumulation of γ-Aminobutyric Acid due to Abiotic Stresses

4.3.1 Water Stress

Water is an important solvent of biologically important solutes such as amino acids, nutrient ions and other small molecules like oxygen. It forms between 70% to 90% of the fresh weight of most non-woody plants (FITTER AND KAY, 1987). Therefore, most plants must have a sufficient water supply to survive the terrestrial environment which forms their habitat. Water stress may arise either from insufficient supply or from excessive supply of water (LEVITT, 1980). The definition used for the purposes of this study refers to the former, a deficit in water. Drought stress is sometimes used interchangeably with water stress. A water deficit may progressively lead to incipient, transient and permanent wilting thus disturbing metabolic activity, growth and reproduction (TRESHOW, 1970).

A four-fold increase in GABA was first reported in excised leaves of *Brassica rapa* due to water loss (THOMPSON, STEWARD AND MORRIS, 1966). The accumulation of GABA continued with the application of stress over 50 h. RHODES, HANDA AND BRESSAN (1986) observed an increase in GABA and alanine pools of cultured *Lycopersicon esculentum* cells adapted to 25%

polyethylene glycol (PEG). An increase in both synthesis and utilization of GABA during water stress in adapted cells was observed. Unadapted cells also catabolized GABA albeit at a lower rate.

GABA, proline and alanine may play a pivotal role in the osmotic adjustment of the cytoplasm due to changes associated with osmotic stress (RHODES AND HANDA, 1989). These authors suggested that analysis of the dynamics of amino acid metabolism due to abiotic stresses could be studied by kinetics of $[^{15}N]H_4^+$ assimilation. Such studies, they argue, require consideration of flux and compartmentation of metabolites involved in nitrogen metabolism and that since about 50% of the proline is located in the cytoplasm it could play a significant role as an osmotic solute. Given the cytoplasmic location of glutamate decarboxylase, GABA could serve a similar role as an osmotic solute in those plants where it forms a major metabolite of the free amino acid pool.

4.3.2 Salinity Stress

Most salt stresses in nature are thought to be caused by sodium chloride (NaCl) (LEVITT, 1980). The stress invariably lowers the water potential thus establishing a relationship between salt and water stresses. STEWART AND LEE (1974)

suggested that the accumulation of proline under saline conditions could be correlated with salt tolerance in some halophytes. Salinity necessitates an osmotic adjustment of the cytoplasm as a result of the uptake of solutes, the synthesis of organic compounds or both (WYNJONES, STOREY, LEIGH, AHMAD AND POLLARD, 1977). Halophytes typically use Na^+ and Cl^- as principal osmotica while glycophytes tend to exclude Na^+ and Cl^- and accumulate organic compounds like GABA for osmotic adjustment.

Amino acids have been referred to as compatible osmotic solutes as they have been shown to play a significant role in osmotic adjustment. Under saline conditions plants are known to sequester the salt to the vacuole, away from active metabolic sites (STEWART AND LEE, 1974). Therefore, osmotic adjustment within the cytoplasm is necessary due to this. Hence, the accumulation of amino acids such as GABA, proline, and alanine (RHODES AND HANDA, 1989). These authors also suggested that such an accumulation could represent a model metabolic system by which plants perceive and transduce signals of osmotic stress to the entire plant. BENZIL, HASEGAWA, RHODES, HANDA, HANDA AND BRESSAN (1987) used cultured tobacco cells adapted to NaCl to show that GABA and proline accumulated substantially when the cells were grown in a medium containing 428 mM NaCl. GABA increased eight-fold while the proline increase was more than 400-fold.

4.3.3 Anaerobiosis

A distinction is usually made between anoxia and hypoxia. The former refers to the total absence of molecular oxygen, O_2, while the latter refers to presence of subnormal levels of oxygen (LEVITT, 1980).

Anaerobic treatment of maize seedlings leads to a pH drop, drastic repression of protein synthesis and the *de novo* synthesis of new proteins unique to such conditions (ROBERTS, HOOKS, MIAULLIS, EDWARDS AND WEBSTER, 1991). The pH drop is due to the production of lactate and a shift to ethanol production is affected. AURISANO, BERTANI AND REGGIANI (1995) observed a 70% decrease in the rate of protein synthesis after 24 h of anoxia in rice seedlings.

Large amounts of GABA were recovered due to incubation of turnip leaves under anaerobic conditions with corresponding loss of glutamate (THOMPSON ,STEWARD AND MORRIS, 1966). It was suggested that the accumulation could be due to repressed transamination of GABA caused by the accumulation of succinic semialdehyde which is metabolized as succinate by the Krebs cycle under aerobic conditions. AURISANO, BERTANI AND REGGIANI (1995) found that 24 h of anoxia in the roots and shoots of rice seedlings induced GABA accumulation. The developing soybean seed was found to be hypoxic. Such conditions caused

the induction of the GABA shunt (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995).

4.3.4 Temperature

Higher plants, unlike homoiothermic animals are unable to maintain optimum physiological temperatures for normal growth and metabolism (FITTER AND HAY, 1987). Temperature is a critical control of metabolic activity since the activity of various enzymes can be reduced due to high or low temperature deviations from the optimum (TRESHOW, 1970). Heat stress may be caused by the transfer of plants to elevated temperatures and is determined by the extremity of the stress, rapidity of change and previous growth conditions. Such temperature extremes may result in death of cells, tissue and organs.

WALLACE, SECOR AND SCHRADER (1984) observed a 100% increase in GABA within five minutes of rapid transfer of soybean plants from 33 °C to 22 °C and a corresponding decrease in glutamate. The accumulation, it is suggested, could be due to an alteration in the compartmentation of glutamate or of some effector molecule which associates with glutamate decarboxylase. The latter is unlikely since glutamate decarboxylase is located in the cytoplasm where glutamate is found.

Fluorescence-ratio imaging with fluorescein diacetate was used by YOSHIDA (1994) to show that low temperature in protoplasts of *Vigna radiata* caused a drop in pH. Such a condition is associated with the activation of glutamate decarboxylase.

4.3.5 Light

Light in higher plants, provides energy which drives the processes responsible for the synthesis of hexose sugars via photosynthesis (TRESHOW, 1970). Although stress due to either a deficit or excess of light may occur it is beyond the scope of this study. The effects due to normal light and dark conditions on GABA synthesis will be examined.

JORDAN AND GIVAN (1979) noticed that glutamine was the main product in leaf discs of *Vicia faba* during incubation in light. However, products of the Krebs cycle which were probably produced via the GABA shunt were predominant in the dark. Observations of GABA accumulation in the dark have also been reported by other workers (SELMAN AND COOPER, 1978; WALLACE, SECOR AND SCHRADER, 1984).

Light has been reported to cause changes in pH across the thylakoid membrane (FELLE, 1988). Such an effect could account

for the variation in the amino acid metabolism resulting in GABA accumulation.

4.3.6 Phytohormones

The accumulation of GABA in cultured rice cells due to addition of ammonium was observed by KISHINAMI AND OJIMA (1980), while glutamine and arginine was reported to accumulate in barley plants (OJI AND IZAWA, 1972). KISHINAMI (1988) observed that GABA accumulation occurred due to ammonium supply in the presence of 2,4-D, NAA and IAA. Such an accumulation was attributed to exogenous auxins in cultured cells.

ABA has been implicated in stress tolerance and also found to be a common factor in response to desiccation, salt, and cold stress. Its response has been comparable to seed development and dormancy since physiological and biochemical processes which occur during seed development may be similar to stress. ABA (100 mM) caused a reduction in protein synthesis and increased the specific activity of GDC by 40% (REGGIANI, AURISANO AND MATTANA, 1993). There was also a two-fold increase in the incorporation of radioactivity from ^{14}C-glutamate to GABA. It has been shown that ABA can stop key steps involved in seed germination and is capable of

reinitiating part of the developmental programme that leads to dormancy (SKRIVER AND MUNDY, 1990). The presence of the GABA shunt in developing soybean seeds was found as a result of the hypoxia associated with seed development (SHELP, WALTON, SNEDDEN, TUIN ORESNIK AND LAYZELL, 1995).

Hormones have been implicated in changes of free intracellular Ca^{2+} and may thus trigger signal transduction pathways (FELLE, 1988). The induction of cold tolerance is mediated by the shift in hormonal balance especially involving ABA. *De novo* synthesis of stress-related proteins have been implicated but it is not known how acclimation to stress and ABA alter patterns of protein synthesis. Some of the proposed models of the mechanism of action of ABA during water stress envisage turgor pressure sensors which are followed by the increase in ABA levels and the subsequent induction of ABA regulated genes (DAUGHERTY, ROONEY, PAUL, VETTEN, VEGAPALAS, LU, GURLEY AND FERL, 1994). No receptors have been characterized yet.

An increase in GABA content due to kinetin and NAA was observed in cultured roots of *Datura stramonium* (FORD, RATCLIFFE AND ROBINS, 1996). The increase was attributed to a probable shift in nitrogen metabolism caused by these hormones.

4.4 Comparisons with Stress-induced Proline Accumulation

Unlike GABA, proline is a protein amino acid. Glutamate provides the carbon skeleton for proline formation (BENDER, 1975). In higher plants, glutamate is oxidized to glutamic-5-semialdehyde which cyclizes to $<^{1}$-pyrroline-5-carboxylate which in turn is reduced to proline. The major pathway for the catabolism of proline is oxidation to glutamate (BROWN, 1995).

Proline has been known to increase as a result of increased salinity to the extent that it becomes the main component of the free amino acid pool. The formation of proline during salt stress is thought to be via the cyclisation of glutamate probably due to a relaxation of the feedback inhibition of its biosynthesis (STEWARD AND LARHER, 1980). Water stress also elicits an accumulation of proline in many plant species and the effect is reversed when normal water relations are restored. Free proline increases more than it decreases, suggesting that proline is not derived solely from proteolysis. Not all species accumulate proline to significant levels due to environmental stress which implies that it is not a universal stress response.

In those plant species where it accumulates significantly, various explanations for its accumulation have been given. It is thought to be a symptom of injury; an adaptive response to

drought; a source of carbon and/or nitrogen; a source of reductant in the post-stress period; an ammonia detoxification product; and a compatible solute in the cytoplasm acting as an osmoticum.

There are obvious similarities between GABA and proline with respect to their biosynthesis and accumulation due to stress. Glutamate is the initial substrate for both synthetic pathways although the routes are different. They both have been shown to increase significantly as a result of the above-mentioned environmental stresses. The physiological significance of the accumulation of both compounds, although not established with any certainty, seems to point to similar roles during stress. It is not known how glutamate is channelled to the respective pathways, particularly during stress.

CHAPTER 5

GENE REGULATION AND EXPRESSION

5.1 Introduction

The observation that the differences in RNA and protein content of different tissues are not always accompanied by differences in their DNA content is indicative of the existence of a process of regulation of gene expression at the level of mRNA synthesis. There are a number of intervening stages between transcription and translation which represent points where regulation can occur. These stages are, the modification of the initial transcript by capping the 5' end with a structure containing a modified guanosine residue; cleavage of the transcript near its 3' end and the addition of a polyA tail, a process called polyadenylation; the removal of the introns which interrupt the coding sequence in a process called splicing; and the final transportation of the RNA molecule to the cytoplasm where it is translated into a protein (LATCHMAN, 1990).

Once in the cytoplasm the relative stability of the mRNA transcript becomes important. Regulation could be achieved by changing the stability in response to some regulating signal (LATCHMAN, 1990). The interaction of highly conserved

sequences in the eukaryotic sequence and specific protein factors ensures accurate initiation of transcription and modify its rate and efficiency (WINNACKER, 1989). Some sequences have been identified as *cis* acting elements which are involved in cold- and drought- regulated gene expression (THOMASHOW, 1989).

GABA accumulation has been associated with an increase in GDC activity. The mechanisms of regulating GDC activity *in vivo* are not known. It is thought that GDC activity may be regulated by such factors as cytosolic pH (CRAWFORD, BOWN, BREITKREUZ AND GUINEL, 1994), abscisic acid (REGIANNI, AURISANO, MATTANA AND BERTANI, 1993) and intracellular Ca^{2+} levels (BAUM, CHEN, ARAZI, TAKATSUJI AND FROMM, 1993).

Signal transduction has been defined by PARKER (1991) as a group of processes that are specifically involved in the translation of selected extracellular changes into programmed intracellular actions. The extracellular changes can range from alterations in cellular contacts (i.e., cell to cell interaction) to binding of diffusible substances (e.g., hormones). Calmodulin (CaM) is known to be one of the principal targets of Ca^{2+} in the control of multiple cellular events. Ca^{2+} binds to CaM, forming a complex which binds to the regulated protein. Calcium usually functions as an

intracellular messenger and couples this role with responses to external stimuli such as light or environmental stress.

The first evidence that plant GDC was stimulated by Ca^{2+}/CaM was provided by LING, SNEDDEN, SHELP AND ASSMANN (1994). The authors showed a 50% stimulation of the *Vicia fava* GDC activity due to addition of 100 µM Ca^{2+}, 100% stimulation with the addition of 100 nM CaM to GDC and no appreciable activation with the addition of CaM in the absence of Ca^{2+}. BAUM, CHEN, ARAZI, TAKATSUJI AND FROMM (1993) showed that although petunia GDC could bind to CaM its activity was not affected by the absence of Ca^{2+}/CaM. It is possible that Ca^{2+}/CaM-induced GDC activation may be influenced directly by other processes acting in concert, such as pH shifts or stress-induced changes in substrate levels (CRAWFORD, BOWN, BREITKREUZ AND GUINEL, 1994). SNEDDEN, ARAZI, FROMM AND SHELP (1995) also showed that soybean GDC was activated two- to eight- fold by Ca^{2+}/CaM *in vitro*.

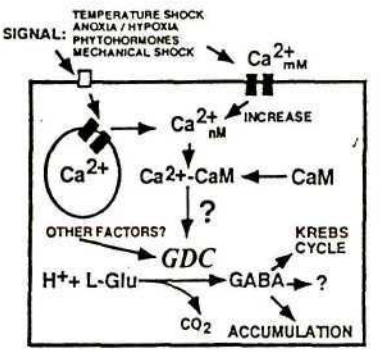

Fig. 5: A model of GABA production in relation to environmental stimuli and Ca^{2+} flux. The *in vivo*

mechanism of GDC regulation and physiological role(s) of GABA are represented by question marks since they are unknown (SNEDDEN, ARAZI, FROMM AND SHELP, 1995).

Since GDC catalyzes the first and irreversible step in the GABA shunt it may provide a regulatory point. Figure 5 outlines the possible action of environmental stress and Ca^{2+}/CaM in the production of GABA.

5.2 Arabidopsis as a Model

MEYEROWITZ (1994) reviewed some of the qualities which make *Arabidopsis thaliana* ideal as a model for genetic and molecular biology studies. *A. thaliana* has a small haploid genome of about 200 Mb but it possesses a gene complement typical of any flowering plant. The plant is also unique among angiosperms because it contains single or low copy number genes which are separated by short stretches of moderately repeated DNA. The nuclear genome is contained in only five types of chromosomes. Therefore, the genome permits repeated screening of genomic libraries which would not require many clones and easier chromosome walks. Importantly though, the genome makes it possible to clone by homology with

known genes, cloning from cDNA libraries and cloning from protein sequences.

The existence of a database of partially sequenced cDNA clones of *A. thaliana* at the Arabidopsis Biological Research Centre, Ohio State, underlies the importance of the plant as a research organism. This database is aimed at facilitating rapid genetic and molecular characterization of the plant (NEWMAN, DE BRUIN, GREEN, KEEGSTRA, KENDE, MCINTOSH, OHLROGGE, RAIKHEL, SOMMERVILLE, THOMASHOW, RETZEL AND SOMMERVILLE, 1994). The database is freely available through the Internet as are various other online databases with specialisations in seed biology, genetics, and morphology. Discussion groups on various subjects pertaining to *Arabidopsis* have also been set up. The plant is also part of the wider human genome project which aims to decipher the entire *A. thaliana* genome.

CHAPTER 6

MATERIALS AND METHODS

6.1 Plant Material

The plant material used in all experiments was *Arabidopsis thaliana* var. Columbia (L.) Heynh grown from seed purchased from Lehle Seeds, Tucson, Arizona. The plants were grown in pots as per guidelines provided by the seed producer. They were grown in a mixture of peat moss/vermiculite/perlite (1:1:1) and watered at least twice a week with a nutrient solution (Appendix I). The pots were kept in a greenhouse where the ambient temperature was about 22±5 °C. Four-week-old seedlings were then transferred to a Conviron, a controlled environment growth cabinet.

The plantlets were allowed to acclimatize in the Conviron for three days at 25 °C, and a photoperiod of 16 h light: 8 h darkness and a relative humidity of 50%. After the acclimatization period, plants underwent stress treatments, as described later, and leaves at the base of the rosette were used for amino acid extraction experiments. The leaves were quickly frozen in liquid nitrogen and stored at -70 °C until further use.

6.2 The Glutamate Decarboxylase Clone

Bacterial strains were grown on Lauria Broth (LB) medium (Appendix I), either solid or liquid, supplemented with 50 $\mu g.ml^{-1}$ ampicillin. Culture stocks were subcultured every 7 days and stored at 4 °C. *Escherichia coli* (DH10B) containing the plasmid pZL1 (amp^r) was kindly provided by the Arabidopsis Biological Research Centre, Ohio State University. The plasmid contained a cDNA of the glutamate decarboxylase gene from *Arabidopsis thaliana* var. Columbia.

The clone was one of over 1500 cDNA clones derived from expressed mRNA pools and thus called expressed sequence tags (EST's). Partial sequences of these cDNA clones are available from the EST database (dbEST) (NEWMAN, DE BRUIN, GREEN, KEEGSTRA, KENDE, McINTOSH, OHLROGGE, RAIKHEL, SOMERVILLE, THOMASHOW, RETZEL AND SOMERVILLE, 1994). The plasmid vector for the clone is shown in Figure 6.

All DNA and RNA samples were quantified with GeneQuant, Pharmacia™ and visualized in a gel using an ultraviolet translumminator, Spectroline (model TC-312A). Molecular weight marker III (Boehringer Manneheim®) was used to size all restricted DNA. All centrifugation of Teflon tubes was done with a Beckman II centrifuge while microfuge tubes were centrifuged in a Sigma 133 centrifuge.

All chemicals used were analytical grade. All enzymes and primers, unless otherwise stated, were purchased from Boehringer Manneheim®. The enhanced chemiluminescence (ECL™, Amersham) system was used for nucleic acid labelling, hybridization and detection.

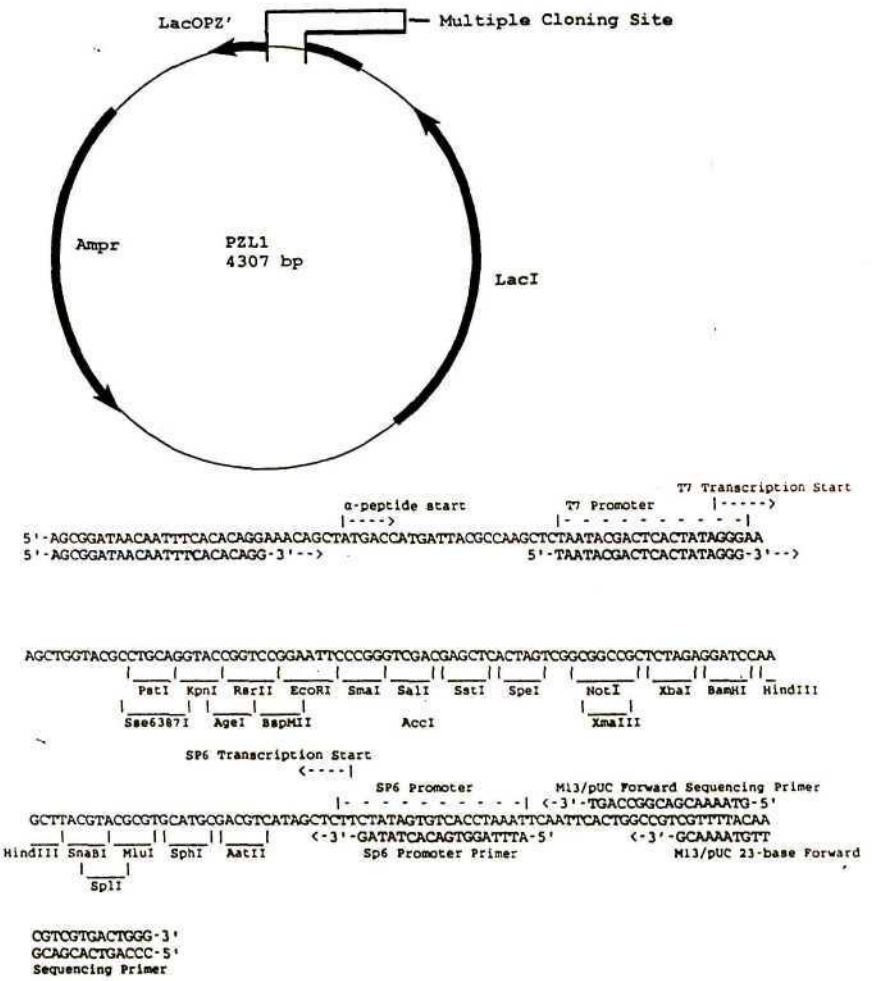

Fig. 6: A plasmid map of the vector, pZL1. The plasmid is 4.3 kb and has the GDC cDNA cloned into SalI and NotI sites of the multiple cloning region. The T7 and SP6 primers flank the multiple cloning region.

6.3 Stress Treatments

6.3.1 Water Stress

During acclimatization both the control and experimental plants were irrigated with the exact volumes of water. Following acclimatization plants were incubated under normal conditions without watering over a period of 24 h. Control plants were watered as before over the same period. Leaf samples were taken after 24 h and frozen until further use.

6.3.2 Temperature Stress

Two temperature regimes were chosen. A set of plants was incubated at 15 °C and the other at 35 °C while controls were placed at 25 °C. The relative humidity in both stress treatments was kept at 50%. Three replicates of each temperature point were made and samples taken every 6 h over a 24 h period.

6.3.3 Salinity Stress

Different concentrations of NaCl were used to determine the effect of salinity stress on the GABA content. The plants were watered with saline solutions at 50 mM, 100 mM, 150 mM 200 mM for 24 h after three days of acclimatization. Thereafter, leaf samples were taken as before.

As a result of the osmotic stress which accompanies saline treatments, it was necessary to determine the accumulation of GABA due to the hyperosmosis. Therefore, polyethyl glycol (PEG) concentrations of equivalent osmotic potential to 50 mM, 100 mM, 150 mM and 200 mM NaCl solutions were used to water the plants over the same period at 25 °C. Three replicates for each of the concentration points were made.

6.3.4 Light and Dark Treatments

Experimental plants were taken at the end of their normal photoperiod and subjected to continuous light and darkness at 25 °C and their accumulation of GABA compared to control plants which were grown under a normal photoperiod of 16 h light: 8 h darkness. The light intensity was 0.5 µmol m^2 s^{-1}. GABA was assayed at 6 h intervals over the 24 h experimental period.

6.4 GABA Assay

In a review of nitrogen metabolism, STEWARD AND POLLARD (1957) acknowledge the role of two-dimensional paper chromatography in the identification and quantitation of amino acids in plants. THOMPSON, ZACCHARIUS AND STEWARD (1951) identified a number of variables such as temperature, humidity, oxygen, solvents and concentration of ninhydrin which influence the reaction of ninhydrin with amino acids. Recovery of amino acids from the paper has been one of the problems associated with paper chromatography.

Factors such as pH and temperature affect recovery of amino acids, but if controlled, could make two-dimensional paper chromatography a reliable quantitative procedure (THOMPSON AND STEWARD, 1951). THOMPSON AND MORRIS (1959) confirmed that two-dimensional paper chromatography is a sensitive and reproducible method for the analysis of the free amino acid pool in plants. The same authors also determined the content of non-protein amino acids such as GABA, asparagine, glutamine and pipeolic acid. The method was also used by VAN STADEN (1966) to characterize the free amino acid pool of the Proteaceae in an attempt to establish whether the distribution of amino acids has any taxonomic significance in this family.

While THOMPSON AND STEWARD (1951) optimised the conditions during the analysis of amino acids for maximum recovery, reagents used in this study obviated the need for complex standardisation procedures.

6.4.1 Amino Acid Extraction

For each extraction about 100 mg of plant material was ground in liquid nitrogen and quickly transferred to a tube containing 2 ml of ice-cold 80% ethanol. An equal volume of chloroform was added to remove pigments; and the tube vigorously shaken. The aqueous layer was transferred to a new tube while the interphase and lower phase were re-extracted with ethanol and chloroform. The two aqueous extracts were combined.

Dowex 50(H^+) resin was immersed in excess 2 M HCl for a few min and then washed several times with excess deionized water. The resin was immersed in excess 2 M ammonium hydroxide for a few min and then rinsed as before. Prior to use, 80% ethanol was passed through the column. A portion of the ethanol-soluble extract was passed through a Dowex 50(H^+) column and the column was rinsed with excess deionized water. Amino acids were eluted with 2 M ammonium hydroxide. The eluate was dried *in*

vacuo at 40 °C or less and then redissolved in 0.5 ml 10% iso-propanol.

The concentratrated amino acid extract was spotted no more than 5 mm wide on a sheet of 3MM chromatography paper. The amino acids were separated firstly in phenol/water (20:5) for about 12 h. The chromatogram was allowed to air-dry for a few hours. Then it was ran in the second dimension in butanol/acetic acid/water (4:1:1) for 18 h. During all stages the ambient temperature was 22±3 °C. Finally, the chromatograms were dried at room temperature and sprayed with 0.2% ninhydrin in absolute ethanol. The chromatograms were placed in an oven at 60 °C until coloured spots appeared (i.e., approximately one hour). The R_f values were determined and the spot corresponding to the purple GABA spot cut out.

The paper was placed in a test tube and the colour eluted by adding 1.5 ml 80% ethanol and leaving the tube for 5 min before vortexing briefly. The paper was removed and the absorbance of the solution was read at 570 nm. Five replicates of each treatment were made.

6.5 Genomic DNA Isolation

The leaf material was washed under tap water to remove dirt. It was then immersed in 70% ethanol for one minute and transferred to a third (a) strength Jik® solution before rinsing in excess water. Approximately 5 g of tissue was used per extraction.

A teflon tube containing 5 ml of extraction buffer (Appendix I) was warmed in a 70 °C waterbath prior to grinding the leaf tissue to a fine pulp in liquid nitrogen. The ground tissue was quickly transferred to a teflon tube containing the extraction buffer and shaken vigourously. The tube was incubated for 5 min at 70 °C with occasional shaking. An equal volume of phenol/chloroform (1:1) was added and thoroughly mixed. The two phases were separated by centrifugation in a Sorvall SS34 centrifuge at 8000 rpm for 10 min.

The aqueous phase was transferred to a new teflon tube and then NaCl and Cetyltrimethylammonium bromide (CTAB) were added to final concentrations of 0.7 M and 1%, respectively. The contents were mixed thoroughly and the tube incubated at 60 °C for 10 min. An equal volume of isoamyl alcohol/chloroform (1:1) was added, gently mixed, and spun in the SS34 rotor for 10 min at 8000 rpm. The step was repeated to remove most of

the polysaccharides. The upper phase was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1).

The upper phase was transferred to a clean teflon tube and 0.1 volumes of 3M Sodium acetate, pH 5.2 and 2.5 volumes of 100% ethanol were added and the nucleic acids precipitated in a Sigma 133 centrifuge at -20 °C for an hour. The DNA was pelleted in a SS34 rotor at 9000 rpm for 10 min. The pellet was washed three times with 70% ethanol. Residual ethanol was aspirated by vacuum drying and the DNA resuspended in 100 µl TE buffer (Appendix I).

The DNA stock was incubated with 10 $\mu g.ml^{-1}$ of DNAse-free RNAse (Appendix I) at room temperature for 15 min to degrade contaminating RNA. The DNA was selectively precipitated by addition of 150 µl of 88% iso-propanol-0.2M potassium acetate solution and left at room temperature for about 10 min. The DNA pellet was finally washed, dried and resuspended as before and then stored at -20 °C.

6.6 Alkaline Plasmid DNA Preparation

The procedure was a modification of the FELICIELLO and CHINALI (1993) method. *Escherichia coli* (DH10B) cells were grown in LB medium supplemented with ampicillin until they reached midlog

i.e., absorbance at 600 nm was 0.6. A midlog culture was used to obtain 2 ml of cell suspension and placed in a microfuge tube and pelleted at 11 000 rpm for 5 min in a microcentrifuge. The pellet was resuspended in 200 µl ice-cold STE buffer (Appendix I) and again pelleted at 11 000 rpm for 2 min. The supernatant was discarded. The pellet was completely resuspended in 100 µl GTE buffer (Appendix I) and then 100 µl of fresh solution II (Appendix I) was added and mixed by inversion. The tube was stored on ice for 5 min. A 150 µl of ice-cold 4M potassium acetate-2M acetic acid solution was added and mixed thoroughly by shaking. The tube was placed on ice for 5-10 min.

After centrifugation at 13 000 rpm for 5 min using a Sigma 133 centrifuge the supernatant was transferred to a clean tube and 140 µl isopropanol was added and mixed by inversion. The tube was centrifuged for 5 min at 10 000 rpm and the supernatant discarded. The pellet was resuspended in 50 µl TE buffer containing 10 $\mu g.ml^{-1}$ RNAse and allowed to stand at room temperature for 15 min. Thereafter, 60 µl 88% isopropanol-0.2M potassium acetate was added and the tube incubated at room temperature for 10 min. RNA was pellet by centrifugation at 13 000 rpm for 10 min and the supernatant removed with a micropipette. Last traces of isopropanol were aspirated in vacuum and the pellet dissolved in 20 µl TE buffer.

6.7 Phenol/Chloroform Extraction of DNA

The DNA that is isolated in the above manner is often contaminated with proteins and thus requires purification. An equal volume of chloroform/phenol/isoamyl alcohol (25:24:1) was mixed thoroughly with the DNA suspension in a microfuge tube. The tube was spun at 13 000 rpm for a minute in a Sigma 133 centrifuge. The upper phase was reextracted twice with phenol/chloroform/isoamyl alcohol as before and transferred to a clean tube.

The DNA was ethanol precipitated overnight as mentioned previously and then washed with 70% ethanol. The DNA was vacuum- dried before resuspending in TE buffer until further use.

6.8 Gene Copy Number Determination

6.8.1 Enzyme Restrictions

Following both plasmid and plant genomic isolations, restrictions with appropriate enzymes were carried out for a restriction analysis of both genomes. Four 100 µl restriction reactions for the plant genomic DNA were set up in microfuge

tubes for EcoRI, HindIII, KpnI and EcoRI&HindIII as shown below:

40 µg DNA

10X Restriction buffer

80 units Enzyme

0.1M Spermidine

dH_2O

The reaction was made up to 100 µl with sterile dH_2O. In case of KpnI 10 µl of 10 $mg.ml^{-1}$ Bovine serum albumin (BSA) was added. Digestion was allowed to take place at 37 °C overnight. The contents were collected at the bottom by spinning briefly and then the tubes were spiked with 80 units of the appropriate enzyme and incubated for a further 8 h.

After incubation the digested DNA was ethanol precipitated by addition of 0.1 volume 3 M sodium acetate, pH 5.2 and two volumes 100% ethanol. The DNA was allowed to precipitate overnight at -20 °C. The DNA pellet was washed three times in 70% ethanol and then dried under vacuum. The DNA was resuspended in 30 µl TE buffer, ready to be loaded onto a 0.8% agarose gel.

The GDC fragment to be used as a probe was excised firstly, by digestion with PstI and then XbaI.These sites are outside SalI

and NotI sites (Fig. 6) into which the GDC fragment is cloned. Following the first restriction with PstI the DNA was precipitated, washed and resuspended as before and the XbaI restriction was performed. The reaction was loaded in a 0.8% agarose gel and the GDC band identified with the aid of a molecular weight marker III® from Promega. The XbaI/Pst-restricted fragment was purified from the agarose using a GeneClean® (Appendix A) kit.

6.9 The Geneclean Protocol for Purifying DNA from Agarose Gel

A DNA band corresponding to the GDC fragment was excised from the agarose gel using sterile blades and placed in a microfuge tube. Care was taken not to expose the DNA to UV radiation for more than a minute. A Geneclean® kit was used to purify the GDC fragment from the agarose gel.

A 500 µl NaI solution was added and the tube incubated in a 50 °C waterbath until the gel dissolved. A glassmilk suspension was added with 5 µl of the suspension added for 5 µg or less of DNA restricted and an additional 5 µl for every 0.5 µg above the original 5 µg. The tube was vortexed for 1-2 min and placed on ice for 5 min to allow the DNA to bind to the silica matrix. The silica matrix and DNA were pelleted by centrifugation for 30 seconds. The supernatant was discarded

and the pellet washed with 200 µl of ice cold New Wash®. The pellet was resuspended in New Wash® (Appendix A) and then spun for 30 seconds at room temperature in a Sigma 133 centrifuge at 13 000 rpm. The supernatant was discarded and the wash repeated twice.

The pellet was resuspended in 10 µl of TE buffer and incubated at 50 °C for 3 min to elute the bound DNA. The glassmilk was pelleted by centrifugation and the supernatant containing DNA was transferred to a clean microfuge tube. The elution was repeated and the two supernatants combined. The DNA was stored at -20 °C until further use.

6.10 Southern Hybridisation

6.10.1 Gel Electrophoresis

Restricted plasmid DNA was heated for 2 min in a boiling waterbath and rapidly cooled on ice. The contents were collected at the bottom of the tube by centrifugation.
Loading buffer (Appendix I) was added to the DNA tube before loading it into the wells of a 0.8% agarose gel. The gel was run in Tris-Acetate (TAE) buffer (Appendix I) at 40 volts until the dye in the loading buffer had migrated a sufficient

distance (about ¾ of the length of the gel) for reasonable DNA separation to occur.

6.10.2 Processing the Gel

The gel was stained with 5 $\mu g.ml^{-1}$ ethidium bromide for 10 min and viewed under UV illumination. The gel was then covered with a depurination solution (Appendix I) and gently agitated until the bromophenol blue dye had turned yellow (about 15 min). The depurinaion solution was discarded and the gel rinsed with distilled water.

The gel was immersed in a denaturation solution for about 15 min or until the yellow colour had turned to the original blue. The denaturation solution was discarded and the gel rinsed with distilled water. The gel was then covered in neutralisation solution (Appendix I) and agitated for 30 min. The neutralisation solution was discarded and the gel agitated in a fresh neutralisation solution for 10 min. The gel was then rinsed in distilled water.

In a glass bowl, 400 ml of 20X SSC solution was poured and two sheets of 3MM Whatmann chromatography paper used as a wick. The gel was placed on a glass platform and a nitrocellulose membrane of the exact size placed on the gel. Three sheets of

3MM filter paper the size of the gel, were soaked in 10X SSC and were placed on top of the membrane. Paper towels, at least 10 cm high were stacked on the gel with a weight placed on top. The stack was left overnight for DNA transfer to occur.

6.10.3 Processing the Membrane

The blotting stack was dismantled and the membrane rinsed in 6X SSC for a minute. A 3MM filter paper was wetted in a 0.4 M NaOH solution. The membrane was placed DNA-side up on the filter paper for 15 min. After DNA fixation the membrane was placed in 2X SSC while 5% blocking agent was warmed up to 42 °C. The membrane was rolled up in miracloth and placed in a hybridization tube. Blocking reagent was poured into the tube and the membrane was allowed to prehybridize for one hour at 42 °C.

6.10.4 Probe Labelling

A GDC fragment excised and recovered from the gel using the Geneclean® kit was labelled for use as probe. The probe was at a 10 $ng.\mu l^{-1}$ concentration and 300 ng of probe used. The DNA was denatured by heating for 5 min in a boiling waterbath and

cooled immediately on ice for 5 min. An equal volume (30 µl) of labelling reagent was added and mixed gently. Then 30 µl of glutaraldehyde was added and the contents of the microfuge collected at the bottom of the tube by spinning briely. The mixture was incubated at 37 °C for 10 min. The labelled probe was added to the hybridization buffer and allowed to hybridize with membrane-bound DNA at constant agitation. Hybridization was carried out overnight at 42 °C in a hybridization oven with constant agitation.

6.10.5 Stringency Washes

Hybridization buffer was discarded and replaced with 2.5 ml.cm^{-2} of prewarmed 5X SSC and washed for 5 min. The solution was replaced with 2.5 ml.cm^{-2} of primary wash buffer and washed for 20 min. The wash buffer was discarded and replaced with an equivalent volume of fresh primary wash buffer and the membrane washed for 10 min. The 10 min wash was repeated. All wash buffers were prewarmed and washes carried out at 42 °C.

The membrane was removed from the hybridization tube and placed in a glass bowl containing excess secondary wash buffer. The membrane was agitated in the secondary wash bath for 5 min at room temeperature. The secondary wash buffer was replaced with fresh buffer and agitated for a further 5 min.

6.10.6 Signal Detection

Equal parts of 0.125 $ml.cm^{-2}$ solutions A and B (ECL™)were poured on the DNA side of the membrane and allowed to stand for 30 seconds. Excess liquid was blotted and the membrane wrapped in Gladwrap®. Care was taken not to include air bubbles and the membrane exposed to an X-ray film for 2 h.

6.11 The Dideoxy Chain-Termination Sequencing

In order to establish the exact base composition of the glutamate decarboxylase gene, the cDNA fragment in pZL1 was sequenced. A commercial variant of bacteriophage T7 polymerase, Sequanase® II (US Biochemicals) was used.

For this purpose 5 µg of phenol/chloroform-extracted pZL1 was used for each primer. The DNA was denatured by addition of 0.1 volume of 3 mM EDTA/0.2M NaOH solution and incubated at 37 âC for 30 min.

A 0.1 volume of 3 M sodium acetate,pH 5.2 was added and the DNA precipitated with absolute ethanol at -70 °C for 15 min. The DNA was pelleted by centrifugation for 15 min and the pellet washed with 70% ethanol. The tube was centrifuged for

10 min, the supernatant discarded, and the DNA dried under vacuum. The DNA was resuspended in 7 µl dH_2O. A separate 10 µl volume of annealing mixture containing either the T7 or the SP6 primer was prepared as below:

Annealing mixture: DNA	7 µl
Sequanase reaction buffer	2 µl
Primer	1 µl
Total	10 µl

The mixture was heated for 2 min in a 65 °C waterbath and then left to cool in the waterbath to 35 °C thus allowing the T7 and SP6 primers to anneal to the template DNA. The tube was chilled on ice until further use.

The DNA was then ready for radioactive labelling and Sequanase® II was added to the DNA in the presence of [^{35}S] dATP. The enzyme was diluted 1:7 before use in the reaction mixture set out below:

Labelling mixture:	Annealed DNA	10 µl
	Labelling mix	2.0 µl
	[^{35}S]dATP	0.5 µl
	Sequanase polymerase	2.0 µl
	0.1 M Dithiothreiotol (DTT)	1.0 µl
	Manganese buffer	1.0 µl
	Total	16.5 µl

The labelling mix and polymerase were diluted five-fold and eight-fold, respectively. The labelling mix was diluted with dGTP while the Sequanase was diluted with sequanase buffer. The mixture was incubated at room temperature for 2-5 min while the polymerase action and labelling took place. In the meantime, four microfuge tubes each containing 2.5 µl of each of the four dNTP's were prewarmed at 37 °C. After the labelling reaction, 3.5 µl was removed and transferred to each of the tubes where DNA strand synthesis would be terminated by the incorporation of ddNTP's. The reaction was stopped after 5 min by addition of 4 µl of stop solution.

The gel was connected to the power supply and prewarmed to 50 °C and the samples were loaded. The samples were heated for 2 min at 75 °C before 3 µl were loaded onto an 8% polyacrylamide gel (Appendix I). The gel was run in Tris borate buffer (TBE) for 3 h with the second set of samples run for another 3 h.

6.12 Sequence Analysis

Once part of the GDC clone had been sequenced it was compared to the EST sequence to determine homology. In this case the EST used was generated with a T7 primer and thus the end of the cDNA sequenced with the T7 primer was compared for homology. The sequences were compared with other sequences in the Genbank database via the Blast electronic mail server at

blast@ncbi.nlm.nih.gov. Further homology studies and restriction site analyses were carried out with the computer program called Seqaid II, Version 3.81.

CHAPTER 7

RESULTS

7.1 GABA Accumulation During Water Stress

Under normal physiological conditions, mature *Arabidopsis* leaves maintain a pool of GABA at about 19.5 $\mu mol.g^{-1}$ of their dry weight (Figs. 7-12). However, when water was withheld over a prolonged period, GABA accumulated significantly. The GABA content of *Arabidopsis* leaves was about 97% more than the normal content after six hours of water stress. GABA continued to increase over the stress period though not significantly more than the initial increase. The highest amount was recorded in the last six hours of water deprivation when GABA levels were over 130% of the control levels. It is also obvious from Figure 7 that these high GABA levels persisted over the 24 h period of water deprivation.

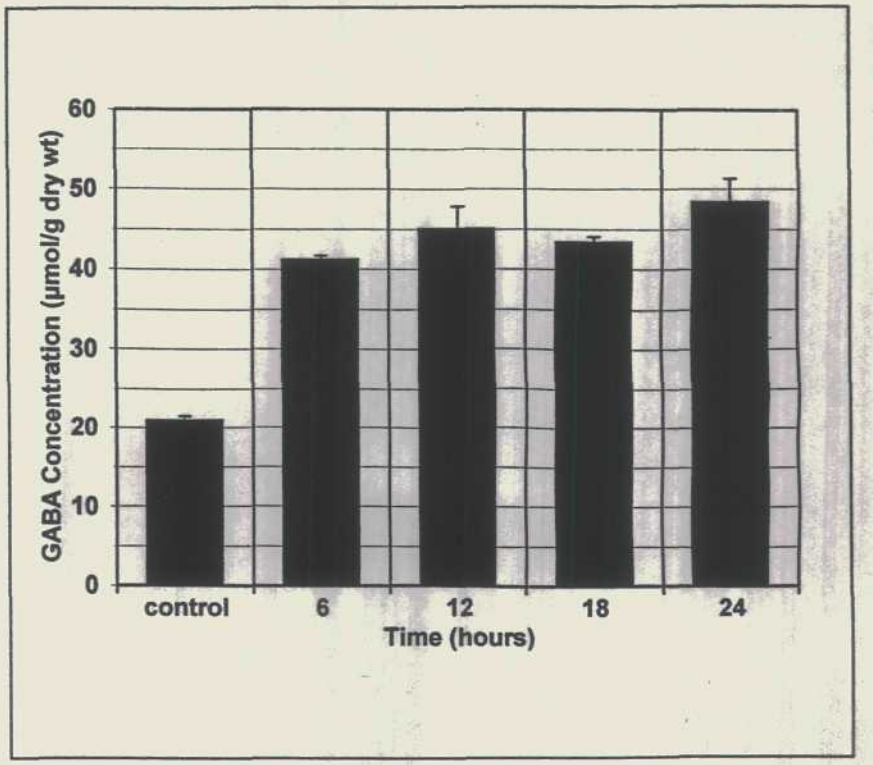

Fig. 7: GABA content of excised *Arabidopsis* leaves at six h intervals after water was withheld over a 24 h period. Each value represents the average of three replicates.The error bar indicates the 95% confidence limit.

7.2 The Accumulation of GABA due to Salinity Stress

An increase in the content of GABA was observed when plants were watered with NaCl solutions of increasing concentration (Fig. 8). An average increase of 31% was noted due to 50 mM NaCl. A further increase of about 15% of the total GABA pool was observed when the NaCl concentration was increased from 50

mM to 100 mM NaCl. The overall increase in GABA due to a 150 mM solution was almost double the amount recorded for control plants after 24 h.

Polyethyl glycol (PEG) was used to determine how much of the GABA accumulated was due to the hyperosmotic stress associated with high salinity. PEG concentrations with equivalent osmotic potential as the NaCl concentrations also caused an accumulation of GABA. PEG elicited higher amounts of GABA at concentrations equivalent to 50 mM and 100 mM NaCl. The GABA contents due to 50 mM and 100 mM NaCl were, respectively, 25% and 17% lower than equivalent PEG concentrations of control levels. Contrary to the trend, the increase in GABA due to 150 mM NaCl was higher and rose by 99% while the equivalent concentration of PEG caused an increase of only 81%.

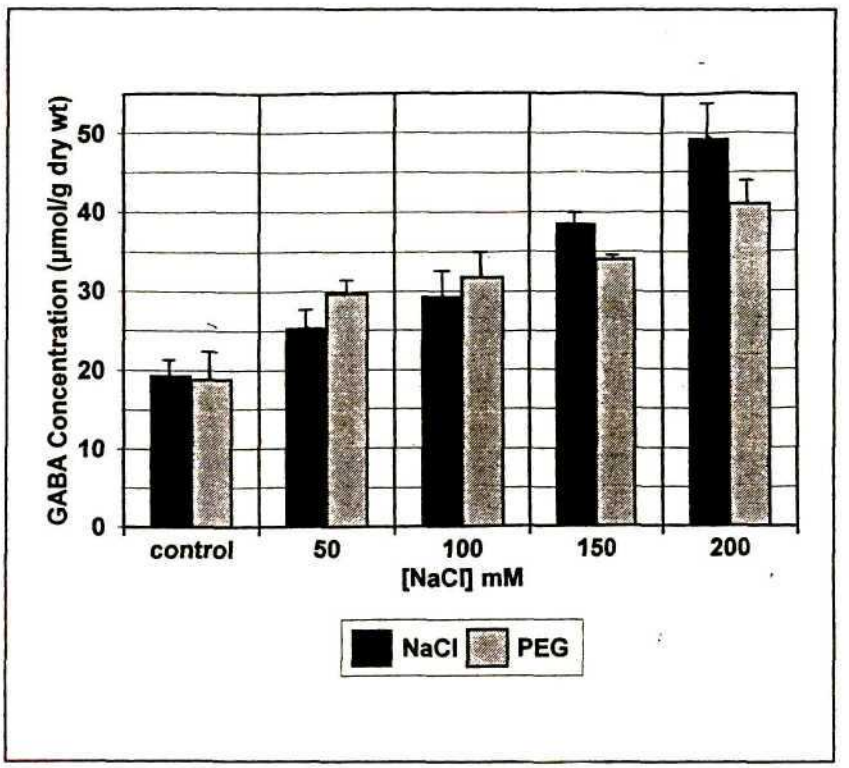

Fig. 8: Changes in the GABA content of excised *Arabidopsis* leaves in plants watered with 50 mM, 100 mM, 150 mM and 200 mM NaCl concentrations. A second set of plants were watered with PEG solutions of equivalent osmotic potential to the NaCl concentrations. The leaves were harvested after 24 h. Each value represents the average of three replicates. The error bar indicates the 95% confidence limit.

7.3 GABA Accumulation due to Temperature Changes

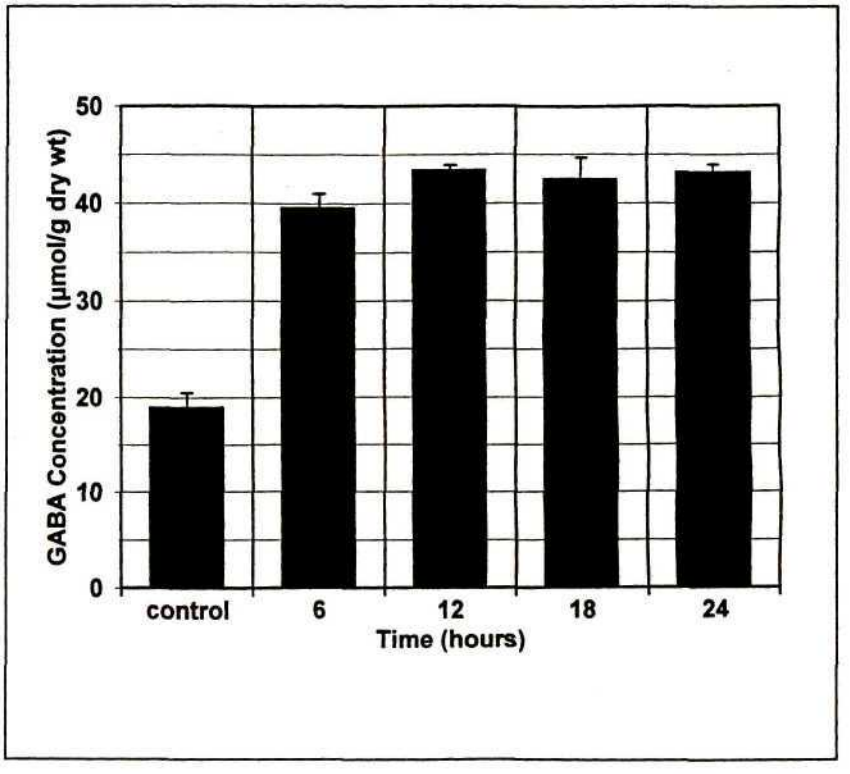

Fig. 9: Changes in the GABA content of excised *Arabidopsis* leaves in plants incubated at 15 °C, harvested at six hour intervals over a 24 h period. Each value represents the average of three replicates. The error bar indicares the 95% confidence limit.

Incubation of plants at 15 °C invoked an increase of 129% of GABA within the first six hours in excised leaves of *Arabidopsis* (Fig. 9). Although other stresses may also induce significantly high levels of GABA during this period, the cold stress seems to induce an even more drastic response with the

GABA content more than double that of control plants. GABA levels did not increase further and seemed to reach a plateau in the remaining 18h of the experiment.

When plants were incubated at 35 °C an increase in GABA from 19 µmol to 30.6 µmol per g dry weight, a 63% increase was observed within the first six hours. A small decrease was noted over the next six hours and then declined sharply (Fig. 10). The amount of GABA in the leaves fell by 31% from its highest level achieved during the 6 h interval.

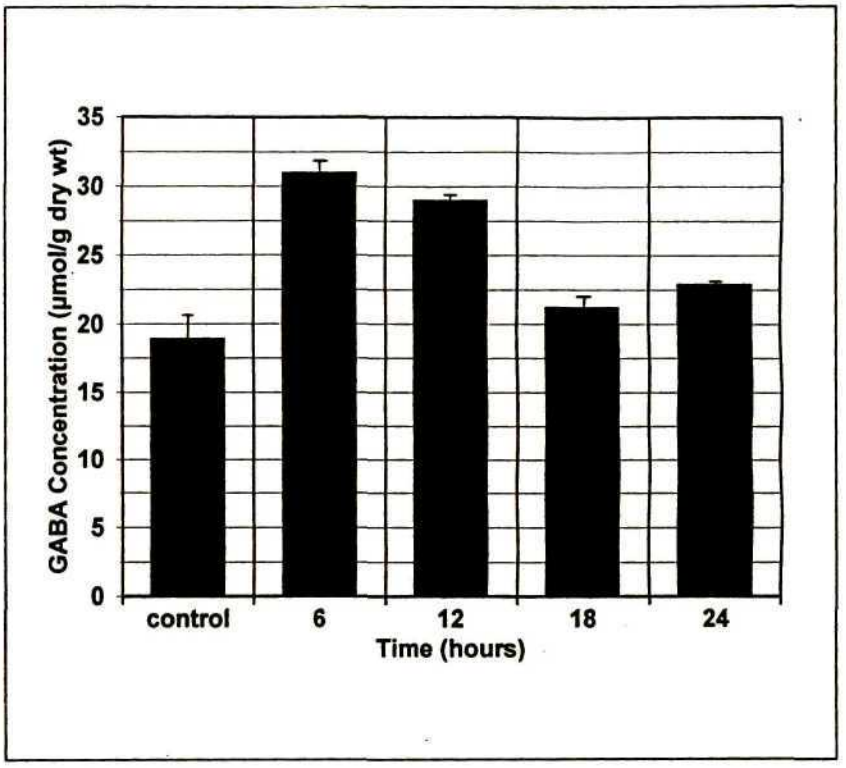

Fig. 10: Changes in the GABA content of excised *Arabidopsis* leaves in plants incubated at 35 °C and harvested every six hours over a 24 h period. Each value represents an average of

three replicates. The error bar indicares the 95% confidence level.

7.4 The Effect of Light on GABA Accumulation

Alteration of the photoperiod during the incubation of plants caused a change in the GABA content of mature *Arabidopsis* leaves.

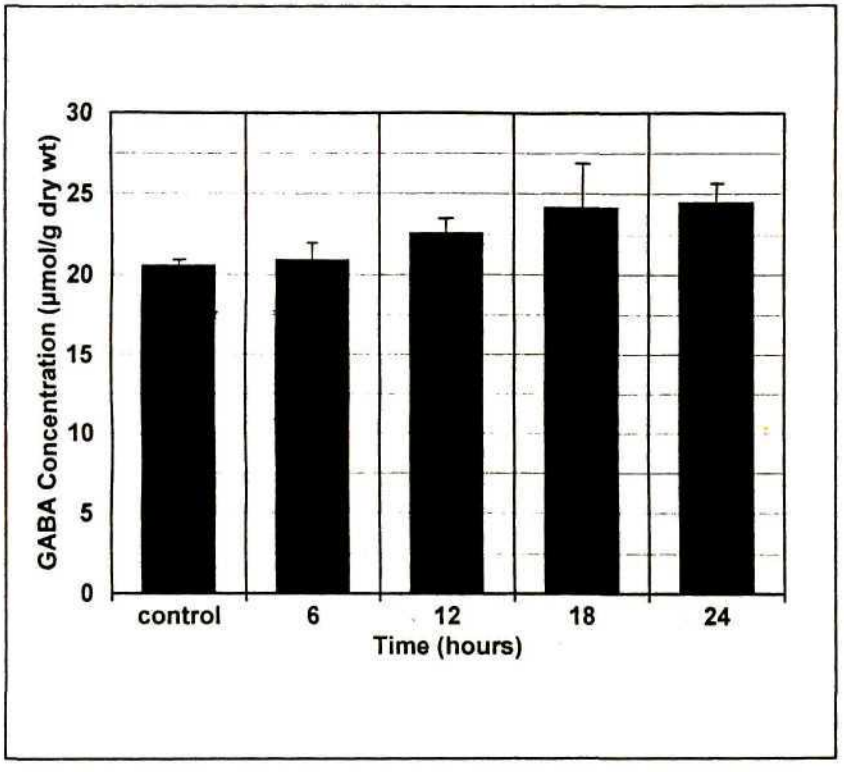

Fig. 11: The GABA content of excised *Arabidopsis* leaves placed under constant light conditions (0.5 µmol m^{-2} s^{-1}) at 25 °C and harvested at six hour intervals over a 24 h period. Each value

represents the average of three replicates.The error bar indicates the 95% confidence level.

The overall increase in the GABA content of leaves subjected to constant light was less than that observed for other stress treatments. Although GABA did not show any significant increase in the first six hours, there was a slight increase after 12 h of incubation. The increase levelled off after an average 18% rise in the last 12 h of constant light (Fig. 11). In plants under continuous darkness, GABA accumulation was noticeably higher in the first 12 h of incubation (Fig. 12). The GABA content then declined significantly during the last six hours of the incubation period.

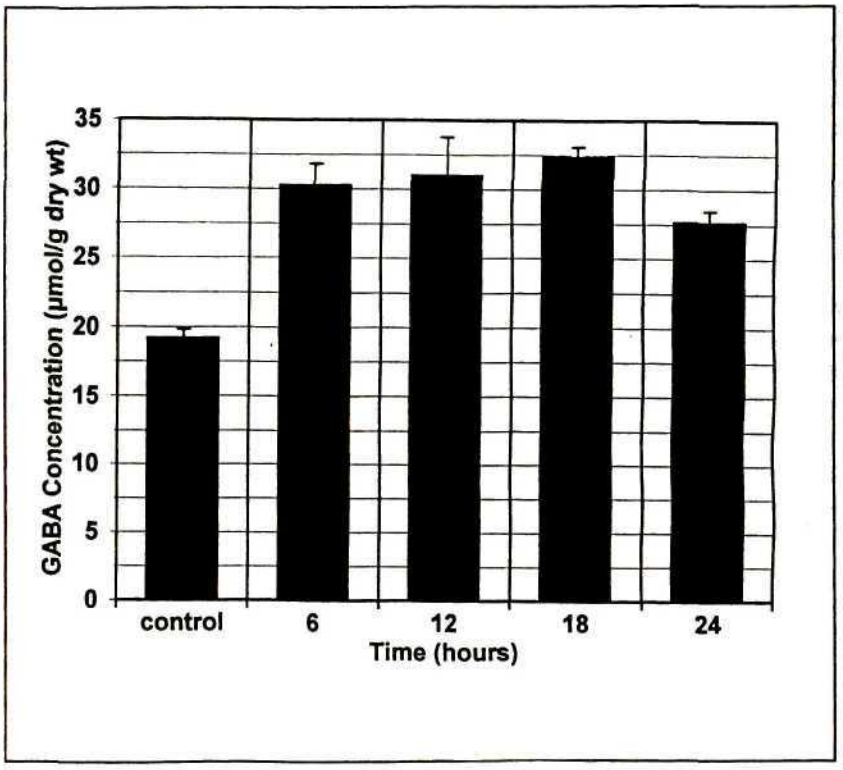

Fig. 12: The GABA content of excised *Arabidopsis* leaves incubated in the dark at 25 °C and sampled every six hours over a 24 h period. Each value represents the average of three replicates. The error bar indicates the 95% confidence level.

7.5 Restriction Analysis of the GDC cDNA Clone

Restriction analysis of the GDC cDNA clone revealed a fragment of about 1.5 kb in size (Fig. 13). The intact fragment could be excised with PstI and XbaI (lane 3) confirming the presence of these sites in the multiple cloning region of the plasmid pZLI. The fragment could also be excised with EcoRI & HindIII and EcoRI & BamHI although EcoRI on both occasions proved to have an internal site within the fragment (Fig.13). EcoRI cleaved the GDC gene into two fragments of about 0.65 kb and 0.85 kb in size which appear faintly in lanes 1 and 2. It was also established that KpnI (lane 4) could only linearise the plasmid due to the presence of a cleavage site within the multiple cloning site but had no cleavage site within the GDC fragment. These observations were used in the subsequent analysis of the gene copy number.

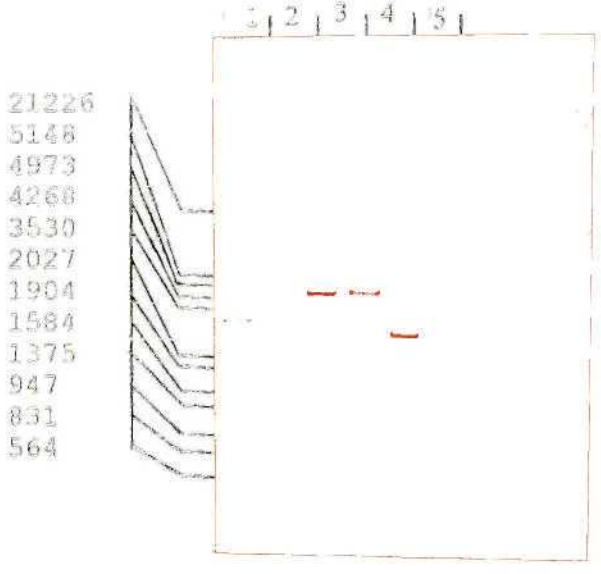

Fig. 13 Restriction analysis of the *A. thaliana* GDC cDNA clone. The vector was restricted with

EcoRI and HindII (lane 1); EcoRI and BamHI (lane 2) KpnI (lane 3); XbaI and PstI (lane 4); uncut (lane 5).

7.6 Genomic Copy Number of GDC Gene

A complete digestion of 40 µg of *Arabidopsis* genomic DNA was obtained following digestions with EcoRI; EcoRI and HindIII; HindIII; and KpnI.

Figure 14 represents an X-ray film of the nylon membrane after chemiluminescent exposure for two hours. The band which showed homology to the 1.5 kb GDC probe in the first lane represents a fragment of about 0.6 kb in size. This lane contained genomic DNA digested with EcoRI only. The second lane in which the DNA was digested with EcoRI and HindIII also produced a band which showed homology to the probe. The fragment size was about 1.0 kb. No signal was detected for the KpnI digestion in lane 4. The exact signal pattern was detected when the experiment was repeated. As a control the probe was hybridized to itself at the top right hand corner of Figure 14.

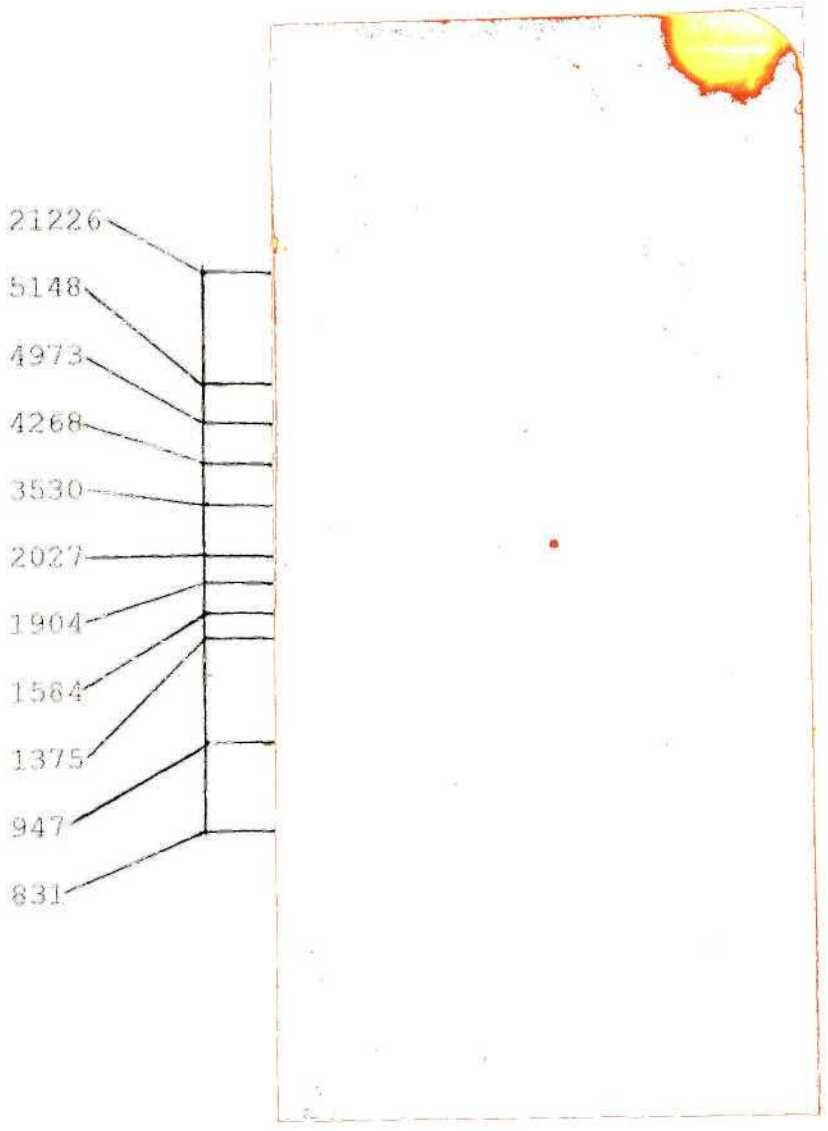

Fig. 14 Homology of the genomic DNA of A. *thaliana* to the 1.5 kb GDC cDNA fragment which was used as probe after hybridisation and signal detection on the X-Ray film.

7.7 GDC Gene Sequence and Homology Comparisons

The dideoxy sequencing revealed the exact sequence of a 204 bp 5' end of the cDNA clone flanked by the T7 primer while 234 bp were sequenced with the SP6 primer at the 3' end of pZL1. A comparison between the sequenced regions of the clone and the GDC EST showed 100% homology with the T7-primed sequence of the clone (Fig. 15(a)). It is also known that the EST for this clone was generated with a T7 primer thus confirming the authenticity of the clone.

CACTTTCTCTCTCTTATCATCATCTCCTGGTATTCTCTCTCTCTCATCTCCGTG**ATGGTG** 60
CTCTCCCACGCCGTATCGGAGTCGGACGTCTCCGTCCACTCCACATTCGCATCACGTTAC 120
GTCCGTACTTCACTTCCTAGGTTCAAGATGCCGGAAAACTCGATTCCTAAGGAAGCGGCG 180
TATCAGATCATCAACGACGAGCTG 204

(a)

ATGCGTGAGCTCGATGAGCTTCCTTCGAGAGTGATTCACAAAATATCACTTGGACAAGAG 60
AAGAGTGAATCTAACAGCGATAACTTGATGGTCACGGTGAAGAAGAGCGATATCGACAAG 120
CAGAGAGATATCATCACTGGCTGGAAGAAGTTTGTCGCCGACAGGAAGAAGACGAGTGGT 180
ATCTGCTAATTCTTTGTTTGTTTCGTTTCTTTGCCGTCTTTTTCCCTTTCTTTC 234

(b)

Fig. 15 The sequenced ends of the GDC cDNA clone. (a) The sequence was obtained with the use of the T7 primer while (b) was an SP6-primed end of the same fragment. The regions shown in bold are homologous to the complete cDNA sequence of *A. thaliana* GDC.

The sequences were compared with complete sequences in the Genbank database via the Blast electronic mail server. Parts of both sequences in Figure 15 showed homology with the *A. thaliana* glutamate decarboxylase cDNA gene identified as GADI (other authors use the abbreviation, GAD instead of GDC). Homology to GADI was from base 55 to 234 for the 5' end (T7-primed) sequence and from base one to 189 for the 3' end (SP6-primed) sequence. The parts of both sequences which showed no homology to GADI could have been part of the vector, pZLI.

Using SEQAIDII to analyse GADI revealed that it had an internal EcoRI site (Fig. 16) which confirmed observations of the restriction analysis of the clone in Figure 12. According to the analysis the EcoRI site cleaves GADI into fragments which are 649 kb and 860 kb in size. These results were consistent with the results depicted in Figure 13.

ATGGTGCTCTCCCACGCCGTATCGGAGTCGGACGTCTCCGTCCACTCCACATTCGCATCA 60
CGTTACGTCCGTACTTCACTTCCTAGGTTCAAGATGCCGGAAAACTCGATTCCTAAGGAA 120
GCGGCGTATCAGATCATCAACGACGAGCTGATGCTTGACGGGAATCCACGGTTGAACTTA 180
GCCTCCTTTGTGACGACATGGATGGAGCCTGAGTGTGATAAACTCATCATGTCCTCCATC 240
AACAAGAACTATGTTGACATGGACGAGTACCCCGTCACCACCGAACTTCAGAACCGATGT 300
GTGAACATGATTGCACATCTATTCAATGCACCGTTAGAAGAGGCGGAGACCGCCGTCGGA 360
GTAGGAACCGTTGGATCATCGGAGGCCATAATGTTGGCCGGTTTGGCCTTCAAGCGTAAA 20
TGGCAGAACAAGCGCAAAGCTGAAGGCAAACCCGTCGATAAACCCAACATTGTCACCGGA 480
GCCAATGTTCAAGTGTGTTGGGAGAAATTCGCTAGGTACTTTGAGGTTGAACTTAAGGAA 540
GTGAAATTGAGTGAAGGATACTATGTGATGGACCCTCAACAAGCTGTTGATATGGTTGAT 600
GAGAACACCATTTGTGTTGCGGACATTCTTGGTTCCACTCTTAATGGAGAATTCGAAGAT 660
GTTAAACTCTTGAACGATCTCTTGGTCGAAAAGAACAAAGAAACCGGATGGGATACACCA 720
ATCCACGTGGATGCGGCAAGTGGAGGATTCATTGCACCGTTTTTGTATCCGGAATTGGAA 780
TGGGACTTTAGACTTCCCTTGGTGAAGAGTATCAATGTGAGTGGTCACAAGTATGGACTT 840
GTGTACGCAGGGATTGGTTGGGTGATCTGGAGAAACAAAGAGGATTTGCCTGAGGAACTC 900
ATCTTCCATATCAATTATCTTGGTGCTGACCAACCCACCTTTACTCTCAATTTCTCCAAA 960
GGTTCAAGTCAAGTCATTGCTCAATACTACCAACTTATCCGATTGGGCCACGAGGGTTAC 1020
AGAAATGTGATGGAGAATTGCAGAGAGAATATGATCGTCCTAAGGGAAGGACTTGAGAAG 1080
ACAGAAAGGTTCAACATCGTCTCAAAGGACGAGGGAGTGCCACTTGTCGCTTTCTCCTTG 1140
AAAGATAGCAGCTGTCACACTGAGTTCGAAATCTCCGACATGCTTCGCAGGTATGGATGG 1200
ATAGTGCCGGCCTACACAATGCCTCCAAATGCACAACACATCACTGTTCTTCGTGTGGTT 1260
ATCAGAGAAGATTTCTCGAGAACACTCGCTGAGAGACTTGTGATCGATATAGAGAAAGTG 1320
ATGCGTGAGCTCGATGAGCTTCCTTCGAGAGTGATTCACAAAATATCACTTGGACAAGAG 1380
AAGAGTGAATCTAACAGCGATAACTTGATGGTCACGGTGAAGAAGAGCGATATCGACAAG 1440
CAGAGAGATATCATCACTGGCTGGAAGAAGTTTGTCGCCGACAGGAAGAAGACGAGTGGT 1500
ATCTGCTAA 1509

Fig. 16 Homology of the sequenced ends of the GDC cDNA clone to the complete sequence of the glutamate decarboxylase cDNA. The start codon ATG is shown in bold and the underlined sequence shows the EcoRI site which cleaves the gene at base 649.

Translation of the SP6-primed end sequence of the GDC cDNA yeilded a 62 amino acid sequence which corresponded with a

portion of the 502 amino acid long peptide of the *A. thaliana* cDNA established by ARAZI, BAUM, SNEDDEN, SHELP AND FROMM (unpublished data, GenBank accession number U10034). The sequence was homologous with a portion from amino acid number 440 to 502. A comparison with the carboxy-terminal sequence of petunia GDC containing the calmodulin-binding domain is shown in Figure 18.

(a) (i) 473 VKKSDIDKQRDIITGWKKFVAD.RKKTSGIC 502

(ii) 470 HKKTDSEVQLEMITAWKKFVEEKKKKTNRVC 500

(b) 473 VKKSDIDKQRDIITGWKKFVADRKKTSGIC 502

HHHHHHHHHHHHHHHHHHHHHHHHHTTTEEE

Fig. 17 (a) A comparison between the carboxy terminals of (i) *A. thaliana* and (ii) petunia GDC's. Identical amino acids are shown in bold. The underlined TrpLysLys motif which is found in some of the known calmodulin targets was present in both sequences. (b) A GOR prediction of the conformation of the carboxy-end of the *A. thaliana* GDC shows a predominantly α-helix forming peptide. H=α-helix, E=β-helix, T=turn. Amino acid codes are explained in Appendix B.

The calmodulin-binding domains of *Arabidopsis* and petunia GDC's showed a 50% amino acid identity although ARAZI, BAUM, SNEDDEN, SHELP AND FROMM (1995) found the overall identity to be over 85%. The 30 amino acid peptide shows an inclination towards an α-helix formation according to the GARNIER, OSGUTHORPE AND ROBSON (1978) predictions (Fig. 17(b)). The sequence also contains residues with an α-helix-forming tendency such as Val, Lys, Ile, Trp and Ala (CHOU AND FASMAN, 1978).

CHAPTER 8

DISCUSSION

8.1 GABA Accumulation due to Hyperosmosis

Mature leaves at the base of the rosette of *Arabidopsis thaliana*, maintain a pool of GABA at about 19.8 $\mu mol.g^{-1}$ of their dry weight under non-stress conditions. A comparative Figure of 18.5 $\mu mol.g^{-1}$ dry wt was obtained by CHIANG AND DANDEKAR (1995) using an Amino Acid Analyser. This amount is increased substantially when the plant is subjected to various abiotic stresses. The observation of GABA accumulation due to water stress, temperature variations, changes to the normal photoperiod and salt stress have been reported for various plant species. This study has confirmed not only the presence of GABA in *A. thaliana* leaves but also its accumulation due to these stresses.

Drought or water stress is one of the major restrictions of plant growth and it results in huge losses in crop production worldwide (DELAUNEY AND VERMA, 1993). At a physiological level, water stress induces a lowering of the osmotic potential as a means of maintaining cell turgor (HEUER, 1994). This osmotic adjustment can be achieved by decreased cell volume or the accumulation of non-toxic solutes within the

cell (DELAUNEY AND VERMA, 1993). The latter response invariably leads to substantial changes in the free amino acid pool of the cell. Proline and glutamate are among the amino acids which accumulate during osmoregulation (HEUER, 1994) while GABA has also been shown to accumulate substantially (CHIANG AND DANDEKAR, 1995). Water deprivation caused an increase in GABA of nearly double the amount observed under non-stress conditions (Figure 7). This accumulation is less than the four- fold increase observed by THOMPSON, STEWARD AND MORRIS (1966) in excised leaves of *Brassica rapa* after 20 h of water stress.

The accumulation of GABA which spanned the entire stress period is also in agreement with THOMPSON, STEWARD AND MORRIS (1966) who reported a continued accumulation after 70 h of water deprivation and RHODES, HANDA AND BRESSAN (1986) who reported an accumulation beyond the 50 h of stress application. In Figure 6 it can be seen that, GABA continues to accumulate throughout the stress application period with the last six hour interval showing a further 15% increase over the earlier values.

When plants are subjected to high levels of NaCl their GABA content increases. The increase in GABA due to NaCl is associated with hyperosmotic stress as shown by the effect of PEG on GABA content. The observation that PEG causes a higher

increase in GABA than NaCl (50 mM and 100 mM) could be attributed to PEG not being absorbed by plants while the salt could be sequestered to the vacuole. The same occurrence was observed for up to 200 mM NaCl for proline in *Arabidopsis* (HARE, 1995). However, this does not explain the opposite response observed for 150 mM and 200 mM NaCl in this study.

In a review of salt tolerance in crop plants, PASTERNAK (1987) loosely classified the effects of salt stress under water relations, nutritional effects and energy costs. The effect of NaCl at 50 mM and 100 mM concentrations could have been an osmotic effect without any major salt toxicity. However, the response observed for 150 mM and 200 mM could have been a result of salt toxicity in addition to the related osmotic stress thus inducing a higher GABA accumulation. Salt toxicity is viewed as a nutritional effect. PASTERNAK (1987) concedes that such classifications are artificial because these processes are interrelated and may vary from one species to another.

Arabidopsis mutants with reduced sensitivity to NaCl have been isolated by WERNER AND FINKELSTEIN (1995). The *rss* (reduced salt sensitivity) mutants could not accumulate compatible solutes such as proline like the wildtype when grown in media with concentrations of up to 150 mM NaCl. In contrast, tobacco cells adapted to NaCl accumulated GABA eight-fold when grown

in a medium containing 428 mM NaCl (BINZEL, HASEGAWA, RHODES, HANDA, HANDA AND BRESSAN, 1987). The literature reviewed in this thesis provides compelling evidence of the involvement of GABA as an osmoprotectant and the observed substantial increase of GABA seems to indicate the same. In addition the high amounts of GABA which persisted for the 24 h duration of sustained hyperosmotic stress under high salinity and water deprivation seem consistent with the role of GABA as a compatible solute.

8.2 GABA Accumulation due to Temperature

Temperature extremes induce an elevation in the amount of GABA in excised leaves of *Arabidopsis*. Naturally, *Arabidopsis* grows in a temperate to cold climate. A temperature decrease to 15 °C, that is 10 °C lower than incubation temperature of control plants, caused a over 100% increase in the GABA pool within six hours. WALLACE, SECOR AND SCHRADER (1984) also found a rapid 100% accumulation of GABA when soybean plants were transferred from 33 °C to 22 °C. GABA started accumulating within five minutes of treatment. It is also important to note that elevated levels of GABA in Figure 8 lasted for the duration of the stress period though without any significant changes.

YOSHIDA (1994) used fluorescence-ratio imaging cryomicroscopy and observed a decrease of 1.1 units in the cytoplasmic pH of cultured *Vigna radiata* cells incubated at 0 °C for 18 h. SNEDDEN CHUNG, PAULS AND BOWN (1992) have reported that the GDC activity is stimulated by cytoplasmic acidification, thus contributing to pH regulation. GABA probably accumulates due to this increased GDC activity at low temperatures. The accumulation of GABA at low temperatures could be the result of altered compartmentation of glutamate (WALLACE, SECOR AND SCHRADER, 1984). However, that suggestion was contradicted by YOSHIDA (1994) by showing that the cytoplasmic acidification was not primarily due to the increase in plasma membrane permeability.

When the temperature in the Conviron was increased from 25 °C to 35 °C a different profile of the GABA pool emerged (Fig. 10). Although a dramatic increase was observed in the first 12 h, a similarly dramatic decline in the last 12 h occurred. There seems to be no report of GABA accumulation due to high temperature to date. A correlation is thought to exist between the reduction in protein synthesis and the increased specific activity of GDC (REGGIANI, AURISANO AND MATTANA, 1993). Changes due to temperature variations could cause alterations in the lipid bilayer leading to plant injury (LYONS, 1973). Temperature changes may shift plant metabolism from protein synthesis to ion transport and amino acid synthesis

(SATYANARAYAN AND NAIR, 1990). Therefore, the transient increase in GABA for the first 12 h could be a response to a slow down in protein metabolism and a build up of a temporary nitrogen sink (AURISANO, BERTANI AND REGGIANI, 1995).

FITTER AND KAY (1987) suggested that plants have developed mechanisms by which they perceive and respond to changes in their environment. GABA has been thought to act as a signal of stress due to its efflux following accumulation (CHUNG, BOWN AND SHELP, 1992). It is possible that the elevation in the incubation temperature of this temperate-growing plant, triggered a stress response leading to GABA accumulation. The decline in GABA levels in the last 12 h of incubation could be due to its metabolism to succinate or into intermediates for amino acid synthesis.

8.3 The Effect of Light on GABA Accumulation

JORDAN AND GIVAN (1979) previously observed an increase in products of the Krebs cycle in the dark and have attributed that to an active GABA shunt. The accumulation of GABA in Figure 11 and a decline in the last interval seems to corroborate this. MIZUSAKI, NOGUCHI AND TAMAKI (1964) also reported an accumulation in GABA, accompanied by its rapid metabolism. Glycine was reported to decrease in the dark

probably due to a cessation of photorespiration and the resulting accumulation of alanine (WALLACE, SECOR AND SCHRADER, 1984). GABA is suggested as a possible amino group donor during alanine biosynthesis. The accumulation of GABA could be followed by its rapid degradation to sustain amino acid biosynthesis in the dark.

Another notable feature of the accumulation in the dark was that the GABA content was of a lower magnitude compared to other stresses. The highest increase was in the order less than 30 $\mu mol.g^{-1}$ dry weight. The accumulation of alanine when soybean plants were transferred to darkness is also indicative of an active GABA shunt with no further increase in GABA during the period of incubation (WALLACE, SECOR AND SCHRADER, 1984), probably accounting for a comparatively low accumulation of GABA in the dark.

Diurnal variations in the rate of GABA accumulation have been reported in tobacco leaves (MIZUSAKI, NOGUCHI AND TAMAKI, 1964). GABA was found to be relatively higher in the daytime than at night. The same authors also observed the rate of incorporation of the glutamate carbon into aspartate via GABA and the TCA cycle to be higher at night. In Figure 11 no major variations in the levels of GABA were observed under constant light when photorespiration would have occurred. The results seem consistent with claims of a relatively small demand on

the need to supply both the glutamate carbon and nitrogen through this route. In contrast the accumulation of GABA in the dark (Figure 12) could be a build up of a temporary carbon and nitrogen sink for the synthesis of organic acids.

8.4 Implications for Nitrogen Metabolism

The nitrogen status of a plant is thought to determine its free amino acid pool. Changes in their cytoplasmic concentration may be involved in the regulation of plant growth and nitrogen uptake (BARNEIX AND CAUSIN, 1996). It is suggested that a decrease in the growth rate due to any cause other than nitrogen deficiency induces an accumulation of amino acids for example, during water stress. These amino acids may be transported to the roots via the phloem where they could contribute to the inhibition of nitrogen uptake and/or fixation rates. The accumulation of GABA as reviewed in the literature seems to provide an indication of the status of the free amino acid pool particularly under stress. Firstly, as one of the predominant amino acids and secondly as a route through which most of the substrate for other amino acids namely, glutamate enters the TCA cycle (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995). The high increase in the GABA content under various stresses as evidenced in

this study could play a direct or indirect role in this process.

Glutamate dehydrogenase (GDH) can synthesize or deaminate glutamate depending on the environmental signals which direct its activity (SIVASANKAR AND OAKS, 1996).The primary role of this enzyme could be the replenishment of TCA intermediates. The GABA shunt is thought to provide alternative entry of glutamate into the TCA cycle while bypassing the GDH reaction, particularly during stress (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995).

8.5 The Glutamate Decarboxylase Gene

The presence of a single EcoRI site within the cDNA clone (Fig. 13) proved useful for determining the copy number of the glutamate decarboxylase gene in the *Arabidopsis* genome. A single copy gene was expected to show two bands corresponding with the 0.65 kb and 0.85 kb bands for the EcoRI and HindIII; and EcoRI digestions after hybridisation to the 1.5 kb probe. For a single copy gene a single band would have been observed for the KpnI digestion. However, in the eukaryote genome, structural genes are known to contain intervening sequences called introns prior to translation (CURTIS, 1983).

The 1.5 kb cDNA fragment excised during the restriction digestions in Figure 13 and also used as a probe did not have these introns. However, the probe showed remarkable homology to the band of about 0.6 kb in the EcoRI digestion and to a band of 0.8 kb in the EcoRI and HindIII digestion. The absence of the 0.8 kb band in the EcoRI digestion and the 0.6 kb band in the EcoRI and HindIII digestion could be the result of fragments too large to be separated properly because of the introns. The results seem to point to glutamate decarboxylase being encoded by a single copy of the gene in the genome of *A. thaliana*.

The sequenced ends of the cDNA clone in Figure 16 confirmed it as a complete cDNA sequence of *A. thaliana* glutamate decarboxylase, GenBank accession number U10034 (ARAZI, BAUM, SNEDDEN, SHELP AND FROMM, unpublished data). Restriction analyses in Figure 12 revealed an internal EcoRI site which cleaved the gene into fragments of about 0.65 kb and 0.85 kb which was confirmed by the sequence analysis of the complete cDNA sequence(Fig. 16).

BAUM, CHEN, ARAZI, TAKATSUJI AND FROMM (1993) showed that it was the carboxy end of a petunia glutamate decarboxylase which interacted with calmodulin. The same authors found the last 30 amino acids of the glutamate deacarboxylase to be important in calmodulin binding. A comparison of this carboxy region with

the partial protein sequence derived from the 3' end DNA sequence of the *A. thaliana* clone (Fig. 17(a)), revealed a 50% amino acid identity between these regions. Such a large deviation in the identities of the carboxy regions is despite an overall 85% amino acids identity between the two glutamate decarboxylases. Amino acid identity was shown to be about 36% between the carboxy terminals if a putative tomato glutamate decarboxylase and petunia while the overall similarity was about 80% (GALLEGO, WHOTTON, PICTON, GRIERSON AND GRAY, 1995).

The carboxy end of *A. thaliana* GDC has been shown to bind calmodulin with similar affinity as petunia GDC (ARAZI, BAUM, SNEDDEN, SHELP AND FROMM, 1995). The TrpLysLys motif which was reported as important to the petunia GDC-calmodulin interaction (BAUM, CHEN, ARAZI, TAKATSUJI AND FROMM, 1993) was also found in the *A. thaliana* carboxy peptide (Fig. 17(a)). The motif has been reported in some of the known calmodulin targets such as the skeletal muscle myosin light chain kinase (MEANS, 1988). Although a putative tomato GDC had a calmodulin-binding site it did not possess this motif (GALLEGO, WHOTTON, GRIERSON, GRAY, 1995).

The Garnier-Osguthorpe-Robson (GOR) prediction of the secondary structure conformations, performed with Seqaid II showed that the peptide was likely to form an α-helix (Fig. 17(b)). The Chou- Fasman analysis for the probability of the

folding patterns of the *A. thaliana* carboxy peptide confirmed this as well. These analyses compare favourably with the predicted α-helical structure of the calmodulin-binding domain of petunia GDC (BAUM, CHEN, ARAZI, TAKATSUJI AND FROMM, 1993) and tomato GDC (GALLEGO, WHOTTON, GRIERSON AND GRAY, 1995). One of the dominant characteristics of proteins known to bind calmodulin/$Ca2^{+}$ is the potential to form amphipathic α-helix structures (MEANS, 1988).

Sequence deviation within the calmodulin-binding regions of glutamate decarboxylases from various species could be the result of the existence of multiple isoforms of calmodulin. LING, PERERA AND ZIELINSKI (1991) identified two calmodulin isoforms in *Arabidopsis* while a further three unique isoforms were described by GAWIENOWSKI, SZYMANSKI, PERERA AND ZIELINSKI (1993). It has been suggested that *Arabidopsis* calmodulin-encoding sequences share more similarities with one another than with other plants due probably to having evolved unique sets of calmodulin sequences (GAWIENOWSKI, SZYMANSKI, PERERA AND ZIELINSKI, 1993).

8.6 GABA Synthesis During Stress: A Molecular Understanding

The content of GABA in basal leaves of *Arabidopsis* increases due to environmental factors discussed in this study. The possibility that GDC is encoded by a single gene in the *Arabidopsis* genome could exclude the occurrence of high amounts of GDC mRNA and ultimately the enzyme due to the presence of multiple genes. Whether GDC gene expression plays a role in GABA synthesis when the plant is subjected to stress still remains to be examined.

GABA synthesis under hyperosmotic stress leads to its accumulation. GDC activity was reported to be higher than GABA:transaminase activity (WALLACE, SECOR AND SCHRADER, 1984) and led SATYANARAYAN AND NAIR (1986) to suggest that it was the slow degradation of GABA that led to its accumulation. This would be important particularly during hyperosmotic stress where GABA serves as an osmoprotectant. However, at high incubation temperatures GABA accumulation probably accedes to other metabolic requirements such as energy needs. The GABA shunt has been thought to play a role in the provision of eneygy during stress (SHELP, WALTON, SNEDDEN, TUIN, ORESNIK AND LAYZELL, 1995).

The cytoplasmic location of GDC where the pH is neutral while its optimum pH is 5.8 implies that the enzyme will be

sensitive to pH decreases (FELLE, 1988). Cytoplasmic acidification has been associated with low temperatures (YOSHIDA, 1994) while glutamate, a GDC substrate has been implicated in the H^+-symport into the cytoplasm (SNEDDEN CHUNG, PAULS AND BOWN, 1992). GDC activity and the synthesis of GABA has been shown to increase due to the lowering of pH. However, GABA accumulation could not always be explained by the reduced cytoplasmic pH (CRAWFORD, BOWN, BREITKREUZ AND GUINEL, 1994).

The stimulation of GDC could also involve changes in the distribution of Ca^{2+}. Some environmental stress conditions such as low temperatures may also involve Ca^{2+}-binding protein, calmodulin (CRAWFORD, BOWN, BREITKREUZ AND GUINEL, 1994). The carboxy terminal of *Arabidopsis* GDC was found to possess characteristics associated with calmodulin-activated proteins. ARAZI, BAUM, SNEDDEN, SHELP AND FROMM (1995) showed that the carboxy end of the *Arabidopsis* GDC could bind to calmodulin. However, the activity of petunia GDC was not affected by the absence of CaM/Ca^{2+} although it could bind to calmodulin. Therefore, whether *Arabidopsis* GDC is regulated by calmodulin as in the case of *Vicia fava* and *Glycine max* GDC's, is still to be established. The evidence that the stimulation of GDC due to CaM/Ca^{2+} and pH shifts as physiological consequences of environmetal stress leading to GABA accumulation is still circumstantial.

The possible involvement of Ca^{2+} in the regulation of GDC could point to signal transduction in the perception, relaying and response to abiotic stress in *Arabidopsis thaliana* and the involvement of GABA in that process. Further examinations should take into account the importance of GABA under non-stress conditions and establish its role in the plant's normal metabolism. Proline also accumulates substantially in many plant species under stress and is also formed from glutamate. Further light has to be shed on the effect of GABA and proline on the glutamate pool particularly during stress. The speculation that GABA and proline synthesis are mechanisms of sequestering glutamate during stress also has to be established as is how the glutamate pool is partitioned between the respective pathways?

REFERENCES

ARAZI, T., G. BAUM, W.A. SNEDDEN, B.J. SHELP AND H. FROMM. (1995). Molecular and biochemical analysis of calmodulin interactions with the calmodulin-binding domain of plant glutamate decarboxylase. *Plant Physiology* 108: 551-561.

AURISANO, N., A. BERTANI AND R. REGGIANI. (1995). Anaerobic accumulation of 4-aminobutyrate in rice seedlings; causes and significance. *Phytochemistry* 38: 1147-1150.

BAGNI, N. AND R. PISTOCCHI. (1992). Polyamine metabolism and compartmentation in plant cells. In: Nitrogen Metabolism of Plants. K. Mengel and D.J. Pilbream (Eds.) Oxford University Press, New York, pp.229-248. ISBN 0-19-857752-4.

BALINT, R., G. COOPER, M. STAEBELL, P. FILNER. (1987). N-Caffeoyl-4-amino-η-butyric acid, a new flower-specific metabolite in cultured tobacco cells and tobacco plants. *Journal of Biological Chemistry* 262: 11026-11031.

BARNEIX, A.J., AND H.F. CAUSIN. (1996). The central role of amino acids on nitrogen utilization and plant growth. *Journal of Plant Physiology* 149: 358-362.

BARNS, R.L., AND A.W. NAYLOR. (1959). The effect of various nitrogen sources on growth of isolated roots of *Pinus serotina*. *Physiologia Plantarum* 12: 82-89.

BAUM, G., Y. CHEN, T. ARAZI, H. TAKATSU AND H. FROMM. (1993). A plant glutamate decarboxylase containing a calmodulin-binding domain. *Journal of Biological Chemistry* 268: 19610-19617.

BEEVERS, L. (1976). Nitrogen nutrition. Nitrogen Metabolism in Plants. Elsevier Publishing Company, New York, pp. 1-27. ISBN 0- 7131-2514-4.

BENDER, D.A., (1975). Amino acids. Amino Acid Metabolism. John Wiley & Sons, London, pp. 80-111. ISBN 0-471-06498-X

BENZIL, M.L., P.M. HASEGAWA, D. RHODES, S. HANDA, A. HANDA AND R.A. BRESSAN. (1987). Solute accumulation in tobacco cells adapted to NaCl. *Plant Physiology* 84: 1408-1415.

BOWN, A.W., I. CHUNG, W. SNEDDEN AND B. SHELP. (1989). Specific glutamate cotransport into mesophyll cells and efflux of the major metabolite 4-aminobutyric acid. In: Plant Membrane Transport: The Current Position. J. Dainty, M.I. De Michelis, E. Marre, F. Rassi-Caldogno (Eds.) Elsevier, New York, pp. 329-334.

BRAY, C.M. (1983). Interconversion of nitrogen. Nitrogen Metabolism in Plants. Longman Group Ltd., London, pp. 1-29. ISBN 0-582-44640-6.

BREITKREUZ, K.E. AND B.J. SHELP. (1995). Subcellular compartmentation of the 4-aminobutyrate shunt in protoplast from developing soybean cotyledons. *Plant Physiology* 108: 99-103.

BROWN, E.G. (1995) Biogenesis of N-heterocyclic amino acids by plants: mechanisms of biological significance. In: Amino acids and their Derivatives in Higher Plants. R.M. Wallsgrove (Ed.) Cambridge University Press, Cambridge, pp. 119-144. ISBN 0-521- 45453-0.

CARROLL, A.D., G.G. FOX, S. LAURIE, R. PHILLIPS, R.G. RATCLIFFE AND G.R. STEWART. (1994). Ammonium assimilation and the role of γ-aminobutyric acid in pH homeostasis in carrot cell suspension. *Plant Physiology* 106: 513-520.

CHEN, Y., G. BAUM AND H. FROMM. (1994). The 58-kilodalton calmodulin-binding glutamate decarboxylase is a ubiquitous protein in petunia organs and its expression is developmentally regulated. *Plant Physiology* 106: 1381-1387.

CHIANG, H.H., AND A.M. DANDEKAR. (1995). Regulation of proline accumulation in *Arabidopsis thaliana* (L.) Heynh during development and in response to desiccation. *Plant, Cell and Environment* 18: 1280-1290.

CHOU, P.Y. AND G.D. FASMAN. (1978). Empirical predictions of protein conformation. *Annual Review of Biochemistry* 447: 251-276.

CHUNG, I., A.L. BOWN AND B.J. SHELP. (1992). The production and efflux of 4-aminobutyrate in isolated mesophyll cells. *Plant Physiology* 99: 659-664.

CRAWFORD, L.E., A.W. BOWN , K.E. BREITKREUZ AND F.C. GUINEL. (1994). The synthesis of γ-aminobutyric acid in response to treatments reducing cytosolic pH. *Plant Physiology* 104: 865-875.

CURTIS, H. (1983). The eukaryotic chromosome. Biology. Fourth Edition.Worth Publishers Inc., New York, pp. 327-346. ISBN 0-87901-186-6.

DAUGHERTY, C.J., M.F. ROONEY, A. PAUL, N. DE VETTEN, M.A. VEGAPALAS, G. LU, W.B. GURLEY AND R.J. FERL. (1994). Environmental stress and gene regulation. In: Arabidopsis.

E.M. Meyerowitz and C.R. Sommerville (Eds.) Cold Spring Harbour Laboratory Press, New York, pp. ISBN 0-87969-428-9.

DAVIES, D.D. (1979). Factors affecting protein turn-over in plants. In: Nitrogen Metabolism Assimilation of Plants. E.J. Hewitt and C.V. Cutting (Eds.) Academic Press Ltd., London, 369- 396. ISBN 0-12-346360-2.

DAVIES, D.D. (1973). Metabolic control in higher plants. In: Biosynthesis and its Control in Plants.B.V Milborrow (Ed.) Academic Press Ltd., London, pp. 1-20. ISBN 0-12-496150-9.

DELAUNEY, A.J. AND D.S VERMA. (1993) Proline biosynthesis and osmoregulation in plants. *The Plant Journal* 4: 215-223.

DIXON, R.O.D. AND L. FOWDEN. (1961). γ-Aminobutyric acid metabolism in plants. Part 2. Metabolism in higher plants. *Annals of Botany* 25: 513-530.

ERLANER, M.G. AND A.J. TOBIN. (1991). The structural and functional heterogeneity of glutamic acid decarboxylases: A review. *Neurochemical Research* 16: 215-226.

FELICIELLO, I. AND G. CHINALI. (1993). A modified alkaline lysis method for the preparation of highly purified plasmid

DNA from *Escherichia coli*. *Analytical Biochemistry* 212: 394-401.

FELLE, H. (1988). Short-term pH regulation in plants. *Physiologia Plantarum* 74: 583-591.

FITTER, A.H. AND R.K.M KAY. (1987). Introduction. Environmental Physiology of Plants. Second edition. Academic Press Ltd., London, pp. 3-20. ISBN 0-12-257764-7.

FORD, Y., R.G. RATCLIFFE AND R.J. ROBINS. (1996). Phytohormone-induced GABA production in transformed root cultures of Datura stramonium: an in vivo ^{15}N NMR study. *Journal of Experimental Botany* 47: 811-818.

FRENEY, J.R. AND A.H. GIBSON. (1975). Accumulation of 4-aminobutyrate in *Trifolium subtarraneum* root cultures: Effect of *Rhizobium trifolii* strain on aminotransferase activity. *Journal of Plant Physiology* 2: 663-668.

GALLEGO, P.P. L. WHOTTON, S. PICTON, D. GRIERSON AND J.E. GRAY. (1995). A role for glutamate decarboxylase during tomato ripening: the characterisation of a cDNA encoding a putative glutamate decarboxylase with a calmodulin-binding site. *Plant Molecular Biology* 27: 1143-1151.

GARNIER, J., D.J. OSGUTHORPE AND B. ROBSON. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* 120: 97-120.

GAWIENOWSKI, M.C., D. SZYMANSKI, I.Y. PERERA AND R.E. ZIELINSKI. (1993). Calmodulin isofroms in *Arabidopsis* encoded by multiple divergent mRNAs. *Plant Molecular Biology* 22: 215-225.

GUERN, J., Y. MATHIEU, M. PEAN, C. PASQUEIR, J. BELOEIL AND J. LALLEMAND. (1986). Cytoplasmic pH regulation in *Acer pseudoplatanus* cells. I ^{31}PNMR description of acid-load effects. *Plant Physiology* 82: 840-845.

HARE, P.D. (1995). A Molecular Characterisation of the gene encoding (Δ^1-pyrroline-5-carboxylate) isolated from *Arabidopsis thaliana* (L.) Heynh. University of Natal, Pietermaritzburg, PhD thesis.

HEUER, B. (1994). Osmoregulatory role of proline in water- and salt-stressed plants. In: Handbook of Plant and Crop Stress. M. Pessarakli (Ed.) Marcel Dekker, Inc., New York, pp. 363-381.

HUNT, S. (1991). Nonprotein amino acids. In: Methods in Plant Biochemistry. Vol. 5. Amino Acids, Proteins and Nucleic acids. L.G. Rogers (Ed.) Academic Press Ltd., London, pp. 1-52. ISBN 0- 12-4461015-3.

INATOMI, K. AND J.C. SLAUGHTER. (1971). The role of glutamate decarboxylase and γ-aminobutyric acid in germinating barley. *Journal of Experimental Botany* 22: 561-571.

INATOMI, K. AND J.C. SLAUGHTER. (1975). Glutamate decarboxylase from barley embryos and roots. General properties and the occurrence of three enzyme forms. *Biochemistry Journal* 147: 479- 484.

JORDAN, B.R. AND C.V. GIVAN. (1979). Effects of light and inhibitors on glutamate metabolism in leaf discs of *Vicia faba* L. *Plant Physiology* 64: 1043-1047.

KISHINAMI, I. (1988). Effect of auxins (2,4-Dichlorophenoxyacetic acid, Indole 3-acetic acid and Naphthaleanic acid) on the accumulation of γ-aminobutyric acid in excised rice roots tips. *Plant and Cell Physiology* 29: 581-585.

KISHINAMI, I AND K. OJIMA. (1980). Accumulation of γ-aminobutyric acid due to adding ammonium or glutamine to cultured rice cells. *Plant and Cell Physiology* 21: 581-589.

KNIGHT, M.R., A.K. CAMPBELL, S.M. SMITH AND A.J. TREWAVAS. (1991). Transgenic plant aequorin reports the effects of touch and cold shock and elicitors on cytoplasmic calcium. *Nature* 325: 524-526.

KOIWAI, A. AND M. NOGUCHI. (1972). Glutamate and γ-aminobutyrate metabolism in cultured tobacco cells. *Plant and Cell Physiology* 13: 395-399.

LÄHDESMÄKI, P. (1968). The amount of γ-aminobutyric acid and the activity of glutamic decarboxylase in ageing leaves. *Physiologia Plantarum* 21: 1322-1327.

LANE, T.R. AND M. STILLER. (1970). Glutamic acid decarboxylation in *Chlorella*. *Plant Physiology* 45: 558-562.

LARHER, F. G. GOAS, D. LERULIER, J. GERARD AND J. HAMELIN. (1983). Bound 4-aminobutyric acid in root nodules of *Medicago sativa* and other nitrogen fixing plants. *Plant Science Letters* 29: 315-326.

LARSEN, P.O. (1980). Physical and chemical properties of amino acids. In: Biochemistry of Plants. Vol.5. Amino acids and derivatives. B.J. Miflin (Ed.) Academic Press Ltd., London, pp. 226-269. ISBN 0-12-675405-5.

LATCHMAN, D. (1990). Regulation at transcription. Gene Regulation: A Eukaryotic Experience. Unwin Hyman Ltd., London, pp. 46-66. ISBN 0-04-445242-8.

LEA, P.J., R.D. BLACKWELL, K.W. JOY. (1992). Ammonia assimilation in higher plants. In: Nitrogen Metabolism of Plants. K. Mengel and D.J. Pilbream (Eds.) Oxford University Press, New York, pp. 153-186. ISBN 0-19-857752-4.

LEVITT, J. (1977). Effect of environmental stress on transport of ions across membranes. In: Regulation of Cell Membrane Activities in Plants. E. Marrè and O. Cifferri (Eds.) North-Holland Publishing Co., Amsterdam, pp.103-119. ISBN 0-7204-0615- 3.

LEVITT, J. (1980). Stress concepts. Response of Plants to Environmental Stresses. Chilling, Freezing and High Temperature Stress. Second edition. Academic Press Ltd., New York, pp. 3-18. ISBN 0-12-445502-6.

LING, V., I. PERERA AND R.E. ZIELINSKI (1991). Primary structures of *Arabidopsis* calmodulin isoforms deduced from the sequences of cDNA clones. *Plant Physiology* 96: 1196-1202.

LING, V. W.A. SNEDDEN, B.J. SHELP AND S.M. ASSMANN. (1994). Analysis of a soluble calmodulin-bindig proteing from fava bean roots: Identification of glutamate decarboxylase as a calmodulin- activated enzyme. *The Plant Cell* 6: 1135-1143.

LYONS, J.M. (1973). Chilling injury in plants. *Annual Review of Plant Physiology* 24: 445-466.

MARAS, B., G. SWEENEY, D. BARRA, F. BOSSA AND R.A. JOHN. (1992). The amino acid sequence of glutamate decarboxylase from *Escherichia coli*. Evolutionary relationship between mammalian and bacterial enzymes. *European Journal Biochemistry* 204: 93-98.

MATHIEU, Y., J. GUERN, M. PEAN, C. PASQUEIR, J. BELOEIL AND J. LALLEMAND. (1986). Cytoplasmic pH regulation in *Acer pseudoplatanus* cells. II Possible mechanisms involved in pH regulation during acid-load. *Plant Physiology* 82: 846-852.

MATSUMOTO, T., I. YAMAURA AND M. FANATSU. (1996). Improved purification and spectroscopic properties of squash glutamate

decarboxylase. *Bioscience Biotechnology and Biochemistry* 60: 889- 890.

MAZAELIS, D. (1980). Amino Acid Synthesis. In: The Biochemistry of Plants. Vol 5. Amino Acids and Derivatives. B.J. Miflin (Ed.) Academic Press Ltd., pp. 1-20. ISBN 0-12-675405- 5.

MEANS, A.R. (1988). Molecular mechanisms of action of calmodulin. *Recent Progress in Hormone Research* 44: 223-265.

MELCHER, I.M. (1986). Glutamate metabolism in intact seedlings and seedling segments of peas. *New Phytologist* 103: 685-688.

MENGEL, K. (1992). Nitrogen: agricultural productivity and environmental problems. In: Nitrogen Metabolism of Plants. K. Mengel and D.J. Pilbream(Eds.) Oxford University Press, New York, pp. 1-15. ISBN 0-19-857752-4.

MEYEROWITZ, E.M. (1994). Structure and organisation of the *Arabidopsis thaliana* nuclear genome. In: Arabidopsis. E.M Meyerowitz and C.R. Sommerville (Eds.) Cold Spring Harbour Laboratory Press, New York, pp. 21-36. ISBN 0-87969-428-9.

MIFLIN, B.J. AND P.J. LEA. (1980). Ammonia assimilation. In: The Biochemistry of Plants. Vol 5. Amino Acids and

Derivatives. B.J. Miflin (Ed.) Academic Press Ltd., pp. 169-202. ISBN 0-12-675405- 5.

MIFLIN, B.J. (1973). Amino acid biosynthesis and its control in plants. In: Biosynthesis and its Control in Plants. B.V Milborrow (Ed.) Academic Press Ltd., London, pp. 49-68. ISBN 0-12-446150-9.

MIZUSAKI, S., M. NOGUCHI AND E. TAMAKI. (1964). Studies of nitrogen metabolism in tobacco plants. VI.Metabolism of glutamic acid, γ-aminobutyric acid, and proline in tobacco leaves. *Archives of Biochemistry and Biophysics* 105: 599-605.

MOORE, S. AND W.H. STEIN. (1954.) Procedures for the chromatographic determination of amino acids of four percent cross-linked sulfonated polystyrene resins. *The Journal of Biological Chemistry* 211: 893-906.

MOORE, S. AND W.H. STEIN. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *The Journal of Biological Chemistry* 211: 907-913.

NATHAN, B., C. HSU, J. BAO, R. WU AND J. WU. (1994). Purification and characterisation of novel form of brain L-glutamate decarboxylase. *The Journal of Biological Chemistry* 269: 7249- 7254.

NEWMAN, T. F.J. DE BRUIJN, P GREEN, K. KEEGSTRA, H. KENDE, L. MCINTOSH, J. OHLROGGE, N RAIKHEL, S. SOMMERVILLE, M. THOMASHOW, E. RETZEL AND C. SOMMERVILLE. (1994). Genes galore: A summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiology* 106: 1241- 1255.

NORDIN, K, P. HEINO AND E.T PALVA. (1991). Separate signal pathways regulate the expression of a low temperature-induced gene in *Arabidopsis thaliana* (L) Heynh. *Plant Molecular Biology* 16: 1061-1071.

OJI, Y. AND G. IZAWA. (1972). Quantitative changes in free amino acids and amides in barley plants during ammonia and nitrate assimilation. *Plant and Cell Physiology* 13: 249-259.

PARKER, P.J. (1991). An overview of signal transduction. In: The Hormonal control of Transcription. Vol 6. P. Cohen and J.G Folkes (Eds.) Elsevier Sscience Publishers BV, Amsterdam, pp. 77-98. ISBN 0-444-80225-8.

PASTERNAK, D. (1987). Salt tolerance and crop production - A comprehensive approach. *Annual Review of Phytopathology* 25: 271- 291.

PATE, J.S. (1989). Synthesis, transport, and utilization of products of symbiotic nitrogen fixation. I: Plant Nitrogen Metabolism. J.E. Poulton, J.T. Romeo and E.E. Conn (Eds.) Plenum Press, New York, pp. 65-115. ISBN 0-306-4332-2.

PEARSON, W.R. AND D.J. LIPMAN. (1988). Improved tools for biological sequence comparison. *Proceedings of the National Academy of Science USA* 85: 2444-2448.

RAINBIRD, R.M., J.H. THOME AND R.W.F. HARDY. (1984). Role of amides, amino acids, and ureids in the nutrition of developing soybean seeds. *Plant Physiology* 74: 329-334.

RAMAGOPAL, S. (1987). Differential mRNA transcription during salinity stress in barley. *Proceedings of the National Academy of Science USA* 84: 94-98.

REGGIANI, R., C.A. CANTU, I. BRAMBILLA AND A. BERTANI. (1988). Accumulation and interconversion of amino acids in rice roots under anoxia. Plant Cell Physiology 29: 981-987.

REGGIANI, R. N. AURISANO, M. MATTANA AND A. BERTANI. (1993). ABA induces 4-aminobutyrate accumulation in wheat seedlings. *Phytochemistry* 34 605-609.

RHODES, D. S. HANDA AND R.A. BRESSAN. (1986). Metabolic changes associated with adaptation of plant cells to water stress. *Plant Physiology* 82: 890-903.

RHODES, D. AND S. HANDA. (1989). Amino acid metabolism in relation to osmotic adjustment in plant cells. In: Environmental Stress in Plants. Biochemical and Physiological Mechanisms. Vol.19. J.H Cherry (Ed.) Springer-Verlag, New York, pp.41-62. ISBN 3-540-18559-3.

ROBERTS, J.K.M, J. CALLIS, D. WEMMER, V. WALBOT AND O. JARDETZKY. (1984). Mechanism of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. *Proceedings of the National Academy of Science USA* 81: 3379-3383.

ROBERTS, J.K.M., M.A. HOOKS, A.P. MAULIS, S.E. EDWARDS AND C. WEBSTER. (1992). Contribution of malate amd amino acid metabolism of cytoplasmic pH regulation in hypoxic maize root tips studied using nuclear magetic resonance spectroscopy. *Plant Physiology* 98: 480-487.

SATYANARAYAN, V. AND P.M. NAIR. (1986). The 4-aminobutyrate shunt in *Solanum tuberosum*. *Phytochemistry* 25: 997-1001.

SATYANARAYAN, V. AND P.M. NAIR. (1990). Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochemistry* 29: 367-375.

SCHNALL, J.A. AND R.S. QUATTRANO. (1992). Abscisic acid elicits the water-stress response in root hairs of *Arabidopsis thaliana*. *Plant Physiology* 100: 216-218.

SELMAN, I.W. AND P. COOPER. (1978). Changes in the free amino acid compounds in young tomato plants in light and darkness with particular reference to γ-aminobutyric acid. *Annals of Botany* 42: 627-636.

SHELP, B.J., C.S. WALTON, W.A. SNEDDEN, L.G. TUIN, I.J. ORESNIK AND D.B LAYZELL. (1995). GABA shunt in developing 11soybean seeds is associated with hypoxia. *Physiologia Plantarum* 94: 219-228.

SIVASANKAR, S. AND A. OAKS. (1996) Nitrate assimilation in higher plants: The effect of metabolites and light. *Plant Physiology and Biochemistry* 34: 609-620.

SKRIVER, K. AND J. MUNDY. (1990). Gene expression in response to abscisic acid and osmotic stress. *The Plant Cell* 2: 503-512.

SNEDDEN, W.A., T. ARAZI, H. FROMM AND B.J. SHELP. (1995). Calcium/calmodulin activation of soybean glutamate decarboxylase. *Plant Physiology* 108: 543-549.

SNEDDEN, W.A., I. CHUNG, R.H. PAULS AND A.W. BOWN. (1992). Proton/L-glutamate symport and the regulation of intracellular pH in isolated mesophyll cells. *Plant Physiology* 99: 665-671.

STEWARD, F.C. AND J.K. POLLARD. (1957). Nitrogen metabolism in plants: Ten years in retrospect. *Annual Review of Plant Physiology* 8: 65-110.

STEWARD, F.C, J.F. THOMPSON AND C.E. DENT. (1949). γ-Aminobutyric acid: A constituent of the potato tuber. *Science* 110: 439-440.

STEWARD, F.C., J.F. THOMPSON AND J.F. POLLARD. (1958). Nitrogen metabolism, respiration and growth of cultured plant tissue. I. Experimental design, techniques and recorded data. *Journal of Experimental Botany* 9: 11-51.

STEWART, C.R., AND J.A. LEE. (1974). The role of proline accumulation in halophytes. *Planta* 120: 279-289.

STEWART, G.R. AND F. LARHER. (1980). Accumulation of amino acids and related compounds in relation to environmental

stress. In: The Biochemstry of Plants. B.J Miflin (Ed.) Academic Press Inc., New York, pp. 609-635. ISBN 0-12-675405-5.

STREETER, J.G. AND J.J. THOMPSON. (1972a). *In vitro* and *in vivo* studies on γ-aminobutyric acid metabolism with the radish plant (*Raphanus sativus* L.). *Plant Physiology* 49: 597-584.

STREETER, J.G AND J.F. THOMPSON. (1972b). Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). *Plant Physiology* 49: 572-578.

THOMASHOW, M.F. 1994. Arabidopsis as a model for studying mechanism of plant cold tolerance. In: Arabidopsis. E.M Meyerowitz and C.S. Sommerville (Eds.) Cold Spring Harbour Laboratory Press, New York, pp. 807-834. ISBN 0-87969-428-9.

THOMPSON, J.F., C.J. MORRIS AND R.K. GERING. (1951). Purification of amino acids for paper chromatography. *Analytical Chemistry* 31: 1029-1037.

THOMPSON, J.F. AND F.C. STEWARD. (1951). Investigations on nitrogen compounds and nitrogen metabolism in plants.II. Variables in two-directional paper chromatography of nitrogen compounds: A qualitative procedure. *Plant Physiology* 26: 421-440.

THOMPSON, J.F. AND C.J. MORRIS. (1959). The determination of amino acids from plants by paper chromatography. *Analytical Chemistry* 31: 1031-1037.

THOMPSON, J.F, C.R. STEWART AND C.J. MORRIS. (1966). Changes in amino acid content of excised leaves during incubation. I. The Effect of water content of leaves and atmospheric oxygen level. *Plant Physiology* 41: 1578-1584.

THOMPSON, J.F., R.M. ZACHARIUS AND F.C. STEWART. (1951). Investigations on nitrogen compounds and nitrogen metabolism in plants. I The reaction of nitrogen compounds with ninhydrin on paper: A quantitative procedure. *Plant Physiology* 28: 375-397.

TRESHOW, M. (1970). The environment. The environment and Plant Response. Mcgraw-Hill Inc., New York, pp. 3-16. ISBN

TUIN, L.G. AND B.J. SHELP. (1994(a)). *In situ* [14C]glutamate metabolism by developing soybean cotyledons. I. Metabolic routes. *Journal of Plant Physiology* 143: 1-7.

TUIN, L.G., AND B.J. SHELP. (1994(b)). *In situ* [14C]glutamate metabolism by developing soybean cotyledons. II. The importance of glutamate decarboxylation. *Journal of Plant Physiology* 143: 1-7.

VAN STADEN, J. (1966). A paper chromatographic survey of free amino acids and related compounds in the Proteaceae. *Journal of South African Botany* 32: 77-86.

VANDEWALLE, I. AND R. OLSSON. (1983). The γ-Aminobutyric acid shunt in germinating *Sinapsis alba* seeds. *Plant Science* 31: 269- 273.

WALLACE, W., J. SECOR AND L.E SCHRADER. (1984). Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiology* 75: 170-175.

WERNER, J.E. AND R.R FINKELSTEIN. 1995. Arabidopsis mutants with reduced response to NaCl and osmotic stress. *Physiologia Plantarum* 93: 659-666.

WINNACKER, E.L. (1989). Tissue-specific gene expression: A summary. In: Tissue Specific Gene Expression. R Renkawitz (Ed.) V.C.H.Verlagsgesellschaff, Weiheim, pp. 1-12. ISBN 0-89573-886-4.

WYNJONES, R.G., R. STOREY, R.A. LEIGH, N. AHMAD AND A. POLLARD.(1977) A hypothesis on cytoplasmic osmoregulation. In: Regulation of Cell Membrane Activities in Plants. E. Marrè and

O. Cifferri (Eds.) North-Holland Publishing Co., Amsterdam, pp.121-136. ISBN 0-7204-0615-3.

YOSHIDA, S. (1994). Low-temperature-induced cytoplasmic acidosis in cultured mung bean (*Vigna radiata* (L.) Wilczek) cells. *Plant Physiology* 104: 1131-1138.

YOUNGS, T.L. AND G. TUNNICLIFF. (1991). Substrate analogues and divalent cations as inhibitors of glutamate decarboxylase from *Escherichia coli*. *Biochemistry International* 23: 915-922.

APPENDIX I

GROWING *ARABIDOPSIS THALIANA* PLANTS

Nutrient Stock Solution (ml stock/litre of irrigation solution)

1 M KNO_3 (5.0)

1 M KH_2PO_4, pH 5.6 (2.5)

1 M $MgSO_4$ (2.0)

1 M $Ca(NO_3)_2$ (2.0)

1.8% Sequestrene (2.8)

micronutrient mix (shown below) (1.0)

Micronutrient Mix

70 mM H_3BO_3

14 mM $MnCl_2$

0.5 M $CuSO_4$

1 mM $ZnSO_4$

0.2 mM $NaMoO_4$

10 mM NaCl

0.01 mM $CoCl_2$

DNA EXTRACTION

Genomic DNA extraction buffer:

100 mM LiCl

100 mM Tris, pH 8.0

50 mM EDTA, pH 8.0

1% CTAB

10 mM ß-mercaptoethanol

TE buffer:

10 mM Tris-HCl, pH 7.0

1 mM EDTA, pH 8.0

Phenol:

Phenol crystals are dissolved by heating. An equal volume of Tris-HCl, pH 8.0 is mixed with the phenol. The mixture is allowed to stand for an hour to allow the two phases to separate and the upper Tris-HCl phase discarded. A fresh solution of Tris-HCl is added until the phenol pH is about 8.0. Then 0.01% hydroxyquinoline is added. The Tris-layered solution is transferred to a glass container covered with tin foil to prevent oxidation due to light.

Enzyme Digestion

40 µg genomic DNA	20 µl
10X Restriction buffer	10 µl
80 units enzymes	2 µl
0.1 M Spermidine	10 µl
dH_2O	58 µl
Total	100 µl

* Bovine serum albumin was added in case of Kpn I

** A double digestion was m

50X TAE buffer stock:

2 M Tris

0.05 EDTA

The pH is adjusted to 8.0 with glacial acetic acid. For use the stock solution was diluted to a 1X solution.

ALKALINE PLASMID MINIPREP

STE buffer

0.1 M NaCl

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

Solution I (GTE buffer)

50 mM Glucose

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

Solution II (NaOH/SDS)

0.2 M NaOH

1 % SDS (Sodium dodecyl sulfate)

88 % isopropanol-0.2 M potassium acetate

Four volumes of 5 M potassium acetate were mixed with 1 volume of 10 M (57 %) acetic acid.

PURIFICATION OF DNA FROM AGAROSE GEL

The GeneClean® Kit

Sodium iodide

Silica

New Wash (Detergent dissolved in 70% ethanol)

SOUTHERN TRANSFER

20x SSC

3 M NaCl

0.3 M Sodium citrate

pH adjusted to 7.0 with 1 M HCl

Depurination solution

250 mM HCl

Denaturation solution

1.5 M NaCl

0.5 M NaOH

Neutralization solution

1.5 M NaCl

0.5 M Tris-HCl

Adjusted pH to 7.5

Primary wash buffer

6 M Urea

0.4 % SDS

0.5x SSC

Secondary wash buffer

100 ml of 20x SSC buffer made up to 1 litre.

DIDEOXY CHAIN-TERMINATION SEQUENCING

Sequencing Gel (150 ml)

30 ml Acrylamide/Bisacrylamide Stock

15 ml TBE buffer

45 ml dH_2O

The mixture is degassed with constant stirring and then add:

1.5 ml Ammonium persulfate

22.5 TEMED

Acrylamide/Bisacrylamide Stock (100 ml)

38 g Acrylamide

2 g Bisacrylamide

Made up to 100 ml with dH_2O.

APPENDIX II

CODES FOR NUCLEIC ACID BASES

A: Adenosine

C: Cytosine

G: Guanosine

T: Thymidine

AMINO ACID CODES

A: Alanine (Ala)

R: Arginine (Arg)

N: Asparagine (Asn)

D: Aspartate (Asp)

C: Cysteine (Cys)

E: Glutamate (Glu)

Q: Glutamine (Gln)

G: Glycine (Gly)

H: Histidine (His)

I: Isoleucine (Ile)

L: Leucine (Leu)

K: Lysine (Lys)

M: Methionine (Met)

F: Phenylalanine (Phe)

P: Proline (Pro)

S: Serine (Ser)

T: Threonine (Thr)

W: Tryptophan (Trp)

Y: Tyrosine (Tyr)

V: Valine (Val)

APPENDIX III

CONVERSION OF NaCl CONCENTRATIONS TO EQUIVALENT PEG SOLUTIONS

Table 1 Water potentials of NaCl solutions between 0 °C and 40 °C

Water Potential (bars)

Molality	0°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C	40°C
0.05	— 2.14	— 2.18	— 2.22	— 2.26	— 2.30	— 2.34	— 2.38	— 2.42	— 2.45
0.1	— 4.23	— 4.31	— 4.39	— 4.47	— 4.54	— 4.62	— 4.70	— 4.77	— 4.85
0.2	— 8.36	— 8.52	— 8.68	— 8.84	— 9.00	— 9.15	— 9.30	— 9.46	— 9.61
0.3	—12.47	—12.72	—12.97	—13.21	—13.44	—13.68	—13.91	—14.15	—14.37
0.4	—16.58	—16.93	—17.27	—17.59	—17.91	—18.23	—18.55	—18.86	—19.17
0.5	—20.70	—21.15	—21.58	—22.00	—22.41	—22.81	—23.22	—23.62	—24.02
0.6	—24.84	—25.39	—25.93	—26.44	—26.94	—27.44	—27.94	—28.43	—28.91
0.7	—29.01	—29.67	—30.30	—30.91	—31.51	—32.10	—32.70	—32.28	—33.85
0.8	—33.20	—33.98	—34.72	—35.43	—36.12	—36.82	—37.51	—38.18	—38.85
0.9	—37.43	—38.32	—39.17	—39.98	—40.79	—41.58	—43.27	—43.14	—43.90
1.0	—41.69	—42.70	—43.66	—44.59	—45.50	—46.40	—47.29	—48.15	—49.01
1.1	—45.99	—47.13	—48.20	—49.24	—50.26	—51.27	—52.26	—53.22	—54.18
1.2	—50.32	—51.60	—52.78	—53.94	—55.07	—56.20	—57.30	—58.35	—59.41
1.3	—54.70	—56.11	—57.42	—58.69	—59.94	—61.19	—62.39	—63.54	—64.71
1.4	—59.12	—60.68	—62.10	—63.50	—64.87	—66.23	—67.54	—68.80	—70.06
1.5	—63.59	—65.29	—66.84	—68.37	—69.86	—71.34	—72.76	—74.11	—75.48
1.6	—68.11	—69.96	—71.63	—73.30	—74.91	—76.52	—78.05	—79.50	—80.07
1.7	—72.60	—74.60	—76.40	—78.20	—80.00	—81.70	—83.30	—84.90	—86.50
1.8	—77.30	—79.40	—81.30	—83.30	—85.20	—87.00	—88.80	—89.40	—92.10
1.9	—81.90	—84.30	—86.30	—88.40	—90.40	—92.40	—94.30	—96.00	—97.80
2.0	—86.70	—89.20	— 91.30	—93.60	—95.70	—97.80	—99.80	—101.60	—103.50

Table2 Concentrations of PEG-6000 Solutions Required for Osmotic Potentials -1 to -20 bars from 15 to 35 C

ψ_s	Centigrade Temperature																				
	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
bars	g PEG-6000/kg H_2O																				
-1	67	68	69	70	71	72	73	74	76	77	78	79	81	82	84	85	87	88	90	91	93
-2	105	107	108	109	111	112	113	115	116	118	119	121	122	124	126	128	129	131	133	135	137
-3	135	137	138	140	141	143	145	146	148	149	151	153	155	157	158	160	162	164	166	168	171
-4	161	163	164	166	168	169	171	173	174	176	178	180	182	184	186	188	190	192	194	197	199
-5	184	185	187	189	191	192	194	196	198	200	202	204	206	208	210	212	215	217	219	222	224
-6	204	206	208	210	212	214	215	217	219	221	224	226	228	230	232	235	237	239	242	244	247
-7	223	225	227	229	231	233	235	237	239	241	243	246	248	250	253	255	257	260	262	265	268
-8	241	243	245	247	249	251	253	255	257	260	262	264	267	269	271	274	276	279	282	284	287
-9	257	259	262	264	266	268	270	272	275	277	279	282	284	287	289	292	294	297	300	302	305
-10	273	275	277	280	282	284	286	289	291	293	296	298	301	303	306	309	311	314	317	320	322
-11	288	290	292	295	297	299	302	304	306	309	311	314	317	319	322	325	327	330	333	336	339
-12	302	305	307	309	312	314	316	319	321	324	326	329	332	334	337	340	343	346	349	352	355
-13	316	318	321	323	325	328	330	333	335	338	341	343	346	349	352	355	357	360	363	366	370
-14	329	332	334	336	339	341	344	347	349	352	354	357	360	363	366	369	372	375	378	381	384
-15	342	344	347	349	352	354	357	360	362	365	368	371	373	376	379	382	385	388	392	395	398
-16	354	357	359	362	364	367	370	372	375	378	381	384	386	389	392	395	399	402	405	408	412
-17	366	369	371	374	377	379	382	385	387	390	393	396	399	402	405	408	411	415	418	421	425
-18	378	381	383	386	388	391	394	397	400	402	405	408	411	414	417	421	424	427	431	434	437
-19	389	392	395	397	400	403	405	408	411	414	417	420	423	426	430	433	436	439	443	446	450
-20	400	403	406	408	411	414	417	420	423	426	429	432	435	438	441	445	448	451	455	458	462