

**PROLINE BIOSYNTHESIS
IN TRANSGENIC
SOYBEAN
PLANTS**

**PROLINE BIOSYNTHESIS IN
TRANSGENIC SOYBEAN PLANTS**

By

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DEGREE OF DOCTOR OF PHILOSOPHY IN THE DEPARTMENT OF
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DECLARATION

I declare that this dissertation is my own work. Experimental work described was carried out at the ARC-Roodeplaat, Vegetable and Ornamental Plant Institute, Pretoria under supervision of Professor J. Van Staden and Dr W.A Cress.

These studies represent original work and have not otherwise been submitted in any form for any degree or diploma to any University.

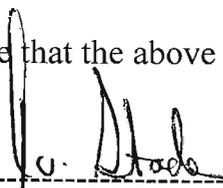


J.A. de Ronde

10.10.2000

date

I declare that the above statement is correct



J. van Staden

Supervisor

10/10/2000

date

Philippians 4: 13

*I have the strength to face all conditions by the power
that Christ gives me*

ABSTRACT

Plants have evolved numerous strategies for the adaptation to drought. Although many investigations reported on the potential value of proline accumulation during environmental stress, it is still unknown whether or not a constitutive higher level of proline accumulation enhances plant tolerance. Thus, it was investigated if underproduction and overproduction of proline will influence the susceptibility to drought stress in soybean plants. This was made possible with the transformation of soybean plants with an L- Δ^1 -pyrroline-5-carboxylate reductase (*P5CR*) gene.

First, an *Agrobacterium*-mediated vacuum infiltration transformation system, using partially germinating Carnia 2233 soybean seed, was established through the assessment of several conditions that can affect transformation efficiency with the use of β -glucuronidase reporter genes. Transformation was confirmed with PCR and Southern blot analysis and results indicated that stable transgenic soybean plants were obtained within one generation with a transformation rate of $\pm 30\%$. This technique was used in the transformation of Carnia 2233 soybean seed with the *P5CR* gene in the antisense orientation under the control of an inducible heat shock gene promoter (IHSP). It was confirmed that the *P5CR*-IHSP gene construct was integrated into the soybean cells and was conserved over three generations. Physiological screening of the antisense *P5CR* transgenic plants in the greenhouse proved that, with activation of the promoter, an under-expression of the *P5CR* gene and subsequent inhibition of the accumulation of proline were experienced during drought and osmotic stress. The decline of the viability of the transgenics with prolonged drought stress, as monitored with a woodenbox screening test, is an indication that proline is needed for survival

of soybean plants under drought stress conditions. The transgenic plants demonstrated a sensitive reaction in contrast to the control plants that displayed a tolerant reaction to osmotic stress in a TTC assay. The underexpression of the *P5CR* gene resulted in a decline protein synthesis due to proline shortage as was observed with the evaluation of the efficiency of protein synthesis. All these results suggest that a decrease in the proline level due to the antisense *P5CR* gene, yielded plants that are more osmotic and drought stress sensitive.

Subsequently, the soybean cultivar Ibis was successfully transformed with the *P5CR*-IHSP construct in the sense and antisense directions in order to test the reproducibility of the transformation process and to assessed the link between the biochemical traits involved in the drought stress mechanism. Three different experiments were conducted: a mild heat and drought stress on “To” transgenic plants exploring changes in chlorophyll fluorescence transients, a mild heat stress on “T1” transgenic plants comparing proline accumulation and chlorophyll fluorescence transients and a severe drought and heat stress on the "T1" transgenic plants comparing proline accumulation, NADP^+ synthesis and chlorophyll fluorescence transients. Chlorophyll fluorescence transients were successfully used as a screening method for transgenic soybean plants during this study. The sense transgenics responded to the mild stresses with a significant decrease in their electron transport, trapping and absorption compared to the antisense plants that displayed significant increases in electron transport and trapping. During the severe stress, the antisense transgenics experienced total photoinhibition indicated by the enormous loss of electron transport but the sense plants had the ability to overcome the stress as is revealed in the increase in the electron transport.

It was demonstrated that although proline accumulation yielded no significant differences during the mild heat stress, the sense plants accumulated substantially more proline than the control and antisense plants during the severe heat and drought stress. It was demonstrated that proline plays an important role in the plant's response to a drought stress as well as in the recovery phase after drought, as the sense plants also had the ability to reduce the accumulated proline during the recovery period in contrast to the antisense transgenics that experienced protein degradation. The transgenics responded to a period of heat and drought stress with a reduction in NADP^+ levels in the antisense plants and increasing levels in the sense plants. The sense plants were able to fully recover after the stress period, thus adaptation to drought may depend on different mechanisms, including the capacity to maintain high levels of proline and to regenerate them through the “reduction” of NADP^+ .

It was possible to alter the drought tolerance of Ibis by transformation with antisense and sense *P5CR* gene constructs, which resulted in respectively more sensitive and more tolerant Ibis plants. It can be concluded that over-expression of *P5CR* during a drought stress resulted in higher proline levels, better photosynthetic efficiency, higher NADP^+ production and thus a more drought tolerant plant. This study gave additional proof that a constitutively higher level of proline accumulation enhances drought tolerance in soybean.

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- DE RONDE JA & CRESS WA, 1998. Patent on transformation process in the name of Protein Research Trust. SA98/9427 (PCT/IB99/01676)
- DE RONDE JA, SPREETH MH & CRESS WA, 2000. Effect of antisense L- Δ^1 - pyrroline-5-carboxylate reductase transgenic soybean plants subjected to osmotic and drought stress. *Plant Growth Regulation* **32**:13-26
- DE RONDE JA, CRESS WA & VAN DER MESCHT A, 2000. *Agrobacterium*-mediated transformation of soybean (*Glycine max* L Merr) seed with the GUS-INT marker gene. *South African Journal of Science* (submitted)

POTENTIAL PAPERS TO BE SUBMITTED

- DE RONDE, JA, CRESS WA & VAN STADEN J, 2001. Interaction of osmotic and temperature stress on transgenic soybean. *South African Journal of Botany*
- DE RONDE, JA, LAURIE RN, CRESS WA & VAN STADEN J, 2001. Can the JIP test be use in screening of transgenic soybean?
- DE RONDE, JA, KRUGER GHJ, CRESS WA & VAN STADEN J, 2001. Interactions between proline, photosynthesis and NADP⁺.
- DE RONDE, JA, CRESS WA & VAN STADEN J, 2001. Phenotypic evaluation of *P5CR* transformed soybean.
- DE RONDE, JA, CRESS WA & VAN STADEN J, 2001. Review on transformation in soybean

LIST OF ABBREVIATIONS

ABS	-	Absorption
Amp	-	Ampicillin
ANOVA	-	Analysis of variance
APH(3')II	-	Aminoglycoside 3'-phosphotransferase II
ARC	-	Agricultural Research Council
AS	-	Acetosyringone
ATP	-	Adenosine triphosphate
bp	-	Base pairs
<i>Bt</i>	-	<i>Bacillus thuringiensis</i>
chl	-	Chlorophyll
chl- <i>a</i>	-	Chlorophyll <i>a</i>
chl- <i>b</i>	-	Chlorophyll <i>b</i>
<i>chv</i> genes	-	Chromosome genes
cyt	-	Cytoplasmic
CO ₂	-	Carbon dioxide
CDNA	-	Complementary deoxyribonucleic acid
CS	-	Cross section
D	-	Days (figures only)
dATP	-	2'-Deoxy-adenosine-5'-triphosphate
dCTP	-	2'-Deoxy-cytidine-5'-triphosphate
dGTP	-	2'-Deoxy-guanosine-5'-triphosphate
DI	-	Dissemination
DNA	-	Deoxyribonucleic acid
DTT	-	Dithiotreitol
dTTP	-	Thymidine-5'-triphosphate

<i>E. coli</i>	-	<i>Escherichia coli</i>
EDTA	-	Ethylene diamine tetra acetic acid
ET	-	Electron transport
F _m	-	Maximal fluorescence intensity when all the RC's are closed
F _o	-	Fluorescence intensity at 50 μs when all RC's are open
F _j	-	Fluorescence intensity at 100μs, 300 μs and 2 ms
F _i	-	Fluorescence intensity at 30ms
g	-	Gram
GMO	-	Genetically modified organism
G6P	-	Glucose-6-phosphate
G6PDH	-	Glucose-6-phosphate dehydrogenase
<i>gus</i>	-	β-Glucuronidase gene
GUS-INT	-	β-Glucuronidase intron
GSH	-	Glutathione
H ₂ O	-	Water
HCL	-	Hydrochloric acid
HI	-	Heat inducible
HSP	-	Heat shock protein
I29	-	Ibis plant with antisense <i>P5CR</i> construct
I39	-	Ibis plant with sense <i>P5CR</i> construct
IHSP	-	Inducible heat shock promoter
JIP test	-	Chl- <i>a</i> fluorescence F _o -J-I-P transient test
<i>kan</i> ^r	-	Kanamycin resistance
kb	-	Kilo base pairs
KCl	-	Potassium chloride
Kda	-	Kilodalton
Km	-	Kanamycin
LB	-	Luria-Broth

LSD	-	Least significant difference
M	-	Molar
MDH	-	Malate dehydrogenase
mg	-	Milligram
MgCl ₂	-	Magnesium chloride
ml	-	Millilitre
mM	-	Millimolar
Mpa	-	Molar Pascal
ms	-	Milliseconds
MTT	-	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MU	-	Methyl umbelliferone
MUG	-	4-Methylumbelliferyl β-D-glucuronide
NAA	-	Naphtalene-acetic acid
N	-	Nitrogen
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NaPO ₄	-	Sodium phosphate buffer
NAD ⁺	-	Nicotinamide adenine dinucleotide
NADP ⁺	-	Nicotinamide adenine dinucleotide phosphate
NADH	-	Nicotinamide adenine hydrogen dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide hydrogen phosphate
ng	-	Nanogram
nm	-	Nanometer
NOS-ter	-	Nopaline synthetase terminator
NPT II	-	Neomycin phosphotransferase II
O-J-I-P	-	Polyphasic fluorescence rise
OPPP	-	Oxidative pentose phosphate pathway
P	-	Phosphorus

PCR	-	Polymerase chain reaction
P5C	-	L- Δ^1 -Pyrroline-5-carboxylase
P5CR	-	L- Δ^1 -Pyrroline-5-carboxylase reductase enzyme
<i>P5CR</i>	-	L- Δ^1 -Pyrroline-5-carboxylase reductase gene
P5CS	-	L- Δ^1 -Pyrroline-5-carboxylase synthetase
PEA	-	Plant Efficiency Analyser
PES	-	Phenazine ethosulfate
PDH	-	Proline dehydrogenase
PHI _(EO)	-	Quantum efficiency flux ratio ET _o /ABS
PI _(CS_o)	-	Performance indexes expressed relative to CS
PI _(ABS)	-	Performance indexes expressed relative to ABS
PSI	-	Photosystem 1
PSI _o	-	Quantum efficiency flux ratio ET _o /TR
PSII	-	Photosystem 2
Q _A	-	Primary quinone acceptor in PS II
Q _B	-	Secondary quinone acceptor in PS II
RC	-	Reaction center
RC's	-	Reaction centers
Rdf	-	Ratio of decrease fluorescence
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
SAAT	-	Sonication-assisted <i>Agrobacterium</i> -mediated transformation
SDH	-	Succinate dehydrogenase
T	-	Transgenic generation
TAE	-	Tris acetate EDTA buffer
TR	-	Trapping
t _{Fmax}	-	Time at which the fluorescence transient reaches the F _m
t RNA	-	Transfer ribonucleic acid

TTC	-	2,3,5- Triphenyl tetrazolium chloride
T-DNA	-	Transferred DNA
Ti	-	Tumor-inducing
UV	-	Ultra violet
v/v	-	Volume/volume
<i>vir</i> genes	-	Virulence region
w/v	-	Weight/volume
X-GLUC	-	5-Bromo-4-chloro-3-indolyl- β -D-glucuronide
μ l	-	Microlitre
μ m	-	Micrometer
Ψ_0	-	Quantum efficiency

CHAPTER 1

LITERATURE REVIEW

1.1. HISTORY OF SOYBEAN

Two plant families of great importance to world agriculture are the *Poaceae* and *Fabaceae*. The *Fabaceae* contains about 650 genera and 18 000 species. Soybean belongs to the genus *Glycine* (HYMOWITZ, 1990). The economic importance of the genus *Glycine* lies within the subgenus *Soja*. Soybean (*Glycine max* (L) Merr) is a summer annual herb that has never been relocated in the wild (HYMOWITZ, 1999). It is believed to be a cultigen from *Glycine ussuriensis* (DUKE, 1983). *Glycine max* has many informal names of which soybean and soyabean are the most common. The diploid soybean genome contains one billion basepairs over 20 chromosomes and an estimated gene total of nearly 25 000 (SINCLAIR & WYNSTRA, 1995).

According to HYMOWITZ (1990), soybean is an ancient food crop of China and was domesticated during the Chou Dynasty which dated back to between the seventh and the 11th Century B.C. As the Chou Dynasty expanded and trade increased, the soybean migrated to South China and Korea (probably by the first century A.D.). The movements of the soybean were associated with the development, consolidation of territories and degradation of Chinese dynasties (HYMOWITZ, 1990). It was later introduced into several countries eg. Japan and South East Asia, and a number of landraces were developed. Soybean reached Europe around 1737 (Netherlands), 1739 (France), and finally in 1790 (England).

Soybean was introduced into the United States in the early 1800s and was grown as a minor forage crop for many years (WILCOX, 1987). The development of a soybean-processing industry in the early 1920s gave soybean cultivation a great impetus, and today soybean is a leading crop in the United States, ranking only behind maize and wheat. By 1930, soybean-breeding programs had been started to hybridise plants and to select progeny better adapted than their parents (WILCOX, SCHAPAUGH, BERNARD, COOPER, FEHR & NIEHAUS, 1979). The United States of America produces about 60% of the world's soybeans, compared to 14% produced by Brazil, 10% by China, and lesser percentages by Argentina, Taiwan, Canada and India. Production in the United States is located chiefly in the Midwest and the lower Mississippi Valley. More than 30% of the United States production is exported.

As soybean is self-pollinated, individuals are highly homozygous, thus the improvement and optimisation of soybean characteristics is very desirable. It is the world's main source of edible vegetable oils and high protein livestock feed (WILCOX, 1987). Legume seeds are richer in protein than cereal grains. Soybean provides humans with a significant amount of their dietary protein requirements. In developing countries, increased cultivation of legumes is the best hope for combating projected shortages in food supplies, especially vegetable protein.

In the oilseed industry, soybean is the clear leader with more than half the of world production of oil. In South Africa, the area planted has increased every year from 1992 to 1999. This is mainly the result of the substitution of soy meal for fish meal as a source of protein in animal feeds. Production is also in a growing trend (AGRIMARK TRENDS, 2000).

1.2. DROUGHT RESPONSE IN SOYBEAN

Understanding plant water relations and predicting water stress responses can have a positive effect on crop production and water use efficiency. Plant growth is characterised by a wide array of anabolic and catabolic processes that are driven primarily by endogenous factors but can be strongly influenced by adverse environmental conditions. When environmental stress is imposed on a plant, several physiological responses are induced or accelerated. Thus, water stress may account for the accumulation of compatible solutes such as proline (VAN RENSBURG & KRÜGER, 1994), betaines (WYN JONES & STOREYS, 1978) and non structural carbohydrates such as sucrose, fructose and glucose (MÜLLER, BOLLER & WIEMKEN, 1996). It may also effect a variety of plant processes including photosynthesis (HOOGENDOORN & ARNTZEN, 1992), NADPH redox (HARE & CRESS, 1997) and anti-oxidative enzymes (EDREVA, 1992). Experiments by SERRAJ, VADEZ, FORD DENISON & SINCLAIR (1999) showed that a key plant response to drought stress is a decline in nitrogen fixation - the process by which soybean plants fix nitrogen from the air.

Components of drought tolerance that could be selected in soybean include: root development, resistance to water loss and recovery from water stress. These components include cellular, developmental and biochemical traits. By selecting for cellular and biochemical traits, it is possible to improve complex traits such as yield under drought conditions. Stem elongation, leaf formation and leaf area increase follow exponential growth curves during early vegetative development under normal water regimes. An opposite effect was observed, during periods of water stress, in which stem elongation and leaf area expansion rates decreased and the new leaves were smaller (HOOGENBOOM, PETERSON & HUCK, 1987). The ability of a crop to recover from a mild or severe water stress and the rate of recovery are linked to drought resistance and water use efficiency of the crop. A

study by WALKER, GOSTERHUIS & EASTHAM (1987) indicated that the intensity and duration of water stress experienced by soybean affects the recovery after water stress has ceased. Water stress at any stage of soybean development can reduce yield but the extent of the yield reduction depends on the stage of development. If the soil water content is below the minimum required for germination, the seed will eventually be damaged and germination will be low (HELMS, DECKARD, GOOS & ENZ, 1996). If the seed water content is below the critical level for germination, the radicle will not emerge from the testa. The negative effects of stress are particularly important during flowering, seed set and seed filling (SIONET & KRAMER, 1977). It can cause an early switch of plant development from the vegetative to reproductive state as was observed by DESCLAUX & ROUMET (1996). Seed filling was earlier and senescence accelerated. Depending on the time of stress, the yield reduction as a result of drought can vary between 13% (early drought, but relieved) to 88% (late drought, unrelieved) (ECK, MATHERS & MUSICK, 1987). If the stress occurs early in seed filling the yield is reduced without reduction of seed number as a result of a shortened seed filling period (DE SOUZA, EGLI & BRUENING, 1997). Water stress during seed filling and physiological maturity has the biggest effect on seed yield reductions (DE BRUYN, PRETORIUS, HUMAN, & DE BRUYN, 1995). This is brought about by accelerating the decline in leaf photosynthetic activity and an increase in the remobilization of C and N to the seed (DE SOUZA, EGLI & BRUENING, 1997). Drought stress seemed to trigger a signal causing the early switching of plant development from vegetative to reproductive growth (DESCLAUX & ROUMET, 1996). Each reproductive phase was shortened under stress. Thus, seed filling and subsequent seed abortion began earlier.

Water use efficiency is higher in drought stressed soybean than in non-stressed plants (CHEN, BEGONIA, ALM, & HESKETH, 1993). Protein and oil content of soybean seed and fatty acid composition of the oil can be altered by

environmental stress. The fatty acid composition of each phospholipid class was altered by drought stress (DORNBOS, MULLER, & HAMMOND, 1989). The linolenic acid proportions increased and the palmitic acid proportions decreased. The plasma membrane of drought stressed soybean hypocotyls were separated from the cell walls and there were more vacuoles in the cytoplasm. With increased drought the protoplasm disintegrated, the nuclear membrane collapsed and the nucleoplasm became disorganised (YIN, WANG & LIU, 1987). These changes were more pronounced in susceptible cultivars than in tolerant cultivars.

Water stress may effect a variety of plant processes, including chlorophyll and protein turnover and photosynthesis (MAJUMDAR, GHOSH, GLICK, DUMBROFF, 1990). In addition to a loss of chlorophyll, the senescence of a leaf is characterised by a progressive increase in the photosynthetic rate per unit of leaf area. Poor correlation between chlorophyll concentrations and photosynthetic activities in senescing leaves of several plant species has been observed (FRIEDRICH & HUFFAKER, 1980). MAJUMDAR, GHOSH, GLICK & DUMBROFF (1990) observed that drought stressed soybean plants experienced a change in chlorophyll levels and chlorophyllase activity with an inverse relationship. A decline in chlorophyll was accompanied by a steady rise in enzyme activity. The photosynthetic activity, transpiration, stomatal conductance, intercellular CO₂, and leaf water potential were also reduced by water stress (PURWANTO, 1994). Reduction in leaf photosynthesis was associated with decreases in leaf stomatal conductance and small increases in leaf intercellular CO₂ concentrations (FREDERICK, ALM, HESKETH & BELOW, 1990). Changes in photosystem II (PSII) activity during drought stress were observed by PLANCHON (1991) by using Rdf (ratio of decrease fluorescence = $F_m - F_o / F_m$) as a parameter. Rdf is also associated with CO₂ exchange.

1.3. TRANSFORMATION

Global population will increase to more than ten billion people by early in the next century. Few other technologies can even approach biotechnology's potential to push back starvation in the next century as traditional plant breeding has in this century. Biotechnology has been around for thousands of years - ever since men and women began selecting the most productive plants to feed their families. Although it has been around nearly as long as human civilisation has produced its own food, it is the modern agriculture biotechnology - genetic manipulation - which is increasingly being recognised as a source of medical and agricultural breakthroughs. Through the centuries, plants were bred and selective crossings were performed, but the outcome was the transfer of hundreds of genes to the offspring. Biotechnology made it possible to transfer only the gene(s) of interest. In the literature, biotechnology has been defined in the following way: "The applied use of living organisms or their components to make or modify products to improve plants or animals or to develop micro-organisms for specific uses." (INDUSTRY CANADA, 1996). Biotechnology does not refer to one specific process but rather a diversity of means for using living organisms or their products and it involves the integration of both science and engineering.

1.3.1. Gene transfer

Gene technology is the transfer of DNA between living cells to produce a specific outcome. In principle, a genetically-based characteristic in one organism can be transferred to a different organism using gene technology. A genetically modified organism (GMO) is thus an organism bearing a gene from a different species or which over expresses or under expresses one of its own genes. Gene technology is made possible because DNA from one organism is chemically similar to DNA from another organism. Biotechnology uses knowledge of how genes and proteins work to transfer the gene sequence(s) that codes for a particular beneficial protein

through a process called genetic engineering (BROWN, 1990). Plant transformation started with a report on successful incorporation of the kanamycin resistance gene into the tobacco genome (FRALEY, ROGERS, HORSCH, SANDERS, FLICK, FINK, HOFFMAN & SANDERS, 1983). Since then successful transformations have been described for over 120 species in 35 different families (BIRCH, 1997).

The general process for manipulation of genes to be transferred into the genome of plant cells is carried out in two phases: Firstly, all the cloning and DNA modification steps are done in *Escherichia coli* and secondly, the plasmid containing the gene construct of interest is transferred by conjugation into *Agrobacterium* or used in particle bombardment.

1.3.2. *Agrobacterium*

Plant transformation involves the selection and application of a delivery system for incorporation of the gene construct into a viable host cell. Recognition of the ability of the soil bacterium *Agrobacterium tumefaciens* (*A. tumefaciens*) to transfer a portion of its DNA to plants was perhaps one of the most important milestones in plant biotechnology (WATSON, CURRIER, GORDON, CHILTON & NESTER, 1975). It was noted that two *Agrobacterium* species could be used as a transformation delivery system, *A. tumefaciens* and *A. rhizogenes* (ZAMBRYSKI, TEMPE & SCHELL, 1989). Transfer and expression of foreign genes in plant cells, has become a major tool to carry out gene expression studies and to attempt to obtain improved varieties of potential agricultural or commercial interest. *A. tumefaciens* causes crown gall tumors, a neoplastic transformation of the wounded tissue of a wide range of dicotyledonous plants (DE CLEENE & DE LEY, 1976). The Ri plasmid of *A. rhizogenes* leads to abundant proliferation of roots (FRIEDBERG, 1999). With the exception of the viruses the *Agrobacterium*-plant cell interaction is the only known natural example of active interkingdom

DNA transport (SHENG & CITOVSKEY, 1996). The transformation process is a unique mixture of several distinct steps, some of which are evolutionarily and functionally related to bacterial conjugation and some of which converge with eukaryotic cellular processes (ZUPAN & ZAMBRYSKI, 1997).

Agrobacterium is a soil dwelling pathogenic bacteria described by BRAUN (1943). All oncogenic *Agrobacterium* strains have a large tumor-inducing (Ti) plasmid. Only a small percentage of natural soil populations of *A. tumefaciens* are found to be oncogenic (ZUPAN & ZAMBRYSKI, 1995). This Ti plasmid is responsible for tumor development. This is due to a natural capacity to transfer, insert and express a particular segment of DNA in the plant cell genome (WATSON, CURRIER, GORDEN, CHILTON & NESTER, 1975). The segment of the Ti plasmid DNA which is transferred from the bacterium and integrated into the plant genome is called the T-DNA (transferred DNA). *Agrobacteria* are classified based on the type of opines specified by the T-DNA. Opines are plant tumor-specific compounds produced by crown gall cells (HOOYKAAS & SCHILPEROORT, 1992). Typical opines present in tumors are octopine or nopaline (HOOYKAAS & BEIJERSBERGEN, 1994). The octopine Ti plasmid contains three T-DNA elements and the nopaline type only one (ZAMBRYSKI, 1992). The T-DNA is a discrete segment of DNA located between two T-DNA borders (25 bp direct repeats) (SHENG & CITOVSKEY, 1996). These borders are the only *cis* elements necessary to direct T-DNA processing (ZUPAN & ZAMBRYSKI, 1995). As the T-DNA element is defined by its borders, the coding region of the wild type T-DNA can be replaced by any other DNA sequence without any effect on its transfer from *Agrobacterium* to the plant genome (SHENG & CITOVSKEY, 1996).

Genetic analysis of the Ti plasmid revealed that in addition to the *onc* genes located on the T-DNA, a large number of other genes are involved in

tumorigenicity (HOOYKAAS & SCHILPEROORT, 1992). Some are located on the Ti plasmid in a 30 kb segment (FRIEDBERG, 1999) called the virulence region (*vir* genes) or they are located on the chromosome (*chv* genes). The *vir* and *chv* genes do not have an essential oncogenic function, but rather determine the transfer procedure. It is known that the *chv* genes are constitutively expressed, but the *vir* genes are silent until activated by certain plant factors (HOOYKAAS & SCHILPEROORT, 1992). The protein products of the *vir* genes respond to these secreted compounds of the wounded plant cells in generating a copy of the T-DNA and mediate its transfer into the host cell (SHENG & CITOVSKY, 1996). Some of these compounds were identified: phenolic compounds such as acetosyringone and hydroxy-acetosyringone (STACHEL, MESSENS, VAN MONTAGU & ZAMBRYSKI, 1985), coniferyl alcohol and sinapinic acid (HOOYKAAS & SCHILPEROORT, 1992) and flavonoids (ZERBACH, DRESSLER & HESS, 1989). Following induction of *vir* gene expression, the T-DNA element is activated to generate a transferable T-DNA copy and the genes are expressed in the plant cell nucleus (BYTEBIER, DEBOECK, DE GREVE, VAN MONTAGU & HERNALSTEENS, 1987). T-DNA insertions into the plant genome are postulated to be mediated by proteins transported from the infected bacterium and/or by host cell factors. The integration initiates at the left border region and T-DNA associated proteins ligate the right border end of the T-DNA to the genomic plant DNA (SHENG & CITOVSKY, 1996). *Agrobacterium* DNA transfer is highly regulated and is triggered only in the presence of susceptible plant cells. When the plant cells are wounded, low-molecular-weight phenolic compounds are produced, which are recognised by *Agrobacterium* as signal molecules that induce the *vir* genes. This expression initiates the mobility and transfer of the T-DNA to the plant cell (ZAMBRYSKI, 1988).

1.3.3. Marker genes

Transformation of plant cells by introducing exogenous genes is an inefficient process as only a small proportion of cells successfully take up, integrate, and express the new genetic material. Because the few cells that do so are not readily distinguishable from the vast majority that do not, researchers frequently link a resistance marker gene to the gene(s) of interest (called the construct) to allow them to distinguish between transformed and nontransformed cells. An antibiotic-resistance marker gene produces a protein that protects plants from a specific antibiotic. When exposed to the antibiotic, cells that have not taken up the new gene are unable to grow and thus die, while transformed cells continue to grow and divide. Use of a marker gene is usually secondary to the principal objectives of the genetic transformation. The marker gene is linked to the gene of primary interest and transferred into the recipient organism at the same time. When both transformed and non-transformed cells are exposed to the antibiotic, only the transformed ones survive. The researchers can thus be confident that the surviving cells contain the gene of primary interest. Selectable marker genes are thus essential for the production of transgenic plants (HARDING, 1995).

Antibiotic resistance genes are present in transgenic plants as a result of the use of marker genes to select transformed plant cells (HARDING, 1995). These genes are under the control of eukaryotic promoters and are expressed in the transgenic plant. Transgenic plants may also contain antibiotic resistance genes that are under the control of prokaryotic promoters, and therefore, not expressed. The latter are incorporated into plant genomes because they are present on constructs used to transform plant cells, having been used for selection in bacteria in earlier steps (SALYERS, 1996).

The kanamycin resistance (*kan^r*) marker gene is one of the most widely used selectable marker genes. The *kan^r* gene, which was originally isolated as a

component of transposon Tn5 from the bacterium *Escherichia coli* (BECK, LUDWIG, AUERSWALD, REISS & SCHALLER, 1982) encodes aminoglycoside 3'-phosphotransferase II (APH(3')II) (GOLDMAN & NORTHROP, 1976). APH(3')II is an enzyme with an apparent molecular weight of 25,000 that catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics including neomycin and kanamycin, thereby inactivating the antibiotics (DAVIES & SMITH, 1978; GOLDMAN & NORTHROP, 1976). Other names used for this enzyme are neomycin phosphotransferase II (NPT II), neomycin phosphotransferase and kanamycin phosphotransferase II.

The expression of these marker genes has raised questions concerning their use and safety in food products. The main concern is that the presence of antibiotic-resistance genes might affect the therapeutic efficacy of antibiotics that are used in human medicine. The fear is that the gene might be transferred to pathogenic bacteria, which would then become resistant to the antibiotic and thus pose a danger to humans. It was suggested that this might occur either through humans eating transgenic plants in which the gene has been incorporated, or through animals eating the plants and subsequently transferring the resistant bacteria to humans. The concern with antibiotic marker genes is that chloramphenicol is commonly used in the treatment of typhoid fever, streptomycin for tuberculosis while hygromycin has important veterinary uses. In the case of kanamycin, the gene product detoxifies kanamycin by phosphorylation (HARDING, 1995). *De novo* phosphorylated metabolites may have consequences on insect life. Thus, crops containing these marker genes raised a food safety issue.

One of the concerns about the safety of these marker genes is the likelihood of gene transfer from plants to micro-organisms into the gut of humans and animals. Such transfer, if any, would likely be insignificant when compared to transfer

between micro-organisms, and in most cases, would not add to existing levels of resistance in bacterial populations in any meaningful way. Transfer to the gut is unlikely because the DNA will be degraded by digestive processes (McALLAN, 1980) and nucleases which degrade DNA and thus provides yet another barrier to transformation (FLINT & THOMPSON, 1990). Even if some were to survive digestion and were transferred, integrated and expressed, epithelial cells are short lived and would be replaced by non-transformed cells. Some experts cautioned that one should assume that DNA can get into the cells lining the gut. However, the critical factor is the lack of selective pressure (SCHUBBERT, RENZ, SCHMITZ & DOERFLER, 1997).

It is highly unlikely that antibiotic resistance genes could be transferred from plant genomes to gut micro-organisms. Firstly, there are no known mechanisms for the direct transfer of plant genomic DNA to micro-organisms. Secondly, there are several barriers to potential transfer. These include degradation by acid and nucleases in the stomach and intestines, the bacterial restriction and modification systems that destroy foreign DNA that enters the cell, the absence of homologous ends for efficient integration into the bacterial genome, and lack of selective pressure (U.S. FOOD & DRUG ADMINISTRATION, 1994). In addition, when any DNA (including antibiotic resistance genes) is integrated into plant genomes, the codon usage may have been altered for more efficient expression in the plant and the gene may have picked up methylation patterns of the plant. If this DNA were now taken up by a bacterium, it would be recognised as foreign and degraded by the micro-organism's restriction endonucleases, thus making integration into the genome and subsequent expression even more unlikely. Moreover, transfer between bacteria, even among broadly different phylogenetic lines, is far more likely than from plants to bacteria. Finally, since uptake is usually not sequence-specific, the antibiotic resistance gene would be competing for transfer into a

bacterium with the rest of the DNA in the plant genome and DNA from other sources in the diet (U.S. FOOD & DRUG ADMINISTRATION, 1992).

The spread of genetic information from a plant to micro-organisms via non-sexual processes (horizontal transfer) is very low. The risk of transfer from a plant genome to soil micro-organisms is not significant as DNA from plant debris would be unavailable for transfer because it would be degraded by nucleases when the plant cell lysed (HARDING, 1995). Transfer from bacteria to bacteria accounts for most dissemination of certain antibiotic resistance markers in soil bacteria.

Another concern about GMOs is the possible production of toxins and the allergenic status of the marker gene. Unlike most allergenic proteins, the kanamycin gene (APH(3')II) is rapidly inactivated by stomach acid and is degraded by digestive enzymes (FUCHS, 1993). APH(3')II does not possess any of the characteristics associated with allergenic proteins such as proteolytic stability, glycosylation, or heat stability (TAYLOR, LEMANSKE, BUSH & BUSSE, 1987).

Transgenic plants have the potential to exchange genetic information with other organisms. The possibility of pollen from a transgenic plant being carried via insects or wind to sexually compatible species exists. The DNA that makes up the marker gene does not differ from any other DNA and does not itself pose a safety concern as a component of food.

1.3.4. Reporter genes

Reporter genes are important tools for the monitoring of the expression of gene constructs as well as for the optimisation of the transformation protocol. Reporter genes are used as indicators for the identification of sequences and factors that control expression at the transcriptional level. Quantification of the protein

activity of a reporter gene, provides indirect measurement of gene expression (BRONSTEIN, FORTIN, VOYTA, JUO, EDWARDS, OLESEN, LIJAM, & KRICKA, 1994).

The bacterial β -glucuronidase (GUS) gene has become the most routinely used reporter gene (THOMASSET, MENARD, BOETTI, DENMAT, INZE & THOMAS, 1996). The system is based on the *E.coli* gene encoding for GUS (the *uidA* locus) which has a monomer molecular weight of about 68.2 kDa (JEFFERSON, BURGESS & HIRSH, 1986). The enzyme is very stable under different physiological conditions and GUS activity can be measured accurately by spectrophotometric, histochemical and fluorometric methods (JEFFERSON, KAVANAGH & BEVAN, 1987). MUG (4-methylumbelliferyl β -D-glucuronide) is cleaved to form the fluorescing product methyl umbelliferone (MU), which is an indication of GUS activity (JEFFERSON, KAVANAGH & BEVAN, 1987).

One major complication in the use of indicator genes together with *Agrobacterium*-mediated transformation, is the fact that the genes are expressed in *Agrobacterium* as well. In order to overcome this problem an intron-containing marker gene was constructed (VANCANNEYT, SCHMIDT, O'CONNER-SANCHEZ, WILLMITZER & ROCHA-SOSA, 1990). In this case, it was a plant intron introduced into GUS. In transgenic plants (containing this reporter gene), the GUS activity was detected, but no activity was observed in *Agrobacterium* cells (VANCANNEYT, SCHMIDT, O'CONNER-SANCHEZ, WILLMITZER & ROCHA-SOSA, 1990).

1.3.5. Promoters

The expression of the foreign gene in the host cell is limited to the activity of its promoter. Some of the commonly used promoters are viral promoters e.g. cauliflower mosaic virus promoters (BENFEY, REN & CHAU, 1989) and

nopaline synthetase of *Agrobacterium* (MORELLI, NAGY, FRALEY, ROGERS & CHUA, 1985) which are always active. In many transformation experiments tissue specific expression is very important and plant specific promoters were isolated. Isolated plant promoters' respond to light (ELLISTON & MESSING, 1989), stress (AINLEY & KEY, 1990) or growth hormones (ELLISTON & MESSING, 1989). The use of these promoters allows control of expression of the foreign gene in the host cell.

1.4. TRANSFORMATION IN SOYBEAN

Soybean genotypes with good agronomic characteristics would be desirable for transformation attempts. An approach for genetic improvement of plants grown under drought conditions will be to identify expression of foreign genes that induces drought and osmotic tolerance in cells subjected to extreme conditions. These genes must then be transferred into crops of agronomic interest.

1.4.1. Tissue culture

The development of procedures by which plants could be regenerated from single cells and organised tissues with specific genes transferred to these plant cells, was the prerequisite for practical genetic engineering for soybean improvement. The limited genetic base in domestic soybean cultivars has restricted the traditional breeding methods to value added traits (HINCHEE, CONNER-WARD, NEWELL, McDONNELL, SATO, GASSER, HELMS, DECKARD, GOOS & ENZ, 1996). The process for the production of transgenic soybean plants is much longer and more labor intensive than those of the model plant systems such as tobacco (LUO, HEPBURN & WIDHOLM, 1994). *In vitro* techniques were thus applied with a view to improvement of soybean plants. Two principle methods

have been identified for soybean regeneration: somatic embryogenesis and shoot morphogenesis.

Somatic embryogenesis is the process whereby embryos develop from either microspores or somatic tissue. Somatic embryos have both shoot and root axes and produce whole plants upon germination. Regeneration via somatic embryogenesis offers great potential for use in mass propagation and in transformation (TRICK, DINKINS, SANTAREM, DI, SAMOYLOV, MEURER, WALKER, PARROTT, FINER & COLLINS, 1997). Early attempts at soybean transformation focused on regeneration of embryogenic suspension cultures. Several studies with somatic embryos were conducted from 1973 to 1983, but developmental progress was made only as far as the torpedo stage (KIMBALL & BINGHAM, 1973; GAMBORG, DAVIS & STAHLQUIST, 1983). CHRISTIANSON, WARNICK & CARLSON (1983) were the first to report successful embryogenic regeneration of soybean. They were able to regenerate one immature embryo from one genotype. Regeneration of complete plants was reported with the use of callus, which was derived from immature embryos (KERN, HR, BARWALE, UB, MEYER, MMJ & WIDHOLM, 1986). Immature cotyledon research experienced a period of growth with the discovery of the multicellular origin of somatic embryos (HARTWECK, LAZZERI, CUI, COLLINS & WILLIAMS, 1988, the apical origin of somatic embryos (FINER, 1988) and proliferated globular embryos in embryogenic cell suspension cultures (FINER & NAGASAWA, 1988). However, up to this stage genotype still determines the efficiency of somatic embryogenesis (KOMATSUDA, KANEKO & OKA, 1990). Factors influencing *in vitro* growth rates of soybean embryos were reported on (LAZZERI, HILDEBRAND & COLLINS GB, 1987a & b; LIPMANN & LIPMANN, 1993). BAILEY, BOERMA & PARROTT (1993a & b) reported on the quantification of the proliferative embryogenesis protocol. A routine method for somatic embryogenesis is now available (PARROTT, DRYDEN, VOGT,

HILDEBRAND, COLLINS & WILLIAMS, 1988; PARROTT, DURHAM & BAILEY, 1995).

Shoot morphogenesis is the process of shoot formation and development. Shoots can be formed from a number of different tissues and can be excised and rooted to generate new plants (TRICK, DINKINS, SANTAREM, DI, SAMOYLOV, MEURER, WALKER, PARROTT, FINER & COLLINS, 1997). Current success stories include the regeneration of pre-existing meristems of cotyledonary nodes of seedlings (CHENG, SAKE & VOQUI-DINH, 1980); 54 cultured genotypes from cotyledonary nodes of immature embryos (BARWALE, KERNS & WIDHOLM, 1986); organogenesis (WRIGHT, KOEHLER, HINCHEE & CARNES, 1986); epicotyl shoot regeneration (WRIGHT, WILLIAMS, PIERSON & CARNES, 1987) and multiple shoot proliferation from shoot tips from immature zygotic embryos (SATO, NEWELL, KOLACZ, TREDI, FINER & HINCHEE, 1993). All of these methods resulted in the recovery of whole plants from the regeneration medium.

Other methods that were also achieved are the isolation, encapsulation and culture of protoplast (TRICOLI, HEIN & CARNES, 1986; WIDHOLM, DHIR & DHIR, 1992) and anther cultures (YE, FU & WANG, 1994).

1.4.2. Transformation processes

1.4.2.1. Direct transformation methods

Stable transformation of soybean callus (CHRISTOU, McCABE & SWAIN, 1988) and meristems of embryonic axes of immature seeds (McCABE, SWAIN, MARTINELL & CRISTOU, 1988) were obtained by bombardment of DNA-coated gold particles. As a result of the invention and optimisation of the particle bombardment technique soybean genetic engineering became a reality as it was shown that whole plants could be derived from single transformed cells by a *de*

novo organogenic pathway (CHRISTOU, SWAIN, YANG & McCABE, 1989). A commercial process was developed by the combination of a genotype-independent regeneration protocol and an electric discharge particle acceleration technique (CHRISTOU, SWAIN, YANG & McCABE, 1990). It was indicated by CHRISTOU & SWAIN (1990) that the co-transformation frequency of linked genes was about 50% and unlinked genes were about 20%.

According to CHRISTOU (1997) particle bombardment is the only method at present, which can be used commercially for the engineering of soybean in a variety-independent fashion. FINER & McMULLEN (1991) also used particle bombardment as a transformation technique with embryogenic suspension cultures as explants. Transient gene expression of the GUS gene was shown using embryogenic suspension cultures (BOND, McDONNELL & GRESSHOFF, 1995). SATO, NEWELL, KOLACZ, TREDO, FINER & HINCHEE, (1993) reported stable transformation via particle bombardment using shoot tip cultures and somatic embryogenesis. Bombardment of shoot tips produced GUS positive sectors in 30% of the regenerated shoots. However, none of the regenerants that developed into plants, produced GUS positive tissue. The bombardment of the embryogenic suspension cultures resulted into GUS positive plants.

Stable transformation of soybean cells has been achieved through direct uptake of DNA into protoplasts that were permeabilised by electroporation (CHRISTOU, MURPHY & SWAIN, 1987; CHRISTOU, SWAIN, YANG & McCABE, 1990; LIN, ODELL & SCHREINER, 1987). A series of articles were published on recovery of transgenic soybean plants using protoplast electroporation (DHIR, DHIR & WIDHOLM, 1991; DHIR, DHIR, STURTEVANT & WIDHOLM, 1991; WIDHOLM, DHIR, & DHIR, 1992; DHIR, DHIR, SAVKA, BELANGER, KRIZ, FARRAND & WIDHOLM, 1992) however, these claims were retracted. *In planta*

gene transfer was reported by electroporation-mediated gene transfer using intact meristems (CHOWRIRA, AKELLA & LURQUIN, 1995).

1.4.2.2. *Agrobacterium*-mediated methods

Soybean was considered a poor host for *A. tumefaciens* (MATTHYSSE & GURLITZ, 1982). The contrary was however proved, with gall formation on some soybean genotypes following inoculation with the octopine type Ti plasmid (OWENS & CRESS, 1985). A number of South African cultivars produced tumours in response to infection with *A. tumefaciens* strain A281 (McKENZIE & CRESS, 1992; AUSTIN & CRESS, 1994). It was indicated that the co-infection with a supervirulent strain or the addition of a phenolic compound could promote transformation of soybean cells (OWENS & SMIGOCKI, 1988). BYRNE, McDONNELL, WRIGHT & CARNES (1987) tested the response of different *Agrobacterium* strains on various genotypes. They reported a large degree of variation between strains and genotypes. The susceptible genotypes displayed a heightened response to nopaline strains of *A. tumefaciens* and *A. rhizogenes*. It was thus demonstrated that tumors form on soybean in response to infection with *A. tumefaciens*, but not to the extent observed in other dicotyledons such as tobacco. Tumorigenesis of soybean is a quantitative trait (BAILEY, BOERMA & PARROTT, 1994) and the heritability estimates are higher than 50% (MAURO, PFEIFFER & COLLINS 1995). This characteristic could easily be transferred to new genotypes. However, genotype differences for tumorigenesis are not necessarily a reflection of the frequency of integration or T-DNA expression (FACCIOTTI, O'NEAL, LEE & SHEWMAKER, 1985), it can be manifested later through oncogenic expression (VAN WORDRAGEN, DE JONG, SCHORNAGEL & DONS, 1992). Predictions of gene integration and expression are thus more accurate using marker genes (VAN WORDRAGEN, DE JONG, SCHORNAGEL & DONS, 1992).

The development of *Agrobacterium*-mediated transformation techniques has been slow in the late eighties to early nineties, but in a few cases transgenic plants have been obtained. The first experiments describing successful recovery of transformed soybean plants using *Agrobacterium* were reported by HINCHEE, CONNER-WARD, NEWELL, McDONNELL, SATO, GASSER, FISCHHOFF, RE, FRALEY & HORSCH (1988). They produced stable transgenics via shoot organogenesis from cotyledons of the cultivar Peking. Cotyledon explants were inoculated with *A. tumefaciens* harbouring plasmids conferring kanamycin resistance and *gus* activity or kanamycin resistance and glyphosate tolerance. This protocol was however, only 6% efficient. Peking was introduced to the USA in 1906. It has limited agronomic value (MAURO, PFEIFFER & COLLINS, 1995) but was selected for its susceptibility to *Agrobacterium* infection. This *Agrobacterium* procedure did not result in the recovery of transformed progeny with varieties other than Peking. A number of other laboratories attempted to reproduce the system with no success (PARROTT, HOFFMAN, HILDEBRAND, WILLIAMS & COLLINS, 1989; CHRISTOU, 1997).

Soybean protoplasts have been transformed at low frequency by using *Agrobacterium* (BALDES, MOSS & GEIDER, 1987). However, no transgenic plants were regenerated from these transformed protoplasts, as regeneration systems were not available. Reports involving infection by needle inoculation with *Agrobacterium* of germinated seeds (CHEE, FABER & SLIGHTON, 1989), *in vitro* grown seedlings (FACCIOTTI, O'NEAL, LEE & SHEWMAKER, 1985) and cotyledons (McKENZIE, 1991) were also reported. Electroporation of intact nodal meristems was also attempted (CHOWRIRA, AKELLA & LURQUIN, 1995).

TRICK & FINER (1997) developed a new and potentially more effective method for delivery of *Agrobacterium* to plant target tissues called sonication-assisted

Agrobacterium-mediated transformation (SAAT). This method mechanically disrupted and wounded cells via brief periods of ultrasound in the presence of *Agrobacterium*. Immature cotyledons and embryogenic suspension cultures were used as explants (TRICK & FINER, 1998). This method was later applied with success on cotyledonary nodes (MEURER, DINKINS & COLLINS, 1998). The SAAT resulted in efficient transformation of the total tissue surface, unlike particle bombardment where DNA-coated particles are delivered only to one side of the target tissue and penetration is limited (TRICK & FINER, 1998). Thus, although success has been reported the *Agrobacterium*-mediated transformation procedure it is still limited by host- and tissue-specificity problems.

1.4.3. Aberrations

DNA integration patterns in transformed plant tissue obtained via particle bombardment tend to be highly variable and multiple or fragmented copies of the introduced gene constructs are common (HADI, McMULLEN & FINER, 1996). In contrast, *Agrobacterium*-mediated transformation results in lower copy number integration (TINLAND, HOHN & PUCHTA 1994). Transgenic plants obtained through the SAAT procedure (TRICK & FINER, 1998) were sterile as a result of the use of long-termed embryogenic suspension cultures as also described previous (HADI, McMULLEN & FINER, 1996). The sterility is the function of the tissue culture process and not the transformation process.

Some aberrations observed in transgenic soybean include: stunted plant growth, leathery dark green leaves, partial to total sterility observed in bombarded embryogenic suspension cultures (SINGH, KLEIN, MAUVAIS, KNOWLTON, HYMOWITZ & KOSTOW, 1998). Chromosomal deletions, trisomics and tetraploidy in some transgenic soybean was also reported (SINGH, KLEIN, MAUVAIS, KNOWLTON, HYMOWITZ & KOSTOW, 1998).

1.4.4. Some examples of genetically engineered soybean

Recent progress in the development of technologies for transformation of soybean has opened the way to genetically engineered soybean. One of the first practical applications of genetic engineering has been the development of tolerance to glyphosate, the active component in the herbicide Roundup (PADGETTE, KOLACZ, DELANNAY, RE, LAVALLEE, TINIUS, RHODES, OTERO, BARRY, EICHHOLTZ, PESCHKE, NIDA, TAYLOR & KISHORE, 1995). Transgenic soybean were obtained with a synthetic Bt CRYIA c gene (STEWART, ADANG, ALL, BOERMA, CARDINEAU, TUCKER & PARROTT, 1996) and a Bt CRYIA b gene (PARROTT, ALL, ADANG, BAILEY, BOERMA & STEWART, 1994) gene of *Bacillus thuringiensis* (*Bt*). Field trials of herbicide resistant soybean generated via particle bombardment are ongoing (CHRISTOU, 1997).

The genetic engineering of soybean with other genes than those conferring herbicide resistance is for example genes to induce higher lysine content (FALCO, GUIDA, LOCKE, MAUVAIS, SANDERS, WARD & WEBBER, 1995) and the bean pod mottle virus coat protein precursor gene (DI, PURCELL, COLLINS & GHABRIAL, 1996)

CHAPTER 2

DEVELOPMENT OF AN AGROBACTERIUM-MEDIATED TRANSFORMATION TECHNIQUE OF GERMINATING SOYBEAN SEED WITH THE GUS-INT MARKER GENE

2.1. INTRODUCTION

Soybean is an important annual seed legume grown for the production of oil and protein. The development of gene transfer techniques for soybean is of commercial interest as it will facilitate the development of cultivars with improved characteristics such as resistance to environmental stress and disease, increased nutritional value, as well as tolerance to specific herbicides. The transfer and expression of foreign genes in plant cells will also facilitate the study of gene expression.

While transgenic soybean has already been produced using several transformation techniques, no single transformation technique has yet become a practical breeding method in soybean. Only a handful of laboratories have had more than sporadic success (TRICK, DINKINS, SANTAREM, DI, SAMOYLOV, MEURER, WALKER, PARROTT, FINER & COLLINS, 1997). The transformation process can only be successful if it is coupled to an efficient and cost effective regeneration process. Only the knowledge of both processes and the ability to combine them will result in the recovery of transgenics (TRICK, DINKINS,

SANTAREM, DI, SAMOYLOV, MEURER, WALKER, PARROTT, FINER & COLLINS, 1997). Techniques for efficient introduction of foreign genes into soybean have been limited by the availability of a regeneration procedure in which transformation can be included.

A number of procedures exist for the introduction of foreign DNA into soybean, e.g. through electroporation (CHRISTOU, McCABE, MARTINELL & SWAIN, 1990, CHOWRIRA, AKELLA & LURQUIN, 1995), particle bombardment (McCABE, SWAIN, MARTINELL & CHRISTOU, 1988, FALCO, GUIDA, LOCKE, MAUVAIS, SANDERS, WARD & WEBBER 1995; SATO, NEWELL, KOLACZ, TREDO, FINER & HINCHEE, 1993; HADI, McMULLEN & FINER, 1996), sonication-assisted (TRICK & FINER, 1997) or direct *Agrobacterium*-mediated transformation (HINCHEE, CONNER-WARD, NEWELL, McDONNELL, FISCHHOFF, RE, FRALEY & HORSCH, 1988; CHEE, FOBER & SLIGHTON, 1989).

Gene transfer is traditionally based on the use of *in vitro* cultured cotyledons (AL-JANABI & SHOEMAKER, 1992; MEURER, DINKINS & COLLINS 1998), protoplasts (CHRISTOU, McCABE & SWAIN, 1988), meristematic tissue (McCABE, SWAIN, MARTINELL & CHRISTOU, 1988) or embryogenic cell suspension cultures (TRICK & FINER, 1998). Such transformation techniques vary in efficiency, from 0.4% with particle bombardment (SATO, NEWELL, KOLACZ, TREDO, FINER & HINCHEE, 1993) to 23% with electroporation (DHIR, DHIR, STURTEVANT & WIDHOLM 1991). These techniques are costly and also require considerable time and specialist knowledge before a stable, transgenic greenhouse plant can be obtained. This restricts genetic engineering of soybean to only a few universities and laboratories.

The development of a method to obtain transformed plants, which is independent of the problems inherent to tissue culture, has been the dream of many laboratories. A non-tissue culture approach to *Agrobacterium*-mediated transformation using germinating seed of *Arabidopsis thaliana* was first reported by FELDMANN & MARKS (1987). This method was improved by BOUCHEZ, CAMILLERI & CABOCHE. (1993). Thereafter, BECHTOLD, ELLIS, PELLETIER (1993) applied a variation of the method to whole plants. CHEE, FOBER & SLIGHTOM (1989) succeeded in transforming soybean seed with an *Agrobacterium*-mediated transformation technique, but with a very low efficiency, only 0.7%.

As confirmation of transformation with any technique may be problematic, use of a reporter system simplifies analysis of transformed plants. Use of an indicator gene that encodes an enzyme not found in the organism under study greatly simplifies the selection and/or screening for transformants. The sensitivity with which the gene activity can be measured is then only a factor of the property of the reporter gene and the quality of the assay. Many reporter genes have been used, but they are all faced with the same problem, that is, they are also expressed in *Agrobacterium* (VANCANNEYT, SCHMIDT, O'CONNER-SANCHEZ, WILLMITZER & ROSCHA-SOSA, 1990). The use of a GUS INT gene allows the expression of the GUS marker enzyme in plants but not in prokaryotic organisms such as *Agrobacterium tumefaciens* (*A. tumefaciens*). The expression of the GUS gene in *A. tumefaciens* is interrupted by insertion of a plant intron into the bacterial gene coding for GUS (VANCANNEYT, SCHMIDT, O'CONNER-SANCHEZ, WILLMITZER & ROSCHA-SOSA, 1990).

South African soybean cultivars can be infected by *A. tumefaciens* and one variety can be regenerated from cotyledon nodes (McKENZIE & CRESS, 1992; AUSTIN & CRESS, 1994). Here an alternative method for obtaining transformed

soybean plants is described, which suggests that *Agrobacterium*-mediated gene transfer can be accomplished at a rate of up to 30% without the need of intermediate tissue culture steps

2.2. MATERIAL AND METHODS

2.2.1. Material

Glycine max (L) Merr, cv. Carnia 2233, was used in all experiments. The plant expression vector pBI121 in *A. tumefaciens* strain LBA 4404 was used for transformation. This binary vector contained the following downstream from the right T-DNA border: *NPTII* gene as selectable marker, nopaline synthetase terminator (NOS-ter), cauliflower mosaic 35S promoter, and as β -glucuronidase reporter genes either GUS (JEFFERSON, KAVANAGH & BEVAN, 1987) or GUS INT (VANCANNEYT, SCHMIDT, O'CONNER-SANCHEZ, WILLMITZER & ROSCHA-SOSA, 1990), followed by the NOS-ter and the left T-DNA border.

2.2.2. Transformation protocol

The transformation procedure was based on a modification of the vacuum infiltration method of BECHTOLD, ELLIS & PELLETIER (1993), developed for the transformation of *Arabidopsis thaliana*. Soybean seed was sterilised for five minutes in 3.5% (v/v) NaOCl, washed twice in sterile water and left to germinate on sterile 0.8% water-agar (Agar-agar) at 29°C in the dark for two days. The germinating soybean seed was sorted into different developmental stages for transformation. The germinating seed used for transformation displayed a visible radicle and shoot tip, an easily removable seed coat and cotyledons that were not appressed against each other.

The pBI121 in LBA4404 (GUS or GUS INT) was cultured at 27°C in 100 ml Luria-Bertani broth (LB) pH 7.00 (SAMBROOK, FRITSCH & MANIATIS, 1989)(Appendix) supplemented with 150 µgml⁻¹ rifampicin and 100 µgml⁻¹ kanamycin until the A₆₀₀ = 0.5. Acetosyringone (AS) (0.01 mgml⁻¹) was added to the *Agrobacterium* culture 24 hours before transformation. Cells were centrifuged at 10 000 rpm for 20 minutes at 10°C. The bacterial pellet was resuspended in 400 ml distilled water supplemented with 0.1% non-oil wetting agent (Break-Thru ®, Goldsmidt Chemical Corporation, Hopewell, VA, USA).

The partially germinated seeds were vacuum infiltrated with the *Agrobacterium*/wetting agent suspension for 20 minutes at 78 millitorr. The treated seeds were incubated for a further 24 hours in the *Agrobacterium* suspension at 25°C, planted in vermiculite and grown in a greenhouse at 25/15 ± 3°C (day/night temperatures). Plantlets with the first trifoliolate leaves fully emerged were transplanted to a soil mixture consisting of soil: sand: vermiculite (5: 5: 3), watered daily and treated fortnightly with 4 gl⁻¹ nutrient mixture (Multi Feed.P^R). Self-pollinated T1 seeds were harvested and germinated on agar plates supplemented with kanamycin for selection of putative transformed seedlings.

2.2.3. GUS detection

When the plants had developed the second set of trifoliolate leaves, a leaf from the main apex was tested for expression of *gus*. A fluorometric GUS assay (modification from JEFFERSON, KAVANAGH & BEVAN, 1987) was used for screening explants for the expression of GUS. Phosphate buffer (100 µl) was pipetted into the wells of a microtiter plate. The assay buffer contained 50 mM Na₂HPO₄ / NaH₂PO₄ buffer (pH 7.00), 10 mM EDTA, 0.1% (v/v) Triton X-100, 10 mM 2-mercaptoethanol and 2 M 4-methylumbelliferyl β-D-glucuronide (MUG) (Sigma).

Small pieces of transformed and untransformed plant tissue, and a streak of bacteria, were crushed in this buffer and incubated overnight at 37°C in the dark. GUS activity was visualised as a blue fluorescence on a long wavelength UV light box (310 nm).

A histochemical assay was also performed in testing the putative transformants according to a modified method of JEFFERSON, KAVANAGH & BEVAN (1987). Tissue from plants that tested positive in the fluorescence assay was incubated overnight at 37°C in the dark in a histochemical staining solution. Untransformed tissue was used as negative control. No background was observed in untransformed tissue. The histochemical staining solution contained 50 mM Na₂HPO₄ / NaH₂PO₄ buffer (pH 7.00), 0.1% (v/v) Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl- β-D-glucuronide (X-GLUC) and 0.5% (v/v) DMSO. The plant tissue was subsequently washed in formalin-acetic acid-alcohol (0.375% (v/v) 95% ethanol, 0.05% (v/v) glacial acetic acid and 0.05% (v/v) 37% formaldehyde) for 10 minutes, followed with a wash in 50% ethanol. The tissue was dehydrated with 100% ethanol to remove chlorophyll and slowly rehydrated to 100% H₂O. Production of a blue precipitate at the site of enzyme activity was visualised microscopically.

2.2.4. Molecular analysis of transformants

DNA of plant tissue (McGREGOR, LAMBERT, GREYLING, LOUW & WARNICH, 2000), putative transformants (T3) as well as untransformed soybean, and plasmids (FELICIELLO & CANAILLI, 1993) were extracted. PCR analyses were performed on T3 plants using GUS and *NPTII* specific primers. The PCR products were separated on a 0.8% agarose gel in 1X TAE buffer, visualised by ethidium bromide staining and sized in comparison to Molecular Weight Marker III (Boehringer Mannheim).

Southern blots were performed on the T3 plants, hybridized with a random primed radioactively labelled ^{32}P probe specific to the GUS gene. Restriction endonuclease digestions were performed on isolated plant DNA separated on 0.8% agarose gels and blotted onto Hybond N+ membranes (Amersham). Hybridization and detection were performed according to SAMBROOK, FRITSCH & MANIATIS (1989).

2.3. RESULTS AND DISCUSSION

Transgenic soybean is a reality, as several transformation techniques have resulted in stable transformants. However, the frequency of plant regeneration does not seem high enough to be applied as a routine breeding method (KANADA, TABEL, NISHIMURA, HARADA, AKIHAMA & KITAMURA, 1997). Although several plant transformation systems are available, an *A. tumefaciens* method with high reproducibility and a high transformation frequency without tissue culture is still needed.

2.3.1. Development of transformation method

A series of experiments were performed in which the optimal conditions for transformation were recorded, in order to develop a reproducible method with a high percentage of transformation success in the transformation of germinating soybean seed. Flocculation of the *Agrobacterium* was overcome by resuspending the *Agrobacterium* cells in distilled water. Although this resulted in some lysed cells, the transformation rate was higher in the water dilution than when a LB culture medium dilution was used. This was due to higher seed mortality as a result of increased bacterial and fungal growth in the presence of LB. The *Agrobacterium* was also diluted to establish the optimum concentration for transformation. The dilutions tested were 1:1, 1:2 and 1:4 respectively. The

transformation frequency was considerably higher with the 1:4 dilution, than with the others. The developmental stage of the seed was of great importance in transformation. The seed was allowed to germinate over a two-day period and then sorted. The highest degree of transformation was obtained with seed containing a minute radicle (about 3 mm) and shoot tip, an easily removable seed coat and cotyledons that were not appressed against each other. A younger developmental stage of the infiltrated seed resulted in damage to the main apex (Figure 1). The amount of this damage can consequently cause either total abortion of the plant (Figure 1A), side growth from the cotyledons (Figure 1B) or in less severely damaged seedlings, early seed production (Figure 1C). The latter two problems did not occur in subsequent generations. At later stages of seed germination, no transformation occurred. The addition of AS to the *Agrobacterium* culture 24 hours before infiltration improved frequencies of transformation. Similar results were also obtained by KATIA, JOAO & BROWN (1993), who observed in tomato transformation that AS enhanced the transformation process, possibly due to stimulation of multiple insertions of the T-DNA into the host genome.

The duration and strength of the vacuum played a role in the percentage of transformants produced. The optimal conditions were found to be 20 minute infiltration under a 78 millitorr vacuum. The efficiency of transformation drastically declined with lower pressures (less vacuum) or lower infiltration times. Transformation efficiency decreased with increasing time or pressure (increased vacuum) as fewer seed survived the vacuum process. The use of a wetting agent in the *Agrobacterium* solution also enhanced transformation, possibly due to a higher penetration percentage of the *Agrobacterium* solution into the cells. The addition of Break-Thru® enhanced the transformation frequency three fold. Transformation percentage increased slightly when the infiltrated seeds were co-cultivated another 24 hours at 27°C in the *Agrobacterium* suspension.

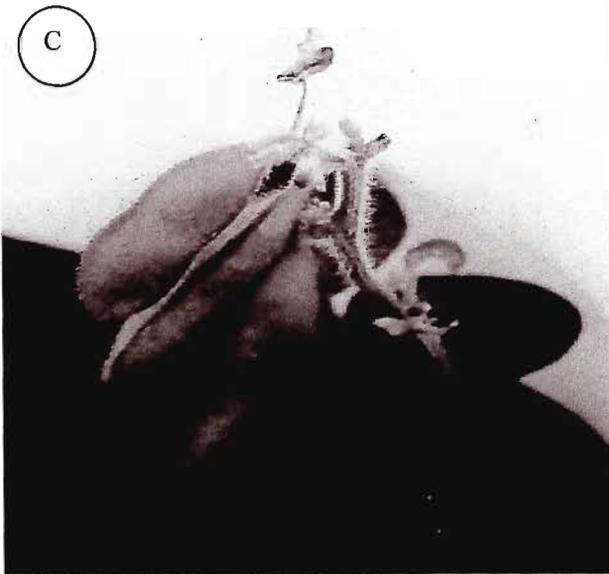
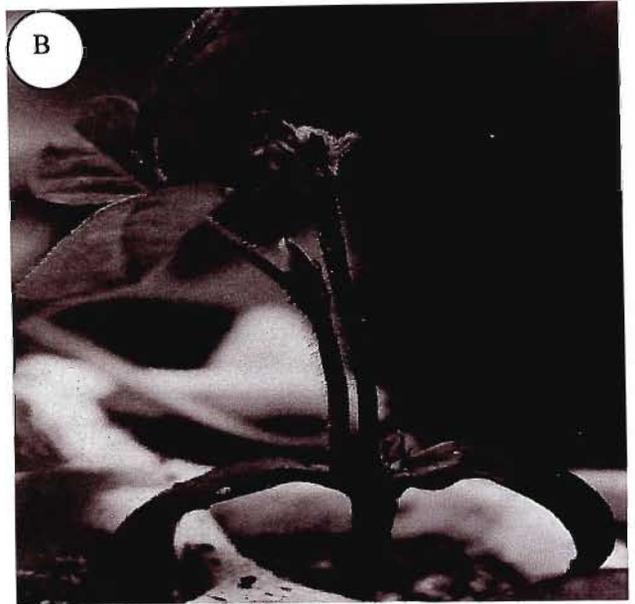
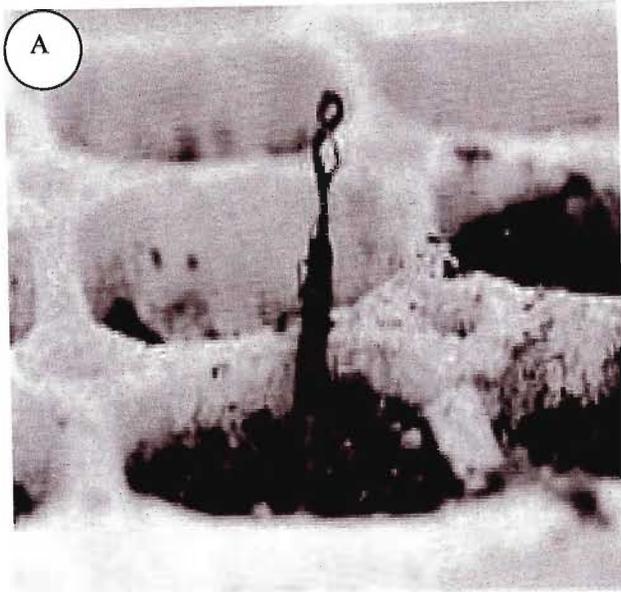


Figure 1: T0 soybean plants displaying damage as a result of an immature developmental stage at infiltration time. (A) Abortion; (B) Side growth; (C) Early seed production

2.3.2. GUS expression

A detailed study of a transformation system requires a visible marker that can be used to assess the degree of transformation as soon as possible after infection. The GUS reporter gene allows the detection of transformed cells by the use of MUG fluorometric and X-GLUC histochemical assays. The histochemical assay gives a blue precipitate at the site of enzyme activity and the fluorometric assay indicates enzyme activity as fluorescence under UV light (310 nm).

An experiment was conducted to determine what the amount of infiltration into the seed was and at what stage transient GUS activity can be observed. Incubated soybean seed was tested for GUS expression 24 hours after infiltration using the histochemical method. Significant GUS enzymatic activity was observed 24 hours after *Agrobacterium* infection in the xylem and phloem of the roots (Figures 2A, B) as well as in the cotyledon (Figure 2C).

The use of the fluorometric and histochemical assays on trifoliolate T0 greenhouse plants indicated transformation of *gus* into the soybean seedlings. The fluorometric assay was used for preliminary screening, as the test is easier, very sensitive and less expensive than the histochemical assay. The MUG fluorometric assay demonstrated fluorescence in leaf tissue of all the putative transformants while there was no fluorescence in the untransformed plants (Figure 3A). Putative transformants identified by this method were again tested and observed under a microscope using the X-GLUC histochemical assay. The putative transformed tissue was incubated for 24 hours in the GUS assay buffer at 37°C. This resulted in blue spots all over the apex leaf (Figure 3B). The penetration of the X-GLUC was subsequently enhanced by a 20 minute vacuum infiltration of the X-GLUC buffer into the cells (Figure 3C).

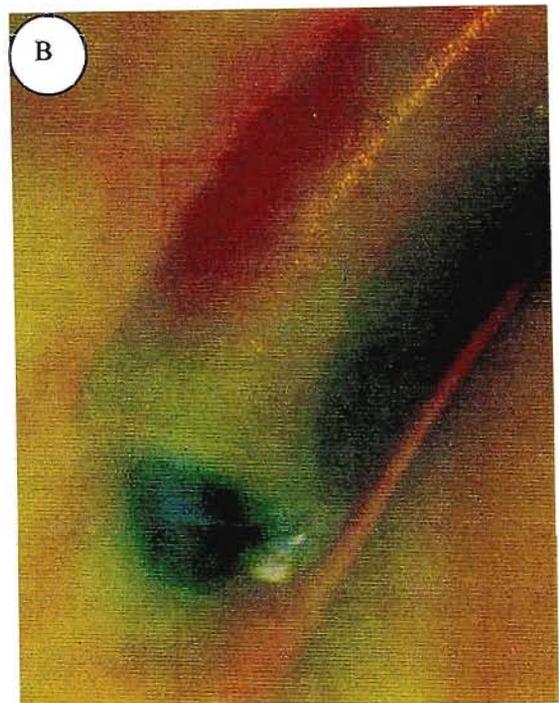
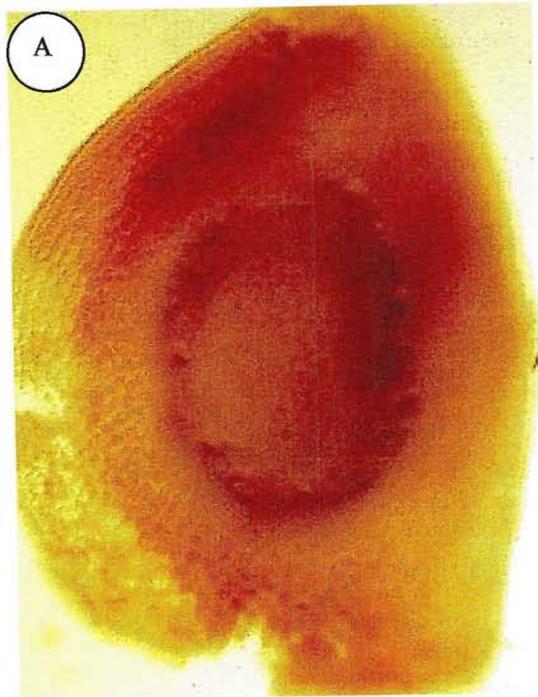


Figure 2: X-GLUC histochemical localisation of transient GUS expression 24 hour after *Agrobacterium* infection seen as a blue precipitant. (A) cross section and (B) side view of the root, (C) cotyledon

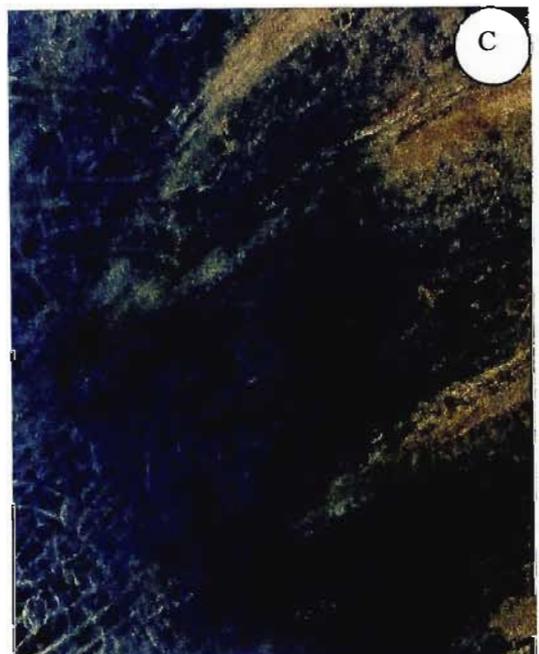
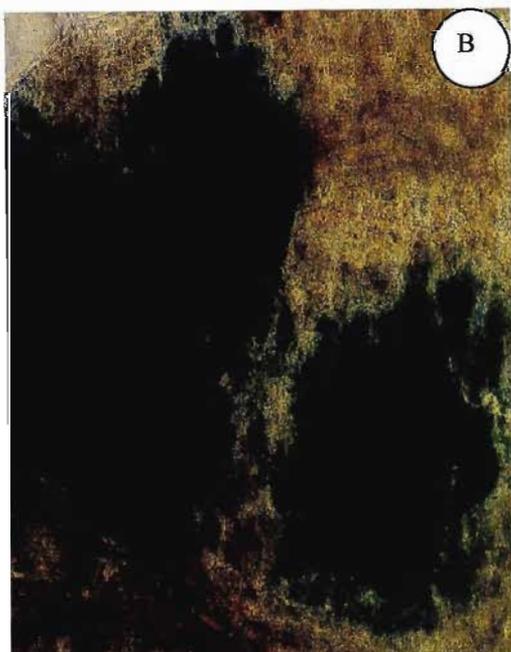
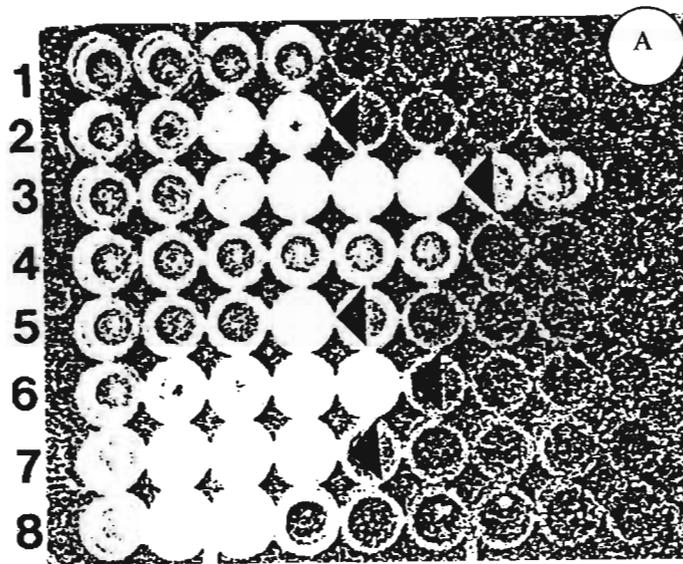


Figure 3: Stable GUS expression of 6-week-old T0 (GUS) soybean plants indicating GUS enzyme activity (A) MUG fluorometric assay; (B) X-GLUC histochemical localisation of blue precipitation in leaf without vacuum infiltration; and (C) X-GLUC histochemical localisation of blue precipitation in leaf with vacuum infiltration

Localisation of GUS expression was found in different organs tested: stem (Figure 4A), stomata (Figure 4B) and veins (Figure 4C) of a trifoliolate GUS INT T0 plant grown in the greenhouse. This indicated that soybean was transformed with the GUS INT gene and that the GUS activity did not arise from endogenous *Agrobacteria*. In Figure 4D the blue precipitate of an immature T1 embryo can be seen, indicating the transfer of *gus* to the progeny.

2.3.3. Kanamycin screening

The presence of the *NPTII* gene in the LBA4404 construct allowed a kanamycin-screening test of the seed. All seed that was produced by the putatively transformed T0 plants was germinated on water-agar plates containing different concentrations of kanamycin. The untransformed seed failed to germinate on the kanamycin plates, whereas the putatively transformed seed germinated on up to 50 mg l⁻¹ kanamycin-agar plates (Table 1). Approximately 50% of the T1 seed produced, demonstrated *NPTII* enzymatic activity, suggesting that at least the *Nos-NPTII* gene region of the binary vector was present in the seed. These kanamycin resistant seeds were planted in the greenhouse and used in subsequent experiments.

Table 1: Germination percentage of transformed and untransformed seed on kanamycin supplemented agar plates

	Kanamycin mg l ⁻¹			
	0	25	35	50
Untransformed seed	100	0	0	0
T1 GUS INT seed	100	75	62	50

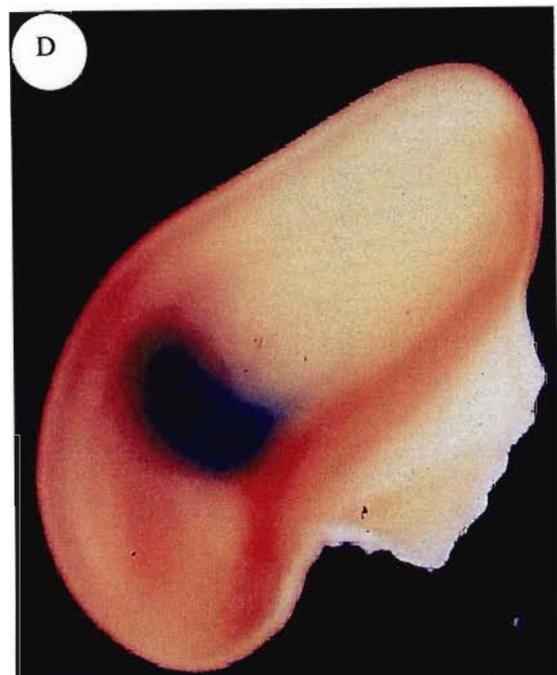
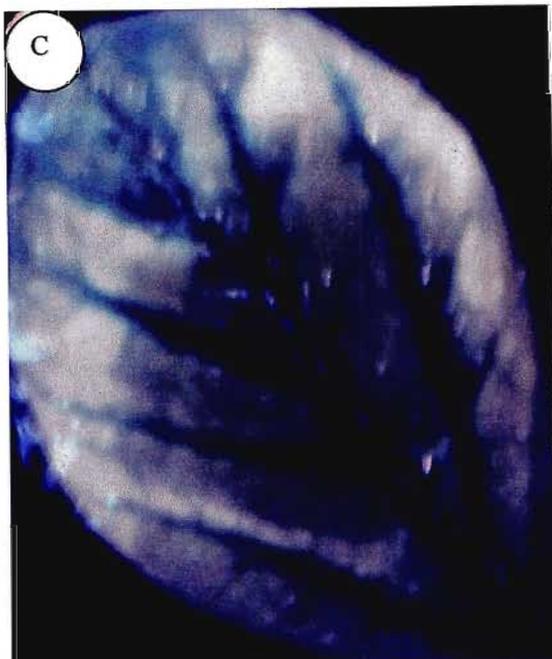
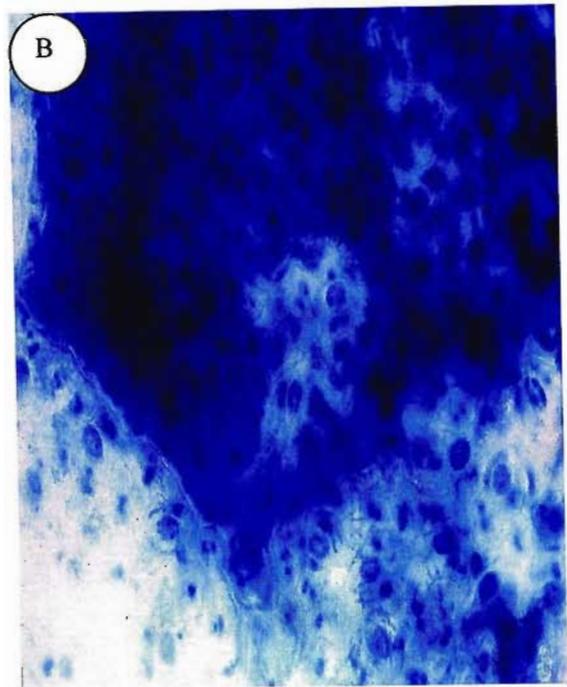
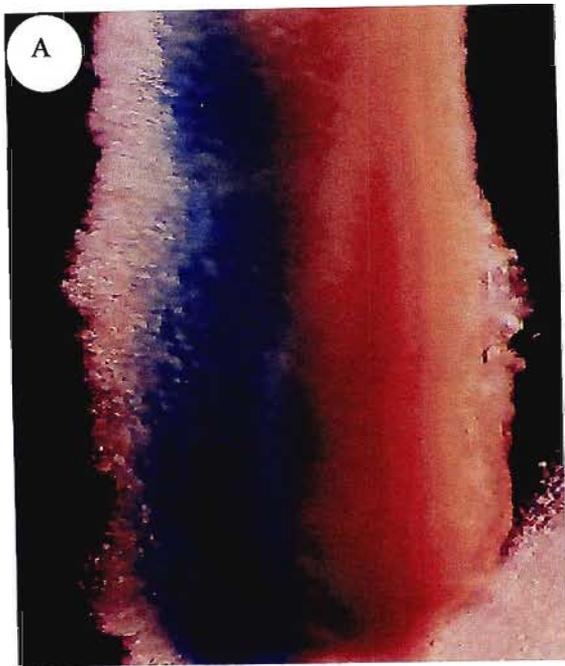


Figure 4: X-GLUC histochemical localisation of stable GUS expression in GUS-INT soybean plants indicating GUS enzyme activity in different organs. Six-week-old T0 plants: (A) Cross section of stem; (B) GUS activity in the stomata; (C) GUS activity in leaf veins. Self-pollinated T1 seed: (D) Immature embryo

2.3.4. Molecular analysis of the screened plants

A further verification of the insertion of the GUS-INT construct into the plant genome, was demonstrated using PCR analysis with GUS (Figure 5A) and *NPTII* specific primers (Figure 5B) on T1 plants. The DNA from the transformed plant tissue and the original *Agrobacteria* strain demonstrated the presence of the gene. No PCR product was observed with the untransformed plant tissue. The *Agrobacteria* containing *gus* without an intron demonstrated a similar result. Southern blot analysis confirmed the results obtained using the PCR reaction. It demonstrated hybridisation of the labelled GUS coding region (Figure 5C) with the transformed plant tissue and LBA4404 (pBI121). No hybridisation was observed with the untransformed plant tissue. The PCR and Southern blot observations confirmed results obtained with the histochemical assays for the presence of the GUS-INT gene in the transformed soybean genome.

It was thus demonstrated that *gus* was conserved in the progeny. The combined results of the GUS assays, the molecular analysis and the kanamycin screening method indicated that the soybean cultivar Carnia 2233 can be transformed with this method at a frequency as high as 30%.

2.4. CONCLUSIONS

A method for transforming germinating soybean seed using *A. tumefaciens* without the need of any tissue culture step was developed. Stable transgenic soybean plants were obtained within one generation. This method is technically easy, rapid to carry out and less expensive compared to conventional transformation procedures. As this method avoids the use of any tissue culture steps and expensive apparatus, the limitations presently connected to the transformation of soybean can be greatly reduced. Selection of the optimum

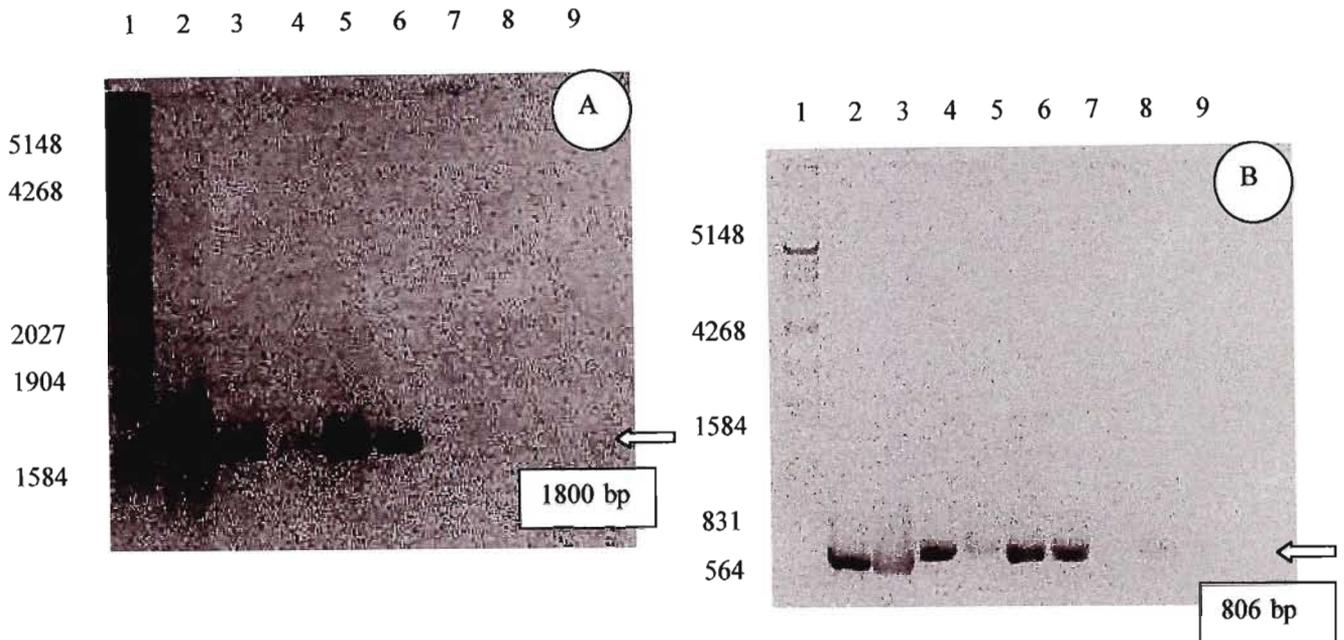


Figure 5: Molecular analysis

(A) PCR analysis of T1 transformants DNA using GUS-specific primers. Lanes: 1: Molecular weight marker; 2: GUS-intron plasmid (positive control); 3-6: Putative transformants; 7: Untransformed plant (negative control); 8: H₂O; 9: Negative control plasmid

(B) PCR analysis of T1 transformants DNA using *NPTII* specific primers. Lanes: 1: Molecular weight marker; 2: GUS-INT plasmid (positive control); 3-6: Putative transformants; 7: Untransformed plant (negative control); 8: H₂O; 9: Negative control plasmid

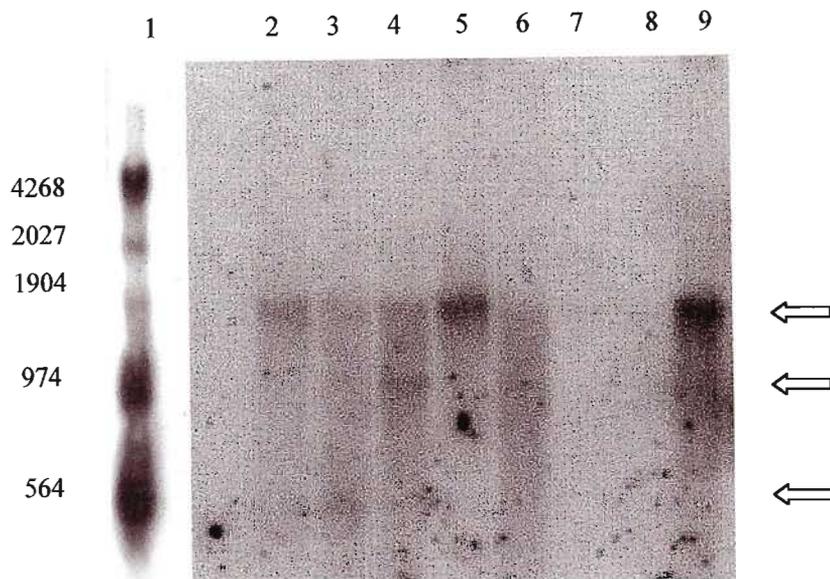


Figure 5: Molecular analysis

(C) Autoradiograph of Southern blot analysis demonstrating hybridisation between the ^{32}P labelled probe (GUS-INT) and DNA extracted from T1 transformed plant tissue. Lanes: 1: Molecular marker; 2–6: Putative GUS transformants; 2: undigested; 3–6: digests; 3: Sac; 4: Eco R1; 5: Eco RV; 6: Hind III; 7: negative plasmid control; 8: Control plant; 9: Positive plasmid control

developmental age of the seed used, addition of acetosyringone and use of a vacuum increased the production of transgenic plants. The addition of Break-Thru[®] had a beneficial effect on the transformation rate. The transgenic nature of these plants was confirmed by expression of the introduced traits, kanamycin resistance and GUS enzyme activity, in the tissue of the T0 plants and their progeny. In consideration of the seed mortality in T0 and the evidence obtained through the screening assays, it can be concluded that the inoculated T0 seeds yielded more than 30% transgenic T1 progeny. This is to date the highest known efficiency of stable transformation obtained through *Agrobacterium*-mediated transformation of soybean. This is in contrast to the methods of CHEE, FOBER & SLIGHTOM (1989), who obtained only a 0.07% transformation and HINCHEE, CONNER-WARD, NEWELL, McDONNELL, SATO, GASSER, FISCHHOFF, RE, FRALEY & HORSCH (1988) who were able to produce only 6% transformation using methods of agrobacterial transformation.

CHAPTER 3

TRANSFORMATION OF CARNIA 2233 WITH ANTISENSE L- Δ^1 - PYRROLINE-5-CARBOXYLATE REDUCTASE GENE

3.1. INTRODUCTION

3.1.1. Transformation

The development of gene transfer techniques for leguminous plants is of commercial importance because it can facilitate the development of new cultivars with improved resistance to environmental stress, disease resistance, tolerance to specific herbicides and increased nutritional value. Transfer and expression of foreign genes in plant cells also enable the study of gene expression. Molecular techniques provide an effective means of analyzing the importance of physiological events. It is likely that the use of such an approach will indicate the relative significance of proline accumulation as a response to drought stress.

Understanding how proline accumulation is regulated at the biochemical and molecular levels is likely to be of great value in defining precisely the role of proline. In order to do this, the genes involved in proline synthesis need to be fully characterised. This may enable the use of transgenic methodology to alter proline metabolism in plants (HARE, 1995).

3.1.2. Proline biosynthesis

DELAUNEY & VERMA (1993) suggest that a direct role for proline as an osmoprotectant is feasible and that possibilities exist for increasing osmotolerance in transgenic crop plants by engineering the overproduction of proline. Much work has been done towards elucidating the proline biosynthetic pathway in plants. Since proline in plants is synthesized from glutamic acid and ornithine (ADAMS & FRANK, 1980), it is meaningful to control the gene coding for the enzyme controlling the common step and last step of both pathways. In the glutamate pathway of proline biosynthesis, glutamic acid is phosphorylated and reduced to glutamyl-5-semialdehyde by a bifunctional enzyme, L- Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), which possesses both glutamyl kinase and glutamic semialdehyde dehydrogenase activities (HU, DELAUNEY & VERMA, 1992). All the pyrroline-5-carboxylate (P5C) producing enzymes, proline oxidase, ornithine aminotransferase and P5CS are mitochondrial, whereas L- Δ^1 -pyrroline-5-carboxylate reductase (P5CR) is cytosolic. Thus, for P5C to be converted to proline, it must emerge from its origin in the mitochondria or pass into cells across cell membrane barriers (PHANG, 1985). The only common step to both pathways occurs at the level of P5C, which is reduced to form L-proline by P5CR (Diagram 1). DELAUNEY & VERMA (1993) have assessed the relative importance of these pathways and suggested that the glutamate pathway for proline synthesis is predominant in plants under stress conditions when available nitrogen is limited. This enzyme is equivalent to the yeast pro3 gene or prokaryote ProC gene. A decrease in proline oxidation frequently accompanies the onset of stress (RAYAPATI & STEWART, 1991).

Genes and cDNAs coding for P5CR and P5CS have been isolated from different plant species, including *Nicotiana tabacum*, *Glycine max*, *Arabidopsis thaliana*, *Pisum sativum* and *Vigna aconitifolia* (LA ROSA, RHODES, RHODES, BRESSAN & CSONKA, 1991; DELAUNEY & VERMA, 1990; HU,

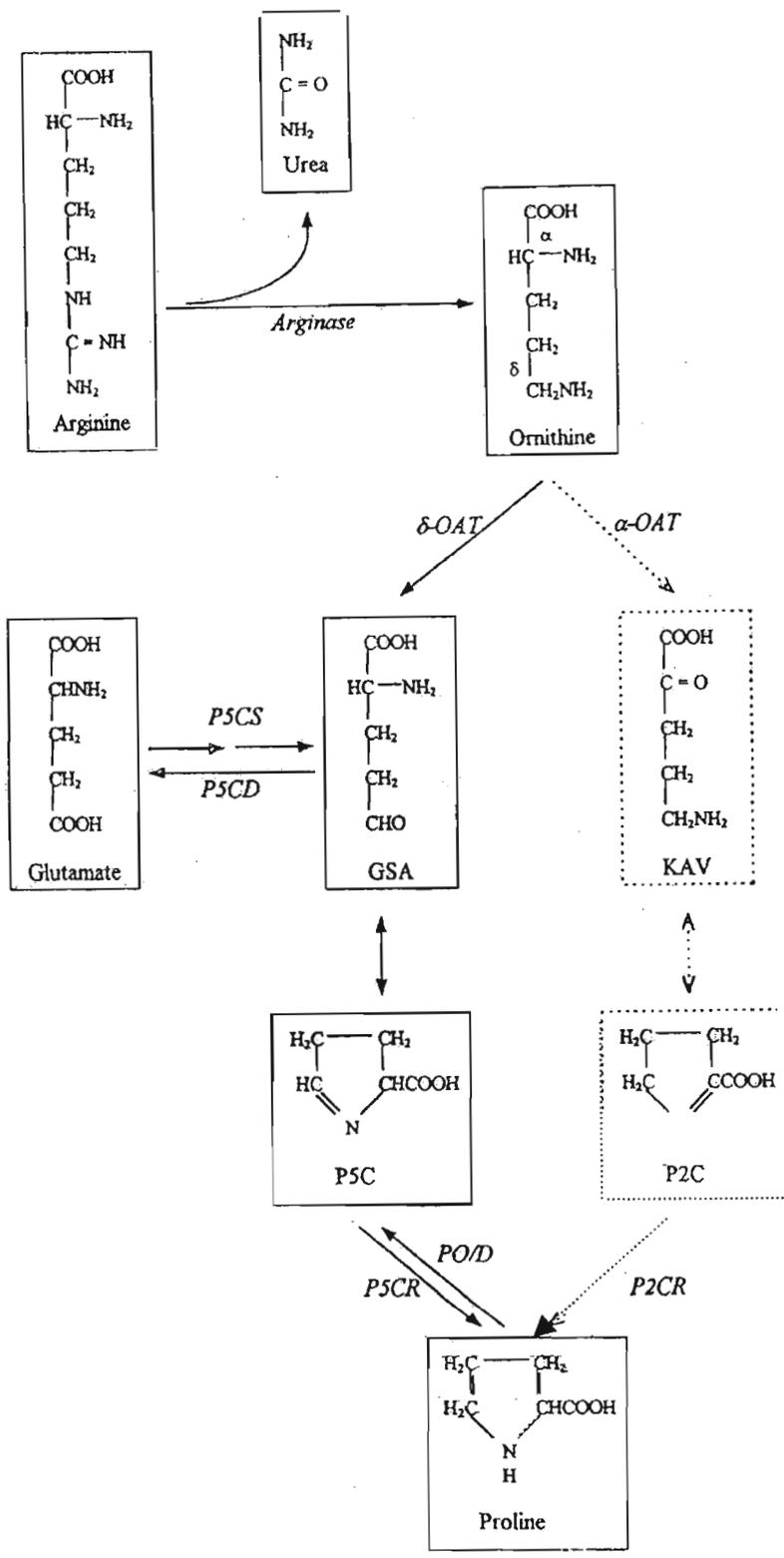


Diagram 1: Proline biosynthesis pathway (DELAUNEY & VERMA, 1993)

DELAUNAY & VERMA, 1992; WILLIAMSON & SLOCUM, 1992; VERBRUGGEN, VIRRAROEL & VAN MONTAGU, 1993; SAVOURE, JAOUA, HUA, ARDILES & VAN MONTAGU, 1995; YOSHIBA, KIYOSHUE, KATAGIRI, UEDA, MIZOGUCHI, YAMAGUCHI-SHINOZAKI, WADA, HARADA & SHONOZAKI, 1995). The activity of P5CR is substantially higher in soybean nodules than that reported for other plant tissue (KOHL, SCHUBERT, CARTER, HAGEDORN & SHEARER, 1988). A possible role for this high P5CR activity might be to allow nodules to produce proline during periods of stress.

Given the sensitivity of N₂ fixation to drought stress, this potential would provide selective advantage if proline accumulation plays a role in protecting nodules from drought. KOHL, KENNELLY, ZHU, SCHUBERT & SHEARER (1991) found that drought stress increases the activity of the pentose phosphate pathway by about 65% in extracts of nodules from old soybean plants, together with a 3-4 times increase in the proline levels. IYER & CAPLAN (1998) indicated in rice, that osmotically induced increases in the intermediates in proline metabolism influenced some of the characteristic responses to drought stress.

3.1.3. Antisense technology

The concept of introducing antisense RNA genes into organisms to control gene expression was initially investigated in prokaryotic systems (GREEN, PINES & INOUE, 1986) and plants (ECKER & DAVIS, 1986). The underlying hypothesis behind the application of antisense genes in plants, is the *in vitro* synthesis of complementary RNA, which subsequently hybridizes to the target mRNA molecule (BOURQUE, 1995). Target and antisense RNA's are generally complementary since they are usually transcribed from promoters in opposite directions on the same DNA segment. Translation of the gene is thus inhibited and gene expression is effectively "switched off" (Diagram 2) (DAY, 1989).

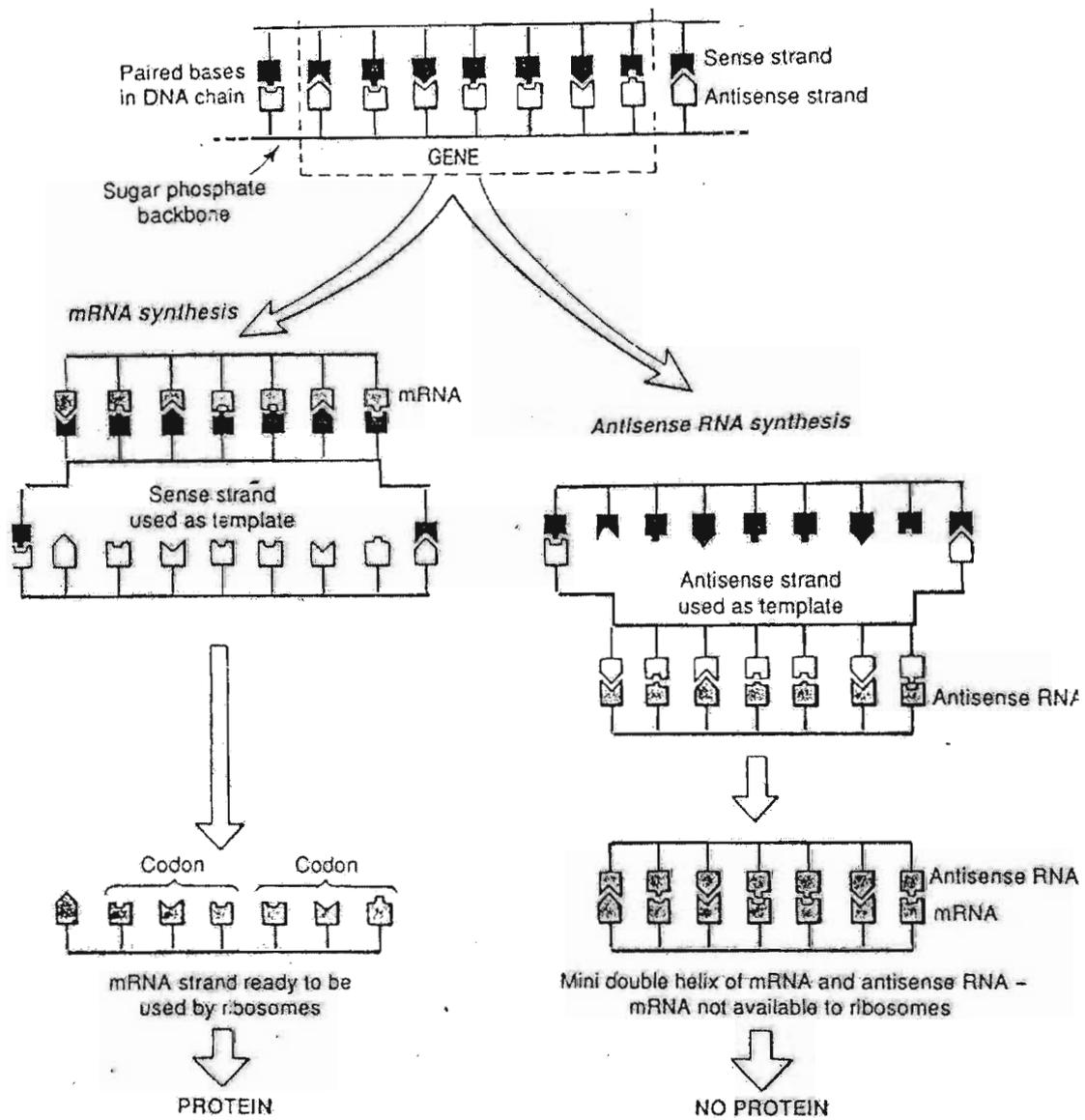


Diagram 2: Potential procedure for switching genes off with antisense technology (DAY, 1989)

A decrease in the steady-state level of target mRNA has been reported, as well as a reduction in the antisense RNA levels (BIRD & RAY, 1991). Antisense technology can thus provide powerful insight into the expression and function of specific genes. In this way it is possible to study the role of a protein or gene whose function was previously unknown.

Although the induction of an antisense gene can result in specific inhibition of the target gene's normal function, the method does not result in null production of the target protein (BOURQUE, 1995) which would in many cases be lethal. However, by comparing, under specific conditions, a plant containing an antisense gene with a plant containing a normal copy of that particular gene, one will be able to better understand the functions of the target gene.

3.2. AIM

The tools of molecular biology, gene cloning and plant transformation have created new ways of studying plant biology. In creating transgenic plants with down regulated genes, we can seek answers to old hypotheses like "What is the role of proline during drought?". The aim of this study was to develop a system in which the role of proline biosynthesis in response to drought stress in soybean can be clarified by the use of antisense gene technology. The *Arabidopsis P5CR* gene was cloned into the heat shock cassette pMA445 for generating antisense soybean plants. An antisense construct consisting of the *P5CR* gene fused to a heat inducible promoter allowed the gene's expression and consequent proline production to be manipulated in plants subjected to environmental stress conditions. This antisense construct was transformed into soybean by means of a vacuum infiltration transformation technique (Chapter 2). The use of genetically transformed soybean plants, may enhance our understanding of the physiology of stress-induced proline accumulation in plants.

3.3. MATERIAL AND METHODS

3.3.1. Material for *Agrobacterium*-mediated transformation of soybean seed with the *P5CR* gene

The *P5CR* gene (AT-P5C1) was obtained from Dr N. Verbruggen, Laboratory of Genetics, University of Gent, Belgium. The gene is a 1029bp-cDNA clone from *Arabidopsis*, inserted into pUC19 in the Bam HI and Eco RI sites (Diagram 3). The *Arabidopsis thaliana* *P5CR* gene used in this study shares over 70% nucleotide sequence homology with the soybean *P5CR* gene (VERBRUGGEN, HUA, MAY & VAN MONTAGU, 1996).

CZARNECKA, GURLEY, NAGAO, MOSQUERA & KEY (1985) determined the DNA sequence of a small heat shock protein (hsE2019) in soybean. A heat shock inducible (HSI) expression cassette, using this soybean gene, was subsequently constructed (AINLEY & KEY, 1990). AN, WATSON, STACHEL, GORDON & NESTER (1985) have constructed a set of small vectors based on the Ti plasmid of *Agrobacterium tumefaciens*, of which pGA470 is one. A derivative of this T-DNA vector (pMA445), containing the HSI expression cassette (AINLEY & KEY, 1990) was used in this study.

3.3.2. Cloning of the *P5CR*-heat shock inducible promoter construct

The insertion of the *P5CR* gene into the vector containing the HSI expression cassette is summarised in Diagram 4. Plasmid DNA was isolated according to FELICIELLO & CHINALI (1993), quantified by the use of the TKO-102 fluorometer and separated electrophoretically for purity testing. Restriction endonuclease digestions were followed by a purification step (GeneClean Bio 101). The fill-in reactions were done according to MANIATIS, FRITSCH & SAMBROOK (1982). Fragments were isolated on a 1.0% low melting point agarose

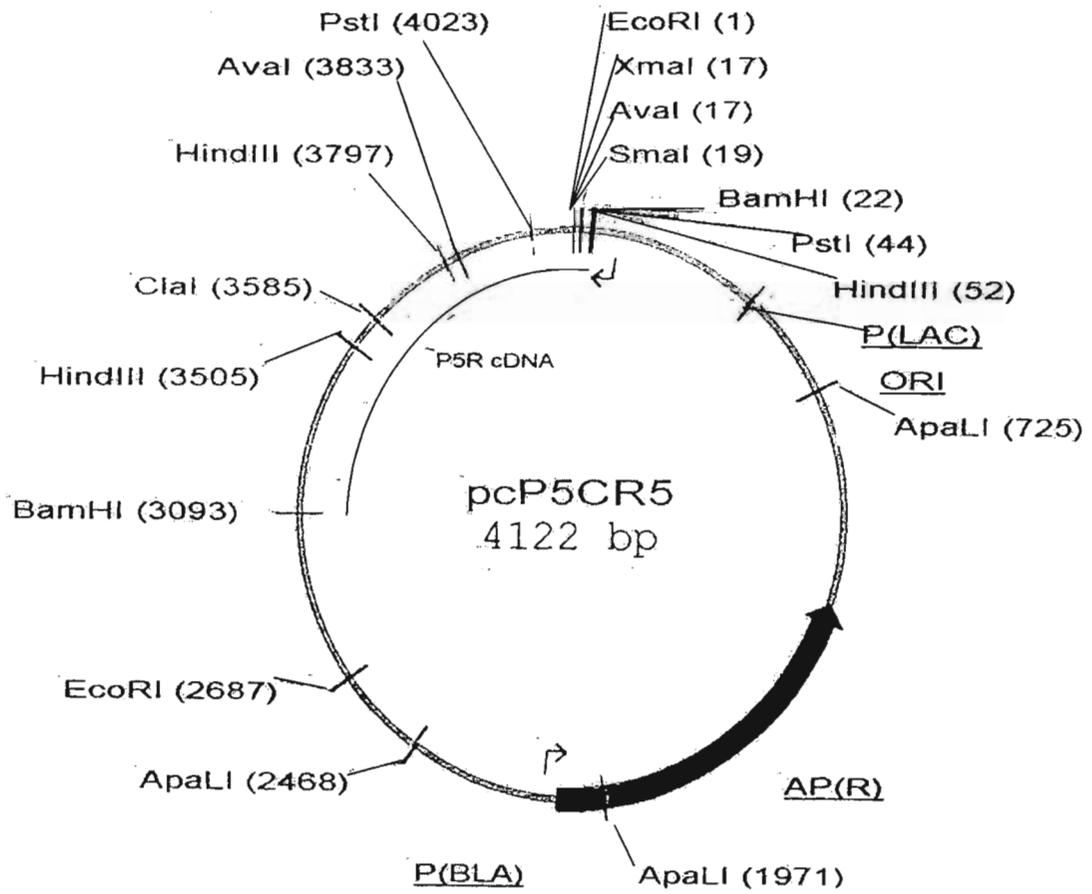


Diagram 3: Construct of *Arabidopsis* P5CR gene AT-P5C1 as found in VERBRUGGEN, VIRRARROEL & VAN MONTAGU, 1993

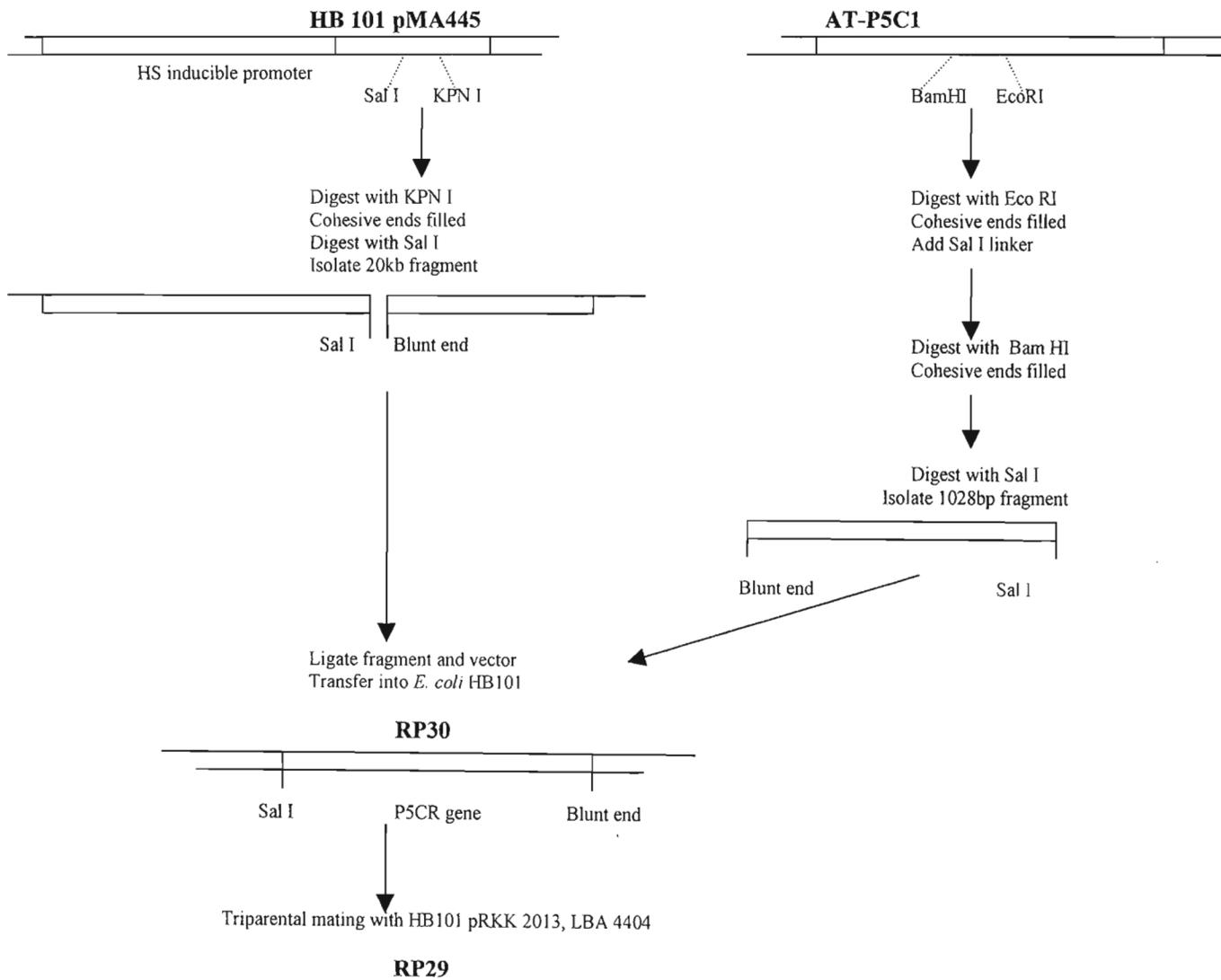


Diagram 4: Construction of the *P5CR* gene from AT-P5C1 into HB 101 pMA445 containing the HS inducible expression cassette

gel. Ligations were done using T4 DNA ligase and T4 DNA ligase buffer together with DTT and dATP overnight at room temperature. Transformations were performed with competent *E. coli* HB101 cells using the ligation reaction mixture (MANIATIS, FRITSCH & SAMBROOK, 1982). Transformed cells were selected on LB plates supplemented with tetracycline (15 μgml^{-1}) and incubated overnight at 37°C. The method of ARMITAGE (1988) was used for the triparental mating procedure. Overnight cultures were established for *A. tumefaciens* LBA 4404 at 27°C, *E. coli* HB 101 (pRKK 2013) at 37°C and RP30 at 37°C. For the triparental mating, all three cultures were mixed in a 2 ml syringe. This mixture was dispensed onto a sterile filter that was placed on top of an LB plate without any antibiotic. The plates were incubated for 48 hours at 27°C. The growth from the plates was streaked onto rifampicin (100 μgml^{-1}) and kanamycin (50 μgml^{-1}) ($\text{Rf}^{100}/\text{Km}^{50}$) supplemented plates. The plates were incubated at 27°C again for 48 hours and single colonies were selected. This triparental-mated construct (RP29) was used in a vacuum infiltration *Agrobacterium*-mediated transformation procedure as described in Chapter 2.

3.3.3. Molecular analysis of transgenics

DNA was isolated from leaf tissue of the putative *P5CR* transgenics (T1-T3) as well as H₂O transformed soybean, using the method of EDWARDS, JOHNSTONE & THOMPSON (1991) as modified by MCGREGOR, LAMBERT, GREYLING, LOUW & WARNICH, 2000. The method of FELICIELLO & CHINALI (1993) was used in the isolation of the plasmid DNA.

In order to verify that transgenes had been integrated into the soybean genome, PCR analysis was performed using *P5CR* or *NPTII* specific primers (Boehringer Mannheim).

The sequence of these primers was as follows:

P5CR left: 5'AGCTCACCCGTCTGAAGC3';

P5CR right: 5'GGCCCATAACCCTTATTTTG3';

NPTII left: 5'GAGGCTATTCGGCTATGACTG3';

NPTII right 5'ATCGGGAGCGGCGATACCGTA3'

The PCR program used was as follows: 1) 35 cycles of stage 1 consisted of the dissociation of dsDNA strands at 94°C for 30 seconds, primer annealing at the T_m for 30 seconds followed by primer elongation at 72°C for 45 seconds. 2) 1 cycle of stage 2 consisted of the dissociation of dsDNA strands at 94°C for 30 seconds, primer annealing at the T_m for 30 seconds followed by primer elongation at 72°C for 5 minutes. The annealing temperature was calculated from the equation $T_m=4(G+C)+2(A+T) \pm 5^\circ\text{C}$ (SCHOBERT & TSCHESCHE, 1978). The annealing temperatures used were 58 and 64°C for the *P5CR* and *NPTII* primers respectively. The PCR products were separated on a 0.8% agarose gel in 1X TAE buffer (MANIATIS, FRITSCH & SAMBROOK, 1982), visualised by ethidium bromide staining and sized in comparison to Boehringer Mannheim Molecular Weight Marker III.

Further analysis of the inserted construct in the soybean plant genome, was performed using hybridization of a radioactively labelled ^{32}P probe, specific to the *P5CR* gene, using the Southern blot procedure. Restriction endonuclease digestions were performed on isolated plant DNA separated on agarose gels and blotted onto Hybond N+ membranes (Amersham). DNA of AT-P5C1 was restricted with Bam HI and EcoRI. The *P5CR* fragment was consequently isolated on a 1% low melting point agarose gel. The isolated fragment was labelled according to the Prime-It[®] RmT Random primer labelling kit (Stratagene[®]), after PCR amplification. Hybridization and detection were performed according to SAMBROOK, FRITSCH & MANIATIS (1989).

3.3.4. Plant material

Putative *P5CR* transformed soybean seeds (T1-T3) and H₂O transformed control seeds (control plant) were planted in a soil mix consisting of soil:sand:vermiculite (5:5:3) (v:v:v) in a greenhouse under controlled temperature and watering conditions. The seeds were reproduced by self-pollination. Plants were grown at 25/15°C (day/night temperatures) and were watered three times a week and fortnightly with 4 gl⁻¹ highly concentrated completely soluble nutrient mix.

Plants, transformed and control, were subjected to a temperature stress of 40°C, in order to monitor any visual reaction of the plants to the activation of the heat shock promoter. After 2 days, water was withheld from the pots for 48 hours to induce drought stress for another 48 hours. Leaves were sampled on the 4th day, quick frozen with liquid nitrogen and freeze-dried immediately.

3.3.5. Kanamycin screening

The T3 seed were tested for their resistance to kanamycin, before planting in the greenhouse. The plant seed (transformed and control) was incubated on agar medium (8 gl⁻¹), supplemented with kanamycin. The following kanamycin concentrations were tested; 0, 25, 35 and 50 mg l⁻¹.

3.4. RESULTS AND DISCUSSION

AINLEY & KEY (1990) observed higher expression of the GUS gene using a heat inducible promoter than with the *CaMV* 35S gene promoter. It was therefore decided to use the inducible heat shock promoter (IHSP) in this antisense study. One problem with an antisense plant is that the expression of the gene under study is either switched off or its expression is greatly reduced. This can be devastating for antisense plants. With an inducible promoter the gene expression and

consequently (in this case) proline production can be manipulated in these transgenic plants, and will thus allow maximum control of gene induction by manipulation of the environmental temperature of the plants. In addition by using an inducible promoter the plant has not had time to adjust physiologically and biochemically to the reduced gene product prior to experimental studies.

The PCR reaction was used as an indication of transformation of the putative transgenics. Figure 6 demonstrates the results obtained during the PCR analysis, using the *P5CR* primer (A) and the *NPTII* primer (B). This indicated that the *P5CR* gene is transferred into the T-DNA vector, the *P5CR*-HS inducible promoter construct and finally into the soybean plants. The PCR amplified the expected band (1029bp) from the DNA of the putative transgenics and positive controls, providing evidence of transformation.

BIRCH (1997) however, claimed that PCR analysis cannot supply sufficient proof of integrative transformation and Southern analysis was subsequently performed. Figure 7 shows the results of an autoradiograph of a Southern blot analysis after an exposure time of 2 days. The figure demonstrated hybridization between the ³²P labelled probe (*P5CR*) and DNA extracted from transformed plant tissue. The arrows in Figure 7 indicated hybridization that occurred between the transformed plant tissue and the labelled probe which did not occur in the control tissue.

The molecular analyses of the T1 transgenics using PCR and Southern blot analysis consistently distinguished between negative and positive controls. This provided proof of the transformation of the soybean plants with the *P5CR*-IHSP construct.

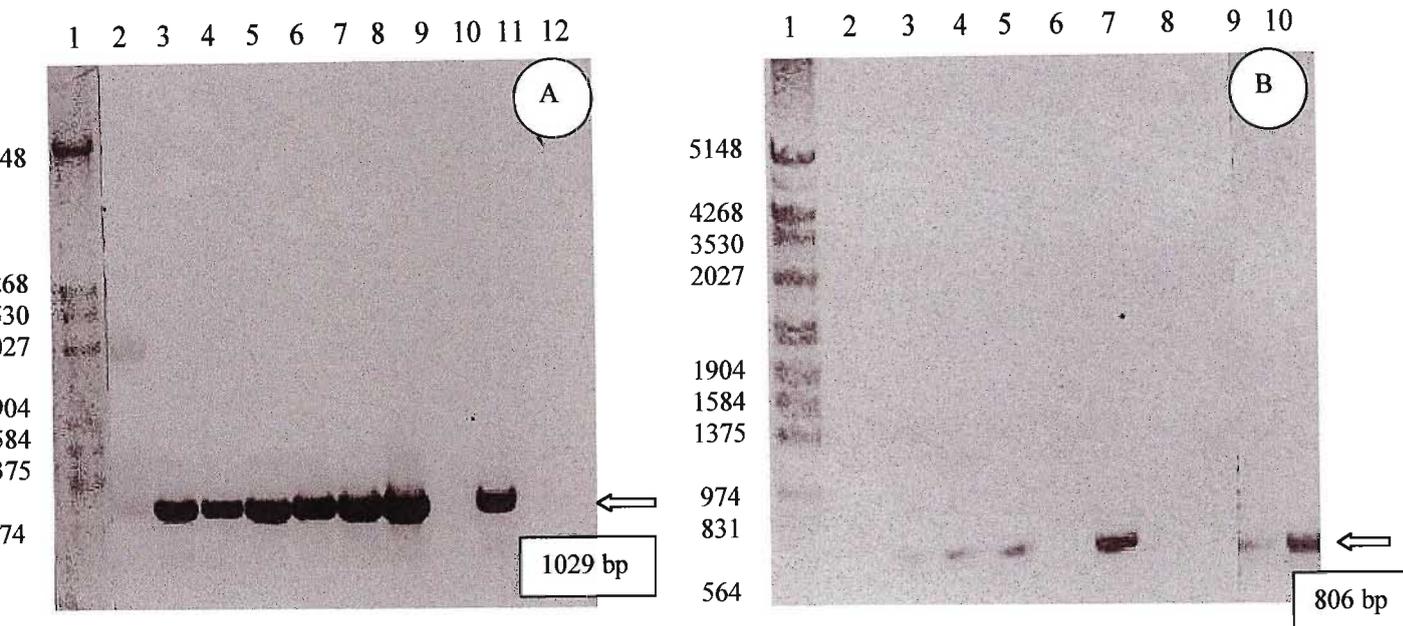


Figure 6: Agarose gel electrophoresis of PCR analysis confirming transformation of the P5CR construct into soybean cells

(A) P5CR specific primers: Lane 1: molecular weight marker; Lane 2: AT-P5C1 (positive control); Lane 3-8: T1 putative transformed plants (T17, 16, 2, 18, 8, 6); Lane 9: control plant; Lane 10: RP29 (positive control); Lane 11: pMA445 (negative control); Lane 12: H₂O.

(B) NPTII specific primers: Lane 1: molecular marker; Lane 2: AT-P5C1 (negative control); Lane 3-5: T1 putative transformed plants; Lane 6: control plant; Lane 7: pMA445 (positive control); Lane 8: H₂O; Lane 9: RP30 (positive control); Lane 10: RP29.

Arrow indicated the expected amplified DNA band at the correct position

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

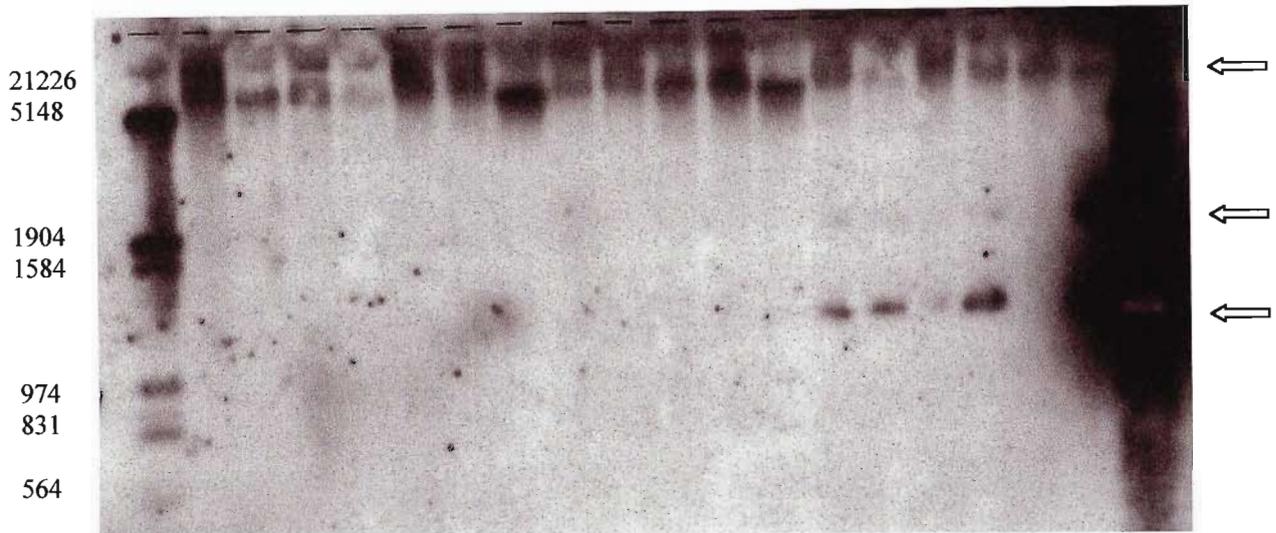


Figure 7: Autoradiograph of Southern blot analysis demonstrating hybridization between the ^{32}P labelled probe (*P5CR*) and DNA extracted from T1 putative transgenic plant tissue. Lane 1: Molecular weight marker; Lanes 2-7: PstI digests; Lanes 8-12, 19: HindIII digests; Lanes 13-18: SAL digest; Lane 20: positive control RP29; Lanes 2, 8, 13: Control plant; Lanes 3, 9, 14: Transformant 2; Lanes 4, 10, 15: Transformant 6; Lanes 5, 11, 16: Transformant 12; Lanes 6, 12, 17: Transformant 16; Lanes 7, 18, 19: Transformant 17

Arrows = hybridization occurring in transformed plant tissue which did not occurred in control plants

T3 seeds were tested for the presence of kanamycin resistance, which would indicate whether they were transformed. Transformed (T3) and control soybean seed were tested for germination percentages on agar plates supplemented with kanamycin. It was observed (Table 2) that with 0 mg l⁻¹ kanamycin all the seed germinated. When the concentration of kanamycin increased, the germination decreased. Some seeds started to germinate but died off before the leaves and roots emerged. The plantlets of some germinated seeds were deformed. At the highest kanamycin concentration none of the control seeds germinated while 37.5 % of the transformed seeds did. Only well developed seedlings were planted in the greenhouse. The resistance to kanamycin indicated that they had most probably been transformed with the *P5CR-NPTII* gene fusion construct.

Table 2: Germination percentage of transformed and untransformed seed on kanamycin supplemented agar plates

	Kanamycin mg l⁻¹			
	0	25	35	50
Untransformed seed	100	0	0	0
T1GUS INT seed	100	80	75	37.5

The T1 plants tested, after 4 days at 40°C and 2 days water stress, reacted to the activation of the IHSP and subsequent drought treatment with retarded growth (Figure 8). The control plants (subjected to the same heat and water stress treatment) showed no visible response to the stress treatment (Figure 8).

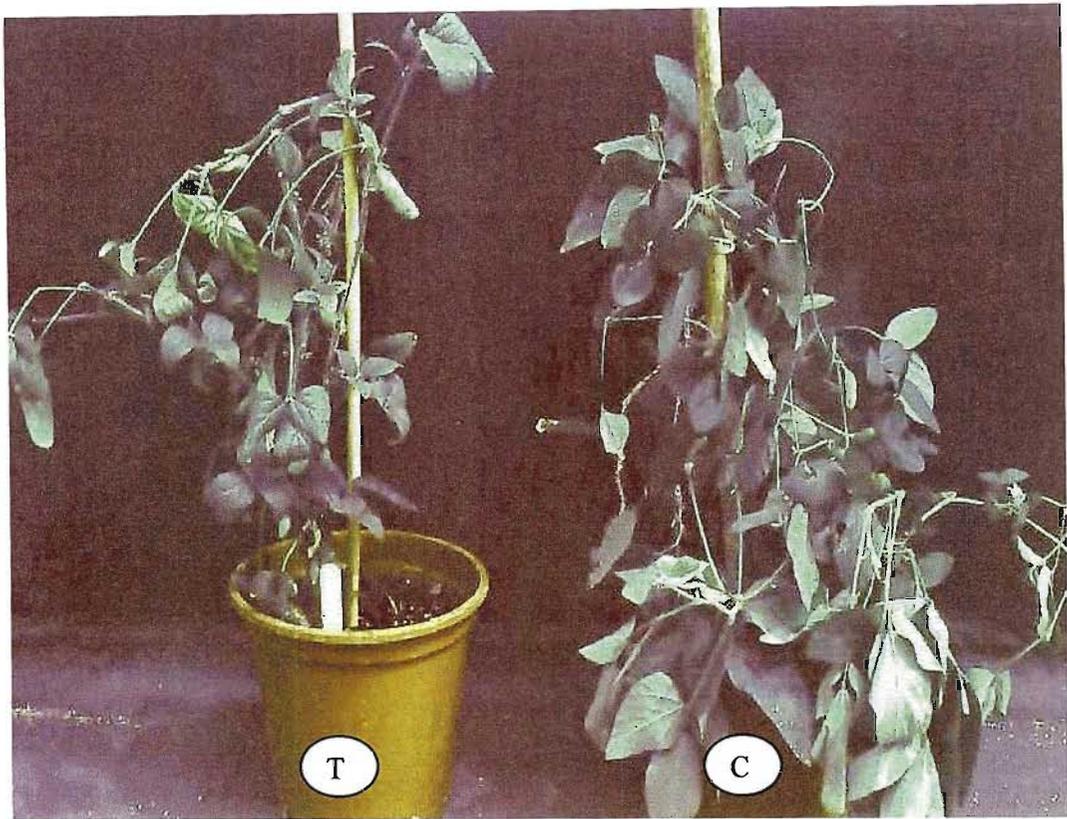


Figure 8: Phenotypic evaluation of soybean plants subjected to a drought stress treatment at 40°C. Growth retardation as a result of proline shortage is indicated in T1 transgenic (T) compared to control plant (C)

3.5. CONCLUSIONS

Study of the biochemical and molecular mechanisms by which plants tolerate environmental stresses is necessary if the genetic engineering of crops to improve their performances under stress is to be successful. By investigating antisense plants under stress conditions, the metabolic pathways and the limits to the enzyme functions can be understood. The screening of antisense *P5CR* transgenic plants, which experienced under-expression of the *P5CR* gene and subsequent inhibition of the production of proline, should provide insight into the correlation between proline accumulation, drought and osmotic stress. It was confirmed that the *P5CR* antisense gene construct was integrated and was conserved over three generations.

CHAPTER 4

EVALUATION OF ANTISENSE *P5CR* TRANSGENIC CARNIA 2233 PLANTS SUBJECTED TO OSMOTIC AND DROUGHT STRESS

4.1. INTRODUCTION

4.1.1. Accumulation of free proline

Water is an essential resource for plant life. Therefore, any limitation in water availability affects almost all plant functions. Water deficit occurs in plants whenever water loss through transpiration exceeds the supply from the soil. The availability of water for all plant biological functions can be impaired by different environmental conditions. Under severe stress, a plant adapts its metabolism and alters its development. Under conditions of water stress, there are changes in many processes as the plant attempts to maintain its metabolism and restore the metabolic conditions needed for growth (BOHNERT, NELSON & JENSON, 1995). One of the most common induced responses in all organisms subjected to water deficit is the accumulation of osmolytes. By lowering water potentials, the accumulation affects virtually all metabolic processes and often results in a severe reduction in plant productivity. The accumulation of compatible osmolytes allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortage within the organism. The amino acid proline is perhaps the most widely distributed “compatible” osmolyte (TAYLER, 1996).

Proline represents a unique class of molecule among the amino acids. With its peptide bond within the pyrrolidone ring, proline confers rigidity and three-dimensional stability to proteins (PHANG, 1985). Because of the secondary amines of the nitrogens, these pyrrolidone imino acids cannot participate in the transamination or decarboxylation reactions common to other amino acids. A specific system of enzymes with special properties has evolved to mediate the metabolism of proline. Another feature of proline as a result of its unusual metabolic feature, is that it easily crosses cellular and organellar barriers (ABRAHAMSON, BAKER, STEPHENSON & WOOD, 1983).

Much remains to be understood concerning the mechanisms whereby free-proline accumulates under stress. KRAMER (1983) warned that mere correlation between the accumulation of proline and the development of stress conditions, does not provide sufficient evidence for any adaptive advantage with regards to the stress. A survey of existing literature on the potential value of proline accumulation during stress, reveals a collection of controversial statements. Some researchers argue that proline accumulation is to the plant's advantage (CHIANG & DANDEKAR, 1995 with desiccation; KUZNETSOV & SHEVYAKOVA, 1997 with NaCl stress; VAN RENSBURG & KRÜGER, 1994 with drought stress; SARADHI, ARORA & PRASAD, 1995 with UV stress), while others suggest the opposite (PEREZ-ALFOCEA & LARHER, 1995 with NaCl and osmotic stress). It is thus still unknown whether or not a constitutive higher level of proline accumulation enhances plant tolerance to environmental stress.

4.1.2. Role of proline accumulation

The extensive accumulation of active oxygen species and their contribution to cell damage induced by water deficit is well known. In order to deal with this effect, plants have evolved a number of protective, scavenging or antioxidant defensive mechanisms. Apart from an enzymatic defensive system (BOWLER, VAN

MONTAGU & INZE, 1992), the accumulation of free proline may also contribute to the scavenging of these active oxygen species by enhancing photochemical electron transport activities (ALIA, SARADHI & MOHANTY, 1991; SARADHI, ARORA & PRASAD, 1995). FLOYD & NAGY (1984) suggested that the accumulation of proline might contribute to the detoxification of the active oxygen species.

Proline can affect the solubility of various proteins due to its interaction with hydrophobic residues on the protein surface (SCHOBERT & TSCHESCHE, 1978). The increase in the total hydrophilic area of the protein stabilises it by increasing its solubility in an environment with low water availability. The proposed role of proline as an osmoregulator (WYN JONES & STOREYS, 1978) can be supported by the involvement of proline in the maintenance of membrane integrity as an adaptation to conditions of reduced water availability (HARE, 1995). The molecules accumulated in the cells during an osmotic stress prevent damage from cellular dehydration by balancing the osmotic strength of the cytoplasm with that of the environment.

During periods of drought and NaCl stress plants increase their pools of free proline far in excess of the demands of protein synthesis. They do this by inducing proline biosynthetic enzymes while repressing further synthesis of catabolic enzymes (VERBRUGGEN, HUA, MAY & VAN MONTAGU, 1996). Although it has been suggested that this increase is a symptom that results from imbalances in other metabolic pathways (PEREZ-ALFOCEA & LARHER, 1995), there is considerable evidence that high levels of proline can be beneficial for stressed plants. The rapid accumulation of free-proline in plant cells during drought stress is well documented for a number of crops. It was reported that drought tolerant barley genotypes showed higher proline accumulation during drought stress at the seedling stage than did the drought sensitive genotypes

(SINGH, ASPINALL & PALEG, 1972). Correlation between field performances and seedling tests encouraged speculation that free-proline accumulation during drought stress is an adaptive response that enhances survival. Proline accumulation appears to be solely controlled by tissue water status and is unaffected by tissue temperature, up to 39°C in barley (CHU, ASPINALL & PALEG, 1974). It was also found in alfalfa that the progressive accumulation of proline was accompanied by a decline in tissue water potential (IRIGOYEN, EMERICH & SANCHES-DIAZ, 1992). A genetic modification that increased the basal level of proline in tobacco reduced the plant's sensitivity to NaCl (KAVI KISHOR, HONG, MIAO, HU & VERMA, 1995).

Under conditions of drought stress, proline is synthesized from glutamate due to a loss of feedback regulation in the proline pathway (BOGGESS & STEWART, 1980). This biosynthesis might be an adaptive mechanism to reduce the accumulation of NADPH that increases as a result of the decrease in the photosynthetic rate of the plant (BERRY & BJÖRKMAN, 1980). High levels of proline during stress play a role in the maintenance of the $\text{NADP}^+/\text{NADPH}$ ratio, as even a small increase in the biosynthesis has a large impact on the NADP^+ pool (HARE & CRESS, 1997). This is the result of stomatal closure which leads to the intercellular decrease of CO_2 as the leaf water stress increases. As the overall protein synthesis declines during drought stress (VAN DER MESCHT & DE RONDE, 1993), proline biosynthesis may substitute for protein synthesis in the turnover of ATP and the oxidation of NADP^+ (HARE, 1995). VAN HEERDEN & DE VILLIERS (1996) observed a higher proline accumulation during drought stress in drought tolerant spring wheat cultivars, than in the more sensitive cultivars.

4.2. AIM

The purpose of this part of the study was to show that proline plays a major role in the drought and osmotolerance mechanisms of soybean and to establish whether increased proline levels are necessary for drought survival. Free proline accumulation was used in the evaluation of antisense *P5CR* transformed soybean in comparison to H₂O transformed control plants subjected to drought or osmotic stresses.

4.3. MATERIAL AND METHODS

4.3.1. Plant material

Putative *P5CR* transformed soybean seeds (T1-T3) and H₂O transformed control seeds (control) were planted and stressed in a greenhouse as described in Chapter 3. Leaf material was sampled on the 4th day, quick frozen with liquid nitrogen and freeze-dried immediately.

4.3.2. Free - proline analysis

Leaves of T3 transformed and control plants (grown under optimal conditions) were harvested in the greenhouse for free proline analysis. Two leaf discs (no. 18 corkborer) were collected and incubated in 3 ml 0.2 M Na₂HPO₄/NaH₂PO₄ buffer for 3 hours at 25°C for acclimation, thereafter the leaf discs were subjected to a series of osmotic stresses for another 20 hours. During this 20 hour period the activation of the promoter, as well as reaction to osmotic stress was tested. Mannitol was used to simulate water stress in the soybean plants. The leaf discs were thus subjected to 3 temperatures; 25, 32 and 42°C and 3 different mannitol concentrations; 0, 0.5 and 1 M. The leaf material was freeze-dried afterwards and stored (-20°C) until needed. Three independent repeats were conducted. The

calorimetric method of BATES, WALDREN & TEARE (1973) was used for proline analysis. Samples (in triplicate) of 50 µg freeze-dried soybean leaves were crushed in liquid nitrogen before adding 5 ml 3% sulphosalicylic acid. The supernatant was collected and vacuum filtered through a Buchner funnel. One ml acid ninhydrin and 1 ml acetic acid were combined with 1 ml of the filtrate. The samples were incubated for 1 hour in a boiling waterbath thereafter the reaction was terminated on ice. Two ml toluene was added to the reaction mixture and vortexed for 15 seconds. Time was allowed for the toluene to separate from the aqueous phase, before the toluene phase was extracted. The absorbance of the samples was determined at 520 nm using a Titertek Multiscan EX. The proline concentration was determined using a standard curve, using L-proline (Sigma) as standard. The µmole proline per g dry weight were determined using the formula $[(\mu\text{g proline/ml} * \text{ml toluene})/115.5 \mu\text{g}/\mu\text{mole}]/[(\text{g dry weight of sample}/5)]$ (BATES, WALDREN & TEARE, 1973).

4.3.4. Woodenbox technique

Self pollinated seeds collected from the transformed plants used in the proline assay, were planted in rows of 12 seeds each, 50 mm apart, in a woodenbox according to SINGH, MAI-KODOMI & TERAO (1999a). The box was made from solid 25 mm thick pine planks with a base of 1300 mm X 650 mm and sides 150 mm high. The sides were firmly attached to the base and the box was lined with a thick plastic sheet. The woodenboxes contained a sandy soil filled up to 120 mm. The seeds were allowed to germinate in a greenhouse at 24/19°C ($\pm 1^\circ\text{C}$) day/night temperature and seedlings were watered every day until the six leaf stage was reached. When this developmental stage was reached, a drought stress was administered by withholding water from the plants. At the same time, a temperature switch of 37/20°C ($\pm 1^\circ\text{C}$) day/night temperature activated the IHSP. The plants were monitored every second day in order to determine the effect of the activated promoter and drought stress. When the first plants died, watering was

resumed at 25°C to determine the recovery percentage of each line. The plants were rated based on percentage wilting and recovering:

Rating	Drought stress	Recovery
0	dead	dead
1	100% wilted	stem 25% hydrated
2	75% wilted	stem 50% hydrated
3	50% wilted	stem 100% hydrated
4	25% wilted	trifoliolate leaves upright
5	0% wilted	alive

4.4. RESULTS AND DISCUSSION

Proline levels in drought stressed *P5CR* transgenics were compared to the control plants (Figure 9). The control plants contained significantly more proline than the transformed plants. This suggested that *P5CR* gene transcription plays a role in proline accumulation during drought stress.

Proline accumulation patterns in leaf discs of the transformed and control soybean plants with different temperatures and mannitol concentrations were also monitored (Figures 10A,B). The transgenics were the offspring of the 3 selected T2 transformed lines which performed best with the PCR and Southern blot procedures. Three of these transgenics were selected to compare to the control, in order to demonstrate the activation and deactivation of the promoter (Figure 10A). No significant difference between transgenics and control plants was observed at the control treatment (25°C/0 M mannitol).

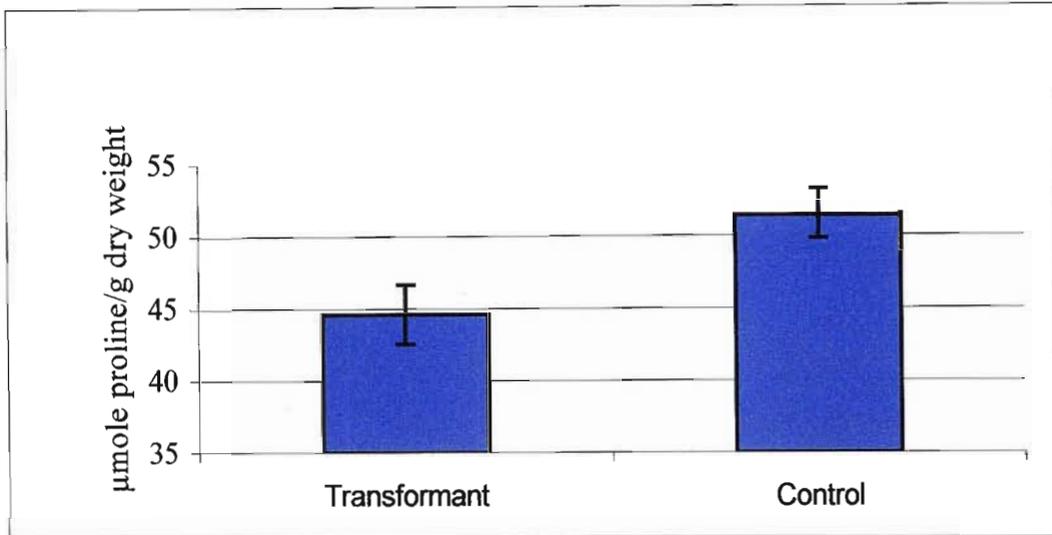


Figure 9: Effect of temperature and drought on the proline synthesis, displaying the manipulation of the IHSP on T1 transformants. Both the transformants and control plants were subjected to a drought and 40°C stress

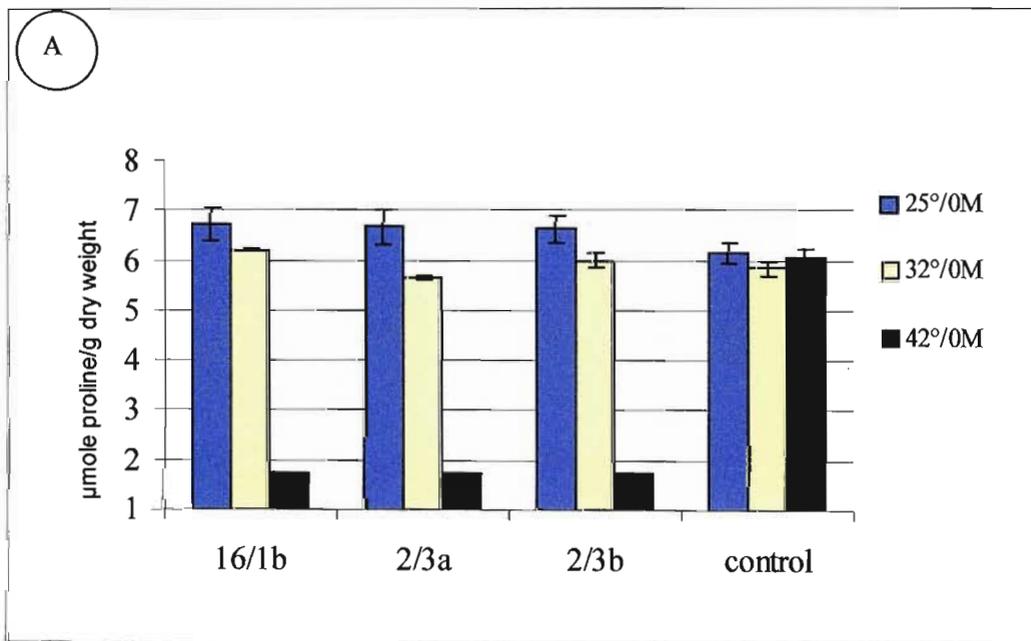


Figure 10: Effect of temperature and mannitol on proline levels, displaying the manipulation of the IHSP and the deactivation of *P5CR* in selected T3 transformants

(A) Effect of activation of inducible promoter at 0M mannitol at 25, 32 and 42°C respectively

This confirms the fact that the IHSP can be manipulated and needs to be switched on for full activation of the *P5CR* antisense gene. With an increase in temperature (32, 42°C), the proline accumulation in the transgenics decreased significantly. This demonstrated that the IHSP was indeed activated at the higher temperature of 42°C and that proline synthesis was switched off or reduced. It is suggested that the *P5CR* gene plays a dominant role during proline synthesis in soybean as was also demonstrated during desiccation stress (CHIANG & DANDEKAR, 1995) and salt stress (MATTIONI, LACERENZA, TROCCOLI, DE LEONARDIS & DI FONZO, 1997). These authors corroborated that the *de novo* synthesis of proline via *P5CR* is the predominant mechanism of proline accumulation in *Arabidopsis thaliana* and durum wheat.

It was however observed (Figure 10B), that only the control plant reacted with an increase in proline to the 25°C/1 M mannitol treatment as expected (KOHL, KENNELLY, ZHU, SCHUBERT, & SHEARER, 1991). The transformant (16/1b) contained significantly lower proline during the 25°C/1 M mannitol treatment (6.03 µmole proline/g dry weight ±0.22) than during the 25°C/0 M mannitol (6.71 µmole proline/g dry weight ±0.32) treatment. This can be explained, as “leakiness” or osmotic induction of the antisense *P5CR* although the IHSP still was not activated by heat. The plant’s proline needs increased with the mannitol stress, but less proline was synthesized and the proline-pool decreased. The control plants were able to sustain the proline levels needed by these plants. This is suggested by the reaction of the transgenics at 32°C/0 M mannitol and 32°C/1 M mannitol, where the IHSP was only partly activated. At 32°C/0 M mannitol (6.19 µmole proline/g dry weight ±0.02), the activation of the IHSP was significant when compared to 25°C/0 M mannitol (6.71 µmole proline/g dry weight ±0.32). However, it becomes even more significant with the 1 M mannitol stress at 32°C in the transgenic antisense line 16/1b, with 4.40 µmole proline/g dry weight ±0.15.

The proline-pool was probably big enough to sustain the proline need without a stress (32°C/0 M mannitol), but with a higher demand for proline during the stress (32°C/1 M mannitol), the plants were unable to synthesize more proline. Significantly less proline was synthesized in the transformant than in the control plant at 32°C/1 M mannitol. The control demonstrated increased proline at 32°C/1 M mannitol (7.49 μ mole proline/g dry weight \pm 0.22) and the 42°C/1 M mannitol (6.79 μ mole proline/g dry weight \pm 0.2) stresses, when compared to 25°C/1 M mannitol (6.24 μ mole proline/g dry weight \pm 0.25). The IHSP was fully activated at 42°C and resulted in a significant decrease in proline production in all of the transgenics when compared to the control. Thus, these findings are in agreement with the observations of AINLEY & KEY (1990), who demonstrated that the expression of the heat shock promoter is temperature dependent. They found that the expression increased approximately 10 fold between 29 and 40°C.

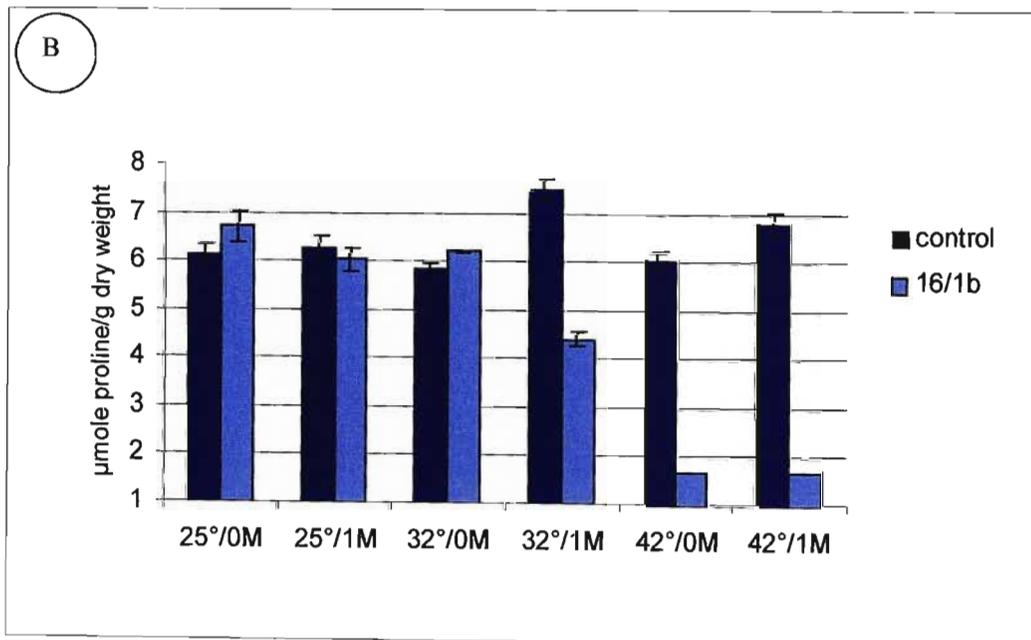


Figure 10: Effect of temperature and mannitol on the proline synthesis, displaying the manipulation of the IHSP and the deactivation of *P5CR* in selected T3 transformants

(B) Effect of either 0M or 1M mannitol at respectively 25, 32, 42°C

When the proline levels of control treatments were subtracted from the levels in the stress treatments, the effect of the IHSP is clearly visible (Figure 10C). The control plants demonstrated a significantly higher proline accumulation than the transgenics. In non-stress environments, a fine balance between proline biosynthesis and degradation maintains a certain proline pool in the plant cells. Under a stress condition, the increase in available proline can be attributed to either an activation of the biosynthesis or a decrease in catabolism or both. It was observed that proline accumulation as a result of an osmoticum (mannitol), became more pronounced at slightly unfavorable conditions in the control plant.

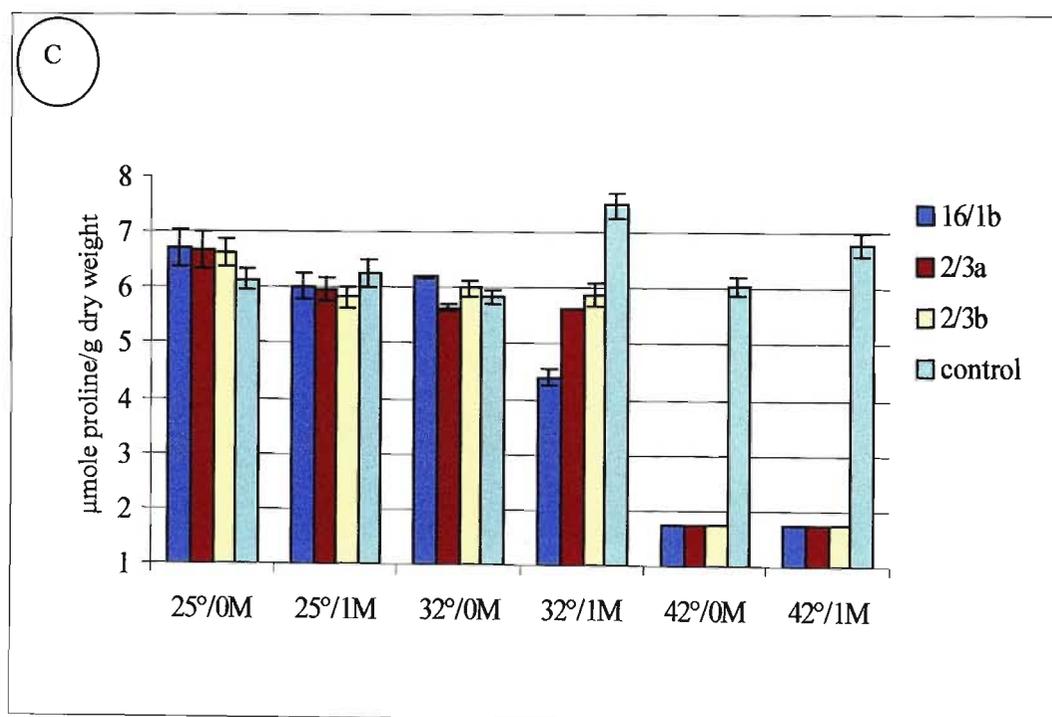


Figure 10: Effect of temperature and mannitol on the proline synthesis, displaying the manipulation of the IHSP and the deactivation of *P5CR* in selected T3 transformants

(C) Indication of the effect of suppressed *P5CR* gene. Proline levels of the control treatments were subtracted from the levels in the stress treatments

At 25°C a minimum increase in proline was observed after the application of a mannitol stress, in contrast there was a significant increase in proline at 32 and 42°C (Figures 10A, B, C). This may indicate that the plant does not need as much proline to survive when an osmotic stress occurs at the optimum growth temperature, as when the stress occurs at a higher temperature. At the optimum temperature, the plant can rely on the proline-pool for the higher proline demand, but at the higher temperature, the proline production must increase to fulfill the demand for proline. This was also demonstrated in a study on cotton, where the heat and drought tolerant cultivar synthesized more proline under a combined drought and heat stress than with just a drought stress (DE RONDE, VAN DER MESCHT & STEYN, 2000).

Thirty transgenics were tested for the activation of the IHSP and subsequent proline decline (Figures 11A, B). It was demonstrated that in some instances the soybean plants produced different amounts of proline with (Figure 11A) and without (Figure 11B) an osmotic stress. When the parameter 42°C/1 M-25°C/1 M was used, the ranking of the transgenics differed from the ranking using the parameter of 42°C/0 M-25°C/0 M. Most of the plants demonstrated a significant proline increase after the imposition of an osmotic stress.

From this data it can be concluded that proline does accumulate as a result of osmotic stress. While the activation of the IHSP resulted in the subsequent inactivity of the *P5CR* gene, demand for proline increased as a result of the osmotic stress. The plant would thus need to find a way of increasing the proline levels and this can be achieved by the inactivation of the proline degradative pathway (KIYOSUE, YOSHIBA, YAMAGICHI-SHINOZAKI, & SHINOZAKI, 1996).

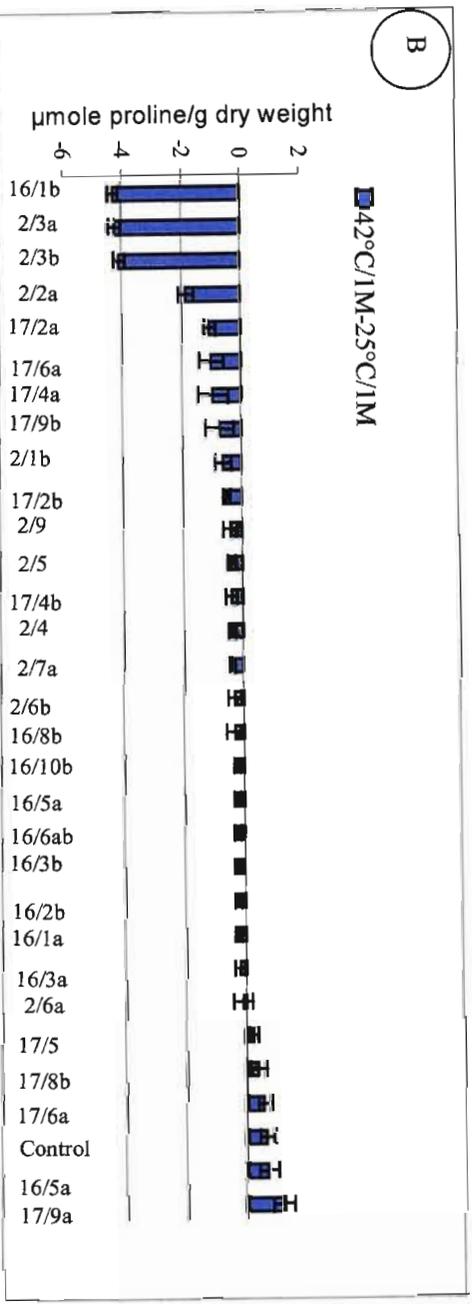
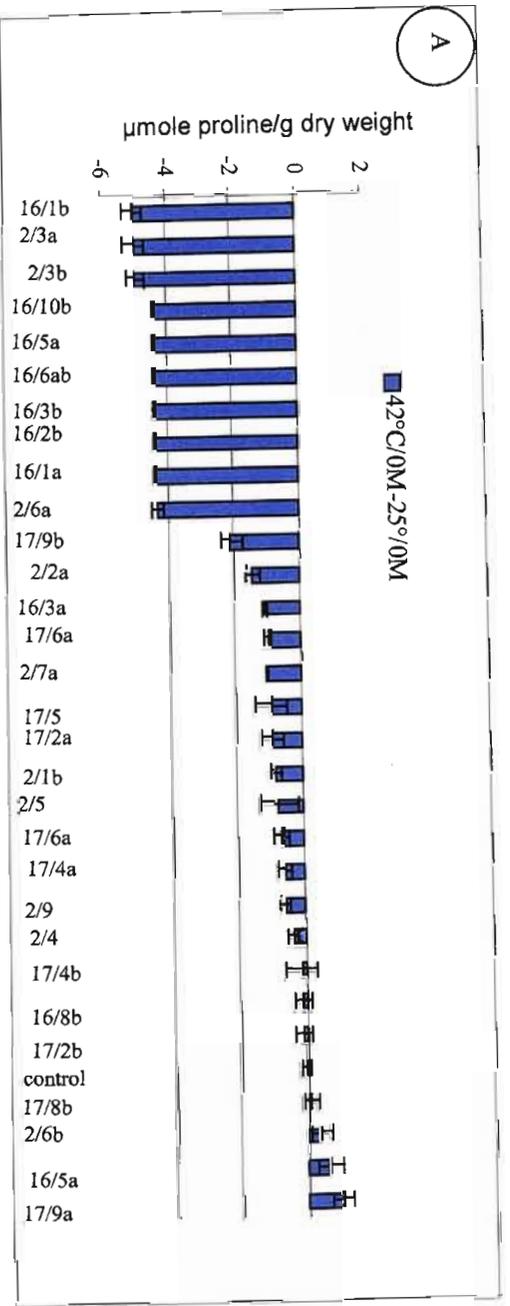


Figure 11: Effect of temperature and mannitol on the proline levels, displaying the manipulation of the IHSP and the deactivation of *P5CR* in all the T3 transformants

(A) Osmotic stress, 42°C/1M-25°C/1M

(B) Without osmotic stress, 42°C/0M-25°C/0M

These results imply a significant inhibition of proline synthesis under osmotic stress in the 25 transgenics compared to the control with the 42°C/1 M-25°C/1 M parameter (Figure 11B). The transgenics that demonstrated a non-significant difference in the concentration of proline in comparison to the control could be escapes of the transformation process or contain fewer copies of the *P5CR* gene. However, loss of expression does not always correlate to transgene loss, but can be the effect of inactivation of the gene (FINNEGAN & McELROY, 1994). The variation in the proline results may be attributable to *P5CR* copy number, integration sites, DNA rearrangement and/or transgene inactivation. The specificity of the antisense sequence for the *P5CR* RNA can also play a role in the level of phenotypic suppression (BOURQUE, 1995).

The self-pollinated offspring of the transgenic plants used in the proline assay was sampled and used in a woodenbox screening experiment. Transformed and control plants were evaluated over a 2 week period in order to determine the effect of temperature and drought (Figures 12A,B,C). The soybean plants were allowed to grow under optimum conditions, until all plants reached the six-leaf stage. Up to this stage, no phenotypic differences were observed between plants. Thereafter a drought stress and a heat stress were applied. As the IHSP is activated between 32 and 42°C, a temperature of 37°C was chosen in order not to apply an intense heat stress. The plants were thus monitored every second day and rated according to a given parameter (Figure 12A). After a 2 day drought stress the soil was still reasonably wet. The plants did not suffer any visual distress symptoms at this stage (Figure 12B). Although the IHSP was already activated, the proline pool was still big enough to supply proline for the plant's need. This may indicate that the plant's proline demand did not exceed the available proline.

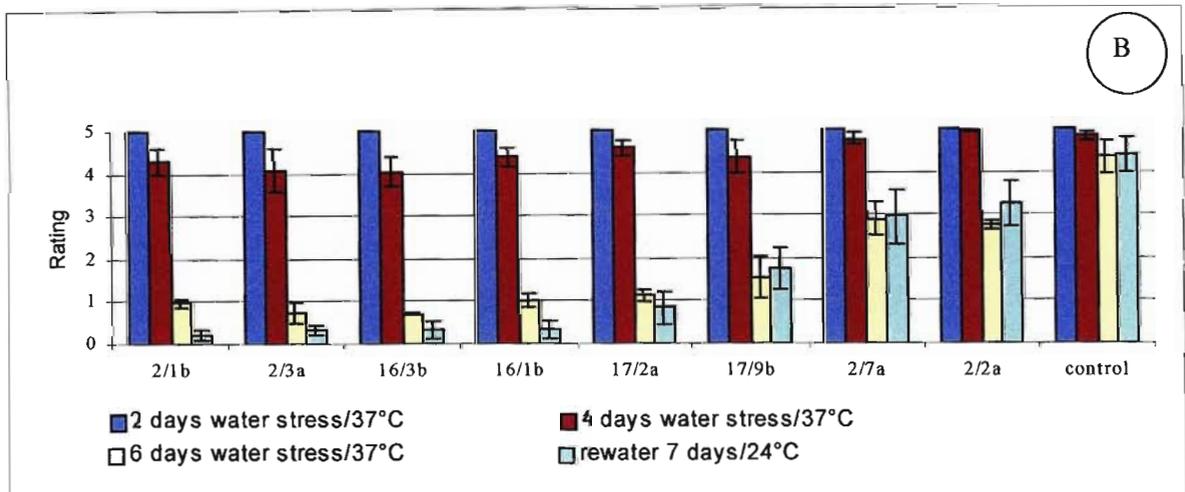
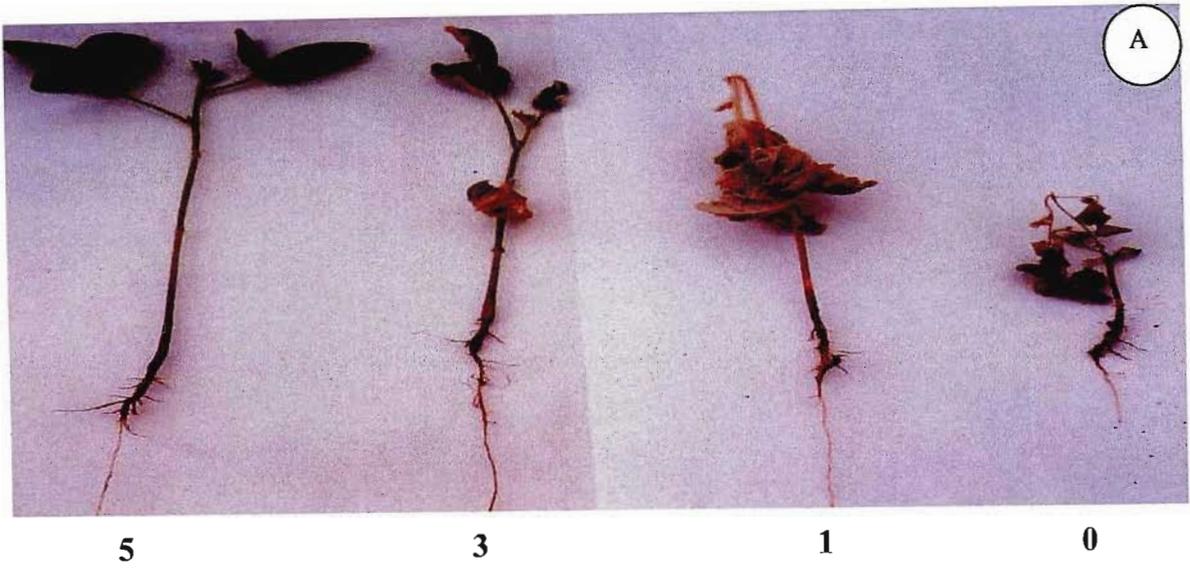


Figure 12: Woodenbox screening of T4 transformants

(A) Parameters used in rating of the seedlings

5 = 100% alive and well, 3 = 50% wilted, 1 = 100% wilted, 0 = dead

(B) The effect of drought stress at 37°C and recovery at 25°C on soybean seedlings

After 4 days without water at 37°C, the plants started to suffer from a drought stress (Figure 12B). It is known that HSP genes are activated not only by heat, but also by unrelated stimuli such as drought. Many treatments that make the intracellular environment less physiological, appear to prompt cells to activate HSP genes (SCHÖFFL, 1988). It appears that the increased drought stress mimicked the heat shock response and was experienced by the plant as an increased heat stress and therefore activated the IHSP to an even greater degree as if the temperature was increased to 42°C. This resulted in a decline in the synthesis of proline in the transgenics. The transgenics 2/1a, 2/3a, 16/3b, 16/1b, 17/2a and 17/9b suffered more than the control, 2/7a and 2/2a and started to die off. After 6 days without water at 37°C, most of the transgenics were severely wilted. The IHSP was now fully activated, as a combined effect of both a heat and drought stress. The control plants were significantly healthier than the transgenics. This demonstrated that the proline demand increased with an increase in drought stress. The transgenic plants lacking the enzyme necessary for proline synthesis could not cope with the drought stress, in comparison to the control plants which did not experienced this shortage.

Some of the plants recovered again after the woodenboxes were rewatered (Figure 12B). The control plants showed the highest number of plants that did not die as a result of the drought stress (Figure 12C). The transgenics 2/2a, and 2/7a demonstrated an ability to sustain the drought stress for a longer period than the other transgenics. This could have been due to a lower copy number of the *P5CR* gene, a decline in the expression of the *P5CR* gene, a decline in the degradation of proline or other survival mechanisms that were activated. The control plants demonstrated an overall better performance than the transgenics, indicating that the *P5CR* gene was transferred to the fourth generation and that these fourth generation plants were less viable than the control plants.

This data indicated that proline must play a definite role in the survival of soybean plants under drought stress. An amino-acid or nitrogen shortage alone could not be responsible for this dramatic reaction of these plants to a 6 day drought stress. The control plants were only mildly stressed after 6 days without water at 37°C, but recovered almost immediately when watering was resumed at 25°C.



Figure 12: Woodenbox screening of T4 transformants

(C) Transformed and control soybean plants after a 6 day drought stress at 37°C and 7 day recovery period at 25°C

4.5. CONCLUSIONS

The understanding of the biochemical processes surrounding proline will enable manipulation of the drought response and may lead to improvement of soybean tolerance to drought stress. It can be concluded from the proline study, that at 42°C the IHSP was fully activated and resulted in a significant decrease in the transgenic's production of proline. The activation of the IHSP resulted in the inactivation of the *P5CR* gene, which resulted in decreased proline synthesis. With the application of a mannitol stress, the control showed a significant increase in proline concentration at 32 and 42°C indicating a role during osmotic stress. This is in contrast to the transgenics that displayed a decrease in proline concentration at 32 and 42°C under mannitol stress. It was confirmed that there is an association between *P5CR* translation and proline accumulation, as the proline accumulation was markedly decreased by the activation of the heat inducible promoter and thus the antisense construct in transformed plants. The proline accumulation documented in this paper provides additional evidence that the increase in proline levels during osmotic stress constitute an adaptive response by the plant. The woodenbox technique indicated that proline synthesis is needed for the survival of plants subjected to a drought stress. The untransformed control plants survived a drought stress longer than the transformed plants, which contained the antisense *P5CR* gene. The viability of the transgenics declined with increasing drought stress. All of these studies confirm that plants need proline levels to increase or to be sustained during a drought or osmotic stress, in order to help the plant survive the stress situation.

CHAPTER 5

INTERACTION OF OSMOTIC AND TEMPERATURE STRESS ON TRANSGENIC SOYBEAN

5.1. INTRODUCTION

Soybean is becoming an increasingly important economic crop in South Africa due to the increase in tonnage (174 800 tons) produced in 1999 (AGRIMARK TRENDS, 2000). The fact that it is cultivated in a number of variable environments, imposes constraints on both growth and development. Among the adverse environmental factors commonly encountered by agricultural plants are extreme temperatures and periods of drought. Much research has been conducted in the attempt to understand these responses (MAJUMDAR, GHOSH, GLICK & DUMBROFF, 1990; BRAY, 1993; PURWANTO, 1994). In nature adverse environmental stress factors are seldom alone, e.g. drought spells in summer are often accompanied by extreme heat. It will thus be important to understand the respond of plants to simultaneous stresses. Proline accumulation is one of the adaptations of plants to osmotic stress, but the response to a simultaneous heat stress is not well understood.

The main focus of proline research has been on the functional aspects as was indicated in Chapter 4. Relatively little is known about the changes in the viability of antisense plants. Reliable determination of plant viability is a requirement for

screening for heat and drought tolerance. A reliable method must be able to differentiate between tolerant and sensitive cultivars and preferably also be able to distinguish between drought and heat stress (BRUCKNER & FROHBERG, 1987).

At present two methods of viability measurement are used: electrolyte leakage for drought (LIN, CHEN & KEY, 1985), heat (INGHAM, 1985), freezing resistance (SULCK, ALBRECHT & DUKE, 1991); and triphenyltetrazolium chloride (TTC) vital staining for heat (VRATSANOS & ROSSOUW, 1991), drought (DE RONDE & VAN DER MESCHT, 1997) and cold resistance (TOWILL & MAZUR, 1974). Electrolyte leakage is a measurement of membrane injury (RUTER, 1993) and has been found to be unreliable in some cases (ZHANG, WILLISON & HALL, 1993). Vital staining with TTC provides information about whether individual cells are functioning physiologically or not.

TTC reduction seems to occur in the mitochondria and the cytosol (NACHLAS, MARGULIES & SELIGMAN, 1960) by the tetrazolium salt accepting electrons from NAD^+ , NADP^+ and FADH_2 . Two of the pathways (amongst others) that may reduce TTC are the succinate oxidase pathway (NACHLAS, MARGULIES & SELIGMAN, 1960) and the NADH dehydrogenase system (COLEMAN & PALMER, 1972). In the succinate oxidase pathway electrons reduce oxygen in a reaction catalyzed by cytochrome oxidase (WHELAN, HUGOSSON, GLASER & DAY, 1995). Cytochrome oxidase is at sufficiently high concentration not to be rate-limiting, but the limiting enzyme in the sequence of electron transport in the succinate oxidase system is known to be succinate dehydrogenase (SDH) (NACHLAS, MARGULIES & SELIGMAN, 1960). SDH provides electrons to the electron transport chain and its activity is an indication of the metabolic state of the organ (AITHAL & RAMASARMA, 1969).

Malate dehydrogenase (MDH) is linked to the electron transport chain via the endogenous NADH dehydrogenase system (COLEMAN & PALMER, 1972) with the reduction of NAD^+ and NADP^+ (BERRIDGE, TAN, McCOY & WANG 1996). MDH catalyzes the interconversion of oxaloacetate to L-malate with the concomitant generation of NAD^+ in the cytosol (CONN & STUMPF, 1976). Malate is then shuttled into the mitochondria by a malate/alpha-ketoglutarate carrier, where malate is converted to OAA. Malate is an intermediate in the tricarboxylic acid cycle (CONN & STUMPF, 1976) and has a dicarboxylate reporter function in the inner mitochondrial membrane whereby malate can be exchanged for succinate and oxaloacetate (MARTINOIA & RENTSCH, 1994).

The exchange of oxaloacetate and malate is a plant specific transport system, which has a high affinity for oxaloacetate but only weakly for malate. Catalysis by malate dehydrogenase favours malate formation (DOUCE & BONNER, 1972). Malate is a characteristic metabolite in the photosynthesis of C4 plants. Furthermore, changes in the intracellular concentration of this organic acid provide part of the osmotic potential for guard cells. Alterations in the malate concentration influence both photosynthetic capacity and stomatal closure (RASCHKE, 1979).

HEDRICH, MARTEN, LOHSE, DIETRICH, WINTER, LOHAUS & HELDT (1994), demonstrated that alterations in the intercellular CO_2 concentration of the guard cells, which control stomatal aperture and thus the transpiration/photosynthesis ratio of leaves, have an effect on the extracellular malate concentrations of *Vicia faba* leaves. Plants respond to changes in the ambient CO_2 concentration by an increase in water-use efficiency, adjusting stomatal aperture in relation to the photosynthetic capacity (RASCHKE, 1979). Malic acid fluctuation increased with decreasing PEG water potential below a threshold of -0.1MPa in *Sedum telephium* (CONTI & SMIRNOFF, 1994).

The viable cells reduce metabolically the water soluble tetrazolium salts into water insoluble highly coloured end products called formazans (BERRIDGE, TAN, McCOY & WANG, 1996). The tetrazolium structure is reduced by the addition of a hydrogen atom, the nitrogen to nitrogen bond is broken with the formazan formation (Diagram 5). Formazan has a red colour and is soluble in ethanol and can be monitored spectrophotometrically.

The ability of viable cells to reduce this tetrazolium salt appears to be a superior measure of heat tolerance for both experimental use and genotype selection (CHEN, SHEN & LI, 1982). In cotton (DE RONDE, VAN DER MESCHT & CRESS, 1995) it was found that a heat-tolerant cultivar was characterized by having a higher formazan value over time in the stress treatment compared to a control treatment. This was in contrast to a heat-sensitive reaction in which the control treatment resulted in higher formazan values over time than under heat stress conditions.

5.2. AIM

The aim of the following experiment was to establish whether there is an interactive response to osmotic and heat stress in antisense *P5CR* soybean plants and to investigate if under-production of proline will influence the viability of soybean plants.

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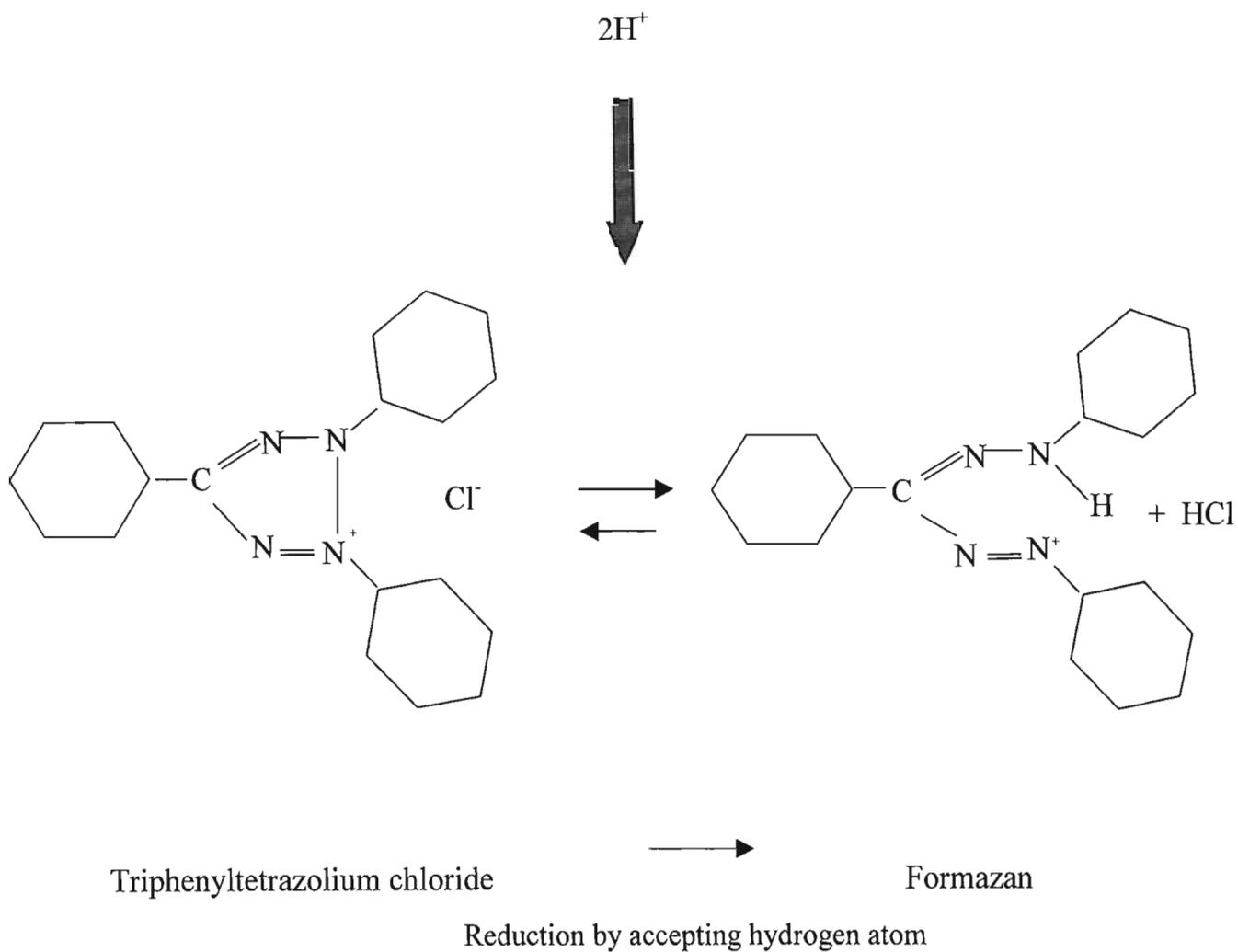


Diagram 5: Reduction of tetrazolium chloride to formazan by accepting of hydrogen (SERVA, 2000)

5.3. MATERIAL AND METHODS

5.3.1. TTC viability assay

Leaf samples were harvested from greenhouse plants (antisense *P5CR* transformed (transgenics) and H₂O transformed (controls)) growing under optimum conditions, in order to evaluate the viability of the transgenics. Mannitol was used as an osmoticum to induce stress. Treated leaf samples, consisted of 5 leaf discs of 7 mm in diameter. They were sampled every 30 minutes for 6 time intervals in the laboratory (Diagram 6). The leaf material was subjected to a control treatment of 3 hours in 3 ml 0.2 M sodium phosphate buffer (pH 6.9) or a moderate stress of 3 hours in 3 ml 0.5 M mannitol (-1.24 MPa) for acclimation, before incubation of both samples in 3 ml 1.0 M mannitol (-2.48 MPa) solution.

The experiment was performed at 25°C for the control evaluations and at 40°C for activation of the inducible heat shock promoter (IHSP) (CZARNECKA, GURLEY, NAGAO, MOSQUERA & KEY 1985). Formazan production was measured by the addition of TTC to the treated leaf discs as described (DE RONDE & VAN DER MESCHT, 1997). The sampled leaf discs were submerged in 3 ml of 0.8 % (w/v) TTC solution dissolved in 0.2 M sodium phosphate buffer, pH 6.9. The leaf discs were vacuum infiltrated for 5 minutes to ensure solution penetration into the tissue prior to a 18 hour incubation at 25°C in the dark. Subsequently, the discs were washed twice with distilled water followed by the addition of 3 ml 95% ethanol. The samples were boiled until dry and resuspended in 3 ml 95% ethanol when cooled. The formazan accumulation was measured spectrophotometrically at 485 nm with a micro titer plate reader.

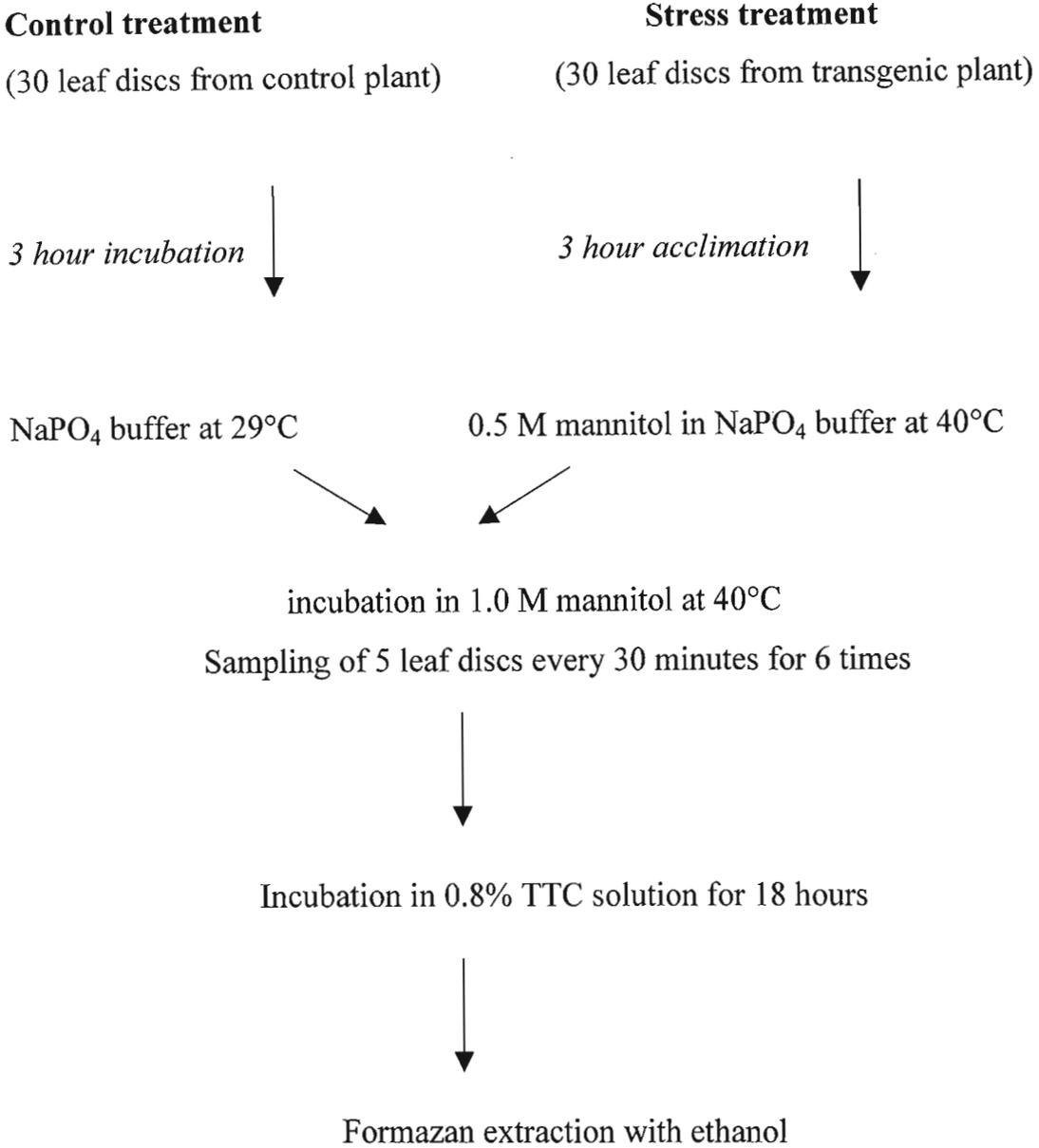


Diagram 6: TTC reduction assay with control and transgenic leaves in order to evaluate the viability of the transgenics

5.3.2. ¹⁴C protein efficiency analysis

Twenty micrograms of leaf tissue (grown under optimum conditions) was pre-incubated at 25 and 42°C for 3 hours in 200µl sodium phosphate buffer, pH 6.9 to overcome the wounding shock (VALLURI, TREAT, NEWTON, COBB & SOLTES, 1988). Osmotic stress was induced by substituting the pre-incubation medium with a sodium hydrogen buffer containing 0.75 M mannitol followed by incubation for 3 hours at either 25 or 42°C. Newly synthesized proteins were labelled by the addition of ¹⁴C-protein hydrolysate (4.25 µCi) at the start of the 3 hour stress period.

Proteins were extracted with an extraction buffer containing 10 ml Tris 0.25 M pH 6.8, 8 ml 10% SDS, 2 ml mercapto-ethanol and 16 ml H₂O (VAN DER MESCHT, VISSER, DE RONDE & VORSTER, 1992). The control reactions did not contain mannitol. A 10 µl sample was mixed with 1.5 ml of scintillation fluid, before the uptake of the radioactive amino acids was measured with a scintillation counter.

5.3.3 Yield measurement

The seeds of the transgenic and control lines were harvested, sorted according to size and counted.

5.3.4. Statistical analysis

Standard error was calculated using Microsoft Excel

5.4. RESULTS AND DISCUSSION

5.4.1. TTC viability assay

A TTC viability assay was used to screen the transgenics for differences in viability after the activation of the IHSP. As plants generally accumulate free proline under osmotic stress conditions (PENG, LU & VERMA, 1996), a mannitol stress was applied to test the hypothesis that the antisense *P5CR* gene in the transgenics will produce plants more sensitive to osmotic stress. It was previously demonstrated that drought simulation by exposure to different osmotic potentials could be monitored by the TTC assay (DE RONDE & VAN DER MESCHT, 1997). This confirmed the finding by IYER & CAPLAN (1998) who correlated osmotic stress with drought stress.

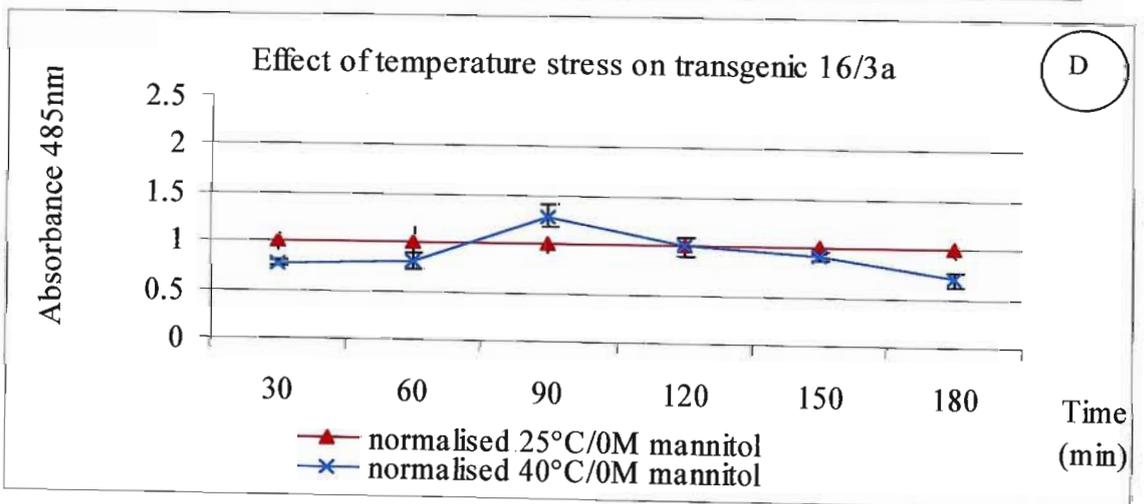
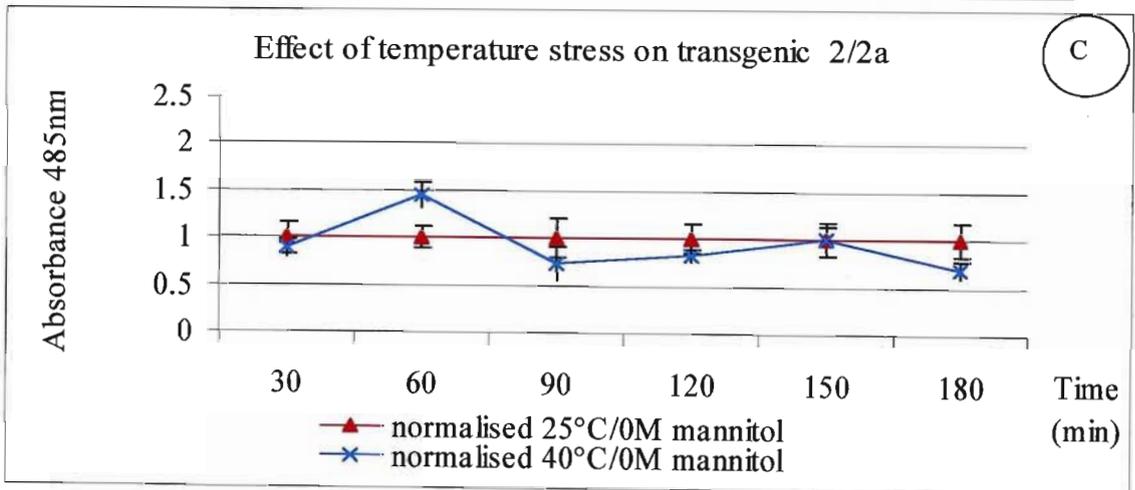
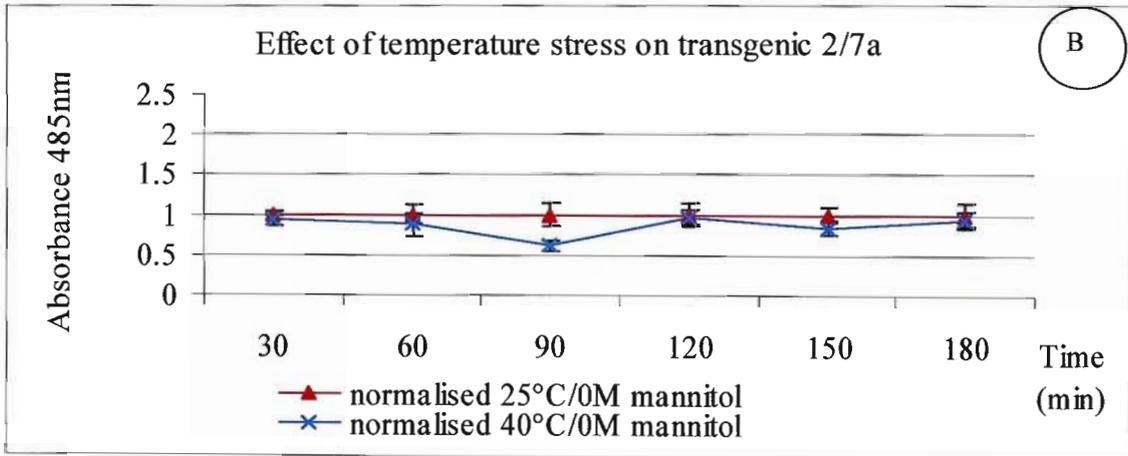
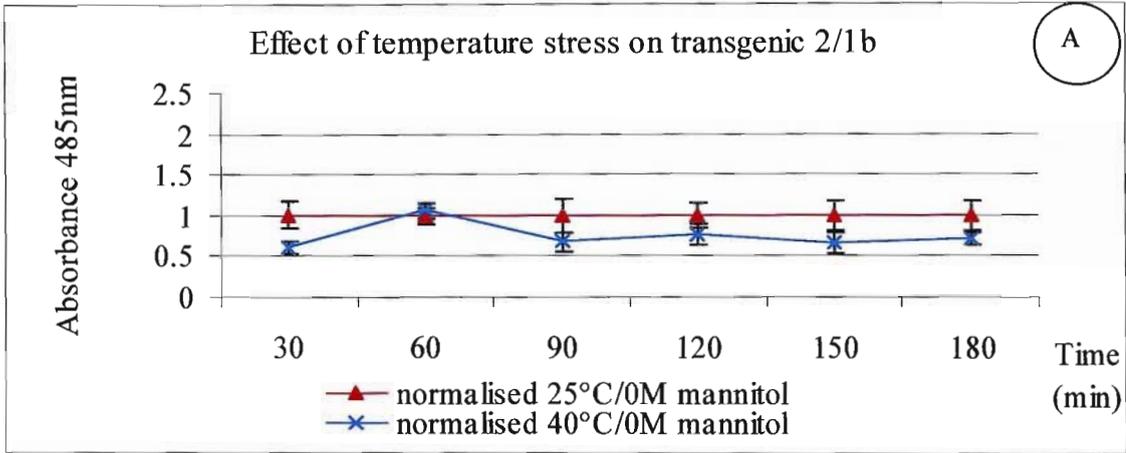
The experiment was conducted at two temperatures (25 and 40°C) and with and without mannitol (0 and 1M) treatments. The 25°C treatment was performed as a control treatment in which the IHSP was not activated. This was in contrast to the 40°C treatment where the IHSP was fully activated (CZARNECKA, GURLEY, NAGAO, MOSQUERA & KEY 1985). The 40°C treatment (stress) was normalised over the corresponding values of the 25°C treatment (control) in order for the differences between these treatments to be better visualised.

The activation of the IHSP as a result of a 40°C heat stress in the transgenics and control under control conditions (0M mannitol), can be seen in Figures 13A to G. A range of responses was detected across the different transgenics. The transgenics (Figures 13A to F) yielded higher or non-significant different formazan values over time in the 25°C treatment, than under the 40°C conditions. It is postulated that the transgenic plants could not activate a tolerance mechanism during the mild stress,

which normally assists with plant survival during the more severe stress treatment. This resulted in high formazan production in the control treatment.

Even the mild stress was experienced as a severe stress in the antisense lines. The control plants in contrast (Figure 13G), yielded higher or non-significant different formazan values over time in the stress treatment than under the control. The control plants had the ability to overcome the severe stress treatment due to activation of a tolerance mechanism, which was triggered during the acclimation treatment. This resulted in high formazan production in the stress treatment. It can be concluded from these results that the transgenics performed as “sensitive” lines. The control plants were however able to activate a tolerance mechanism during the mild stress and had the ability to withstand the more severe stress. The activation of the IHSP resulted in lower proline levels in the transgenic plants and thus a lower viability.

The effect of the activation of the IHSP under a stress treatment (1M mannitol) can be seen in Figures 14A to G. Much the same variation was observed as in Figure 13. The transgenic lines (Figures 14A to F) yielded higher or non-significant different formazan values over time in the control treatment, than under the stress conditions. The control plants in contrast (Figure 14G) yielded higher or non-significant different formazan values over time in the stress treatment than under the control. The transgenics experienced the mannitol and 40°C stress as being more severe than the 40°C alone.



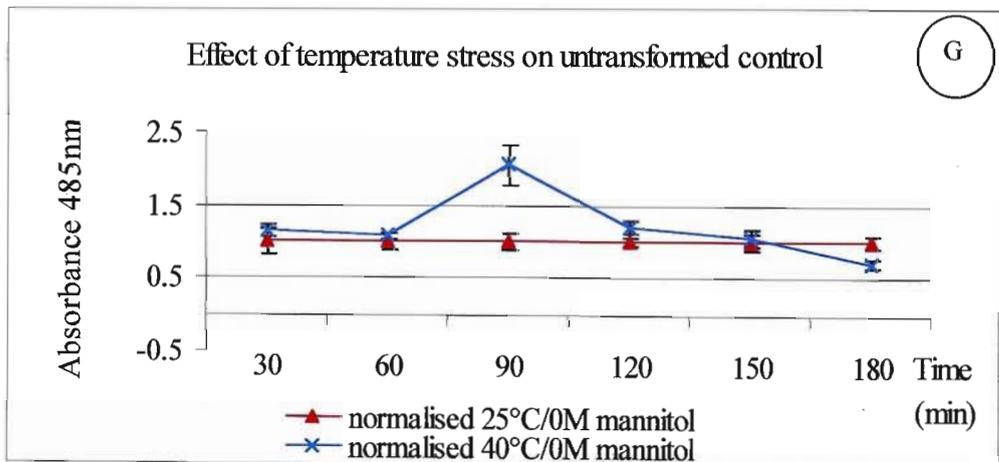
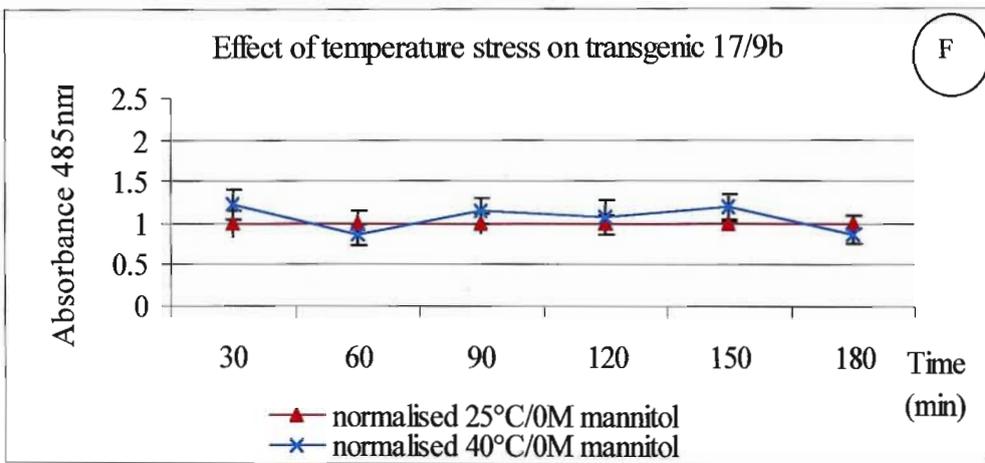
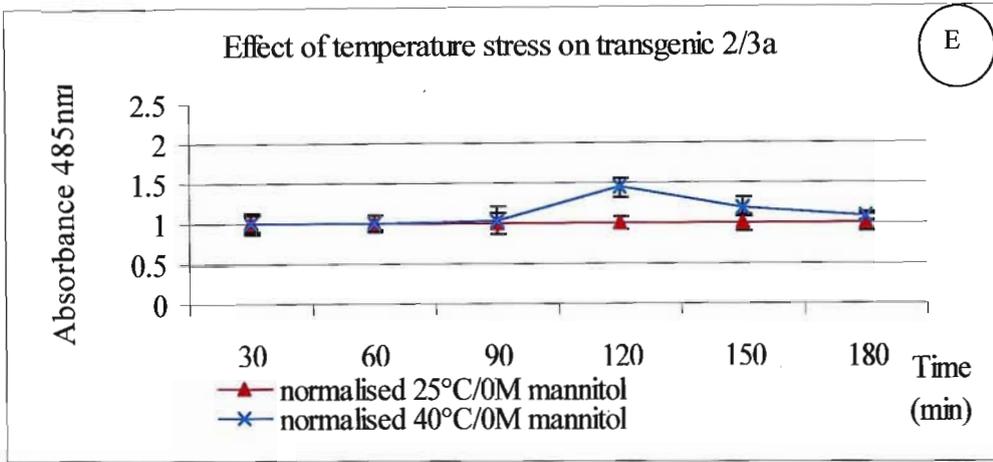
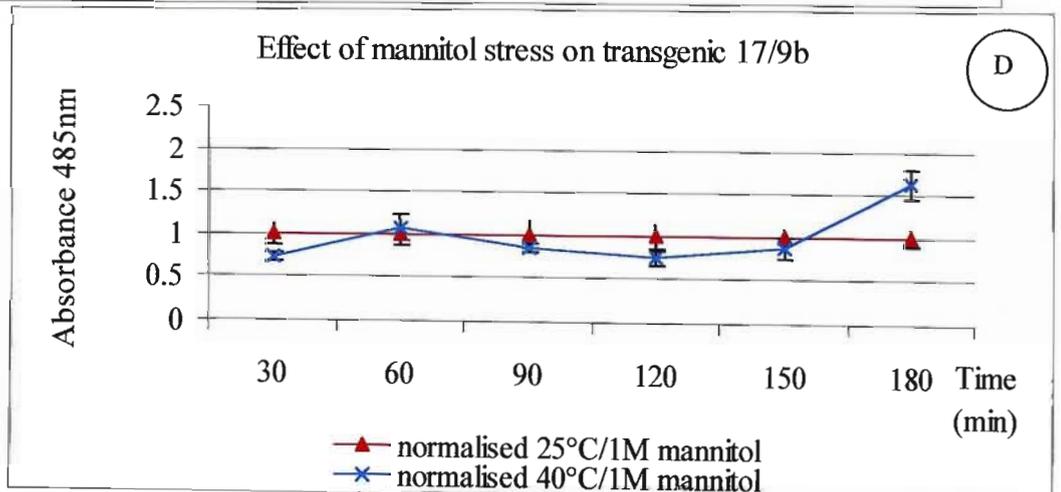
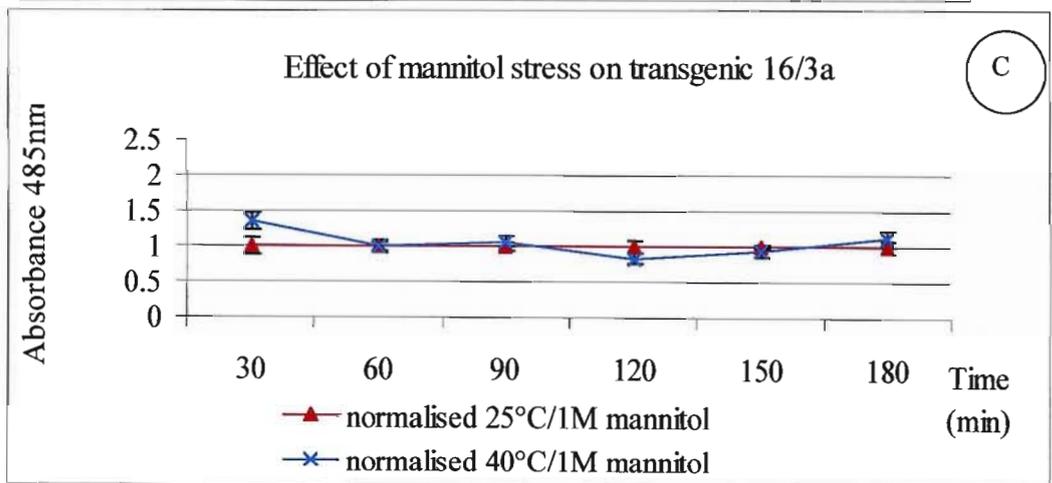
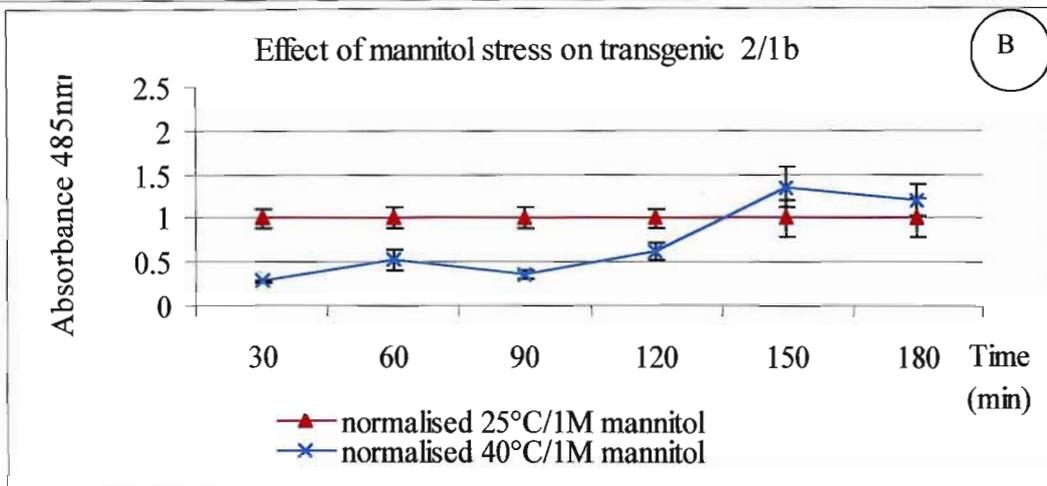
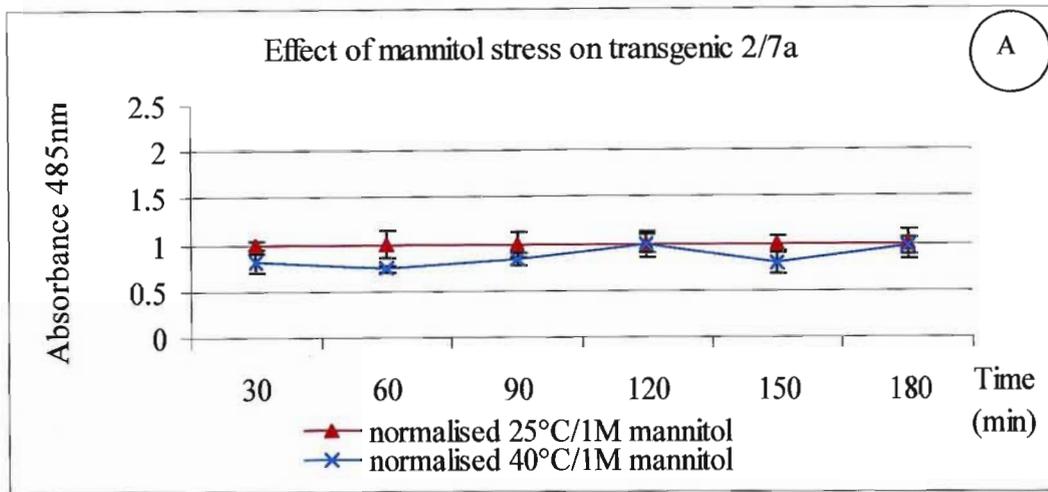


Figure 13: The effect of activation of the IHSP, as a result of a 40°C heat stress, on antisense *P5CR* transgenics (A to F) and untransformed control plants (G), as observed through formazan production in a TTC viability assay



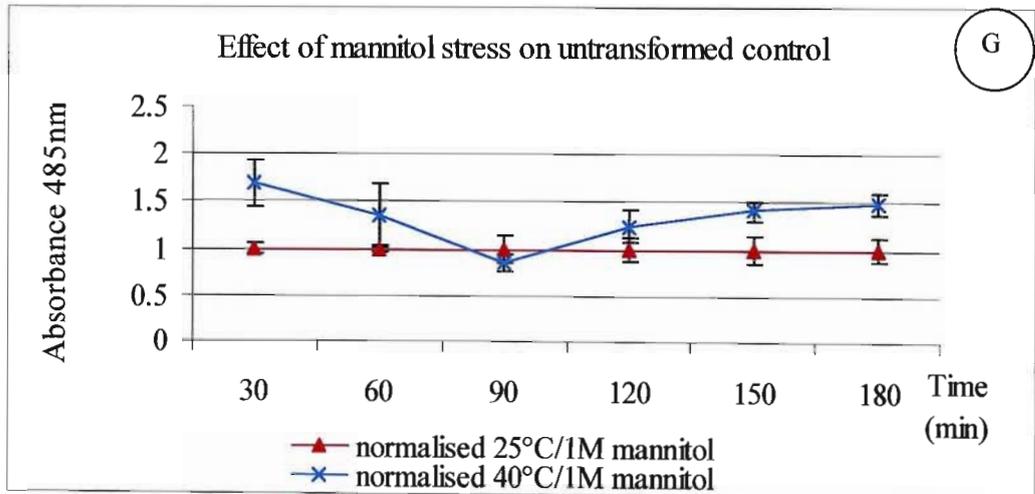
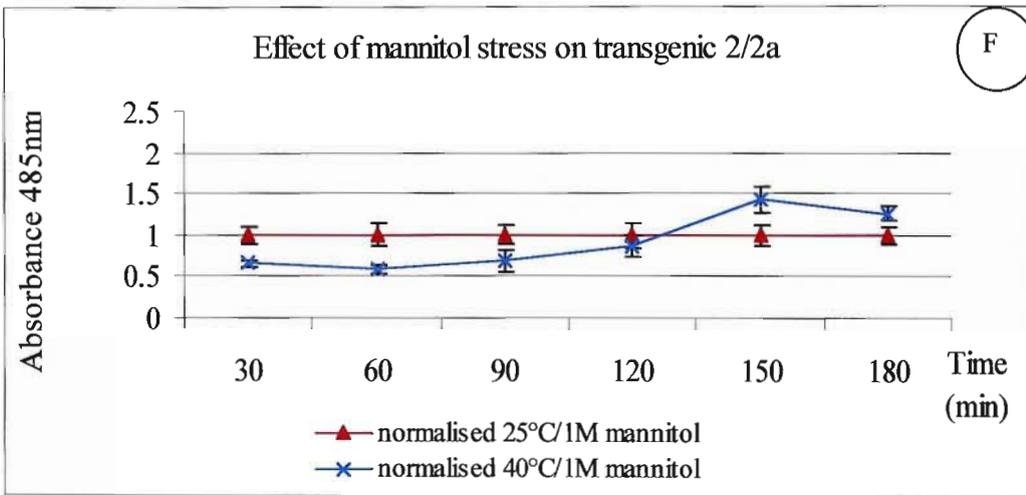
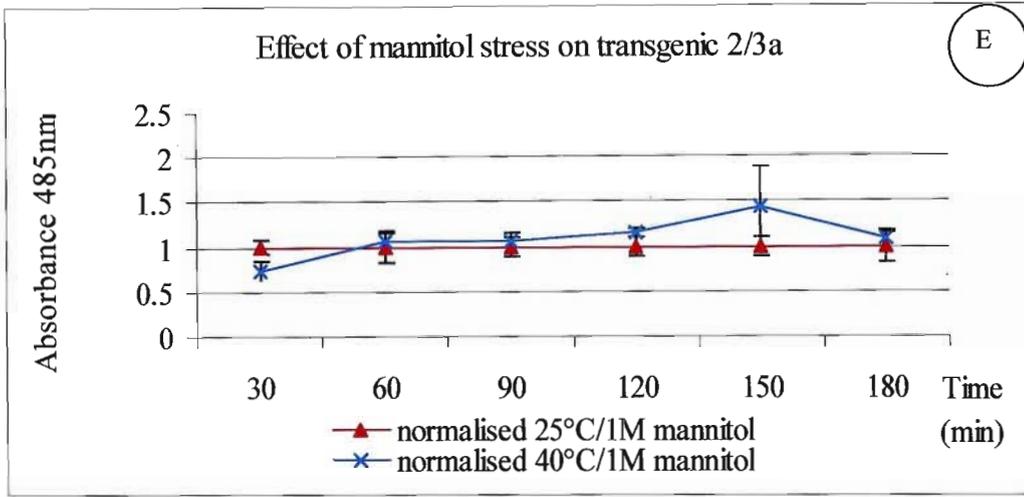


Figure 14: The effect of activation of the IHSP, as a result of a 40°C and 1M mannitol stress, on antisense *P5CR* transgenics (A to F) and untransformed control plants (G), as observed through formazan production in a TTC viability assay

The antisense soybean transgenic plants reacted as sensitive and the control plants as being tolerant. The difference between the mean values of the stress treatments (40°C/1M mannitol - 25°C/1M mannitol) and the mean values of the control treatments (40°C/0M mannitol - 25°C/0M mannitol) were used as an indicator of the viability of the transgenics. This allowed ranking the lines in increasing order of viability (Figure 15). This ranking made it possible to observe the effect of the antisense *P5CR* gene in soybean on the viability of the plants. The control plants were more viable than the transgenic plants, confirming the findings that proline plays an adaptive role during osmotic stress as lower levels of proline resulted in less viable plants. The antisense transgenics were not able to withstand the osmotic stress as a result of the decline in the proline levels (Figures 10A,B,C) due to the antisense *P5CR* gene.

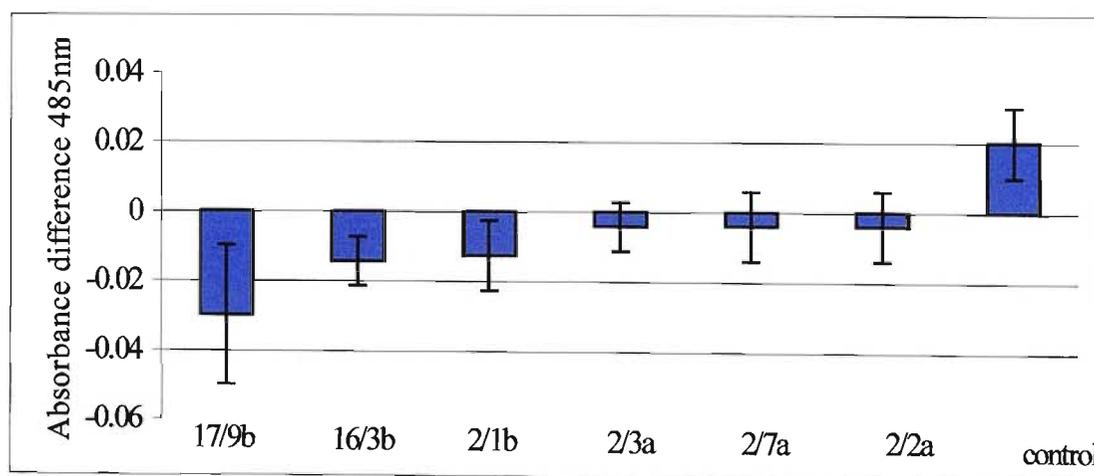


Figure 15: The viability ranking of the antisense *P5CR* transformants and untransformed control. The difference between the mean absorbance values of the stress treatments (40°C/1M mannitol - 25°C/1M mannitol) and the mean values of the control treatments (40°C/0M mannitol - 25°C/0M mannitol) were used as an indicator of the viability of the transgenics

5.4.2. C¹⁴ Protein efficiency analysis

When the IHSP was not activated (25°C), there was no significant difference between the controls and the transgenics in the efficiency of protein synthesis (Figure 16A). This indicates that the transformation procedure did not effect the normal protein efficiency negatively.

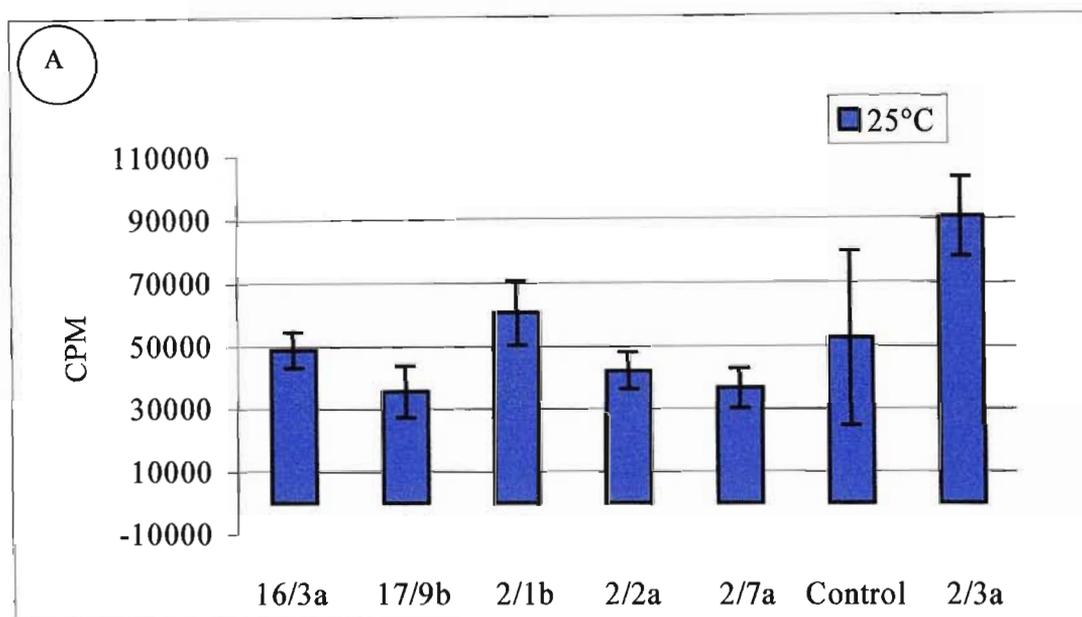


Figure 16: Comparison of transgenic soybean plants and control plants using ¹⁴C protein efficiency analysis at a 25°C treatment (A)

However, when the IHSP was activated at 42°C, the control plants used less of the ¹⁴C amino acids than the transgenics (Figure 16B). The control plants have the ability to synthesize amino acids without using the radioactive amino acid mixture, as there was no proline shortage. This was in contrast to the transgenics, which demonstrated a proline decline. This decline in proline, when the IHSP is activated can effect amino acid synthesis negatively. The decline is also more pronounced during an osmotic stress.

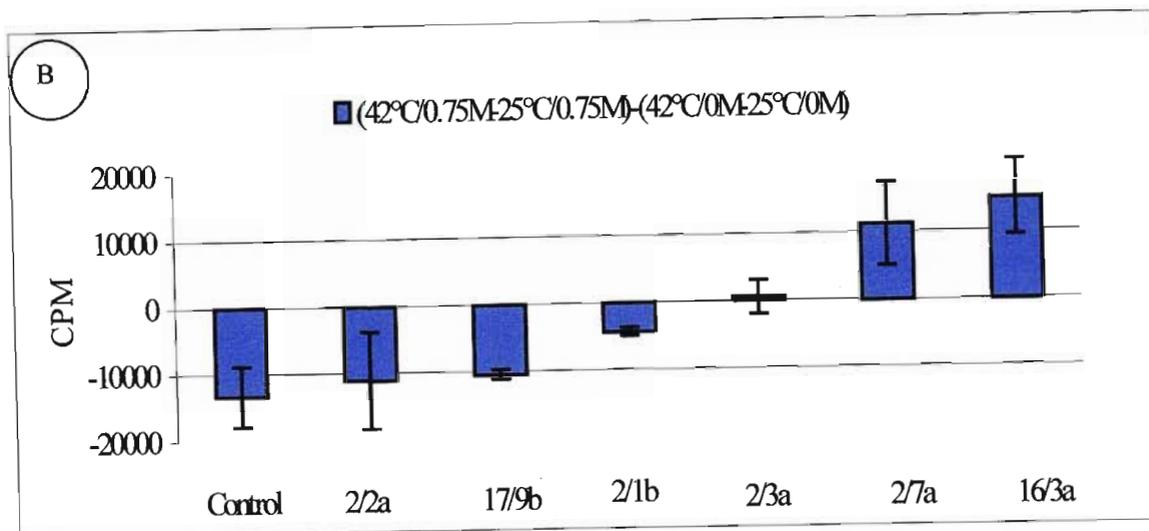


Figure 16: Comparison of transgenic soybean plants and control plants using ^{14}C protein efficiency analysis at a 42°C and mannitol treatment (B)

5.4.3. Yield measurements

The seed of the transgenics and control lines were harvested and it was observed that the transgenics produced a lower yield than the control plants (Table 3). This could be the result of occasional temperatures exceeding 25°C in the greenhouse. This increased temperature could have activated the IHSP and the decline in proline would influence the seed formation negatively.

5.5. CONCLUSIONS

The TTC assay is based on the ability of viable cells to metabolically reduce tetrazolium salts into formazan. The TTC viability assay was thus used as a method to screen the viability differences of the antisense transgenics. It was possible to detect differences between the transgenics and the control plants using this method. The transgenics performed a sensitive reaction in that the formazan production is the highest during the control treatment, but the control plants

performed a tolerant reaction in that the formazan production is the highest during the stress treatment.

The efficiency of protein synthesis in the transgenic plants was lower than in the control plants when the IHSP was activated. This indicated that underexpression of the *P5CR* gene resulted in a decline in protein synthesis due to proline shortage. The transgenics experienced a lower seed production as the control plants indicated that the antisense *P5CR* gene also influenced seed production negatively.

Results from the screening methods of the transformants are summarized in Table 3. This showed that the control plants were the most viable of the plants tested. The decrease in the proline level due to the presence of the antisense *P5CR* gene, yielded plants that were more sensitive to osmotic stress. These findings confirm that proline plays an adaptive role during osmotic stress as lower levels of proline in the transgenic plants resulted in less viable plants. It is postulated that the transgenics were not able to withstand the osmotic stress as a result of the decline in the proline synthesis.

Table 3: Summary of screening the antisense transgenic plants indicating the difference between antisense *P5CR* and control plants. The lowest number indicated most tolerant and the highest number indicated most sensitive reaction to the applied screening method

Lines	yield	¹⁴C protein efficiency	TTC	Summary
Control	1	1	1	3
2/7a	2	6	4	12
2/2a	7	2	3	12
16/3b	4	7	2	13
17/9b	6	3	7	16
2/3a	8	5	5	18
2/1b	9	4	6	19

CHAPTER 6

EFFECT OF SENSE AND ANTISENSE GENES ON CHLOROPHYLL FLUORESCENCE FUNCTIONS DURING STRESS

6.1 INTRODUCTION

Photosynthesis is the mechanism used by plants to convert solar into biochemical energy. The basic reaction(s) of photosynthesis were outlined in the 1800's by Jean Senebier and Theodore de Saussure as $\text{CO}_2 + \text{H}_2\text{O} + \text{light energy} \rightarrow (\text{C H}_2\text{O})_n + \text{O}_2$ (LIMP, RODAL & WILLIS, 1998). The overall process can be divided into two parts, the light and dark reactions. The light reactions consist of light absorption, production of ATP and reduction of NADP^+ . The dark reactions consist of the utilization of ATP and NADPH as well as CO_2 in the production of O_2 and glucose (LIMP, RODAL & WILLIS, 1998). Photosynthesis takes place in the chloroplasts: the light reactions in the grana of the thylakoid membranes and the dark reactions in the stroma.

Chlorophyll fluorescence measures the changes occurring in a plant leaf during the light reactions and more precisely during photosynthesis II (PSII). It measures the absorption of sunlight by the pigment antennae, containing the chlorophyll (chl) *a*

and *b* pigments (KRÜGER, TSIMILLI-MICHAEL & STRASSER, 1997). The chl-*a* and *b* pigments efficiently absorb light at the red and blue end of the light spectrum. PSII uses a form of chl-*a* known as P680. Photophosphorylation occurs when the electrons from water are excited by light in the presence of P680. If a pigment absorbs light energy, the energy is dissipated as heat, emitted as a long wave length known as fluorescence emission or trapped in the reaction center (STRASSER, SRIVASTAVA & TSIMILLI-MICHAEL, 1997). The pigment antennae channel the absorbed energy into the reaction center of PSII. The absorbed energy moves the electron through a redox chain to generate ATP and NADPH. Water splits and oxygen is released in the process. The electron eventually attaches to an electron acceptor in photosystem I (PSI). The electron is passed through different redox reactions to form NADPH and sugar (FARABEE, 2000) (Diagram 7).

PSII plays a central role in energy transduction and in the redox mechanisms involved in signal transduction for acclimation to environmental stresses (ANDERSON, PARK & CHOW, 1997). Photosynthesis is also associated with decreases in leaf stomatal conductance and small increases in leaf intercellular CO₂ concentrations (FREDERICK, ALM, HESKETH & BELOW, 1990). Changes in PSII activity during drought stress were observed by PLANCHON (1991) by using Rdf (ratio of decrease fluorescence = $F_m - F_o / F_m$) as a parameter.

Chl-*a* fluorescence has proved to be a useful, non-invasive tool in quantification of the behavior of PSII and can be used to provide a dynamic description of any given sample at any physiological state (KRÜGER, TSIMILLI-MICHAEL & STRASSER, 1997). Photosynthetic material displays a polyphasic fluorescence rise during the first second of illumination. These phases have been labelled O-J-I-P (Diagram 8) (STRASSER, 1996).

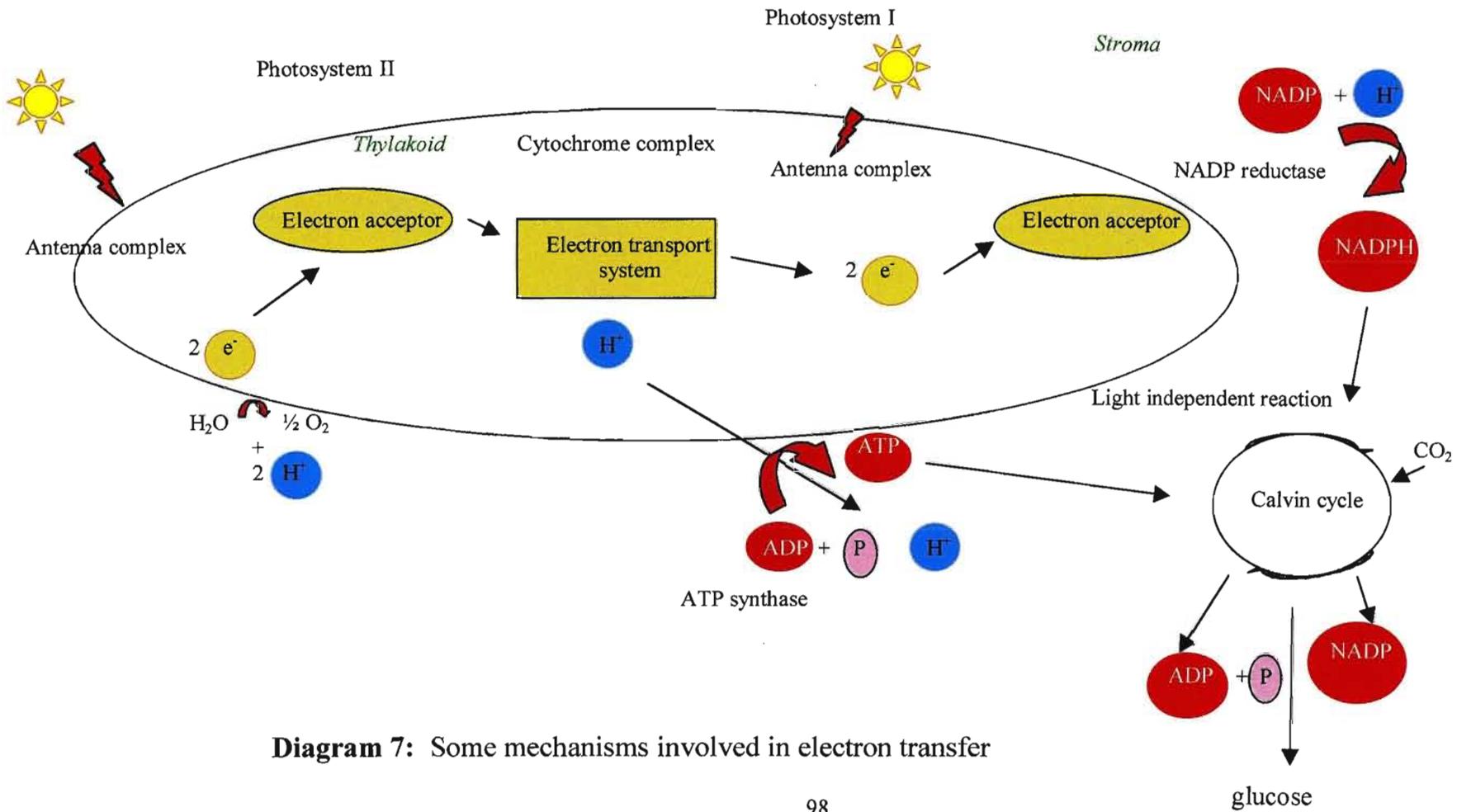


Diagram 7: Some mechanisms involved in electron transfer

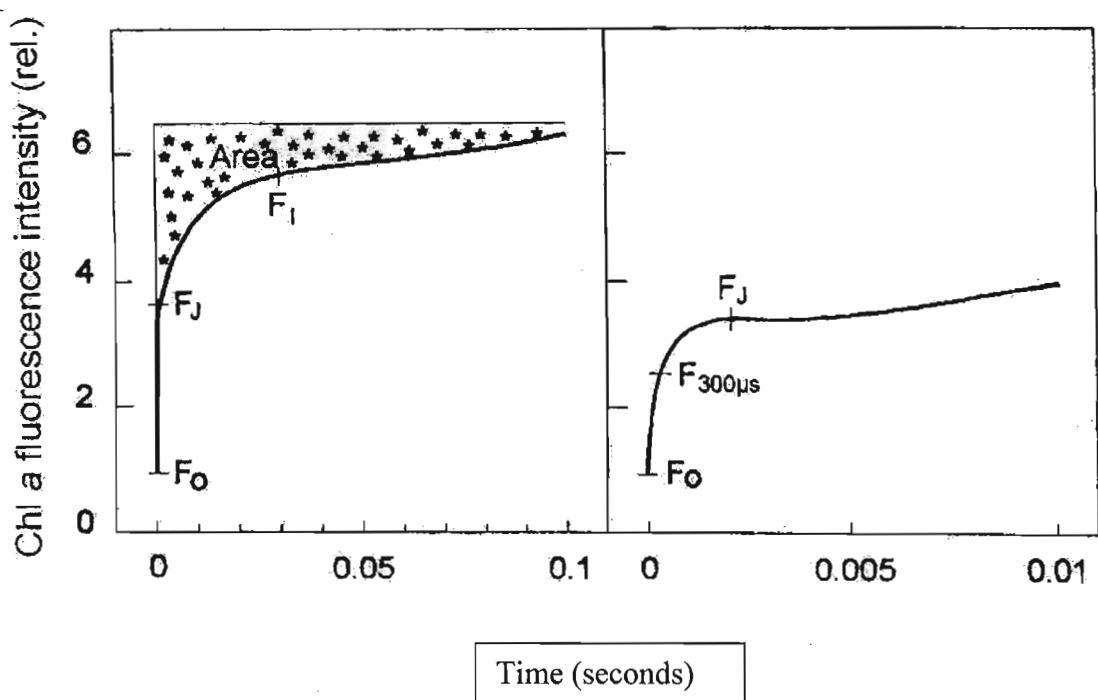
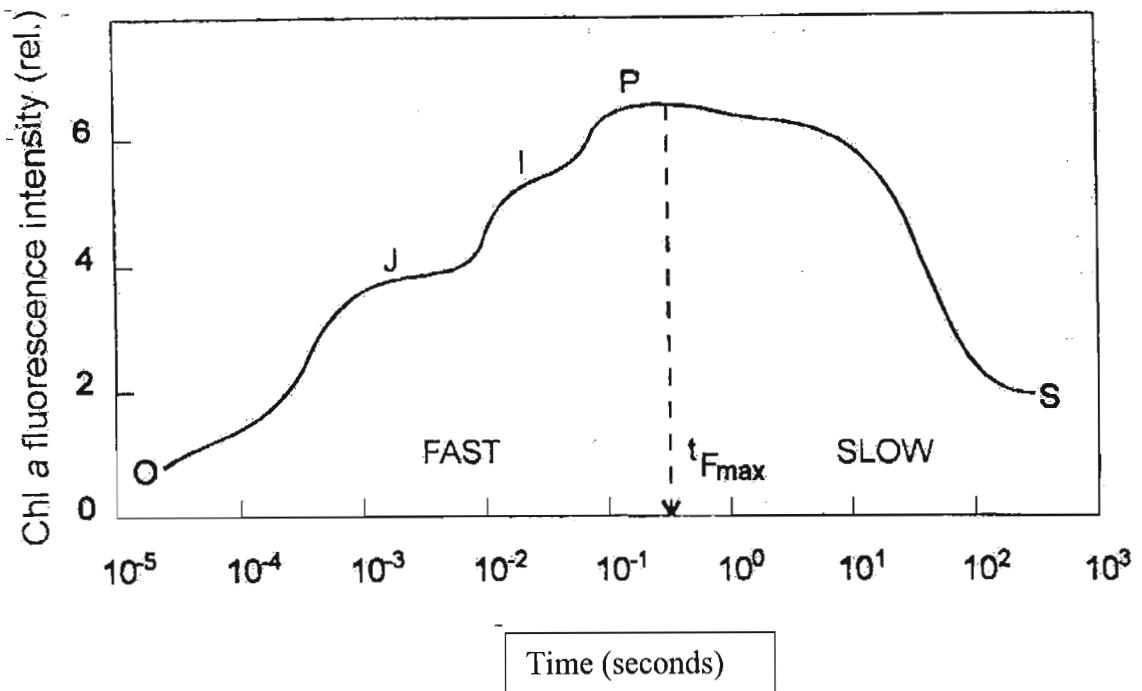


Diagram 8: O-J-I-P polyphasic rise of chl-*a* fluorescence as measured by a Plant Efficiency Analyser (STRASSER, 1996)

A detection method using the O-J-I-P polyphasic rise of chl-*a* fluorescence provided even more insight into a plant's reaction to stress (STRASSER, SRIVASTAVA & GOVINDJEE, 1995). This method permits precise detection of different parameters during the O-J-I-P transient. The information obtained through the test can be used in quantification of the effect of the stress on plant.

6.2. AIM

In the first series of experiments, the transformation process was successfully completed with a GUS-INT gene and an antisense *P5CR* gene in the breeding line of soybean Carnia-2233. In order to test the reproducibility and the general applicability of the transformation process further, the soybean cultivar Ibis was transformed with the *P5CR* gene in the sense and antisense directions. The transformation process results in many putatively transformed plants, which must be tested for the presence of the *P5CR* gene. Molecular analysis must be performed on all of these plants to determine whether they are transformed. The procedure is expensive and time consuming and thus another method for the screening of putative transgenics was developed.

Chlorophyll fluorescence has been used extensively by plant stress physiologists in monitoring changes observed in the photosynthetic apparatus of plants subjected to environmental stress (ÖGREN, 1990). EU, LEE, CHANG, RHEW, LEE & LEE (1998) also used chlorophyll fluorescence in screening for kanamycin resistance in transgenic plants. They were able to distinguish between transgenics and non-transgenics containing the *NPTII* gene on a selective medium. It was hypothesised that if the kanamycin gene could be detected under kanamycin stress, the proline gene could be detected under a drought stress. Thus, the chl-*a* fluorescence O-J-I-P transient (JIP) test was performed on putative transgenics (sense and antisense *P5CR*) in order to test this hypothesis.

6.3 MATERIAL AND METHODS

6.3.1. Molecular cloning, transformation and analysis

The *P5CR* gene (AT-P5C1) was used in this study. This gene was cloned, in sense and antisense (see Chapter 3) orientation, into HB101 pMA445, a derivative of the T-DNA vector pGA470, containing the heat shock inducible expression cassette (AN, WATSON, STACHEL, GORDON & NESTER, 1985). Plasmid DNA was isolated, quantified with the use of a TKO-102 fluorometer and tested electrophoretically for purity (see Chapter 3). Vector pMA445, containing the heat shock inducible expression cassette, had a multiple cloning site which enable cloning of DNA sequences into the cassette. The directional cloning of *P5CR* cDNA into pMA445 requires a multi-step ligation (Diagram 9). The gene was cloned in the sense direction. The 5' HIND III site and the 3' SAL I site made this directional cloning of the *P5CR* gene possible. Vector pMA445 plasmid DNA was digested with HIND III, purified (geneclean Bio 101) and the cohesive ends were filled in. The vector DNA was digested with SalI before it was isolated on a 1% low melting point agarose gel. The DNA concentration was quantified.

The *P5CR* gene in puc19 has a 3' EcoR1 site and a 5' BamHI site. A compatible cloning site with the vector was obtained through a SalI linker, which was blunt end ligated onto the EcoR1 site. The DNA concentrations were quantified before overnight ligation. Thereafter the plasmid was digested with BamHI and blunt ended to create a blunt ended 5' site. Subsequently the insert was digested with SalI to create a 3' SalI site. The insert was purified by electrophoresis and cut out of a low melting point agarose gel. The DNA concentration was quantified and the ligation reactions planned accordingly.

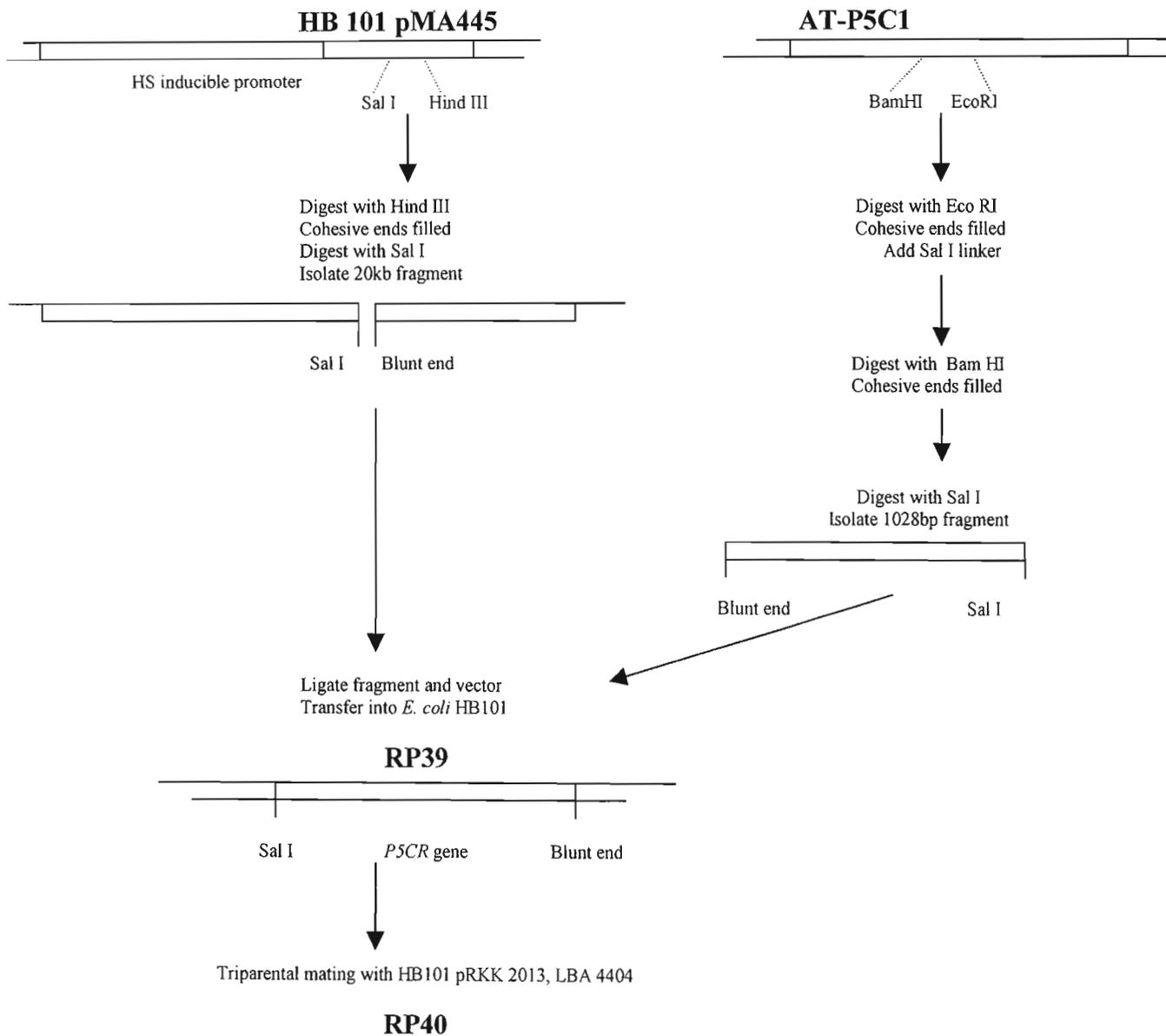


Diagram 9: Construction of the *P5CR* gene in a sense direction into a vector containing the heat inducible promoter

Competent *Escherichia coli* strain HB101 cells were transformed with the ligation reaction mixture (MANIATIS, FRITSCH & SAMBROOK, 1982). Transformed cells were selected on LB plates supplemented with tetracycline after a 37°C overnight incubation. A negative control, competent cells without added DNA, and a positive control, competent cells with only pMA445, were run simultaneously. Transformed cell colonies were picked and maintained on selective plates. Plasmid DNA of the single colonies was isolated and analysed by restriction digestions and gel electrophoresis.

The triparental mating procedure of ARMITAGE (1988) was used on plasmid colonies containing the *P5CR* insert. The *Agrobacterium*-mediated vacuum infiltration transformation procedure described in Chapter 2 was used to produce sense transgenic plants.

6.3.2. Chlorophyll fluorescence transients

Soybean plants (cultivar Ibis) were grown in the greenhouse under optimum conditions. Plants were subjected to a 36/25°C ±2°C day /night heat treatment for 2 days. This was done in activating the heat shock promoter. It was shown that the IHSP was partly activated at 32°C, but fully activated at 42°C (DE RONDE, SPREETH & CRESS, 2000). At the same time, watering was withheld. Plants evaluated were putative transgenics containing the RP29 construct (antisense plants) or the RP40 construct (sense plants) and non-transgenic control plants (non transgenic plants).

Chl-*a* fluorescence transients were measured using a Plant Efficiency Analyser (PEA, Hansatech). It is based on the JIP-test (STRASSER, 1996) which refers to the main steps for F_O-J-I-P. The fluorescence transients were induced by red light of 600 μmol m⁻² s⁻¹ intensity (excitation intensity) provided by 6 light-emitting diodes. The PEA has the ability to monitor the polyphasic fluorescence rise during the first second of illumination with 1200 points.

During the first 2 ms a point is recorded every 10 μ s. Up to 1 second, a point is recorded every 1 ms. The points chosen to represent the whole transient are: F_m (maximal fluorescence intensity when all the reaction centres (RC's) are closed); F_o (fluorescence intensity at 50 μ s when all RC's are open); F_j (fluorescence intensity at 100 μ s, 300 μ s and 2 ms); F_1 (fluorescence intensity at 30ms); $t_{F_{max}}$ (time at which the fluorescence transient reaches the F_m ; area (area between the fluorescence curve and the level of F_m (STRASSER & STRASSER, 1995). These data were used to calculate 44 ratios and expressions.

An experiment was conducted with leaves which were dark adapted for a period of 1 hour before the measurements were taken with the PEA. This was done with specially provided clips that fit onto the leaves. The measurements were taken 3 times, the day before the start of the stress (control readings) and for the next 2 days. The measurements were taken between 10H00 and 12H30 and the data were transferred to the computer where all calculations were done with the use of the Microsoft Excel program. Plotted values were the mean of 4 replicates per leaf of 3 different plants. Readings were taken each day on a marked leaf.

6.3.3. Molecular analysis

PCR analysis was performed as outlined in Chapter 3.

6.4. RESULTS AND DISCUSSION

PCR reaction was used as an indication of the cloning of the constructs into *Agrobacterium* and transformation of the putative transgenics. Figures 17 and 18 show the results obtained during the PCR analysis, using the *P5CR* primer and the *NPTII* primer. The bacteria containing the *P5CR* gene, in the sense and

antisense directions, corresponded with the results of the plasmids containing the *P5CR* gene (Figure 17). The transformed plant tissue demonstrated a similar result using the NPTII gene (Figure 18). No homology was observed with the untransformed plant tissue. This indicated that the *P5CR* gene was transferred into the T-DNA vector, the *P5CR*-IHS inducible promoter construct and into soybean plants. The PCR amplified the expected *P5CR* band (1029bp) and the expected NPTII band (806bp) from the DNA of the putative transgenics and positive controls, which is evidence for transformation. The molecular analyses of the T1 transgenics using PCR analysis consistently distinguished between negative and positive controls. This serves as proof for the transformation of the soybean plants with *P5CR*-IHSP constructs.

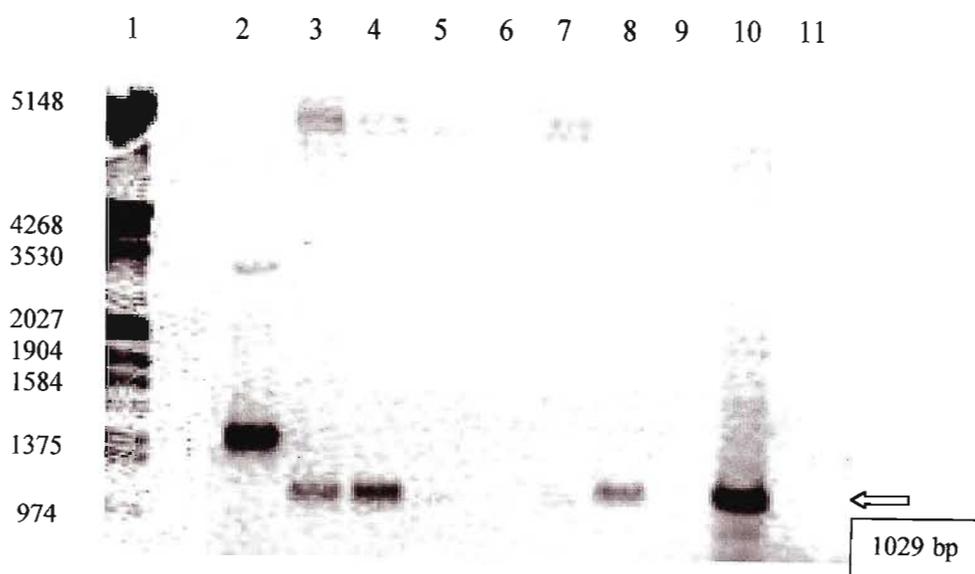


Figure 17: Agarose gel electrophoresis of a PCR analysis with *P5CR* specific primers confirming transformation of the *P5CR* construct into *Agrobacterium tumefaciens*

Lane 1: molecular weight marker; Lane 2: pBI121; Lane 3-5: sense constructs ; Lane 6-9 antisense constructs; Lane 10: AT-P5C1 (*P5CR* gene); Lane 11: H₂O

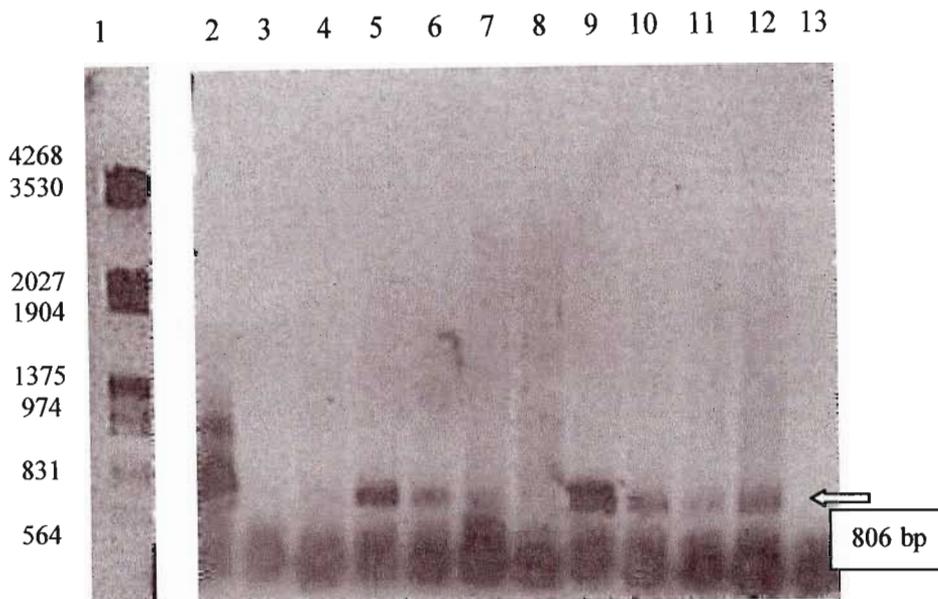


Figure 17: Agarose gel electrophoresis of a PCR analysis with NPTII specific primers confirming transformation of the *P5CR* construct into soybean cells: Lane 1: molecular weight marker; Lane 2: pBI121; Lane 3: AT-P5C1; Lane 4: non-transformed plants; lane 5-10: T1 putative sense plants; Lane 11-12: T1 putative antisense plants; Lane 13: H₂O

As the photosynthetic activity, transpiration, stomatal conductance, intercellular CO₂ and leaf water potential were known to be reduced by water stress (PURWANTO, 1994), chlorophyll fluorescence transients were chosen as a parameter in evaluating the transgenic plants. The aim of this experiment was to detect differences between the sense and antisense transgenics.

Based on the analysis of the JIP test several expressions were calculated. The quantum efficiency or flux ratio ψ_0 or electron transport (ET) per trapping (TR), is the ability that a trapped exciton can move an electron into electron transport. It is the ratio of ET and TR at time zero. The specific fluxes are

absorption (ABS), dissemination (DI), TR and ET. ABS is the photon flux absorbed by the antenna pigments. TR is the rate by which the excitation energy flux is trapped resulting in the reduction of the electron acceptor Q_A to Q_A^- . The ET is the electron transport that left PSII by reoxidizing Q_A^- to Q_A . DI is the dissipated energy flux, which is wasted per RC as heat or transfer to other systems. The maximum values are at zero time when all RC's are open. Each sample can be seen per RC, this component deal with the specific energy fluxes, or per cross section (CS), this component deal with the fluxes per event. RC/CS is an indication of the number of active RC's (density) per CS (STRASSER & STRASSER, 1995). Utilising all the parameters of the JIP test, the values of 10 selected functional (ABS/RC, TR_0/RC , ET_0/RC , DI_0/RC , RC/CS_m , ABS/CS_m , TR_0/CS_m and ET_0/CS_m) and structural parameters (RC/CS_0 and ψ_0) which seem to be most affected by activation of the inducible heat shock promoter (IHSP), were normalised over the corresponding control values and plotted using spider-plot presentations. The putative transgenics were normalised over the non transgenic plant's data at 0 days (control) treatment in demonstrating the effect of the IHSP (Figure 19).

In the control treatment, 25°C and with fully hydrated plants, no significant differences can be monitored between the transgenic and the non transgenic plants. The IHSP was not activated and no stress was applied. The stress and control values are summarized in Table 4 and explain the differences between the three types of plants. The different plants (non transgenic, antisense and sense) responded differently in their OJIP reaction to water and heat stress. The antisense transgenics displayed much the same reaction to the stress as the non transgenic plant. The ABS/RC increased in the non transgenic and antisense plants during the stress. This also coincided with an increased trapping of the excitons by the RC's in the non transgenic and antisense plants. This is in contrast with the sense plants that displayed a decrease in both ABS/RC and TR_0/RC with stress.

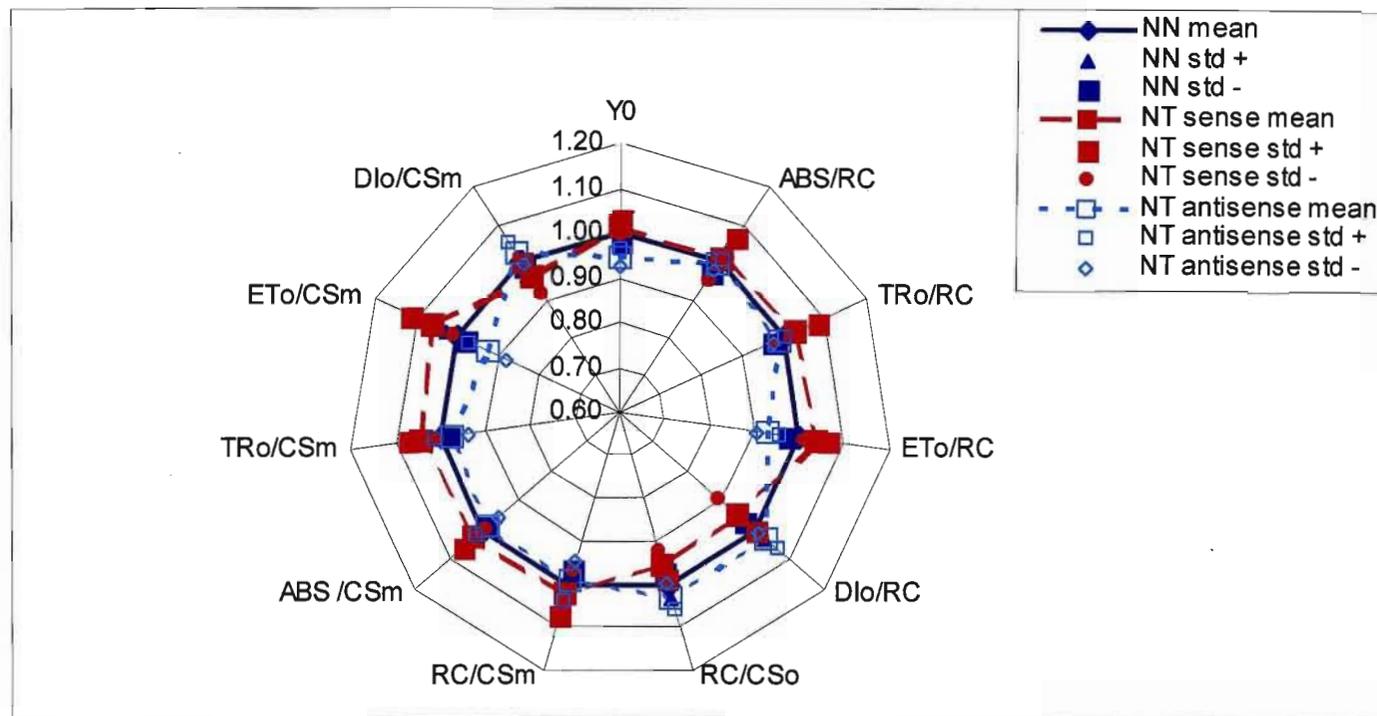


Figure 19: Normalisation of data from putative transgenics with non transgenic plants data at 25°C and well watered, demonstrating the effect of the IHSP inactivation

RC = reaction center, CS_m = cross section, ABS = absorption, TR_0 = trapping, ET_0 = electron transport, DI_0 = dissemination, RC/CS_m = RC density, ψ_0 = quantum efficiency

Table 4: A summary of the values \pm SE of selected parameters, which seem to be most affected by activation of IHSP

	Plant type	ABS/RC	TR ₀ /RC	ET ₀ /RC	DI ₀ /RC	RC/CS _m	ABS/CS _m	TR ₀ /CS _m	ET ₀ /CS _m	RC/CS ₀	DI ₀ /CS _m
Stress	Non transgenic	2.07 ± 0.04	1.67 ± 0.03	1.06 ± 0.01	0.40 ± 0.01	1602 ± 44	3308 ± 40	2667 ± 41	1698 ± 50	310 ± 4	641 ± 9
Control		1.86 ± 0.05	1.49 ± 0.04	0.78 ± 0.02	0.37 ± 0.01	1854 ± 64	3430 ± 39	2746 ± 35	1440 ± 35	370 ± 11	684 ± 8
Stress	Antisense	2.03 ± 0.02	1.65 ± 0.02	1.09 ± 0.04	0.38 ± 0.01	1652 ± 26	3350 ± 32	2719 ± 28	1793 ± 66	311 ± 8	631 ± 14
Control		1.85 ± 0.03	1.46 ± 10.02	0.73 ± 0.02	0.38 ± 0.01	1834 ± 81	3383 ± 111	2681 ± 99	1331 ± 68	380 ± 11	702 ± 19
Stress	Sense	1.2 ± 0.03	0.95 ± 0.03	0.62 ± 0.04	0.26 ± 0.01	1428 ± 42	1716 ± 65	1352 ± 60	881 ± 75	302 ± 9	363 ± 13
Control		1.87 ± 0.1	1.52 ± 0.08	0.81 ± 0.02	0.35 ± 0.02	1893 ± 98	3519 ± 108	2860 ± 88	1527 ± 75	658 ± 26	658 ± 26

The ET per RC showed a decrease in the value for the non transgenic and antisense plants, but an increase was observed in the sense plants. The dissipated energy fluxes per RC increase slightly in the non transgenic plants, there was no difference in the antisense plants, but it decreases in the sense plants. The ABS of photons per CS displayed a slight decrease in the non transgenic plants, no difference in the antisense plants and a severe decrease in the sense plants.

The sense plants also experienced a decrease in the TR_o/CS_m . The ET_o/CS_m shown an increase for the non transgenic and antisense plants, but a decrease was experienced in the sense plants. All the plants experienced a decrease in the density of RC's, but it was more pronounced in the sense plants. This was attributed to the decrease in DI_o/CS_m which was also greater in the sense plants. The profiles indicate the effect of simultaneous drought and heat stress on the tested Ibis plants. The non transgenic profile is typical of stressed leaves, with the RC's closed coinciding with increasing ABS, TR and ET/RC values as compensation to the applied stress. The antisense plants closed down even more RC's as the plants suffered more from the stress. The sense plants on the other hand experienced the stress under more favourable circumstances and reacted with decreased ABS, TR and ET/RC even though the RC/CS declined.

The stress values were normalised with the control values of the specific plant. The normalised values of the transgenic plants were subsequently also normalised with the already normalised values of the non transgenic plants to demonstrated the specific effect of the over expression (sense) (Figure 20) or under expression (antisense) (Figure 21) of the *P5CR* gene. In this way the effect of the stress on the non transgenic plant is eliminated and only the effect of the under or over production of the *P5CR* gene is noticeable.

The ψ_0 and ET/RC of the antisense transformant (Figure 20) increased significantly together with the DI_o/RC which decreased significantly in

comparison to the non transgenic plant. The antisense plants compensate for the stress situation by increasing the overall electron transport and thus decrease the amount of wasted energy. The ABS and TR also increased, although they are not significantly different from the non transgenic plants.

The sense transformants (Figure 21) in contrast, slow down the whole Chl-*a* fluorescence process. It closes down some of the RC/CS at time max where all the RC's are closed. The RC/CS at time zero, with all the RC's open, remained the same as in the non transgenic plants. Together with this effect, the ABS, TR, ET and DI decreased. The wasted energy per CS was lower in the sense plants than in the antisense plants. The quantum efficiency did not change from the non transgenic plant response. This may be an indication that the electron transport yield was not negatively effected by the stress.

6.5 CONCLUSIONS

Some insight into the biology of Chl-*a* fluorescence was possible with the OJIP type transient. The detection of differences between the sense, antisense and non-transformed Ibis plants was possible after only 2 days stress at 36°C. No visual differences could be seen at this early stage of stress. The sense plants did not have to work as hard in the stress situation as the antisense plants. The sense plants utilised their energy much better than the antisense plants. The decrease in the ET, ABS and TR may have resulted in this energy saving. These reactions may be the result of the higher copy number of the *P5CR* gene and subsequent higher synthesis of proline. The sense plants performed thus as more tolerant to drought and heat than the non transgenic plants and the antisense plants were more sensitive.

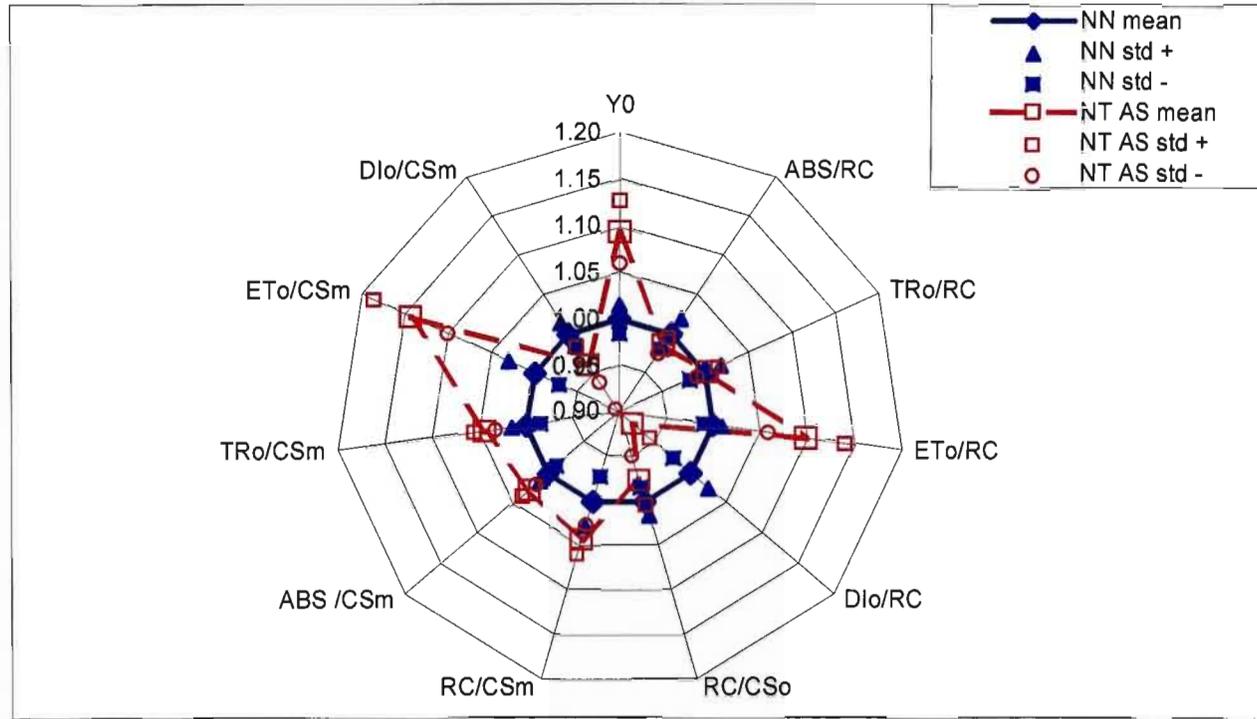


Figure 20: The normalised values of the transgenic plants after 2 days of stress, were normalised with the already normalised values of the non transgenic plants at 2 days stress to demonstrate the specific effect of the under expression (antisense) of the *P5CR* gene RC = reaction center, CS_m = cross section, ABS = absorption, TR₀ = trapping, ET₀ = electron transport, DI₀ = dissemination, RC/CS_m = RC density , ψ_0 = quantum efficiency

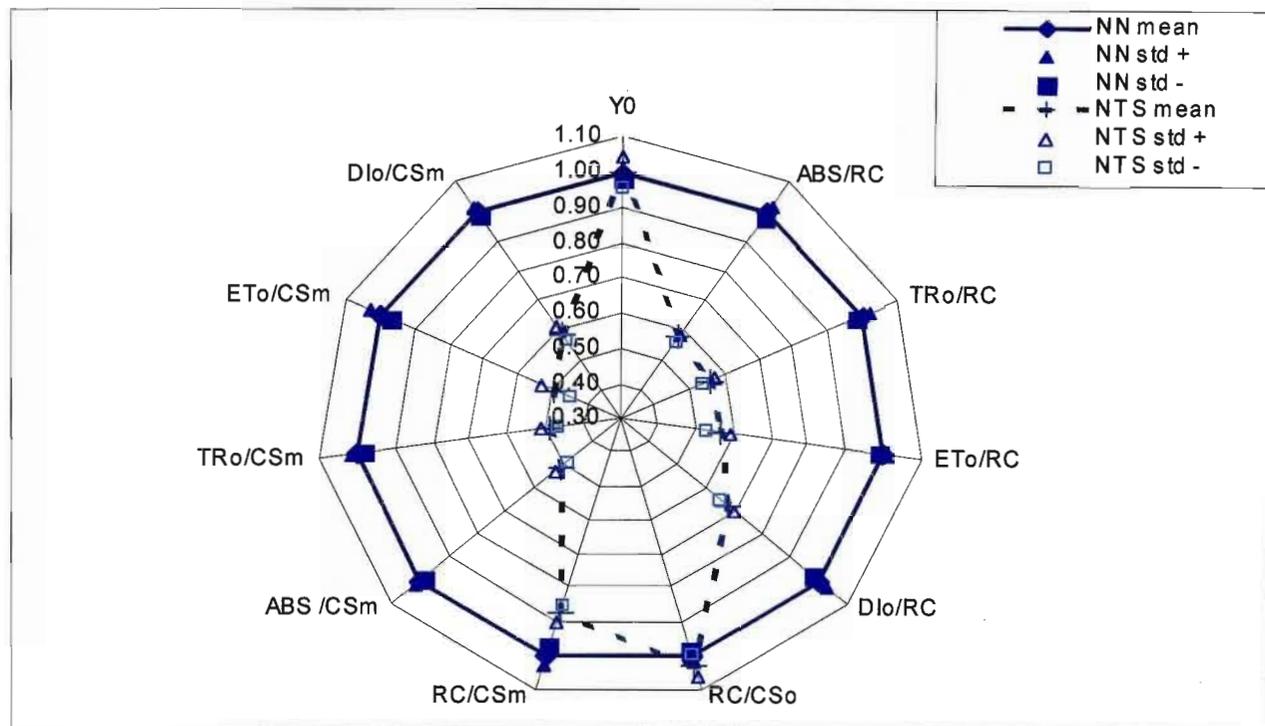


Figure 21: The normalised values of the transgenic plants after 2 days of stress, were normalised with the already normalised values of the non transgenic plants at 2 days stress to demonstrate of the specific effect of the over expression (sense) of the *P5CR* gene
 RC = reaction center, CS_m = cross section, ABS = absorption, TR_0 = trapping, ET_0 = electron transport, DI_0 = dissemination, RC/CS_m = RC density , ψ_0 = quantum efficiency

Considering all the parameters, but especially the electron transport and dissipation, it could be possible to perform an early non-destructive screening of putative transgenics. Plants, which tested 'positive' with this method, can be used in a PCR test to confirm transformation. Thus, with the *P5CR* gene construct, is it possible to use this cheaper non-destructive method for initial screening.

CHAPTER 7

INTERACTIONS BETWEEN PROLINE, PHOTOSYNTHESIS AND NADP⁺

7.1. INTRODUCTION

Drought stress is defined as a prolonged and abnormal moisture deficiency. To counteract drought, some plants accumulate proline. Many roles were proposed for proline during drought stress (see Chapter 4), such as balancing of the NADPH/NADP ratio (ALIA & SARADHI, 1993). According to HARE, CRESS & VAN STADEN (1998) proline synthesis may improve the accumulation/regeneration of NADPH in plant cells.

The pyridine nucleotide coenzymes nicotinamide adenine dinucleotide phosphate (NADP) and nicotinamide adenine dinucleotide hydrogen phosphate (NADPH) are synthesized from nicotinamide and are the most important coenzymes in the cell. In cells, most oxidations are accomplished by the removal of hydrogen atoms. NADP⁺ can be reduced by two electrons, however only one proton accompanies the reduction. The other proton is oxidised in the surrounding media. NADP⁺/NADPH is thus an electron and proton shuttle as they are the principal mobile carriers of reducing equivalents between soluble dehydrogenase enzymes and the respiratory chain (KIMBALL, 1999). As a major currency in biological energetics, the NADP⁺/NADPH redox state has been exhaustively studied and shown to be important in several metabolic processes including gluconeogenesis and urea formation (CORNELL, LUND & KREBS, 1994). NADPH is produced

by the light reactions of photosynthesis to be consumed in the Calvin cycle (HERREID, 1977). ATP and NADPH provide the energy and electrons to reduce CO₂ to glucose and fructose (KIMBALL, 1999). NADP⁺ is produced by glutathione (GSH) metabolism with glutathione reductase responsible for the transfer of electrons from NADPH to oxidised glutathione an important component of the ascorbate-glutathione cycle that participates in the removal of hydrogen peroxide (FOYER, LELANDIAS & KUNERT, 1994).

Redox, reduction and oxidation describe the class of chemical reactions that transfer electrons or hydrogen atoms. All cells use redox reactions in the conversion or production of energy. Photosynthesis consists of a series of light-driven redox reactions. Via reduction of ATP and NADPH, carbon metabolism affects the proton gradient and the redox state of PSII (ÖGREN, 1990). The photochemical reactions of photosynthesis catalyse electron transport across the thylakoid membrane of the chloroplasts. The cyclic electron flow serves to control the pH in the thylakoid lumen and to regulate PSII activity (SCHELLER, 1999). The tight regulation of electron transport ensures that overreduction of the photosynthetic apparatus does not take place under stress conditions (i.e. restricted carbon dioxide assimilation) (SCHELLER, 1999).

PSII thus plays a central role in the molecular redox mechanism involved in signal transduction during environmental stress (ANDERSON, PARK & CHOW, 1997). The photosynthetic pigments must absorb light energy in order for it to be available for photosynthesis. When light is absorbed by a photosynthetic pigment, the molecule is elevated to an excited state (HERREID, 1977). Thus, light absorbed in the peripheral antennae is transferred through the inner antennae to the reaction centers (RC's) which can capture this energy to form an excited molecule (GILMORE, 1997). The RC in turn loses an electron to a series of electron carrier molecules. The last electron carrier molecule in the chain is NADP⁺.

Thus, for each photon absorbed, an electron is ejected (HERREID, 1977). Limitations in photosynthetic CO₂ uptake, when plants are subjected to environmental stress, may arise from both stomatal and non-stomatal effects (TERASHIMA, 1992). Although stomatal closure under drought limits both net CO₂ uptake and leaf transpiration, it could also increase the water use efficiency of net photosynthesis (FISHER & TURNER, 1978). Plants must maintain an effective balance between the supplied energy and the dissipated energy (ANDERSON, PARK & CHOW, 1997). Photosynthesis coordinates interactions between light harvesting, energy conversion, electron transport, proton translation and carbon fixation. The chloroplasts play the role of redox sensors together with other signal transduction pathways in the elicitation of physiological and molecular responses as a result of environmental changes (ANDERSON, CHOW & PARK, 1995). The stomata control the resistance to the diffusive transfer of water vapor and CO₂ between the leaf and the ambient air, and thus the photosynthetic rate which is dependent on the concentration of CO₂ in the leaf intercellular spaces (BERRY & BJÖRKMAN, 1980).

In the absence of water stress as the ambient temperature increases, plants open their stomata, or let them remain open, in a joint action with the demand for CO₂ (RASCHKE, 1970). Thus, the heat inhibition of CO₂ fixation is not caused by stomatal closure (BERRY & BJÖRKMAN, 1980). BERRY & BJÖRKMAN (1980) postulated that heat inactivation is partly caused by the uncoupling of phosphorylation from electron transport (ET) as well as the inactivation of ET. Differences in photosynthetic acclimation to high temperatures may be attributable to differences in the heat stability of the photosynthetic apparatus (BERRY & BJÖRKMAN, 1980). In heat-adapted plants, the threshold of heat shock is shifted towards higher temperatures and the damage to PSII activity can be diminished (SCHUSTER, EVEN, KLOPPSTECH & OHAD, 1988). Exposure of cells to temperatures above the normal growth temperature induces the expression of heat

shock proteins (KIMPEL & KEY, 1985). The HSPs 22, 25 and 29 bind to the chloroplasts and prevent inactivation of PSII during heat shock (SCHUSTER, EVEN, KLOPPSTECH & OHAD, 1988). Drought stress inhibited photosynthesis through alterations in the proportion of photochemical and energy-dependent quenching as a result of inhibition of the enzymatic sites that consume ATP and NADPH (ISMAELOV, ZULFUGAROV & ALIEV, 1998). The fluorescence quenching, predominantly caused by photochemical and energy-dependent mechanisms, is strongly influenced by the utilization of NADPH and ATP in photosynthesis (KRAUSE & SOMERSALO, 1989).

HARE & CRESS (1997) postulated that the $\text{NADP}^+/\text{NADPH}$ redox system may be important in metabolic regulation, as a small change in the intracellular $\text{NADP}^+/\text{NADPH}$ ratio mediated by the production of proline from glutamate might have a large effect on a redox-sensitive pathway such as the oxidative pentose phosphate pathway (OPPP). During proline biosynthesis, two molecules of NADPH are oxidised, one in the mitochondria and one in the cytosol. The interconversion of P5C and proline could mediate the transfer of reducing equivalent from cytosolic NADPH into the electron transport chain (HARE & CRESS, 1997). PHANG (1985) postulated that these interconversions could shuttle redox between cellular compartments, transfer electrons from NADPH to NADP^+ , couple the oxidation of NADPH to mitochondrial electron transport and serve as a mechanism for energy production.

KOHL, SCHUBERT, CARTER, HAGEDORN & SHEARER (1988) proposed that *P5CR* plays a major role in transfer of energy to the location of biological N_2 fixation and the production of NADP^+ to drive the oxidative pentose phosphate pathway (OPPP). YEH & PHANG (1988) also demonstrated that P5C enhanced the activity of the OPPP. Thus, a role for the high levels of proline synthesis during environmental stress may be in maintenance of the NADP^+ levels.

Glucose-6-phosphate dehydrogenase (G6PDH) is the enzyme that catalyses the first oxidation/reduction reaction in the OPPP (YOSHIDA & BEUTLER, 1986) (Diagram 10). The main function of the OPPP is the generation of ribose sugars for nucleic acid and fatty acid synthesis (COPELAND & TURNER, 1987). In addition to this, G6PDH also has an important role in the provision of NADPH inside the cell. Since the flux through the OPPP is tightly regulated with the intracellular concentration of NADP^+ , the flux increases when NADP^+ becomes more available (KREBS & EGGLESTON, 1974). Thus, HARE & CRESS (1997) proposed a linkage between proline accumulation and the regulation of cellular redox potential.

7.2. AIM

The tolerance mechanism of transgenic soybean plants, cultivar Ibis, was evaluated. This was accomplished by the comparison of antisense with sense transgenics, containing the *P5CR* gene, through evaluation of different physiological techniques. The following physiological screening methods were used: free proline accumulation, NADPH accumulation and chlorophyll fluorescence measurements. The polyphasic rise in chl-*a* fluorescence was used to investigate the behavior of photosystem II as a result of heat stress or a combination of heat and drought stress. Analysis of the fast fluorescence rise (OJIP) provided a quantification of the applied stress. It was hoped to assess whether there is a link between some traits that play a role during drought stress.

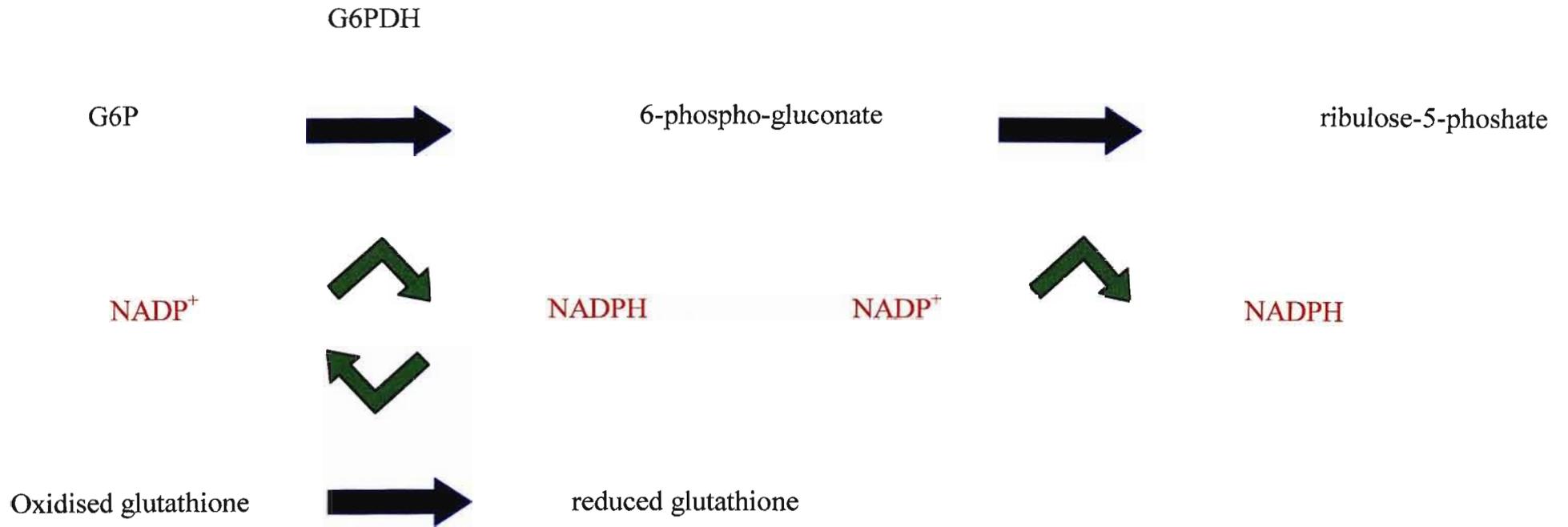


Diagram 10: Role of NADP⁺/NADPH in the oxidative pentose phosphate pathway (YOSHIDA & BEUTLER, 1986)

7.3. MATERIALS AND METHODS

7.3.1. Heat treatment

Plants were grown in a greenhouse under optimum conditions until they were ten weeks old. Plants evaluated consisted of: 6 different antisense plants (I29: T0 containing the RP29 construct), 6 sense plants (I39: T1 containing the RP40 construct) and 3 non transgenic control Ibis plants. The plants were subjected to a 36/15 $\pm 2^{\circ}\text{C}$ day /night heat treatment for 2 days while well watered. This treatment was performed to test the activation of the heat shock promoter over time. The heat stress was lifted after 2 days, when the first symptoms of wilting occurred. Three leaves from every plant were sampled for 3 days; the day before stress plus 2 days of heat stress and then quick-frozen with liquid nitrogen and freeze-dried for proline studies.

7.3.2. Heat and drought treatment

The same plants utilized above were subjected to a heat and drought combination stress four weeks later after the plants had recovered from the mild heat stress. The plants were subjected to a 38/25 $\pm 2^{\circ}\text{C}$ day /night heat treatment for 2 days together with the withholding of water. The plants were rewatered and the heat stress was removed. Three leaves from every plant were sampled every day. Sampling took place at the day before stress, 2 days of combination stress as well during the first day of recovery. Leaves were quick-frozen with liquid nitrogen and freeze-dried for the proline and NADP⁺ studies.

7.3.3. Chlorophyll fluorescence

Chl-*a* fluorescence transients of intact leaves of the plants were measured by a Plant Efficiency Analyser (PEA, build by Hansatech Ltd, King's Lynn, Norfolk, UK). Data were recorded up to 1 second, with a rate of 10 μs for the first 2 ms

and 1 ms thereafter (STRASSER, SRIVASTAVA & GOVINDJEE, 1995). The transients were induced by red light of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ intensity at 650 nm provided by an array of six light-emitting diodes. Four measurements per plant were taken as described in Chapter 6 on the heat stressed and combination stressed plants.

7.3.4. Proline analysis

Proline was analysed as outlined in Chapter 4 on the freeze-dried material.

7.3.5. NADP

The spectrophotometric method of GIBSON & LARHER (1997) was used to measure $\text{NADP}^+/\text{NADPH}$. This is an improvement of the method of MATSUMURA & MIYACHIO (1983). The method involves 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) as the terminal electron acceptor with phenazine ethosulfate (PES) as an electron carrier and NaCl as a terminating solution (Figure 21).

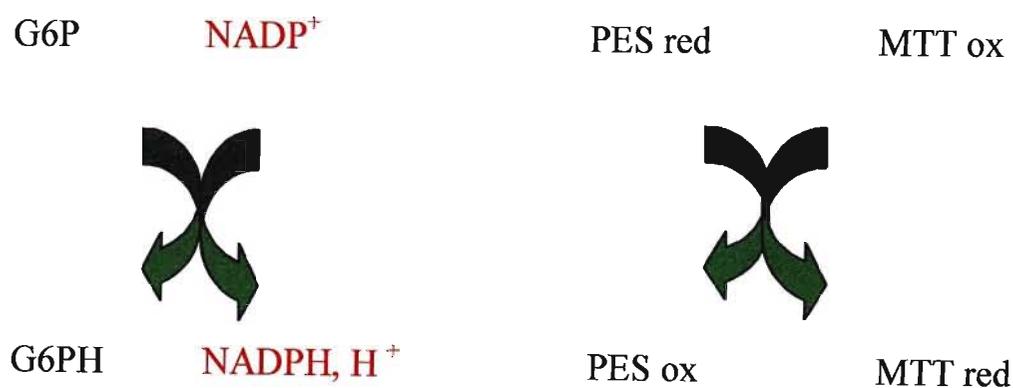


Figure 21: Enzyme cycling assay (GIBSON & LARHER, 1997)

Freeze-dried material was homogenized with a mortar and pestle in 1 ml of 0.1M HCl for NADP⁺ determination. The homogenates were heated in a boiling water bath for 5 minutes, cooled in an ice bath and centrifuged for 10 minutes at 10 000g. Supernatants were neutralized with 0.1M HCl and centrifuged for 10 minutes at 4°C at 10 000g. Supernatants were kept on ice. Equal volumes of 0.1 M Tricine – NaOH buffer (pH 8.00), 40 mM EDTA (di-sodium salt), 4.2 mM MTT, 16.6 mM PES and 25 mM glucose-6-phosphate (G6P di-potassium salt) were mixed just before the assay. One hundred µl of this mixture was transferred to tubes. Four µM NADP⁺ or biological samples 0-20 µl (less than 160 pmol NADP⁺ in the final concentration) were added to the mixture. The volume was brought up to 180 µl with 0.1 M NaCl. The tubes were incubated for 5 minutes at 37°C in a water bath. Enzyme cycling was initiated by adding 20 µl glucose-6-phosphate dehydrogenase (G6PDH) solution (14 units/ml G6PDH in 0.1M Tricine – NaOH buffer (pH 8.00)) for NADP⁺ determination.

A blank reaction was determined by adding 20 µl 0.1 M Tricine–buffer instead of enzyme to a duplicate biological sample. Cycling time was 40 minutes. The reaction was terminated with the addition of 100µl 6 M NaCl (2 M final concentration). The reaction tubes were centrifuged for 5 minutes at 4°C at 10 000g. This enabled the precipitation of the formazan as it separated easily from the assay medium. The pellet was solubilised in 1 ml 96% ethanol. NADP⁺ of each biological sample was measured at 570 nm against a blank reaction.

7.3.6. Statistical analysis

The significance of differences between mean values obtained through the different techniques was determined with either Microsoft Excel (proline and NADP⁺) or Biolizer (STRASSER, 1996) (chlorophyll fluorescence).

7.4. RESULTS AND DISCUSSION

Living organisms have managed to survive many diverse environments including drought and heat. Different techniques and assessment methods with varied time scales relating to these fields, have been developed and are being used to identify the real extent, severity, and effect of droughts. Long-term indices of moisture shortage that relate to hydrological drought are especially difficult to quantify. There is a need to investigate drought indicators, to clarify the use of existing indicators and focus on appropriate methods, and to develop new indices.

7.4.1. Proline under different stress conditions

7.4.1.1. Heat stress

Free proline accumulation was determined in transgenic and non transgenic plants subjected to a heat stress of 36°C for 2 days (Figure 23). No significant differences were obtained between the sense, antisense and control plants. This indicated that although the IHSP must have been activated (DE RONDE, SPREETH & CRESS, 2000), proline did not accumulate as a result of short term heat stress. Similar results were also observed during a study using cotton (DE RONDE, VAN DER MESCHT & STEYN, 2000).

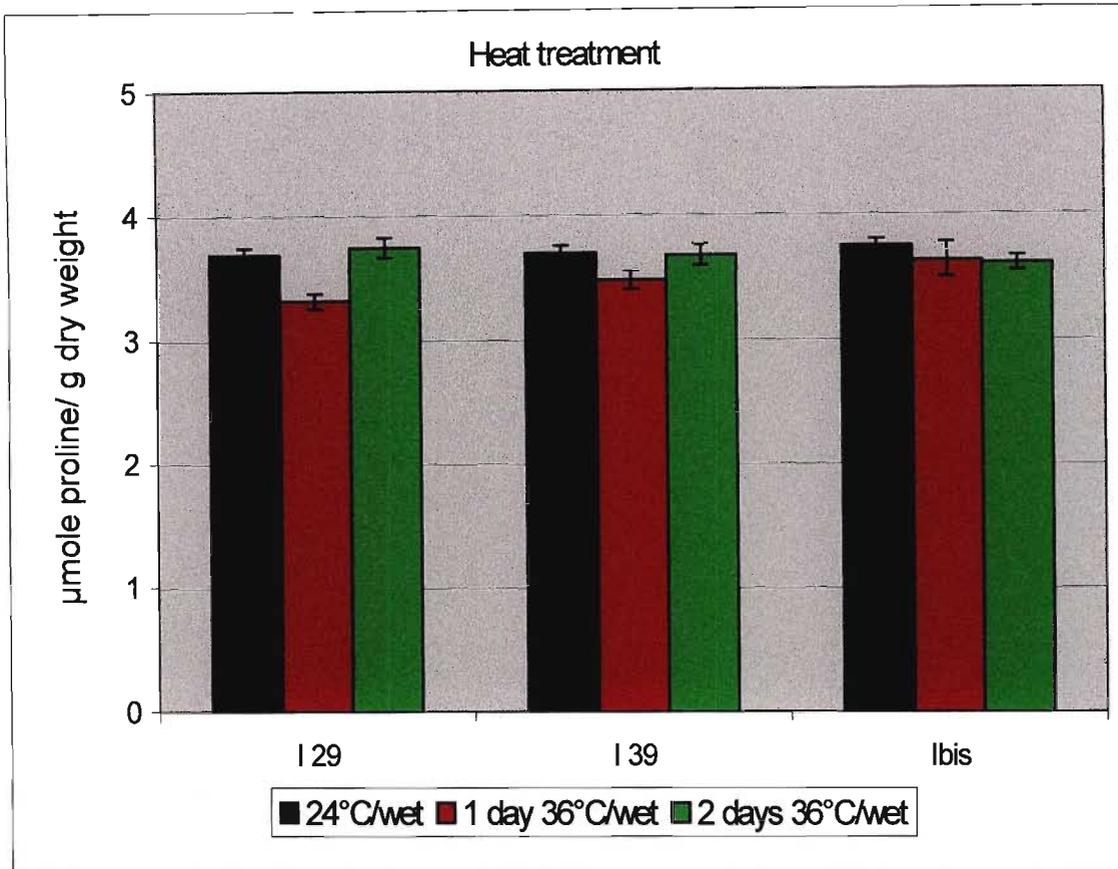


Figure 23: Free proline accumulation as a result of a 2 day heat stress on antisense (I29) and sense (I39) transgenic plants compared to non transgenic control plants (Ibis)

7.4.1.2. Combination stress

The transgenic plants were subsequently subjected to a combination of heat and drought stress and free proline accumulation was determined (Figures 24A, B). It was noted that at 1 day combination stress, the plants did not accumulate significantly more proline than at the control treatment. However, when the stress was more severe at 2 days, the proline accumulation increased dramatically in all the plants. It was found that individual antisense plants increased with different amounts, some significantly less than the control plant, others not (Figure 24A).

It is postulated that some of the accumulated proline may have resulted from protein breakdown as were observed by BECKER & FOCK (1986). In all cases the increases in the sense plants were significantly more than those observed in the control plants. There were however, differences among the individual sense plants (Figure 24B). This is in contrast to the results of SZOKE, MIAO, HONG & VERMA (1992) who demonstrated that sense transgenic *P5CR* tobacco plants did not accumulate significantly higher proline levels than control plants. The present results are in agreement with those of a study on *P5CS* transgenic tobacco plants, which accumulated 10-fold more proline than the control plants (KAVI KISHOR, HONG, MIAO, HU, VERMA, 1995). In some sense transgenic plants at least a 2-fold increase compared to the controls was experienced. The mean of all the plants (Figure 24C) demonstrated that the sense plants accumulated substantially more proline than the control and antisense plants. However, some of the proline in the antisense plants might be breakdown products.

A plant uses two separate enzymes to degrade proline: proline dehydrogenase (PDH) (also known as proline oxidase) and P5C dehydrogenase (KIYOSUE, YOSHIBA, YAMAGUCHI-SHINOZAKI & SHINOZAKI, 1996). When the proline levels were determined upon removal of the stress, it was found that the levels returned to normal within 1 day in all the sense plants (Figure 24B).

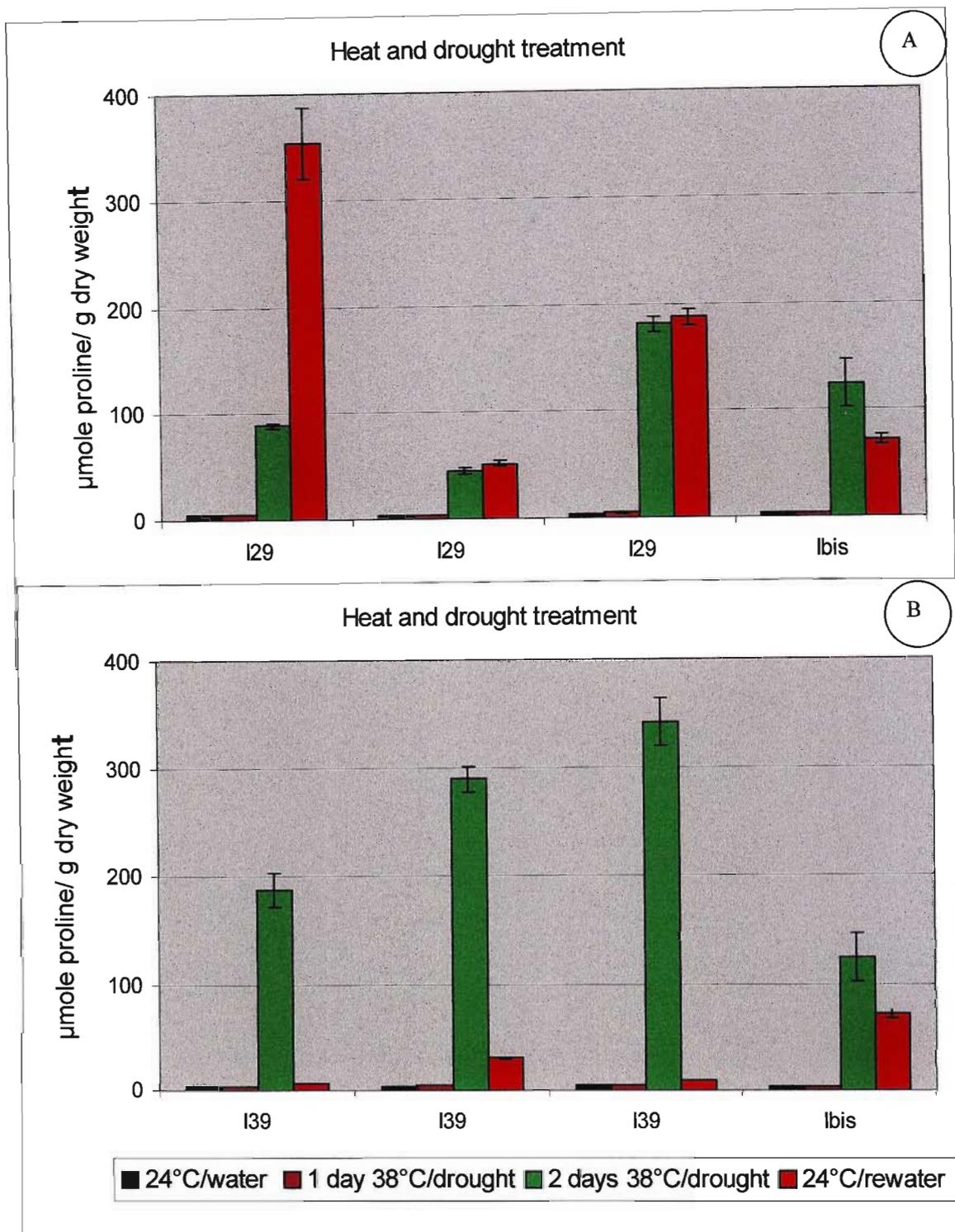


Figure 24: Free proline accumulation as a result of a combined heat and drought stress on antisense transgenic (I29) plants (A) and sense transgenic plants(I39) (B) compared to non transgenic control plants (Ibis)

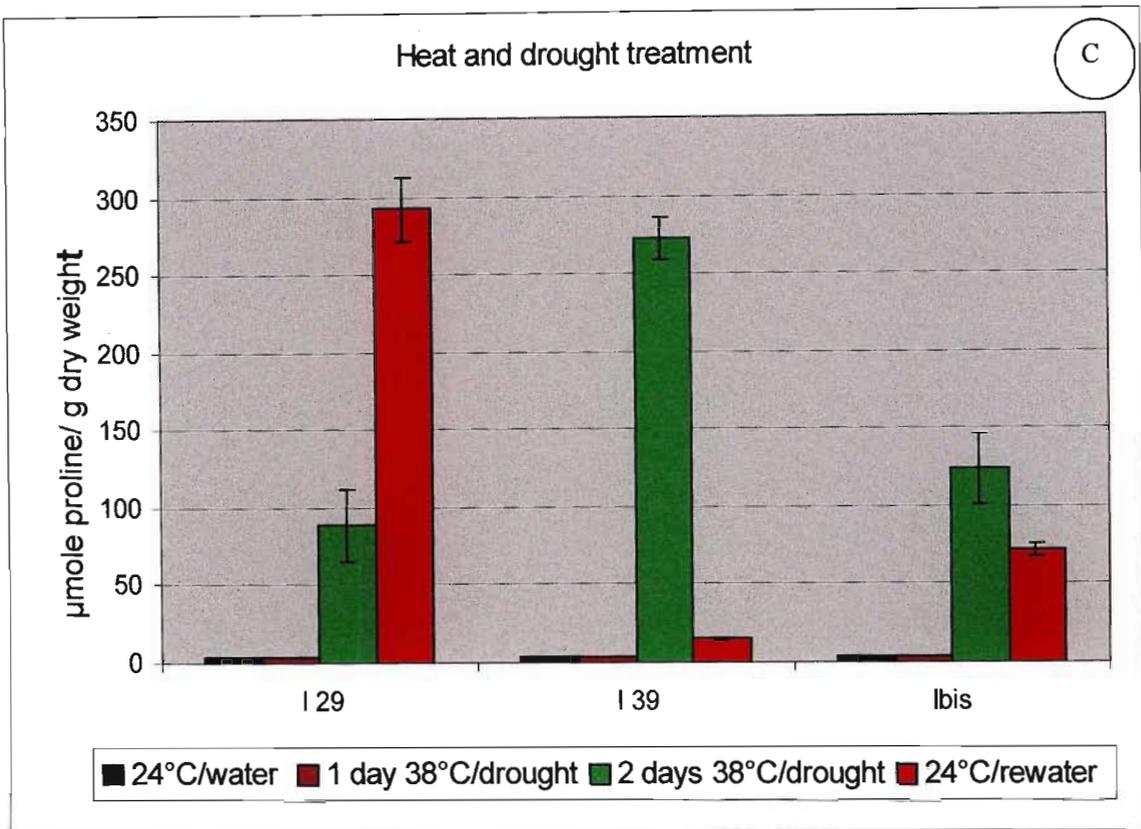


Figure 24C: Mean free proline accumulation as a result of a combined heat and drought stress on antisense (I29) and sense (I39) transgenic plants compared to non transgenic control (Ibis) plants

The antisense plants (Figure 24A) failed to reduce their proline levels and sustained or increased proline levels were observed. The control plants were able to reduce their proline level, but not to the unstressed level. There are significant differences in the recovery of the different transgenic plants (Figure 24C). Proline degradation is only active during the recovery period as was observed by RAYAPATI & STEWART (1991) who found the PDH enzyme activity in water stressed plants was only 11% of the enzyme activity found in the control plants. Similar results were also observed in a study with PDH in *Arabidopsis* (NAKASHIMA, SATOH, KIYOSUE, YAMAGUCHI-SHINOZAKI & SHINOZAKI, 1998). PENG, LU & VERMA (1996) also found that the expression of the PDH gene was low under salt and drought stress, but significantly induced by the removal of the stress. Removal of the excess proline after a stress appears to be important for the recovery from the stress and the oxidation of proline may provide extra energy during the recovery period as accumulated proline is rapidly oxidized to glutamate (VERBRUGGEN, HUA, MAY & VAN MONTAGU, 1996).

This may be an indication that the amount and time of proline accumulation is not necessarily the only important aspect during an environmental stress, but more so the ability of the plants to decrease the proline levels after removal of the stress. Thus, the sense plants may have performed as more tolerant to the applied stress than the antisense and control plants, as they accumulated significantly more proline and had the ability to reduce the accumulated proline during the recovery period, providing extra energy for the plant to help with the recovery.

7.4.2. Photosynthesis under different stress conditions

7.4.2.1. Heat stress

After the 2 day heat stress of 36°C, and the activation of the IHSP was monitored in sense and antisense plants, differences in chlorophyll fluorescence transients

were observed in the sense, antisense and control plants. The plants had adequate time to acclimate to the stress, as the night temperature was low (15°C). No substantial differences were observed between the plant types at the control treatment (24°C/watered), which indicated that the IHSP was not activated. This reaction may be the result of the carboxylase reaction that is dominant at optimum growth temperatures producing efficient photosynthesis reactions in all plants. Differences between the antisense, control and sense plants were however noticed as the IHSP become activated due to changes in the balance between oxygen and carbon dioxide and the carboxylase reaction which became less dominant (LIMP, RODAL & WILLIS, 1998).

These differences were evaluated with the use of the PEA and were visualised using the energy pipeline models of the photosynthetic apparatus as proposed by KRÜGER, TSIMILLI-MICHAEL & STRASSER (1997). The manifestation of the energy fluxes by the transgenics (average of 6 different plants and 4 repeats of each plant) are demonstrated as arrows, either in specific energy flux (per RC = membrane model) or phenomenological fluxes (per cross section (CS) = leaf model) (Figures 25A, B, C). The antisense transgenics increased their ABS/RC, DI₀/RC, ET₀/RC and TR₀/RC with increased heat (Figure 24A) as visible in the membrane model as increased arrow widths. The antenna size thus increased with heat stress, as the ABS/RC is an indication of antenna size (TSIMILLI-MICHAEL, KRÜGER, & STRASSER, 1996).

The leaf model of the antisense plants indicated that some of the RC's become inactive as a result of the heat stress (active = open circles or inactive = closed circles). The DI/RC increased significantly as a result of more RC's that become inactive. Less excitation energy is available for primary photochemistry, as the inactive RC's dissipate all their trapped energy (KRAUSE, SOMERSALO, ZUMBUSCH, WEYERS & LAASCH, 1990).

antisense	ABS/RC	TR _o /RC	ET _o /RC	DI _o /RC	RC/CS _o	ABS/CS _o	TR _o /CS _o	ET _o /CS _o	DI _o /CS _o
25°C/wet	2.06	1.67	0.94	0.39	361.22	745.28	602.26	339.25	143.02
SE	0.09	0.07	0.05	0.03	18.68	37.63	21.90	14.71	15.94
1 day 36°C/wet	2.26	1.80	0.97	0.46	327.51	741.40	589.12	318.01	152.28
SE	0.12	0.08	0.05	0.04	10.88	24.06	14.30	16.34	10.24
2 days 36°C/wet	2.24	1.81	1.15	0.43	320.36	716.79	578.73	367.26	138.05
SE	0.10	0.07	0.03	0.03	10.69	11.22	6.89	9.076	4.943

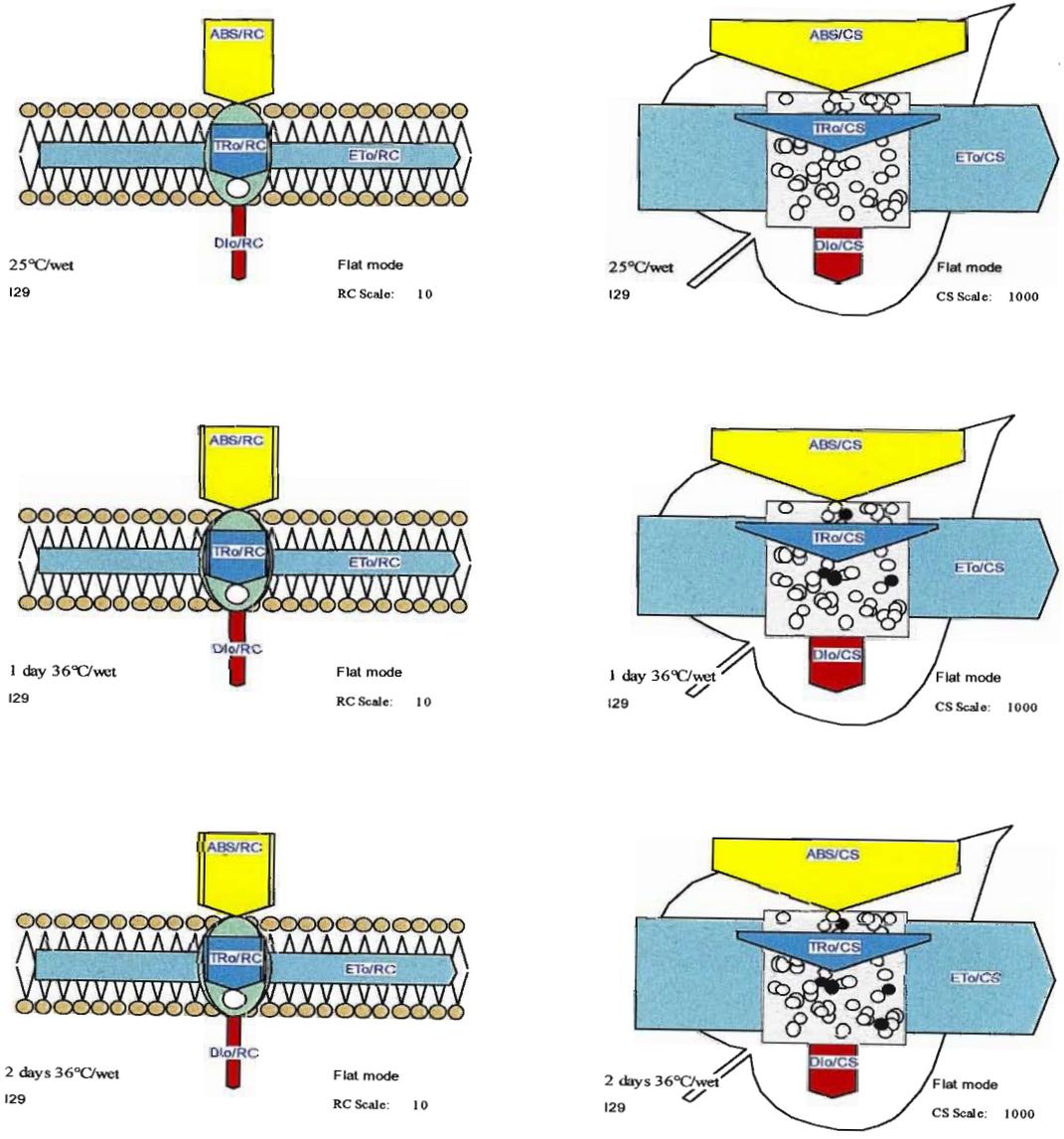


Figure 25A: The manifestation of the energy fluxes by the antisense transgenics(I29), at a heat stress, are demonstrated either in specific energy flux (per RC = membrane model) or phenomenological fluxes (per CS = leaf model)

No significant differences were observed in TR_o/CS , ABS/CS and DI_o/CS between stress and control treatments. The ET_o/CS is significantly higher after 2 days of stress than at 1 day of stress. This increase in ET_o per RC and CS and TR_o/RC confirmed the results obtained with a mild heat and drought stress (see Chapter 6).

In the control plants (Figure 25B) a reaction similar to that seen in the antisense plants was observed. The antenna size increased with application of the heat stress, together with increasing TR_o/RC and DI/RC . Some RC's become inactive, but less so than that observed in the antisense plants. In the sense plants (Figure 25C), the effect of the activated HSP manifests at 1 day of heat stress in the membrane model with a decrease in the ET_o/RC as well as an increase in the DI_o/RC . The leaf model shows the significant decrease in ABS/CS , TR_o/CS and ET_o/CS together with an increase in DI_o/CS . This corresponded with the observation during a mild heat and drought stress where the ET_o/CS , TR_o/CS and ABS/CS also displayed a significant decrease (see Chapter 6).

Decreasing ET might be seen as a reflection of photoinactivation, but it is postulated that in this case it is not a damaging process, but part of a photoprotective acclimation strategy as was observed by ANDERSON, PARK & CHOW (1997). This hypothesis is further substantiated by the different reaction of the sense plants at 2 days of heat stress compared to the control and antisense plants as well when a comparison is made to the control treatment's reaction. Some RC's become inactive as a result of the stress, but significantly less than in the other plant types. The ABS/CS and TR_o/CS increased significantly, but less than that observed for the control treatment. The ET_o/CS increased to significantly higher levels than the control treatment. It appeared as if the sense plant 'recovered' after an acclimation period to even better photosynthetic performances than before the stress.

Ibis	ABS/RC	TR _o /RC	ET _o /RC	DI _o /RC	RC/CS _o	ABS/CS _o	TR _o /CS _o	ET _o /CS _o	DI _o /CS _o
25°C/wet	2.13	1.72	1.00	0.41	329.28	702.30	566.12	328.97	136.18
SE	0.09	0.06	0.02	0.03	10.38	16.52	11.91	12.83	6.56
1 day 36°C/wet	2.42	1.91	0.96	0.51	316.66	766.47	604.90	303.60	161.57
SE	0.09	0.05	0.04	0.04	5.53	26.63	13.85	13.52	13.06
2 days 36°C/wet	2.31	1.83	1.03	0.49	305.80	708.18	559.18	315.82	149.00
SE	0.06	0.04	0.03	0.02	5.58	13.19	8.06	9.74	5.98

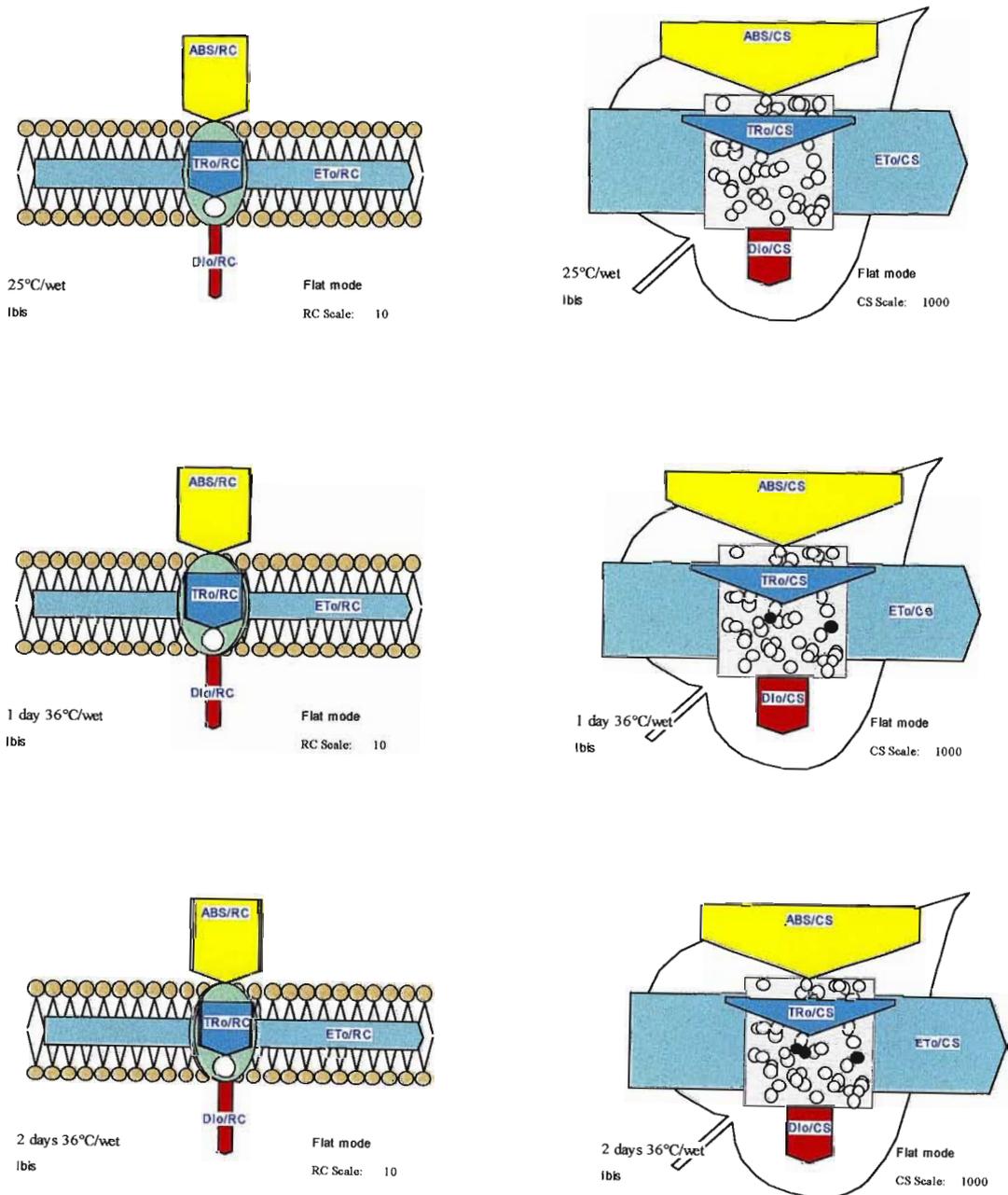


Figure 25B: The manifestation of the energy fluxes by the non transgenic control plants (Ibis), at a heat stress, are demonstrated either in specific energy flux (per RC = membrane model) or phenomenological fluxes (per CS = leaf model)

Sense	ABS/RC	TR _o /RC	ET _o /RC	DI _o /RC	RC/CS _o	ABS/CS _o	TR _o /CS _o	ET _o /CS _o	DI _o /CS _o
25°C/wet	2.30	1.85	1.00	0.45	327.51	752.42	605.70	326.96	146.72
SE	0.092	0.06	0.03	0.03	7.44	15.89	10.46	7.78	6.40
1 day 36°C/wet	2.48	1.88	0.76	0.60	326.12	808.80	612.83	248.19	195.97
SE	0.11	0.08	0.05	0.04	8.26	23.15	12.83	13.65	10.75
2 days 36°C/wet	2.35	1.89	1.13	0.47	316.52	744.72	597.11	357.79	147.60
SE	0.10	0.07	0.04	0.03	7.54	18.54	11.35	7.99	7.47

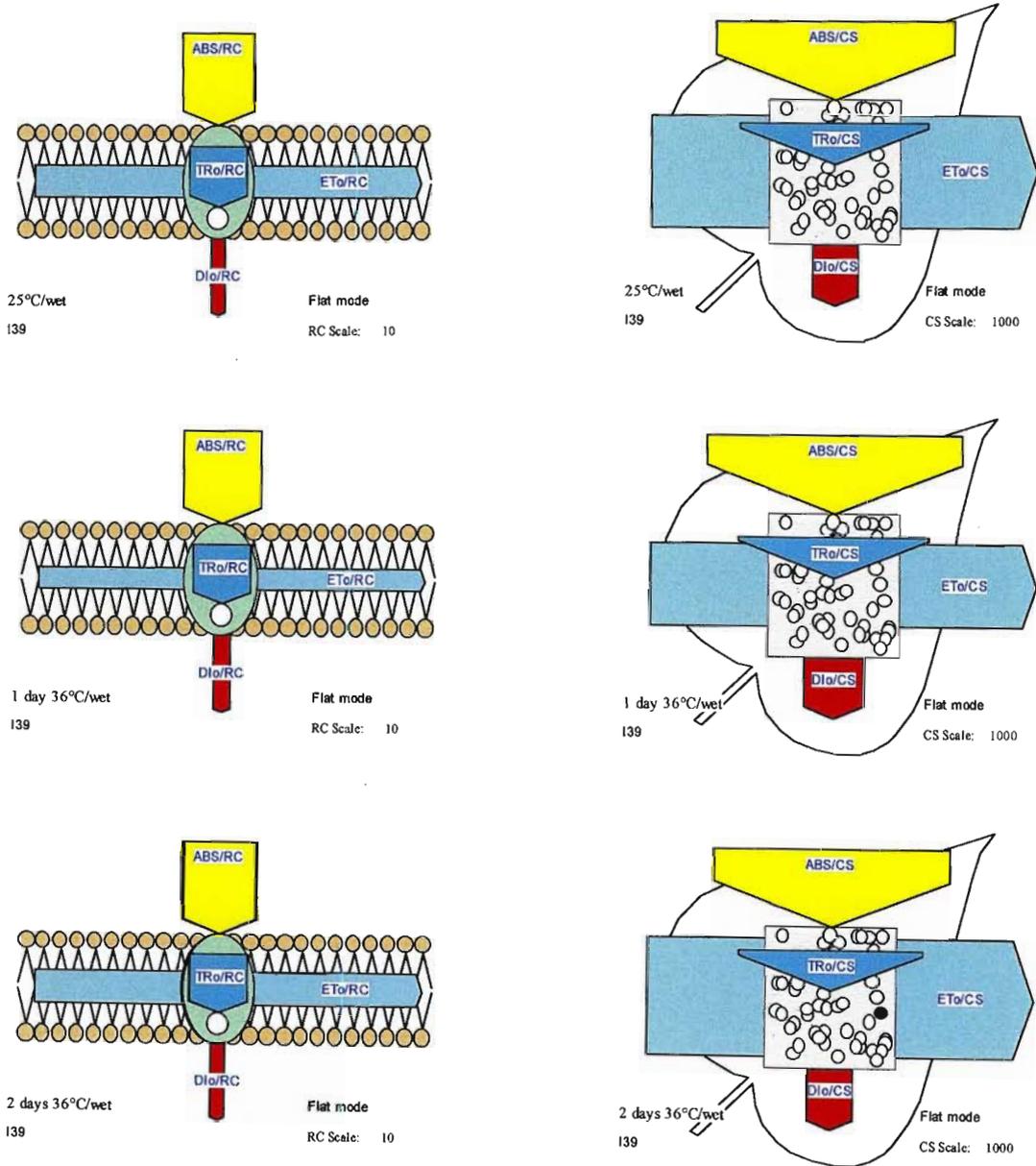


Figure 25C: The manifestation of the energy fluxes by the sense transgenics(I39), at a heat stress, are demonstrated either in specific energy flux (per RC = membrane model) or phenomenological fluxes (per CS = leaf model)

A marked increase in F_0 appeared when the plants reached a critical heat stress after 2 days of stress (visible symptoms of wilting) (Figure 26). There was however, no difference between the different plants. HAVAUX, ERNEZ & LANNOYE (1988) postulated that an increased F_0 might reflect the physical dissociation of the RC's. This can be confirmed with the coinciding of the higher RC/CS as well as DI_0/CS with increased stress (Figure 25). The quantum yield in potato leaves, expressed as a fraction of the value measured before temperature stress, declined simultaneously with an increase in the F_0 (HAVAUX, 1993, SCHREIBER & BERRY, 1977).

A prolonged heat stress of 2 days was characterised by a decrease in the quantum efficiency as well as the performance indexes (PI) (Figure 25). The quantum efficiency $PHI_{(EO)}$ (flux ratio ET_0/ABS) showed a significant increase in activity from the control treatment at 1 day of stress in all plants (no significant difference between the transgenics) (Figure 26). The antisense plants as well as the control plants experienced a significant decline in the flux ratios at 2 days of stress, in contrast to the sense plants that show no significant decrease. The quantum efficiency PSI_0 (flux ratio ET_0/TR) (Figure 26) also increased at 1 day of stress and declined at 2 days of stress in the antisense and control plants.

The PI can be per absorption flux (ABS) or by excited leaf area (CS) as a reference (STRASSER, SRIVASTAVA & TSIMILLI-MICHAEL, 1999). The $PI_{(CS_0)}$ of the antisense and control plants was significantly lower than that of the sense plants at 2 days of stress (Figure 26). The $PI_{(ABS)}$ declined in all the plants from day 1 to day 2 of stress, with the sense plant's value still significantly higher than the others (Figure 26). High temperature affects the rate of the chemical reactions associated with the photosynthetic functions as seen with the present fluorescence indicators.

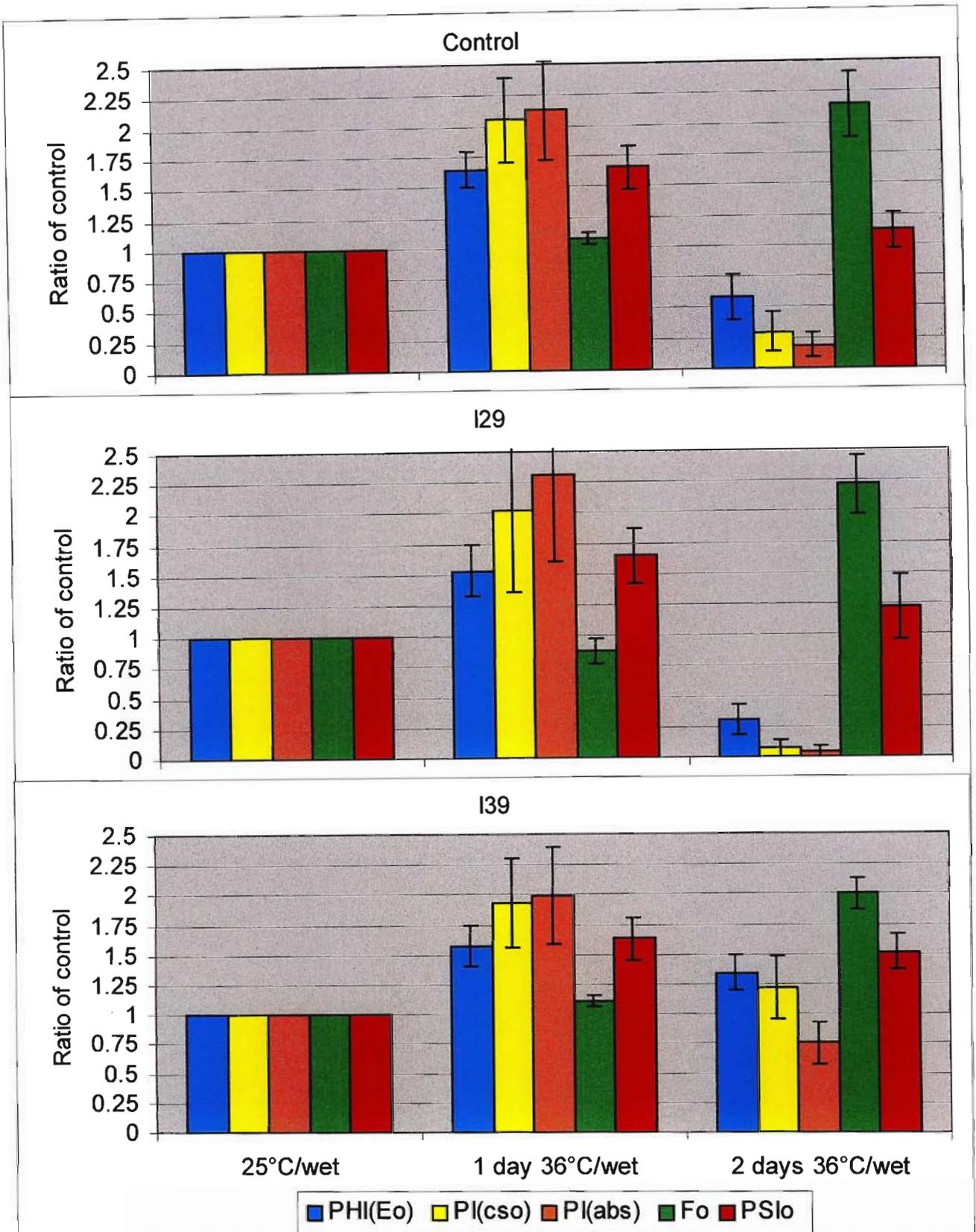


Figure 26: Some fluorescence indicators displaying differences between sense (I39) and antisense (I29) transgenics during heat stress

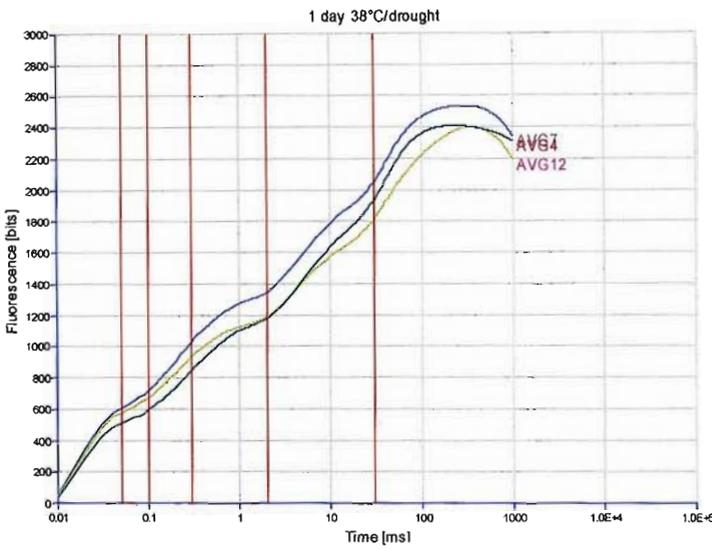
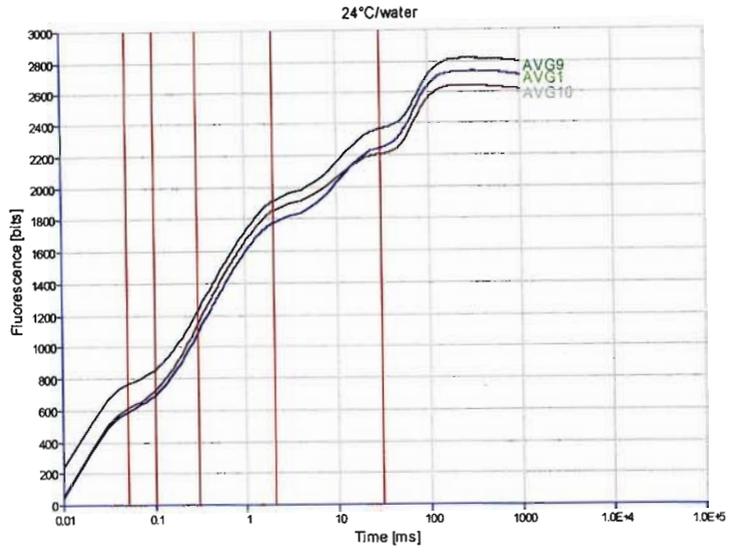
Chlorophyll fluorescence transients can be used to readily quantify the response of the different transgenics towards their reaction to heat stress and rank them accordingly. The antisense plants reacted as more sensitively to the heat stress than the control plants. The sense plants in turn were more tolerant to the heat stress. This reaction might be the result of the higher (sense) or lower (antisense) proline levels as the IHSP became activated at 36°C.

7.4.2.2. Combination stress

The response of leaf photosynthesis to a severe case of environmental stress is described: 2 days at 38/25 ±2°C day /night together with the withholding of water. The plants did not have adequate time to acclimate to the stress. When the leaves were exposed to the stress, inhibition of some of the photochemical processes occurred. This resulted in substantial changes in the characteristics of the chlorophyll fluorescence curves seen as the average of 6 different plants and 4 repeats of each plant (Figures 27A, B, C). At 24°C/watered, little differences were observed between the antisense, control and sense plants (Figure 27A). The HSP was not activated and all the plants performed the same as the control. The rate of photosynthesis declined after 1 day stress was administered, but still little difference was observed between the plants (Figure 27B). This decline increased after the 2nd day of stress to almost no activity in the antisense plants (Figure 27C). Although these changes can serve as easy and rapid indicators of the stress condition, it is however, not an indicative of the total extent of the changes which occurred during the photosynthesis process.

The energy pipeline models were used to reveal the changes that take place in the photosynthetic apparatus as a result of the combination stress (Figures 28, 29, 30). The antisense transgenics reacted in response to a mild stress (1 day) by increasing the ET_0/RC and TR_0/RC ratios significantly (Figure 28) as is visualized in the membrane model. The stressed transgenics also closed some RC's down.

A: Control treatment
 24°C/watered:
 9=sense, 1=antisense,
 10=Ibis



B: 1 day stress at 38°C/drought:
 7=Ibis, 4=antisense, 12=sense

C: 2 days stress at
 38°C/drought: 9=I39, 8=Ibis,
 3=I29

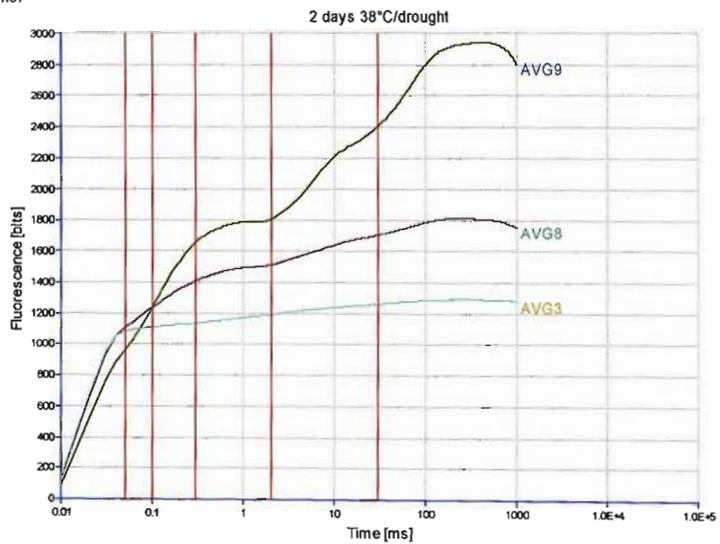


Figure 27: Changes in the chlorophyll fluorescence curves as a result of a combination of heat and drought stress

Antisense	ABS/RC	TR _o /RC	ET _o /RC	DI _o /RC	RC/CS _o	ABS/CS _o	TR _o /CS _o	ET _o /CS _o	DI _o /CS _o
24°C/water	2.25	1.73	0.71	0.52	267.92	591.00	455.64	191.66	135.36
SE	0.25	0.14	0.05	0.12	17.54	39.48	14.42	22.49	26.16
1 day 38°C/drought	2.56	2.01	1.30	0.54	200.02	511.07	402.88	260.94	108.19
SE	0.20	0.15	0.08	0.05	11.67	45.70	36.18	17.75	10.07
2 days 38°C/drought	11.66	1.94	0.94	9.71	92.66	1080.40	180.21	87.24	900.19
SE	8.87	0.15	0.13	8.72	35.13	35.37	61.00	21.98	76.76

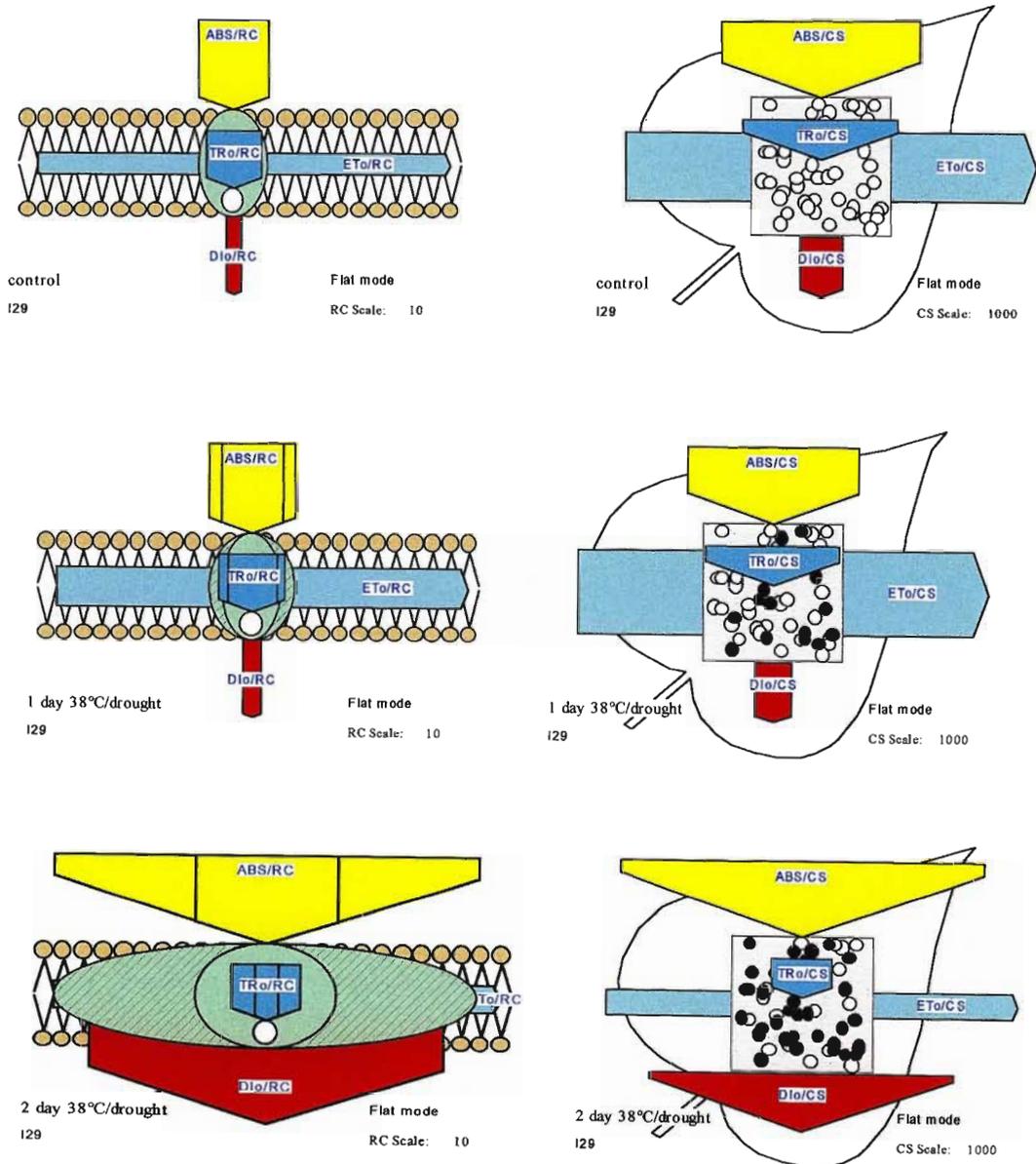


Figure 28: The manifestation of the energy fluxes by the antisense transgenics, at a combination stress, are demonstrated either in specific energy flux (per RC = membrane model) or phenomenological fluxes (per CS = leaf model)

It was postulated by GREER (1998) that plants that close down some RC's were more susceptible to photoinhibition than plants that do not close their RC's. This closure also coincides with an increase in the ET_0/CS_0 (Figure 28). A 2 days stress resulted in an enormous loss of electron transport activity as photoinhibition occurred, ET_0/RC as well as ET_0/CS_0 (Figure 28). This is in agreement with results of RICHTER, BOTHIN & WILD (1992), who coupled electron transport loss with photoinhibition.

The stress slows down the rate of photosynthesis, causing an increase in the proportion of closed RC's. This coincides with increasing ABS and DI. Photoinhibition occurred at 2 days of stress in the antisense plants. According to JANDA, SZALAI, KISSIMON, PALDI, MARTON & SZIGETI (1994) photoinhibition during drought is the result of an irradiance exposure which is higher than the plants can utilize in photosynthesis. This results in damage to the photosynthetic apparatus as a result of limited CO_2 uptake and leaf transpiration (FISHER & TURNER, 1978). Thus, in the antisense plants there was not only temporary stress damage but also photoinhibition.

The leaf and membrane models of the reactions of the control plants are visualised in Figure 29. The ABS/RC , TR_0/RC , ET_0/RC and DI_0/RC increased significantly after 1 day of stress, which was similar to the result in the antisense plants. The closing down of the RC/CS_0 coincides with a significant increase in the ET_0/CS_0 . When the stress was continued for another day, the differences became more pronounced. The reaction per CS was much the same as experienced in the antisense plant, but with less RC's shut down. The ET_0/CS decrease was synchronized with an increase in the DI_0/CS and ABS/CS_0 . The plant reacted in response to the closing of the RC's with an increasing in the ABS/RC , TR_0/RC and DI_0/RC . The ET_0/RC also declined as a result of the stress, but not as severely as in the antisense plants.

It was observed by LOGGINI, SCARTAZZA, BRUGNOLI & NAVARI-IZZO (1999) that drought caused a more pronounced inhibition in the growth and photosynthetic rates of the more sensitive wheat cultivar than in the drought tolerant wheat cultivar. The antisense plants (Figure 28) performed thus more as drought and heat sensitive than the control Ibis plants (Figure 29). This sensitive reaction might be the result of the activation of the IHSP, and subsequent loss of proline cycling, in the antisense plant. The sense plants responded to the 1 day stress in much the same manner as the control plants (Figure 30).

The closure of some RC's is not necessarily a damaging process, it can also be a photoprotective acclimation strategy for plants (GILMORE, 1997). The main discrepancies between the transgenics became evident after a 2 day stress. The sense plants behaved in a different manner than the control or antisense plants in that the ET_0 increased as a result of the intense stress together with more open RC/ CS_0 . The sense plants had the ability to restore some RC as the stress mechanism was already activated after 1 day of stress. The ABS, TR_0 and DI_0 increased as was observed in the antisense and control plants, but to a lesser extent. The increased DI_0 is the result of the PSII unit that down-regulates the delivery of excess excitation to the RC's by increasing heat dissipation in the inner antennae (GILMORE, 1997). As a dramatic decrease in ET_0 is a symptom of photoinhibition (RICHTER, BOTHIN & WILD, 1992), it is postulated that photoinhibition was less in the sense plants than in the other plants. The sense plants performed thus as tolerant to stress, most probably as a result of the higher proline levels as the IHSP was activated by the stress.

Ibis	ABS/RC	TR _o /RC	ET _o /RC	DI _o /RC	RC/CS _o	ABS/CS _o	TR _o /CS _o	ET _o /CS _o	DI _o /CS _o
24°C/water	2.26	1.73	0.68	0.52	271.24	611.83	470.44	183.52	141.39
SE	0.07	0.04	0.04	0.04	6.10	16.81	6.15	9.24	11.30
1 day 38°C/drought	3.01	2.29	1.42	0.72	202.01	607.38	462.46	286.26	144.92
SE	0.19	0.11	0.10	0.08	8.57	16.91	9.30	7.86	9.60
2 days 38°C/drought	7.57	2.97	1.30	4.60	146.51	1109.21	435.36	190.25	673.84
SE	1.96	0.28	0.18	2.07	20.47	75.25	84.79	45.30	107.72

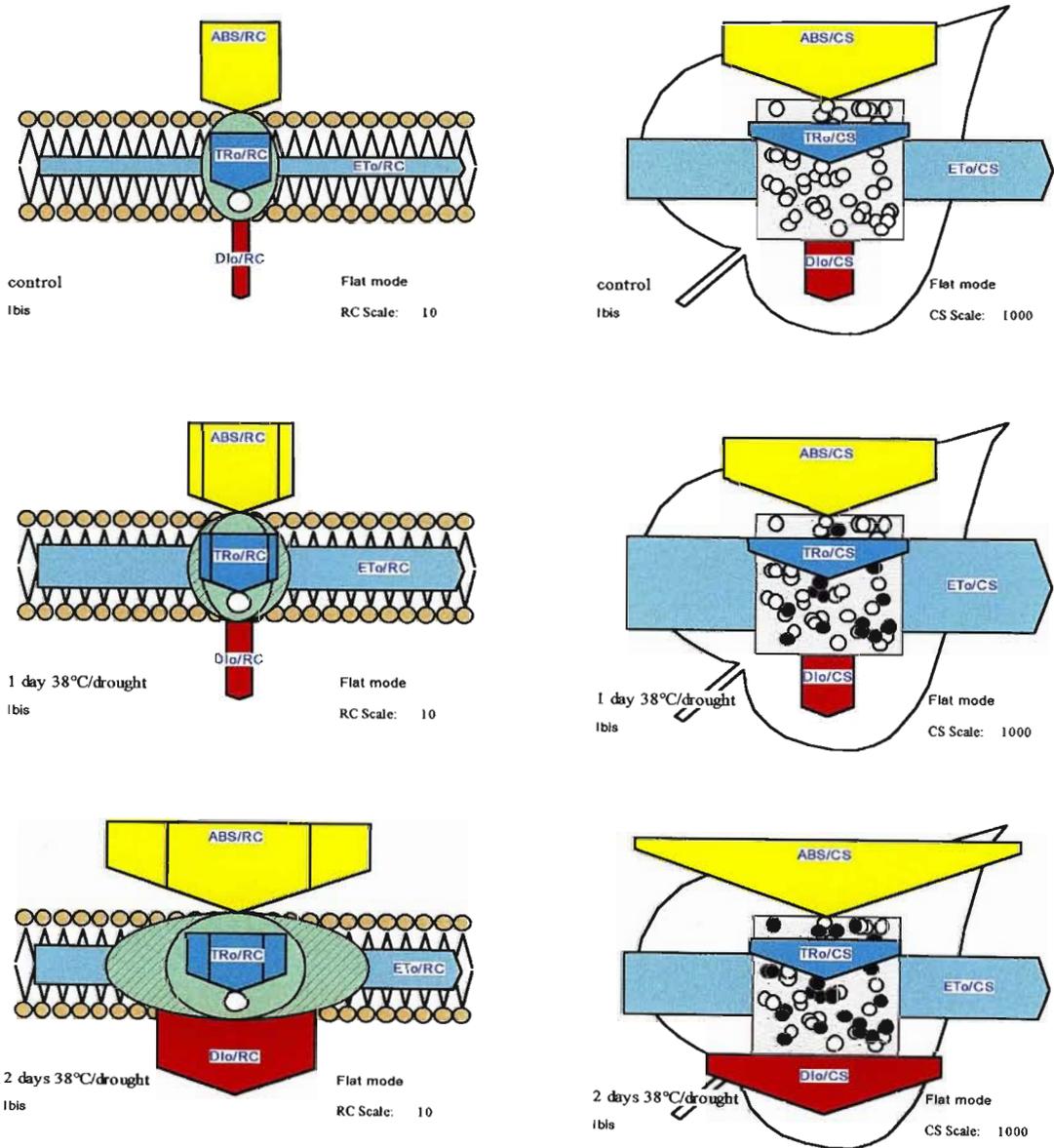


Figure 29: The manifestation of the energy fluxes by the control plants, at a combination stress, are demonstrated either in specific energy flux (per RC = membrane model) or phenomenological fluxes (per CS = leaf model)

Sense	ABS/RC	TR _o /RC	ET _o /RC	DI _o /RC	RC/CS _o	ABS/CS _o	TR _o /CS _o	ET _o /CS _o	DI _o /CS _o
24°C/water	2.31	1.81	0.76	0.50	252.75	583.27	456.41	191.92	126.85
SE	0.08	0.05	0.06	0.04	8.72	11.95	7.62	15.57	6.96
1 day 38°C/drought	3.07	2.33	1.56	0.73	188.14	576.95	438.95	292.56	138.01
SE	0.17	0.11	0.12	0.07	8.14	15.48	12.07	9.93	6.94
2 days 38°C/drought	4.88	3.29	1.90	1.59	197.33	963.81	649.41	374.06	314.39
SE	0.32	0.17	0.19	0.16	6.49	41.65	16.39	25.20	26.49

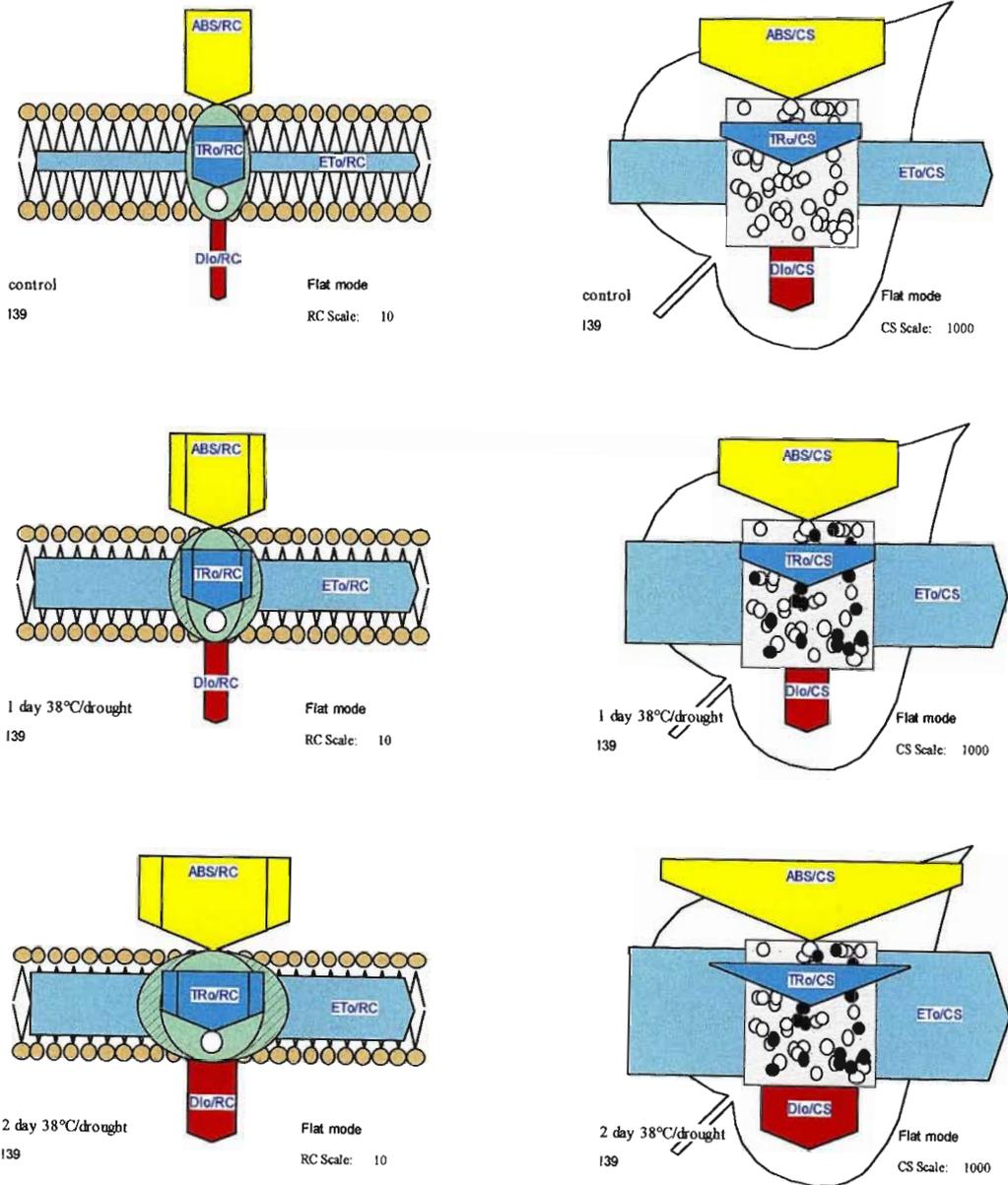


Figure 30: The manifestation of the energy fluxes by the sense transgenics, at a combination stress, are demonstrated either in specific energy flux (per RC = membrane model) or phenomenological fluxes (per CS = leaf model)

The F_0 values increased in all plant types when a combination stress of 2 days was applied (Figure 31). No difference between the different plants was recorded. The quantum efficiency PSI_0 increased after 1 day of stress in the antisense plants and even more so in the control and sense plants (Figure 31). The antisense and sense plants maintained the same PSI_0 levels at 2 days of stress, but the control plants decreased. The PSI_0 of the sense plants at 2 days stress was significantly higher than in the other plant types. As PSI_0 is the efficiency of the movement of an electron into the transport chain via a trapped exciton (STRASSER, SRIVASTAVA & TSIMILLI-MICHAEL, 1999), then a higher PSI_0 in the sense plants produces less photoinhibition and subsequently more tolerance to the applied stress.

The quantum efficiency $PHI_{(EO)}$ and performance indexes ($PI_{(CS_0)}$ and $PI_{(ABS)}$) differed significantly from the control treatment at 1 day of stress in both sense and control plants but to a lesser extent in the antisense plants (Figure 30). The increase in the antisense plants was significantly less than in the other two plant types. After 2 days of stress the antisense plants as well as the control plants experienced a dramatic decline in all the above activities, in contrast to the sense plants that experienced only a slight decrease in the $PHI_{(EO)}$. All of the plants had a significant decline in their performance indexes. The decreases of the sense plants were however significantly less than the decline observed with the antisense and control plants (Figure 31). As a decrease in the quantum efficiency of photosynthesis can be seen as damage to PSII (OSMOND, 1994), it can be postulated that the sense plants were more tolerant to the applied stress than the antisense and control plants.

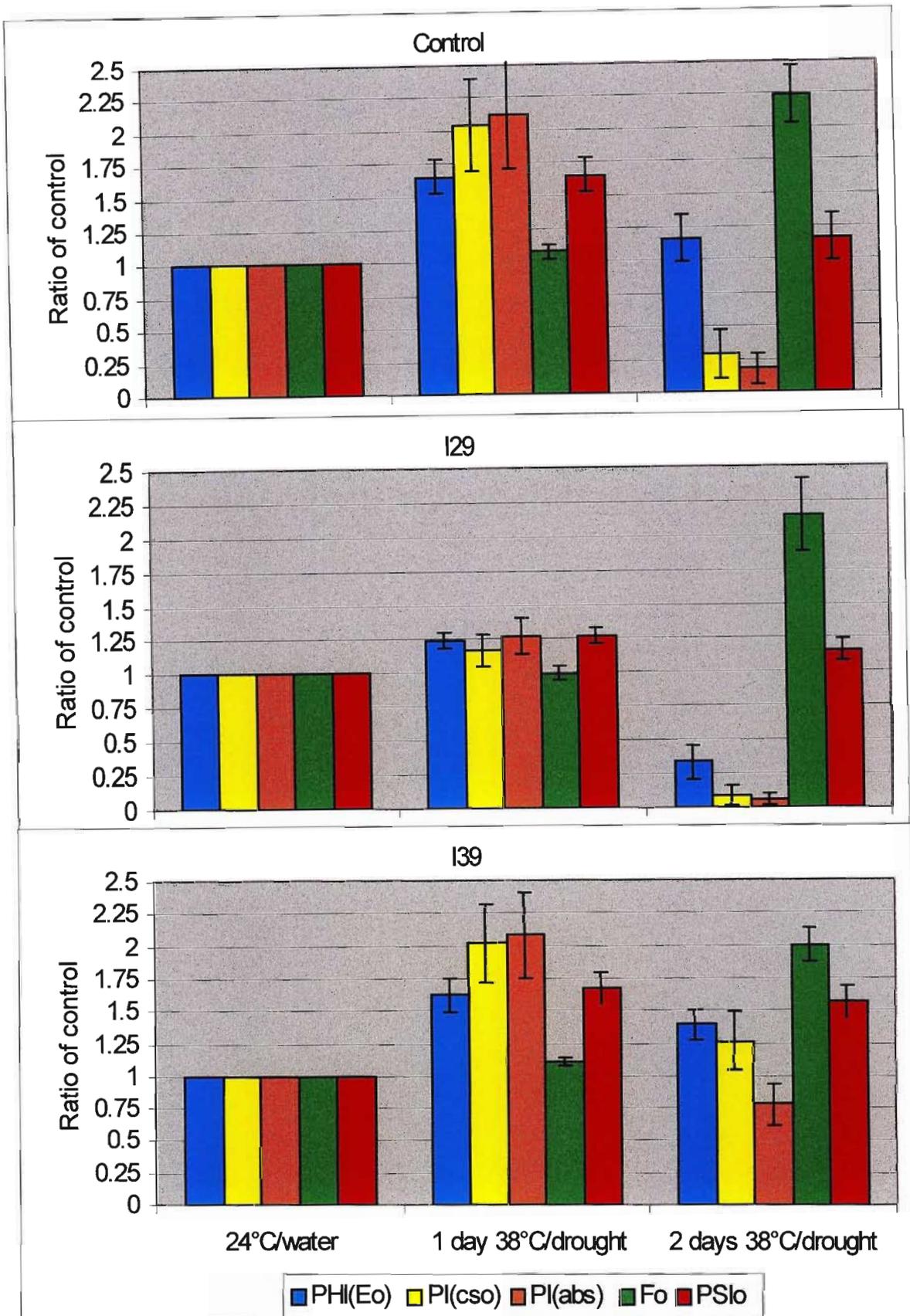


Figure 31: Some fluorescence indicators displaying differences between sense (I39) and antisense (I29) transgenics during combined heat and drought stress

7.4.3. NADP⁺

The NADP⁺ levels of all plant types were low during the control treatment (Figure 32). The plants are in equilibrium with the environment at the optimum growth temperature and water status. After 1 day of combination stress, the antisense and control plants sustained their levels of NADP⁺ in contrast to the sense plants that regenerated significantly higher NADP⁺. After 2 days of stress the NADP⁺ levels decreased in all plant types. In the antisense plants the decrease was more than that observed in the control and stress treatments. As the ABS/CS increased at 2 days of stress (Figures 27, 28, 29), the NADP⁺ concentration became limited under conditions of continued photon absorption. When the plants were subjected to a recovery treatment, the antisense and control plants experienced a decline in activity, in contrast to the sense plants that increased their levels. This increase in the sense plants may be the result of the higher proline accumulation at 2 days of stress (Figure 26) and subsequent NADP⁺ production. This NADP⁺ is thus available the following day for recovery. The antisense and control plants did not have as high proline accumulation and thus not the increase in the NADP⁺. The increased NADP⁺ levels after 1 day of stress can also be utilised during chlorophyll fluorescence as was seen in the increased ET_o/CS at 2 days stress (Figure 30). Thus, the sense plants are able to increase the NADP⁺ levels after a mild stress, in order to maintain physiological processes such as photosynthesis. The antisense and control plants do not have this mechanism and experienced photoinhibition as a result of the stress.

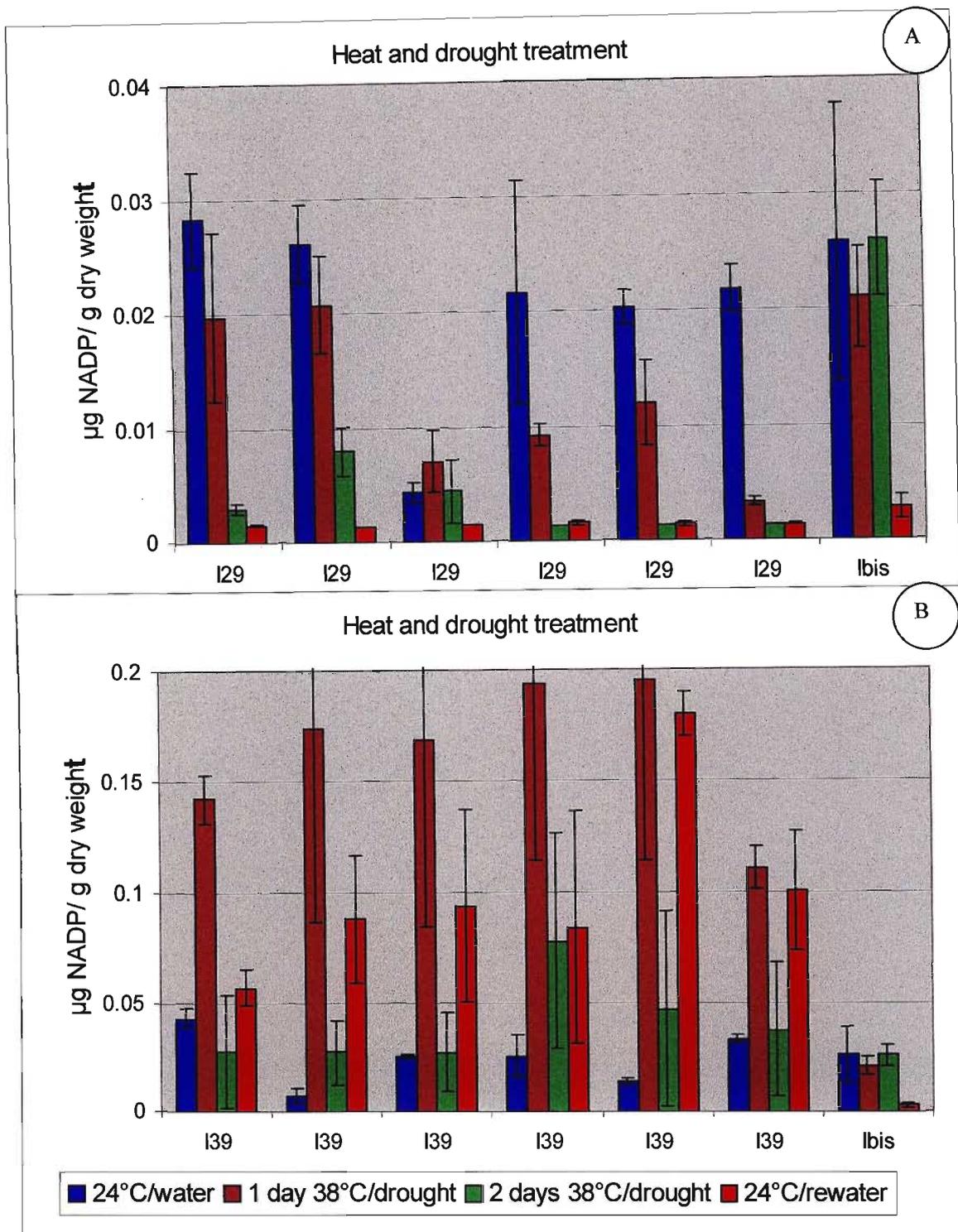


Figure 32: Accumulation of NADP⁺ as a result of a combined heat and drought stress on antisense (I29) (A) and sense (I39) (B) transgenics compared to non transgenic (Ibis) plants

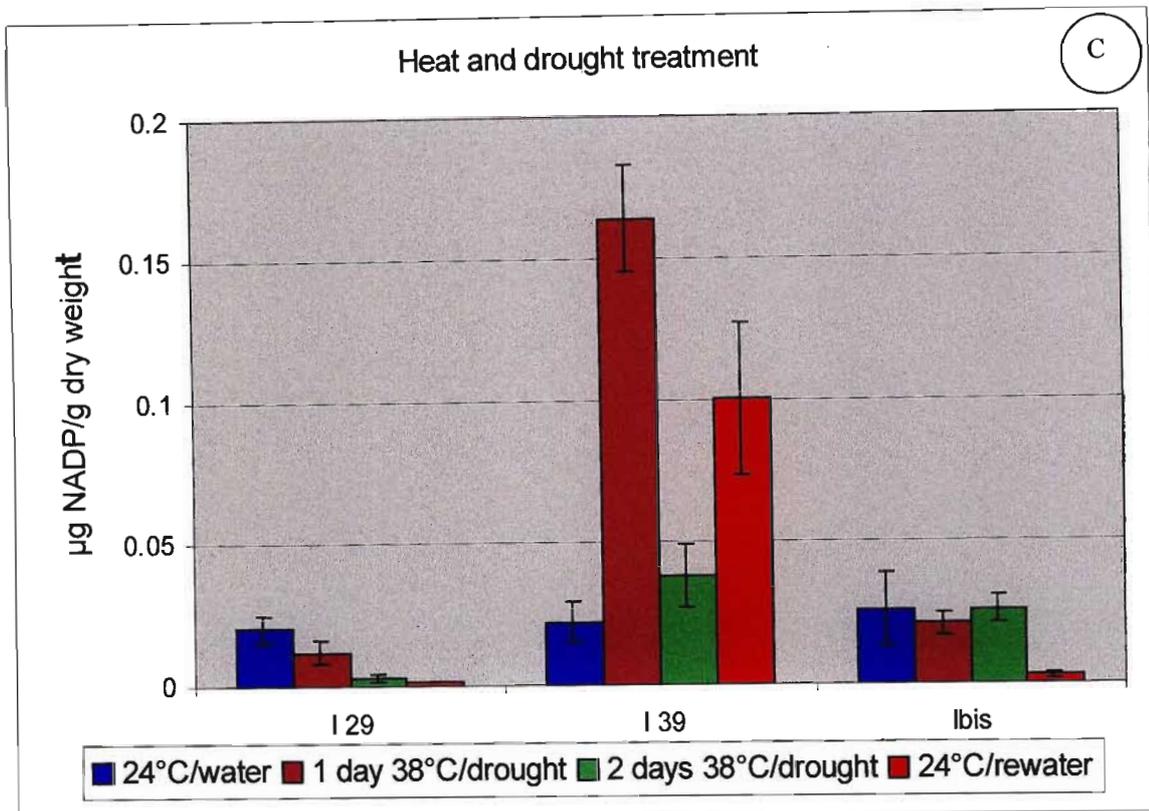


Figure 32C: Mean accumulation of NADP^+ as a result of a combined heat and drought stress on antisense (I29) and sense (I39) transgenics compared to non transgenic (Ibis) plants

7.5. CONCLUSIONS

Abiotic stress is expressed in plants by a series of physiological and morphological changes, which adversely affect plant growth and productivity. A number of plant biological processes might be associated with stress tolerance. It is possible that stress tolerance is not exclusively related to the accumulation of any individual component, but to the achievement of optimal homeostasis among the various components present in the cell.

The proton gradient and the redox state of the primary electron acceptor of the photosystem are affected by consumption of ATP and NADPH during carbon metabolism. Proline on the other hand might play a role in the maintenance of the $\text{NADP}^+/\text{NADPH}$ ratio (HARE & CRESS, 1997). The more drought-sensitive antisense plants responded to a period of heat and drought stress by reducing photosynthetic efficiency, increasing proline (but only slightly) and reducing NADP^+ levels as their defense mechanisms become activated. The defense mechanisms were however not able to prevent the plants from irreversible damage as proline degradation and photoinhibition occurred, as well as decreased NADP^+ levels that dropped lower than before the stress. On the other hand, the more drought-tolerant sense plants seemed to increase their proline levels, maintaining a high photosynthetic activity and increasing the NADP^+ levels as their defense system become activated. It is remarkable that the proline levels returned to control levels rapidly upon rehydration. Adaptation to drought may thus depend on different mechanisms, including the capacity to maintain high levels of proline and to regenerate them through the “reduction” of NADP^+ .

CHAPTER 8

PHENOTYPIC EVALUATION OF *P5CR* TRANSFORMED SOYBEAN

8.1. INTRODUCTION

Ever since farming began thousands of years ago, there has been a constant struggle to produce enough food for an ever growing population. This challenge has become even more acute with the world's population having passed 6 billion in 1999 (UNFPA, 1999). Soybean contains three times as much protein per kilogram than wheat and four times as much as maize. Soybean is responsible for more than 60% of the world's total protein consumption (CALLANAN, 1996). Soybean contributes apart from its use as animal feed, to human consumption in the form of margarine, cooking oils, milk and meat replacements.

Soybean is known to have been produced in South Africa from the beginning of the 20th century, but the first cultivars adapted to local growing conditions were not introduced until the 1950s. Soybean production increased after 1976, with the introducing of a market scheme under control of the oilseeds board (SMIT, 1992). The national soybean cultivar trial program conducted by the ARC Grain Crops Institute has made a major contributions towards the testing of promising foreign cultivars and the breeding of new cultivars adapted to the environmental conditions of South Africa (SMIT, 2000). Developing soybean with high yield potentials through identifying drought tolerance mechanisms is important for increasing yield in dry areas, as environmental variation is the major factor affecting soybean yield.

Osmotic adjustment is an effective component of drought resistance in several crops. It involves the accumulation of solutes in plant cells as a result of the fall in leaf water potential (CHANDRA BABU, SAFIULLAH PATHAN, BLUM & NGUYEN, 1999). Accumulation of proline is but one compatible solute that may contribute to osmotic adjustment (CARCELLER, PRYSTUPA & LE McOFF, 1999). It was reported by VOETBERG & SHARP (1991) that increased proline concentrations may account for as much as 50% of the osmotic adjustment in maize. Osmotic adjustment may allow turgor-dependent processes such as growth and stomatal activities to continue under lower leaf water potential (BLUM, 1996).

Heterogeneity within soil such as organic carbon content and physical soil properties, could cause problems with selection. These variations may affect the grain yield as well as other characteristics such as plant height, seed size and protein oil content of soybean. (VOLLMANN, WINKLER, FRITZ, GRAUSGRUBER & RUCKENBAUER, 2000). Treatment effects could be masked by these variations (SCHARF & ALLEY, 1993). Selection for grain yield under drought conditions is difficult due to differences in the intensity of the stress throughout the field as well as genotype and environmental interactions (DENCIC, KASTORI, KOBILJSKI & DUGGAN, 2000).

Water supply and atmospheric evaporative demand largely determine water consumption of plants. It is well-known that root, as well as shoot growth, are influenced by environmental variables. The modification of the phenology of a plant during drought stress has an effect on the growth and productivity of the plant (BLUM, 1996). A most common observation concerning the roots, is an increase in root/shoot dry matter ratio. Seedling mortality is a common problem as a result of drought, thus a screening method for seedling resistance can be of great value. The woodenbox screening method accurately discriminates between tolerant and susceptible cowpea plants at seedling stage (SINGH, MAI-KOMOMI

& TERA0, 1999a). It is a method that has the ability to screen for shoot drought tolerance eliminating the root effect and permitting non-destructive identification of drought tolerant plants at the seedling stage. SINGH, MAI-KOMOMI & TERA0 (1999b) observed a close correspondence between woodenbox screening, field screening and pot screening in 12 cowpea cultivars. Two types of drought tolerance mechanisms were observed in cowpea through this method (MAI-KODOMI, SINGH, MYERS, YOPP, GIBSON & TERA0, 1999). This same group demonstrated that segregating populations for drought tolerance can successfully be evaluated through this method and the survivors can be transplanted afterwards. (MAI-KODOMI, SINGH, TERA0, MYERS, YOPP & GIBSON, 1999).

8.2. AIM

In this part of the study, transgenic soybean lines were evaluated for tolerance to drought stress using pot-screening and woodenbox-screening methods. Pot screening was achieved by a combined drought and heat stress experiment where the IHSP was activated by increasing the temperature to 38°C. The box experiment was conducted at 25°C, which enabled us to study the possible activation of the IHSP by drought stress. It was investigated whether the IHSP can be activated without a heat treatment.

8.3. MATERIALS AND METHODS

Transgenic soybean of different nature, transformed with either the sense *P5CR* construct or antisense *P5CR* construct, were used in this study. They were screened for drought tolerance in plastic pots and in wooden boxes.

8.3.1. Pot screening

Seeds of transgenic (I29: T0 containing the RP29 construct (antisense), I39: T1 containing the RP40 construct (sense)) and control plants, were planted in plastic pots (25 cm) in a soil mixture consisting of soil: sand: vermiculite (5: 5: 3) in a greenhouse under controlled temperature and watering conditions. Plants were grown at 25/15°C (day/night temperatures) and were watered three times a week and fortnightly with 4 g^l⁻¹ nutrient mix. Fourteen-week-old plants were subjected to a heat and drought combination stress. Plants were subjected to a 2 day 38/25°C ±2°C day/night heat treatment together with the withholding of water, thereafter the plants were rewatered and the heat stress was relieved. The effect of the combined stress on the plants was rated after 1 day of recovery, with 100% wilted = 0 and 100% recovery = 5.

8.3.2. Wooden box screening

One hundred and forty four soybean plants were planted in randomized complete block designs with four replicates in a greenhouse with day/night temperatures of 25/15°C ± 2°C. Each box was comprised of 1 row of 12 plants each (which constituted one repeat) of 12 different soybean "lines". The "lines" were: untransformed Ibis plant, different sense and antisense *P5CR* T2 Ibis plants. The boxes were watered once daily from the time of planting of the seed until the emergence of the first trifoliate leaves (two weeks after emergence). Watering was stopped at this stage to impose a drought stress in all the boxes. The effect of the drought stress was observed on the trifoliate leaves and on the growing tips of each plant. The lines were rated for drought tolerance every second day until all the plants of the most susceptible line appeared dead after 6 days. Watering was subsequently resumed to obtain the recovery rate of the plants. The effect of plant recovery was rated, using wilting of the leaves as well as new growth of the growing tips as indicators.

8.4. RESULTS AND DISCUSSION

As drought is one of the major constraints affecting yield in soybean, soybean seedling and adult plants were evaluated for drought stress and a combination of drought and heat stress. Fourteen-week-old plants, transgenic and control, were subjected to a severe heat stress of 38°C and no water for 2 days. The activation of the IHSP resulted in under production of proline in the antisense plants (I29) and an overproduction in the sense plants (I39) (see Chapter 7). The antisense plants (Figure 33A) were severely effected by this treatment, the control plants (Figure 33B) were able to endure the stress better and the sense plants looked as if they were not stressed at all (Figure 33C).

When the plants were rewatered and the heat stress removed, 16% the antisense plants recovered fully, 65% of the control plants and 100% of the sense plants (Figure 34). There is thus a definite correlation between the rate of recovery of adult transgenic plants after a combination of heat and drought stress and the activation of the IHSP.

The box screening method enabled screening for shoot drought tolerance, as the method discriminates between the different transgenics after a 6 days drought stress (Figure 35). There was no heat stress applied, thus the IHSP was only activated as a result of the drought stress. This was possible with the drought stress mimicking the production of HSP70 (see Chapter 4). The plants showed no visual effect after 2 days of drought stress (Figures 36A, B). After 4 days of drought stress, small differences were observed between the different plant types, the control rated ± 2.8 , the antisense (I29) rated between 2.5 and 1.8 and the sense plants (I39) rated between 2.3 and 3.2 (Figures 36A, B).

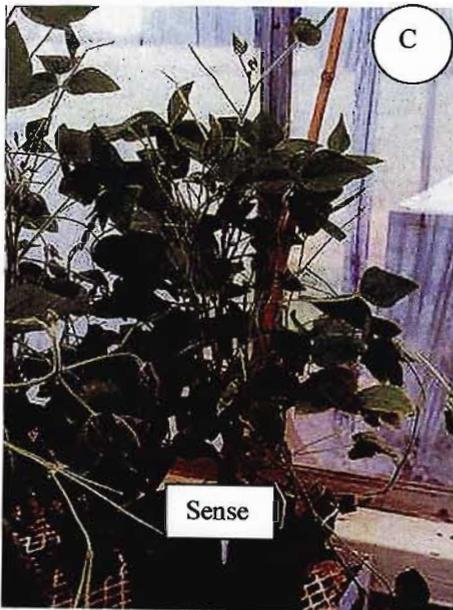
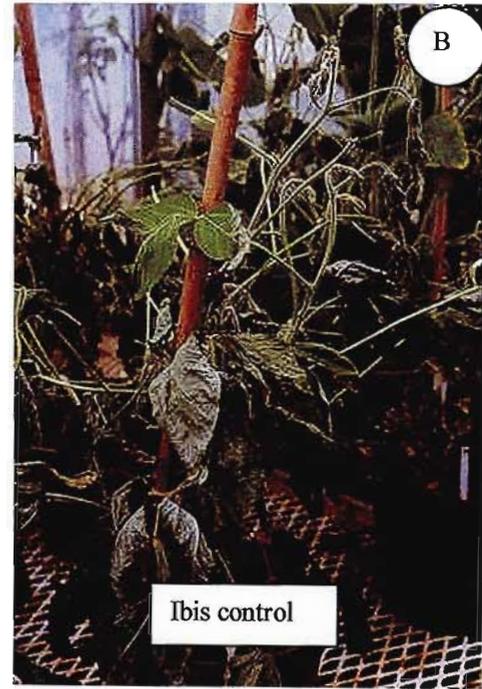


Figure 33: Transgenic and control plants subjected to a severe heat stress of 38°C and no water for 2 days

- A) Antisense
- B) Control
- C) Sense

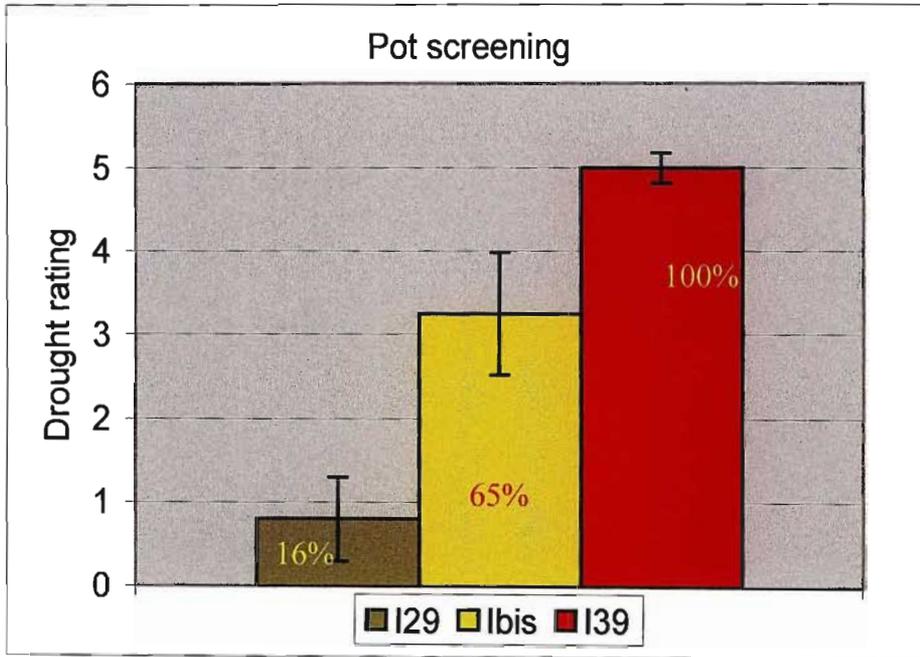


Figure 34: Transgenic and control plants subjected to a recovery period after a severe heat stress of 38°C and no water for 2 days

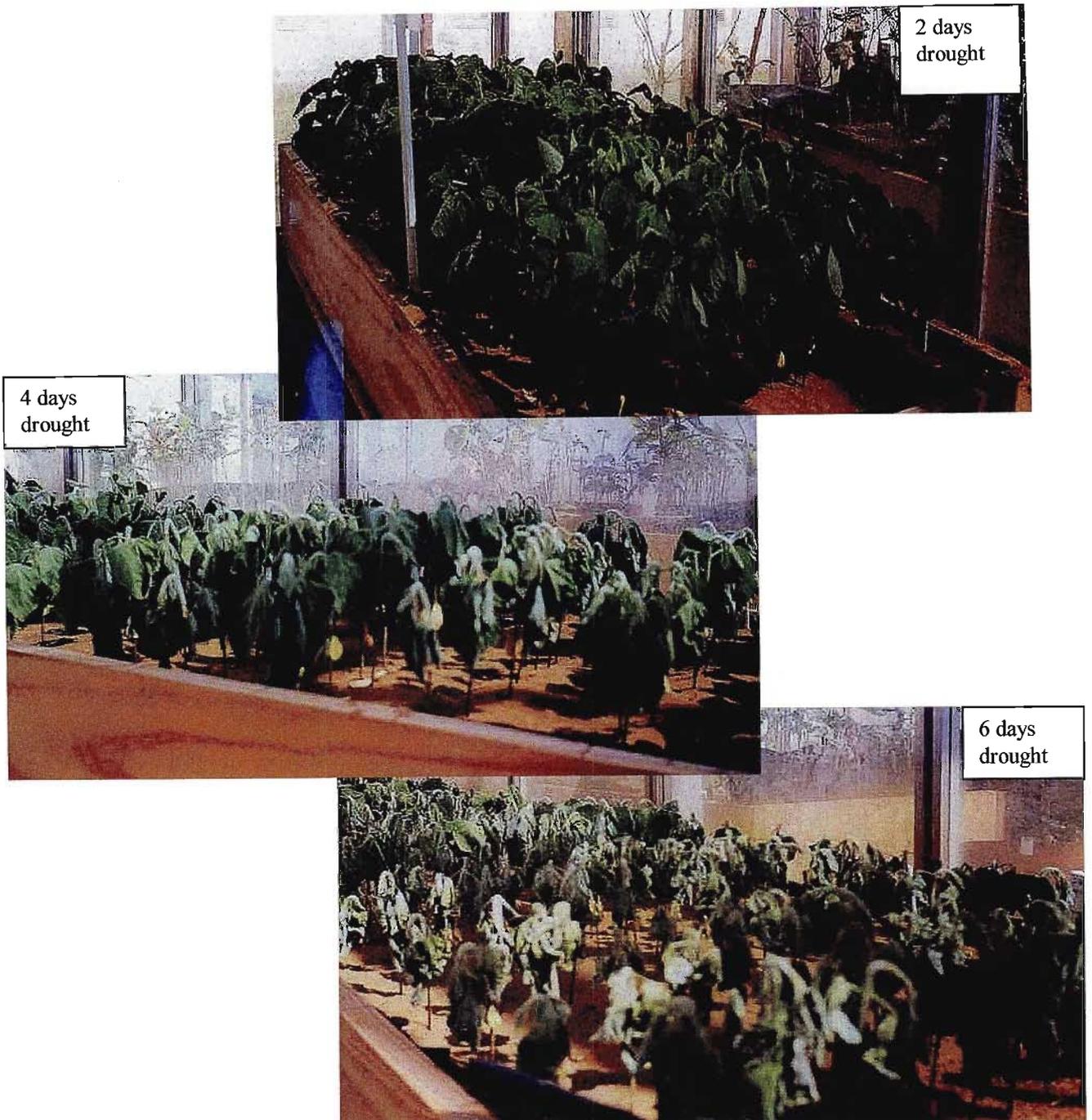


Figure 35: Shoot drought tolerance screening, through a woodenbox method

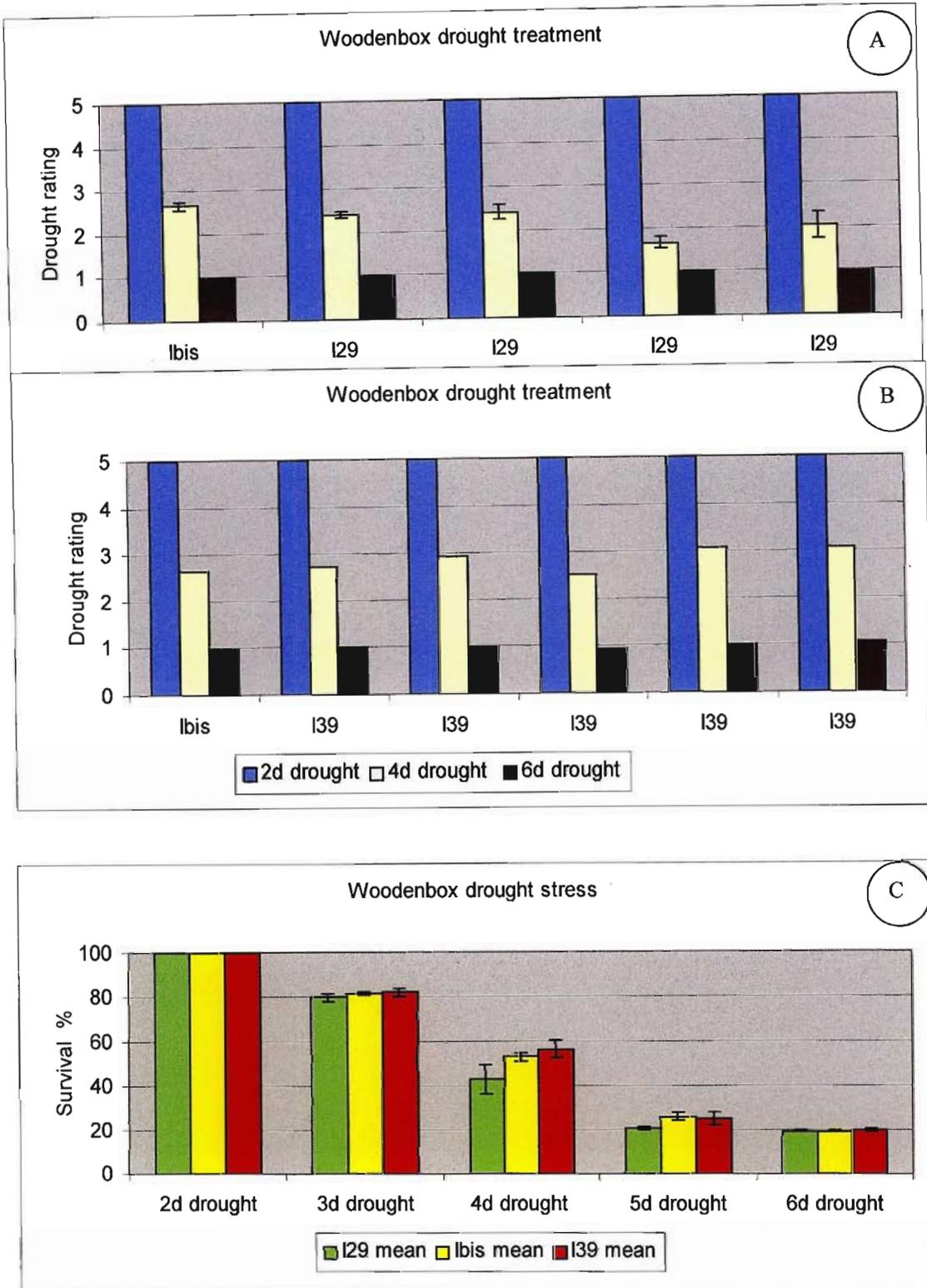


Figure 36: Drought screening of antisense (I29) (A) and sense (I39) (B) transgenic plants compared to control (Ibis) plants using a woodenbox technique. The survival of these plants during the drought stress was expressed as a percentage of the highest possible rating (C)

After 6 days of drought stress all the plants rated 1. Thus, the drought stress did not activate the IHSP quickly enough for the sense plants to be more tolerant to the drought stress than the antisense plants, as the stress period was too short. During stage I of drought stress the plants met their transpirational demand by reducing leaf water potential and leaf tissue impairment resulted in the plants without osmotic adjustment (BLUM, 1996).

With a 6 day stress from 100% turgor to almost 0% turgor, the plants moved through the different stages too quickly for adequate adjustment to the stress. For osmotic adjustment to take place, the plants need to have time to adjust (about a 12 day long stress) as fast reduction of the plant water status does not allow enough time to adjust (BLUM, 1996). Solute accumulation, like proline, is probably the most distinctive feature of adaptation during stress. The sense plants, having a higher production of proline, might be able to adjust osmotically. However, this must be tested before such assumptions can be made. In a study comparing different crops SINGH, MAI-KODOMI & TERA0 (1999b) indicated that a sandy loam soil mixture can lengthen the time of soybean stress to 19 days, this may give the soybean plants adequate time to adjust osmotically.

The mean drought ratings of the transgenics were expressed as a percentage of the highest possible rating (Figure 35C). It indicated that all the transgenics decline in the survival rate as the drought stress progresses, with 4 days of stress being the only time period in which differences between the different plant types were evident. After 6 days of stress, the sense, antisense and control plants all respond as if there would only be a 20% survival.

The real differences between the transgenic plants became evident in the recovery phase (Figures 37A, B, C). The control plants showed some recovery after rewatering, but the damage to the plants was such that at 14 days after rewatering the plants only rated a mean of 0.5 (Figures 38A, B). The antisense plants failed totally to recover after the drought stress and their survival rate declined steadily from 6 days of drought stress through the recovery phase (Figure 38A).

The sense plants displayed an immediate response to rewatering and even at 2 days recovery the rating doubled (Figure 38B). In most of the sense plants the recovery rating increased with time. The mean recovery ratings of the transgenics were expressed as a percentage of the highest possible rating in Figure 38C. Even after 1 day of rewatering, the difference between the antisense and sense plants was significant. This difference increased over the 2 week evaluation period. The difference between the control and sense plants became significant after 2 days of rewatering.

All the plants that were apparently still alive, were transplanted in pots and evaluated after 2 months (Figure 39). At this stage it became clear that only 2% of the antisense plants planted in the woodenbox recovered completely from the stress and the control plants demonstrated a 13% recovery rate. This was in contrast to the sense plants where 35% of the plants recovered from the stress. These results indicate that the sense plants were more drought tolerant than the control and antisense plants.

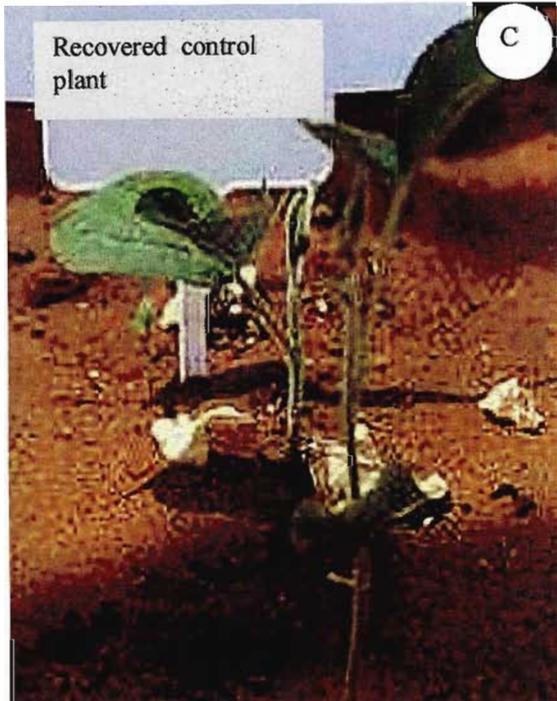
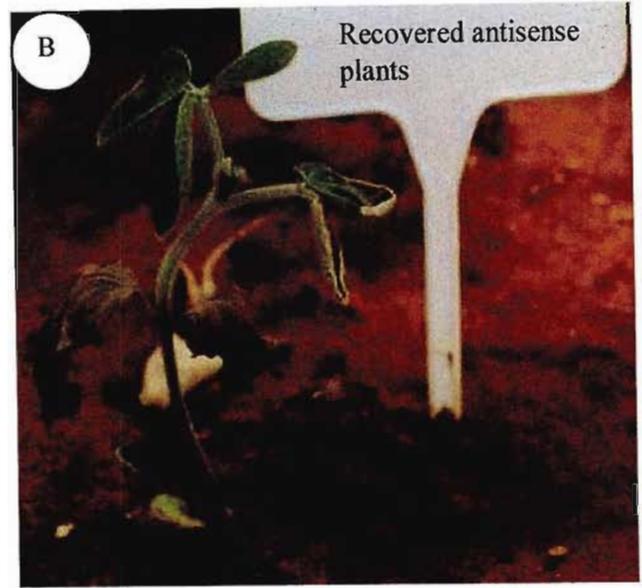
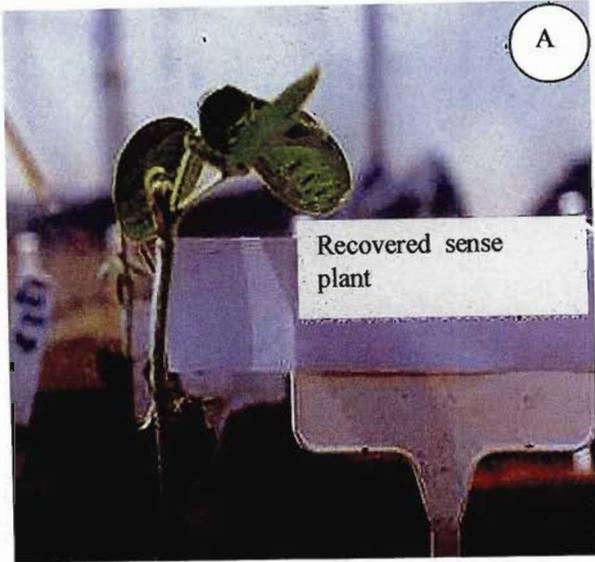


Figure 37: Differences between transgenics after rewatering

- a) Recovered sense plants
- b) Recovered antisense plants
- c) Recovered control plants

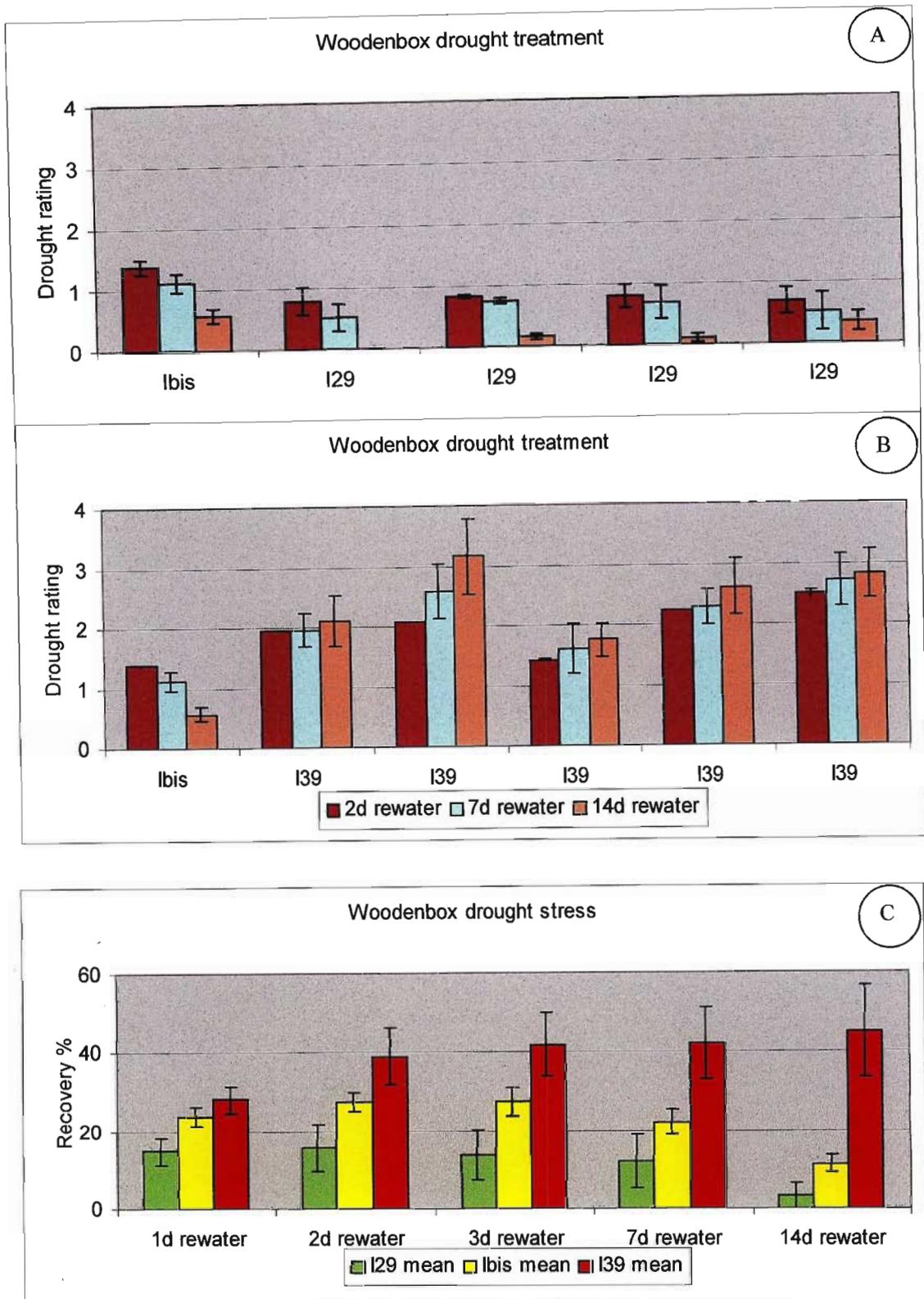


Figure 38: Differences between antisense (I29) (A) and sense (I39) (B) transgenic plants compared to control (Ibis) plants, as observed during a recovery phase of a woodenbox technique. The survival of these plants was expressed as a percentage of the highest possible rating (C)

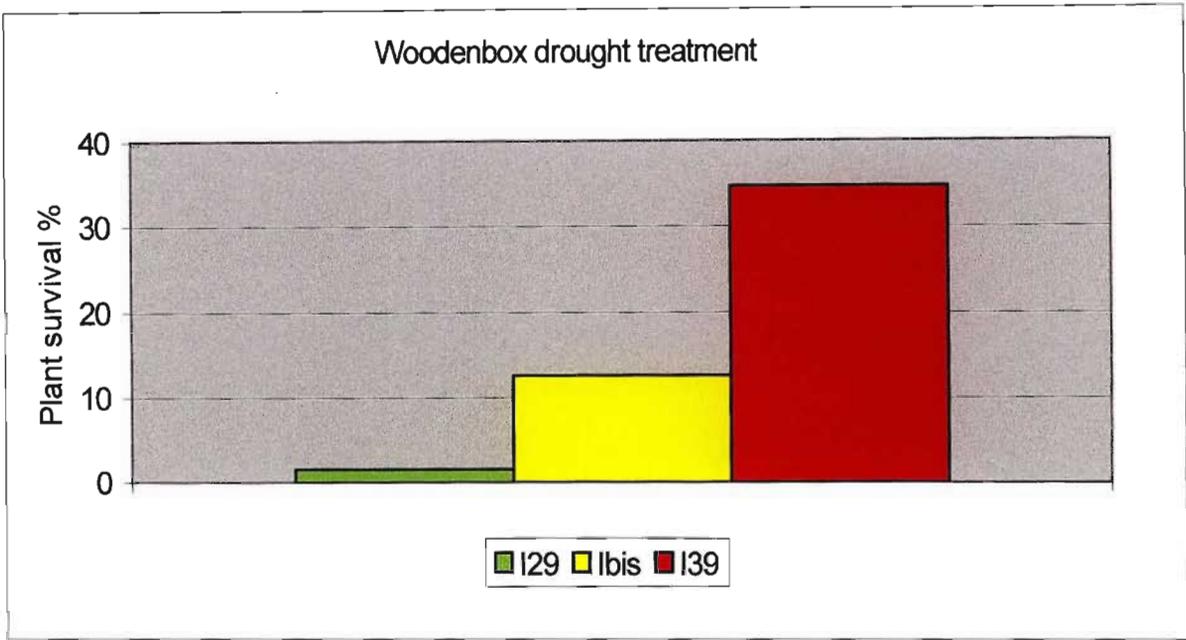


Figure 39: Transgenic plants that survived the woodenbox screening method after 2 months recovery

8.5. CONCLUSIONS

Although traditional breeding is used to develop soybean cultivars with enhanced drought tolerance, the use of biotechnology can enhance our search for a more drought tolerant crop. Different transgenic lines were tested in a woodenbox and pot trial and it became evident that some of the sense *P5CR* plants performed better to a drought or combination of drought and heat treatment than the control plants. The differences between susceptible and tolerant lines became evident with the combination stress in the pot screening and after the recovery period of the box screening. The sense plants were able to activate the IHSP thus increasing proline synthesis/cycling during the combination stress and were able to survive the harsh stress. They were thus more drought tolerant than the control and antisense plants. This result correlates well with more than twice as many sense plants surviving a box drought stress treatment than the control plants. The results from the box experiment also indicated that the transgenics did not require heat stress for activation of the IHSP. A drought stress can also activate it.

CHAPTER 9

DISCUSSION

We live in a world where the population (expected to reach seven billion in 25 years) is outstripping agricultural productivity (growth of $\pm 1.8\%$ annually) (ALTMAN, 1999). Sustainable food production is uncertain, especially in developing countries because of climatic diversity, thus increasing food production is a must to provide food for the rural poor. Since this can no longer be achieved by traditional methods alone, merging of classical breeding with plant biotechnology is a necessity. Doubts about the benefits of biotechnology are however, presently impeding the acceptance of genetically modified plants in many areas of the world. Transgenic crops have much to offer the world's food production, but the industry still lacks the permission to operate in society.

While plant biotechnology has been successfully applied to pest control, it is not the case for abiotic stress conditions although drought and salinisation are the most common natural causes of lack of food in arid and semiarid areas of the world (ALTMAN, 1999). Incorporation of genes conferring drought resistance into commercial soybean varieties has been a goal of considerable importance. Together with an increased need for soybean, there has been an upsurge in plant breeding over the past 20 years, with new varieties giving higher yield (SMIT, 2000). However, the developing of new varieties is still a key factor in the support of the greater biodiversity. Biotechnology may be at the heart of this breeding program in future. Conventional breeding for yield improvements under dryland conditions is time consuming and laborious. Considering also the fact that there can be a decrease in the heritability of yield components with increasing

environmental stress, the use of biotechnology could prove a useful tool in plant breeding (RIBAUT, HOISINGTON, DEUTSCH, JIANG, GONZALEZ-DE-LEON, 1997). Introduction of foreign genes of interest into soybean will increase the value of soybean, when transgenic soybean lines can be developed routinely.

This prerequisite has spurred the development of different gene transfer techniques for soybean, which utilise different regenerable tissue explants (Chapter 2). The development of a method to obtain transformed soybean plants, independently from problems inherent in regeneration techniques, has been one aim of this study. Several conditions that can effect transformation efficiency were assessed before an *Agrobacterium*-mediated vacuum infiltration transformation system was established (Patent pending SA98/9427 & PCT/IB99/01676). Wounding of the plant tissue is a critical step in *Agrobacterium*-mediated transformation, as it allows the bacteria to enter the host cell. In this method, the concentrated *Agrobacterium* solution was infiltrated into germinated seed under vacuum. It is proposed that the negative pressure forced the bacteria into the plant cells, thus increasing the susceptibility of the cells. The vacuum process stimulates the wounding response and increases the virulence of the bacteria. Stable transgenic soybean plants were obtained within one generation with a transformation rate of $\pm 30\%$. This is to date the highest known efficiency of stable transformation obtained through *Agrobacterium*-mediated transformation of soybean.

The transformation of soybean facilitated the transfer of a proline gene into soybean in an attempt to study the role of proline biosynthesis in response to drought stress by means of antisense gene technology. The general pathway for synthesis of proline under stress is the glutamate pathway (DELAUNEY & VERMA, 1993) with P5C being reduced to L-proline by P5CR. Soybean plants were successfully transformed with a *P5CR* gene in the antisense orientation under the control of an inducible heat shock gene promoter. It was confirmed that the

gene construct was integrated into the soybean cells and was conserved over three generations.

Screening of these plants confirmed that the antisense *P5CR* transgenic plants, with activation of the promoter, experienced an under-expression of the *P5CR* gene and subsequent inhibition of the production of proline. At 42°C the IHSP was fully activated and resulted in a significant decrease in the production of proline. Inhibition of proline accumulation in drought stressed plants resulted in declined growth and increased drought sensitivity reactions. With the application of a mannitol stress, the control plants showed significant increase in proline accumulation at 32 and 42°C indicating a role during osmotic stress. This was in contrast to the transgenic plants, which displayed a decrease in proline concentration at 32 and 42°C under mannitol stress. It was thus confirmed that there is an association between *P5CR* translation and proline accumulation during an osmotic stress, as the proline accumulation was markedly decreased by activation of the antisense *P5CR* construct. The magnitude of proline accumulation during the osmotic stress was however lower as during the drought stress. This can be explained in that the drought stress was performed in a greenhouse and the osmotic stress in a laboratory.

The woodenbox screening technique demonstrates that the control plants can endure a drought stress longer than the antisense *P5CR* transgenic plants. The decline of the viability of the transgenics with prolonged drought stress, is an indication that proline is needed for survival of soybean plants under drought stress conditions. The viability of the transgenics was also evaluated with the use of a TTC assay. The transgenic plants demonstrated a sensitive reaction in contrast to the control plants that displayed a tolerant reaction to the osmotic stress.

The underexpression of the *P5CR* gene resulted in declined protein synthesis due to proline shortage as was observed with the evaluation of the efficiency of protein synthesis. The transgenics experienced a lower seed production than the control plants. This indicated that the antisense *P5CR* gene also influenced seed production negatively. These results suggest that a decrease in the proline level due to the antisense *P5CR* gene, yielded plants that are more osmotic stress sensitive. These findings confirm that proline plays an adaptive role during osmotic stress, as lower levels of proline in the transgenic plants resulted in less viable plants. The control plants were the most viable of the plants tested providing additional evidence that increasing proline levels during osmotic stress constitute an adaptive response by the plant.

The soybean cultivar Ibis was successfully transformed with the *P5CR* gene in the sense and antisense directions in order to test the reproducibility of the transformation process and to assess the link between the biochemical traits involved in the drought stress mechanism. The effect of a mild heat and drought stress on “To” transgenic plants was evaluated with the chl-*a* fluorescence JIP test. Differences in the chlorophyll fluorescence transients were detected before any visual effects were observed. The differences between the control and sense plants were more pronounced than between the control and antisense plants. The control plants compensate for the applied stress, with closure of the RC's together with increasing ABS, TR and ET/RC. The antisense plants closed down even more RC's because the plants suffered more from the stress. The sense plants utilised their energy much better than the antisense plants by reacting with a decreased ABS, TR and ET/RC even though the RC/CS declined. The effect of a mild heat stress on “T1” transgenic plants was evaluated comparing proline accumulation and chlorophyll fluorescence transients. Although the proline accumulation yielded no significant differences, the energy fluxes of the photosynthetic apparatus indicated differences between the sense and antisense transgenics. The

ABS/RC, DI_o/RC , ET_o/RC and TR_o/RC increased in the antisense transgenics together with inactivation of the RC's. This resulted in less excited energy for primary photochemistry in the antisense plants (KRAUSE, SOMERSALO, ZUMBUSCH, WEYERS & LAASCH, 1990). In the sense plants, the heat stress manifests with decreasing ET_o/RC , ABS/CS, TR_o/CS and ET_o/CS and increasing DI_o/RC and DI_o/CS . The performance indexes, $PI_{(CS_0)}$ and $PI_{(ABS)}$, are also valuable parameters for screening, as both indexes were significantly lower in the antisense and control plants compared to the sense plants. As the performance indexes are an indication of the vitality of the plant (KRÜGER & VAN DER RIET, 2000), it indicated that the sense plants performed as drought tolerant. High temperature thus affects the rate of the chemical reactions associated with the photosynthetic functions to a greater extent in the control and antisense plants than in the sense plants. The F_0 levels of all the plants tested increased, as was reported in the literature (YAMANE, SHIKANAI, KASHINO, KOIKE & SATOH, 2000). Although the levels were slightly lower in the sense plants than in the antisense plants, it was not significantly different. The F_0 levels could thus not be used as an indication of tolerance in the transgenic soybean as was observed in a tobacco study (VAN RENSBURG, KRÜGER, EGGENBERG & STRASSER, 1996).

It will be possible to use chlorophyll fluorescence transients as a screening method for transgenic soybean plants using a mild heat or heat and drought stress since the results of both the heat and the heat and drought stress experiments were correlated with each other: with the sense transgenics having a significant decrease in the ET_o/CS , TR_o/CS and ABS/CS compared to the antisense plants that displayed significant increases in ET_o/RC , ET_o/CS and TR_o/RC .

The effect of a severe drought and heat stress on the "T1" transgenic plants was evaluated comparing proline accumulation, $NADP^+$ synthesis and chlorophyll fluorescence transients. The mean of all the plants demonstrated that the sense

plants accumulated substantially more proline than the control and antisense plants, as a result of the higher (sense) or lower (antisense) *P5CR* levels in the plant upon activation of the IHSP. The antisense plants did not have the mechanism to increase the proline when stressed and irreversible damage occurred in the plant cells as protein degradation took place. The sense plants had the ability to reduce the accumulated proline during the recovery period. As oxidation of proline is activated during the rewatering phase (KIYOSUE, YOSHIBA, YAMAGUCHI-SHINOZAKI & SHINOXAKI, 1996), a decline of free proline in the sense plants occurred and extra energy was provided to the plant as the accumulated proline is rapidly oxidized to glutamate (VERBRUGGEN, HUA, MAY & VAN MONTAGU, 1996). This indicated that proline plays an important role in the plant's response to a drought stress as well as in the recovery phase after drought.

The antisense plants reacted to the heat and drought stress with a decrease in the RC/CS , ET_0/RC , ET_0/CS_0 as well as an increase in the ABS and DI. Irreversible photoinhibition occurred to the photosynthetic apparatus as a result of ET loss (RICHTER, BOTHIN & WILD, 1992) and a dramatic decrease in active RC's (GREER, 1997). This is in contrast to the sense transgenics which responded to the severe stress with increased ET. At two days of stress the antisense plants as well as the control plants experienced a dramatic decline in quantum efficiency and performance indexes, in contrast to the sense plants that experienced only a slight decrease in these parameters. This also showed that photoinhibition occurred in the antisense plants. As a decrease in the quantum efficiency of photosynthesis resembles damage to PSII (OSMOND, 1994), it is proposed that the sense plants are more tolerant to the severe stress than the antisense and control plants.

The sense plants have a mechanism to increase NADP⁺ levels during a mild stress, in contrast to the antisense and control plants where the levels decreased or remained stable. Increased reduction of P5C to proline, as a consequence of the sense *P5CR* construct, increased the availability of NADP⁺. This NADP⁺ can thus be utilised in physiological processes such as photosynthesis (LIMP, RODAL & WILLIS, 1998) and the oxidative phosphate pathway (KOHL, LIN, SHEARER & SCHUBERT, 1990). The generated NADP⁺ in the sense plants could have an additional role in regulation of cytosolic redox cycling (HARE, CRESS & VAN STADEN, 1998) as well as energy transfer for N₂ fixation (KOHL, LIN, SHEARER & SCHUBERT, 1990), they could assist the plants in its resistance to drought stress. The NADP⁺ shortage in the antisense plants may in turn have contributed to the experienced photoinhibition.

The antisense transgenics thus responded to a period of heat and drought stress with a slight increase in free proline together with a reduction in photosynthetic efficiency and NADP⁺ levels. This resulted at the end in irreversible damage as proline degradation and photoinhibition occurred which led to death of the plants. The sense plants responded to the same stress with increased proline levels, maintenance of photosynthetic activity and increasing NADP⁺ levels. These plants were able to fully recover after the stress period. Sense plants in a woodenbox drought experiment also performed as more drought tolerant than the control and antisense plants. Throughout all the experiments it became evident that the sense plants were more tolerant to drought/(drought and heat) than the control plants and that the antisense plants were more sensitive.

It was thus possible to alter the drought tolerance of soybean cultivar Ibis by transformation with antisense and sense *P5CR* gene constructs, which resulted in respectively more sensitive and more tolerant Ibis plants. It can thus be concluded that over-expression of *P5CR* during a drought stress resulted in higher proline

levels, better photosynthetic efficiency, higher NADP⁺ production and thus a more drought tolerant plant. This study provided additional proof that a constitutively higher level of proline accumulation enhances drought tolerance in soybean. Information obtained also attributes to the understanding of proline function in plants.

In future studies the biochemical processes involved in the entire proline biosynthetic pathway and the degradative pathway need to be explored. The effect of enhanced proline in the transgenic plants must also be tested under other environmental stresses like salt, aluminium and cold stress. Finally field studies should be carried out to determine the value of enhance proline synthesis to drought and heat stress under field conditions.

CHAPTER 10

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APPENDIX

Methods not in text

Media for bacterial growth

LB broth (SAMBROOK, FRITSCH & MANIATIS, 1989)

10 g tryptone

10 g yeast extract

10g NaCl (pH 7.00)

Molecular analysis

Plasmid DNA miniprep (FELICIELLO & CHINALI, 1993)

Inoculation of single colony into 20 ml LB supplemented with antibioticum

Incubation at 37°C overnight

Centrifugation at 10 000 x g for 10 minutes

Resuspend cells in 2 ml STE buffer

Cell suspension were halved and centrifuge at 6 000 x g for 1 minute

Pellet was resuspend in 250 µl solution I

Add 500 µl solution II, mix and keep on ice for 5 minutes

Add 750 µl solution III, mix and keep on ice for 10 minutes

Centrifuge at 12 000 x g for 15 minutes

Add 700 µl isopropanol for every 1.4 ml supernatant and keep on ice for 10 minutes

Centrifuge at 12 000 x g for 15 minutes

Resuspend pellet in 250 µl TE containing 1 µl Rnase

Keep at room temperature for 20 minutes

Add 1.2 µl solution IV for every 1 µl TE

Mix and keep at room temperature for 10 minutes

Centrifuge at 12 000 x g for 15 minutes

Wash pellet with 70% ethanol

Dry and resuspend in 100 µl H₂O

STE buffer

0.1 M NaCl

10 mM Tris (pH 8.00)

1 mM EDTA

Solution I

50 mM glucose

10 mM Tris (pH 8.00)

1 mM EDTA

Solution II

0.2 M NaOH

1% SDS

Solution III

4 Volumes 5 M potassium acetate

1 Volume 10 M glacial acetic acid

Solution IV

1 Volume 5 M potassium acetate

2 Volumes H₂O

22 Volumes iso-propanol

TE

10 mM Tris (pH 8.00)

1 mM EDTA

50 X TAE buffer

242 g Tris

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA pH 8.00

Final volume 1000 ml

Plant DNA isolation (McGREGOR, LAMBERT, GREYLING, LOUW & WARNICH, 2000)

Grind 2 leaf discs together with Carborundum (400 grit) with a glass grinder

Incubate at 60°C for 30 minutes in 400 µl prewarmed Supaquick buffer

Add an equal volume of chloroform:isoamyl alcohol

Mixe and centrifuge at 10 000 x g for 10 minutes at room temperature

Add 0.6 volumes ice-cold isopropanol to the aqueous phase

Precipitate DNA for 30 minutes at -20°C, centrifuge (10 000 x g; 10 minutes; 4°C)

Wash with 70 % ethanol.

Dissolve DNA in H₂O and store at -20°C until use.

Supaquick buffer

200 mM Tris-Cl (pH 7.5)

250 mM NaCl

25 mM EDTA

0.5% SDS

Southern blot analysis (SAMBROOK, FRITSCH & MANIATIS, 1989)

Separate digested DNA on 0.8% agarose gel at 25 volts overnight at 4°C

Depurinate gel for 10 minutes in 0.25 N HCl

Denaturate gel twice for 15 minutes in 1.5 M NaCl and 0.5 M NaOH

Neutralise gel twice for 15 minutes in 3 M NaCl, 0.5 M Tris pH 7.5

Blot gel in 0.025 M NaPO₄ buffer at 4°C overnight

Incubate blot for 2 hours at 80°C

Prehybridise blot for 3 hours at 40°C in 20 ml hybridisation buffer containing 200 µl (10 mg/ml) denaturated herring sperm DNA

Hybridise overnight in fresh hybridisation buffer containing denaturated ³²P probe DNA

Wash blot twice with 2 X SSC for 15 minutes at room temperature

Wash blot twice with 2 X SSC and 0.1 % SDS for 15 minutes at 65°C

Wash blot twice with 0.1 X SSC and 0.1 % SDS for 15 minutes at 65°C

Hybridisation buffer

30 ml 20 X SSC (0.3 M Na₃Citrate and 3 M NaCl (pH 7.00))

5 ml 100 X Denhardts (2% BSA, Ficoll and PVP 1:1:1)

5 ml 10% SDS

10 ml H₂O

50 ml formamide

Cloning (MANIATIS, FRITSCH & SAMBROOK, 1982)

Fill in reaction

5 µg DNA

12.5 units T4 DNA polymerase

250 µM dNTP

5 µl 10X DNA polymerase buffer

Final volume 50 µl

Incubate at 11°C for 20 minutes

Incubate at 70°C for 10 minutes

Presipitate at -20°C overnight with 0.3 M ammonium acetate and 2 volumes ethanol

Blunt end ligation

15 units T4 DNA ligase

1 µg DNA

5 µl T4 DNA ligase buffer

1 mM dATP

1 µl 0.2 M DTT

Final volume 50 µl

Incubate at room temperature overnight

Stop reaction at 65°C for 15 minutes

Physiological analysis

Acid ninhydrin for free-proline analysis (BATES, WALDREN & TEARE 1973)

Dissolve 1.25 g ninhydrin in 30 ml glacial acetic acid by warming

If cooled, add 20 ml 6 M phosphoric acid

(prepared fresh every day)

Tricine-NaOH buffer for NADP cycling (GIBSON & LARHER, 1997)

Dissolve 1.971g Tricine in 80 ml distilled H₂O

Adjust pH to 8.00 with NaOH

Full up to a volume of 100 ml with distilled H₂O