MOLECULAR AND PHYSIOLOGICAL APPROACH TO DROUGHT AND HEAT TOLERANCE FOR SELECTED CROPS

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Project leader: Anette van der Mescht

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LIST OF ABBREVIATIONS

β	*Beta*
μCi	Micro Curie
^{14}C-protein hydrolysate	^{14}Carbon-protein hydrolysate
^{32}P-dCTP	Phosphorus 2^{1}-Deoxy-cytidine-5^{1}-triphosphate
A. tumefaciens	*Agrobacterium tumefaciens*
ABA	Abscisic acid
AD	Activation domain
ADC	Arginine decarboxylase
ARC	Agricultural Research Council
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	base pair
cDNA	Complimentary deoxyribonucleic acid
CO_2	Carbon dioxide
cpm	counts per minute
Cu/Zn	Copper / Zinc
dATP	2'-Deoxy-adenosine-5'triphosphate
DCIP	2,6 Dichloroindophenol
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-Deoxy-guanosine-5'triphosphate
DIG	Digoxigenin
DNA	Deoxyribo nucleic acid
DNA-BD	DNA - binding domain
DTT	dithiothreitol
dTTP	2'-Deoxy-thymidine-5'-triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid disodium salt
F0	Minimal fluorescence
FAA	Formaldehyde acetic acid

Fe-SOD	Iron - Superoxide dismutase
Fm	Maximal fluorescence
GCA	General combining ability
GR	Glutathione reductase
GUS	Glucoronidase
h^2	heritability
$HCLO_4$	Perchloric acid
hfl	High frequency lysogeny
HMW	High molecular weight
HPLC	High pressure liquid chromatography
HS-like	Heat shock like
HSPs	Heat shock proteins
HSR	Heat shock response
II Fv/Fm	Maximum quantum efficiency
K_2HPO_4	di potassium hydrogen orthophosphate
KCN	Potassium cyanide
LB	Luria-Bertani
LGP	Long growth period
LMP	Low melting point
LMW HSPs	Low molecular weight heat shock proteins
mCi	Milli Curie
MDH	Malate dehydrogenase
MGP	Medium growth period
mg	milligram
min	minute
ml	millilitre
mM	millimolar
Mn-SOD	Manganese - Superoxide dismutase
MPa	Molar Pascal
mRNA	Messenger Ribonucleic acid
MS	Murashige and Skoog nutrient medium

NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide hydrogen phosphate
NaOCl	sodium hypochloride
ODC	Ornithine decarboxylase
PCR	Polymerase chain reaction
PO_4	Phosphate
Poly(A)+RNA	Poly adenosine RNA
PPT	*Phosphinotricin*
PS II	Photo system II
Put	Putrescine
PVPP	Polyvinyl-polypyrrolidone
qQ	Quenching
RIAT	*In vitro* regeneration medium for potato plantlets
RNA	Ribonucleic acid
rpm	revolutions per minute
rRNA	Ribosomal RNA
SCA	Specific combining ability
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate gel electrophoresis
SGP	Short growth period
SOD	Superoxide dismutase
Spd	Spermidine
Spm	Spermine
SSC	Sodium citrate buffer
TCA	Tricarboxylic acid
TE	Tris EDTA
TTC	2,3,5-Triphenyltetrazolium chloride
tRNA	Transfer RNA
v/v	volume/volume
w/v	weight/volume
µl	micro litre

EXECUTIVE SUMMARY

MOLECULAR ASPECTS OF DROUGHT TOLERANCE

In South Africa, where drought is a severe problem, the value of drought tolerance in economic important crops cannot be under estimated. Since most agronomically important plants can only survive limited drought, an understanding of how water stress affects their growth, metabolism, development and yield is of practical value. Losses caused by extended drought can amount to millions of rands. These losses can be divided into direct and indirect losses. Direct losses result from reduced yields while indirect losses include losses from crops not planted, abandonment of land and land use changes following the drought. Agricultural industries absorb the primary losses but eventually the cost is spread over the whole nation. This happens when the government makes relief grants to the agricultural sector and results in higher consumer prices due to shortage of commodities.

Little attention has been paid to plants with a high degree of drought tolerance. For economic reasons, it is important to explore the mechanisms of drought tolerance. The general aim of the project was to identify and characterise the genes, which are involved in drought tolerance in plants, and to transfer such genes to drought sensitive plants. Different approaches were followed with different crops and these are outlined below according to the crops involved.

OBJECTIVES AND MAJOR RESULTS

Tobacco

The original goals were as follows:

- Test proline incorporation into proteins synthesized during osmotic stress in tobacco leaves.
- Determine the efficiency of protein synthesis in tobacco leaves.
- Identify drought-related proteins.
- Screen different tobacco genotypes in South Africa for differences in their genomic DNA's.

- Obtain probes identifying drought stress tolerance characteristics in tobacco genotypes.
- Clone cDNA from mRNA into an appropriate vector.
- Transform tobacco plants to create transgenic drought tolerant plants.

Since drought tolerance is not of major economic importance in the South African tobacco industry, research on tobacco was terminated prematurely and investigations into physiological aspects of drought tolerance in the remaining crops intensified. However, the following experiments were completed:

- Test proline incorporation into protein synthesized during osmotic stress in tobacco leaves.
- Identify drought-related proteins.
- Measure 2,3,5-Triphenyltetrazolium chloride reduction.
- Determine free proline and polyamine levels during drought stress.
- Determine fresh weight and dry weight of leaves and roots during a water gradient method.

From the results it was evident that free proline accumulation titres and the water gradient method could be used as reliable methods for selection of drought tolerance in tobacco.

Potatoes

The original goals were as follows:

- Test proline incorporation into proteins synthesized during osmotic stress in potato leaves.
- Determine the efficiency of protein synthesis in potato leaves and tubers.
- Identify drought stress-associated proteins in tubers.
- Screen different potato genotypes in South Africa for differences in their genomic DNA's.
- Obtain probes identifying drought stress tolerance characteristics in potato genotypes.
- Clone cDNA from mRNA into an appropriate vector.
- Transform potato plants to create transgenic drought tolerant plants.

As a result of the early termination of the research on tobacco, the following experiments were added to the potato research:

- Evaluation of chlorophyll fluorescence as a screening method for drought tolerance.
- Determination of the importance of the enzymes from the antioxidative system during drought tolerance.
- Evaluation of the free proline accumulation and polyamine levels during drought stress.
- The reduction of tetrazolium salt as a possible indicator of drought- and heat tolerance in potato.

The original as well as the new goals were achieved. Firstly, in our attempts to develop a screening method for drought tolerance, we found that Cu/Zn superoxide dismutase (SOD) activity, spermine synthesis and free proline accumulation should be determined to successfully identify drought tolerant potato genotypes. Secondly, the Cu/Zn SOD gene was transferred to the cultivar Aviva via *Agrobacterium tumefaciens* mediated gene transfer. Four transformed lines were evaluated using Cu/Zn superoxide dismutase activity, greenhouse trials and triphenyltetrazolim chloride reduction as indicators of drought tolerance. It was confirmed that the four transformed lines were more drought tolerant compared to Aviva.

Cotton

The original goals were as follows:

- Test proline incorporation into proteins synthesized during osmotic stress in cotton leaves.
- Determine the efficiency of protein synthesis in cotton leaves.
- Identify drought stress associated proteins in leaves.
- Screen different cotton genotypes in South Africa for differences in their genomic DNA's.
- Obtain probes identifying drought stress tolerance characteristics in cotton genotypes.
- Clone cDNA from mRNA into an appropriate vector.
- Transform cotton plants to create transgenic drought tolerant plants.

The additional goals added to the cotton research included:

- Evaluation of chlorophyll fluorescence as a screening method for drought tolerance.
- Determination of the importance of the enzymes from the antioxidative system during drought tolerance.
- Evaluation of free proline accumulation and polyamine levels during drought stress.
- Determination of the importance of the enzymes from the polyamine pathway during drought tolerance.
- Evaluation of the reduction of tetrazolium salt as a possible indicator of drought and heat tolerance in cotton
- Determination of the importance of some dehydrogenases in the reduction of tetrazolium salt.

We showed that 2,3,5,-triphenyltetrazolium chloride reduction (viability assay) is a reliable screening method for drought- as well as heat tolerance in cotton, especially when combined with a study of the dehydrogenases involved in the reduction of the tetrazolium salt. From our results we were not able to identify gene(s) involved in drought tolerance of cotton with the strategy as outlined in the original proposal. Subsequently, the combining ability and heritability of the viability assay were determined. Results from these experiments showed that the trait is successfully transferred to the offspring. Thus viability assays can be incorporated in a breeding program. Additionally we were able to determine the correlation between drought and heat tolerance in the cultivars used. This is of great economic importance in terms of benefits to the breeding program and predicting the optimum locality for a specific cultivar.

Maize

The goals were originally stated as follows:

- Physiological and gene expression responses to water stress in maize would be studied.
- The physiological responses would be determined through the measurement of parameters such as diffusive resistance, transpiration rate, leaf water potential and proline and abscisic acid concentrations.
- The genes involved in the response to water stress would be identified and characterized.

- cDNA would be synthesized from mRNA.
- cDNA would be cloned into an appropriate vector.
- Maize plants would be transformed.
- Transgenic maize plants with increased drought tolerance would be regenerated.

Only one goal was added to the maize research namely:

- Evaluation of 2,3,5-triphenyltetrazolium-chloride reduction as a measure of drought- and heat tolerance in maize inbred lines.

The goals as outlined were achieved. The differential screening isolated nine novel cDNAs that were more abundant in the water stress treatment and three of these were isolated more than once. Two of these genes were sequenced completely. One of them showed characteristics of dehydrins in its derived amino acid sequence, while the other showed homology to mammalian chloride channel proteins. A maize inbred line was transformed successfully with the Cu/Zn superoxide dismutase (SOD) gene using the particle gun method of direct gene transfer. Maize plants were regenerated and plantlets tested positive for the transformation of the SOD gene into the maize genome. As a result of a *Baccilus sp* contamination, the transformed lines could not yet be tested for increased drought tolerance.

CONCLUSION

There are no genes for drought tolerance as such, only genes for traits that contributes to drought tolerance. Thus, the traits involved in drought tolerance offered the opportunity to develop a screening method. From these results it can be defined that the mechanisms of drought and heat tolerance involved a series of anatomical and physiological traits, but that the importance of these traits differs between species and stresses. In tobacco, the proline pathway is crucial in sustaining drought tolerance. A balanced antioxidative enzyme ratio was found to be important in sustaining drought tolerance in potato. The levels of the enzymes involved in the antioxidative pathway; Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase, must all be high to achieve the maximum advantage for the cultivar

during drought tolerance. With cotton it was observed that the dehydrogenases involved in the triphenyltetrazolium chloride reduction assay play a vital role in the drought and heat tolerance mechanisms. It was also observed that proline metabolism cannot be used as an indicator of heat tolerance. The maize study established that the tolerant cultivar responded by growing a greater amount of roots in the deeper, wetter soil and was thus able to maintain a higher transpiration rate for longer than the sensitive cultivar. It is thus evident that physiological and anatomical screening methods can be used in distinguishing between sensitive and tolerance cultivars. Screening for different traits involved in tolerance enhanced our knowledge of the genes contributing to the tolerance of the cultivars tested and is of great economic importance in terms of benefits to the breeding program and predicting the optimum locality for a specific cultivar.

Genetic manipulation enabled us to transfer genes, contributing to traits involved in drought tolerance, to plants. The transformation of potato cultivar Aviva with the Cu/Zn SOD gene, resulted in transformed plants which survived drought conditions in the glasshouse longer than the untransformed plants. Previously, it was believed that as drought is a polygenic trait, plants would not benefit from the transformation of only one gene. The increased copy number of the SOD gene influenced the enzymes and products involved in the antioxidative pathway and resulted in increased drought tolerance in potato. Maize embryos were also successfully transformed with the Cu/Zn SOD gene. Transgenic plants will subsequently be evaluated for increasing drought tolerance.

The transformation of single genes contributing to drought tolerance, is extremely important in existing breeding programmes, as the transfer of drought genes to already valuable cultivars will enhance the breeding process. This enabled us to alter the genepool for drought tolerance resulting in more tolerant cultivars.

RECOMMENDATION FOR FUTURE RESEARCH

Future research will include the increase of the transformed potato lines in field plots. The transformed lines will then be evaluated for drought stress using line source irrigation

experiments, Cu/Zn SOD activity, spermine levels and free proline accumulation. An application for field testing of transformed potato lines was forwarded to the South African Community for Genetic Experimentation (SAGENE).

A maize two - cultivar library has been obtained which uses a different reporter system and it is planned to continue the work of studying the set of genes that have been isolated using differential screening of the maize cDNA.

It is recommended that maize plants be transformed with the isolated gene(s) under the control of a constitutive promoter to obtain over expression. Alternatively, plants can be transformed with the antisense sequence to switch off or decrease the level of expression of the gene. The phenotype resulting from both these strategies could help elucidate the gene function.

It is further recommended that the use of the isolated genes as selectable markers for breeding for drought resistance can be investigated. This can be done by taking several drought tolerant and drought sensitive maize genotypes, digesting their genomic DNA with a selection of restriction endonucleases, running the resulting fragments on a gel and doing a Southern blot, using the isolated cDNAs as probes. If polymorphisms exist that correlate with drought tolerance, then the gene can be used as a selectable marker.

Future work on the maize transformation will be to obtain a new seed batch showing no contamination to *Bacillus sp* and repeat the transformation procedure with the Cu/Zn superoxide dismutase gene. The transformed plants will then be evaluated for drought stress using Cu/Zn superoxide dismutase activity and chlorophyll fluorescence levels.

TECHNOLOGY TRANSFER

Two MSc dissertations and two Ph.D. theses were completed. Fifteen scientific papers were published, three have been submitted and six are in preparation. Thirty papers and nineteen posters were delivered at national and international conferences and workshops.

PUBLICATION LIST FROM DROUGHT RESEARCH

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CHAPTER 1
INTRODUCTION

1.1. EFFECT OF DROUGHT

Drought is a major factor limiting crop production. Drought thus continues to be a challenge to agricultural scientists, not only due to the economic importance of the problem, but also due to the complexity of factors affecting crop response to drought (Ceccarelli and Grando, 1995). Losses caused by extended periods of drought can amount to millions of rands. Direct losses occur as a result of reduction in yield, while indirect losses include crops not planted, abandonment of land and land-use changes following the drought. Agricultural industries absorb primary losses but eventually the entire nation pays. This happens when the government makes relief grants to the agricultural sector and shortage of commodities resulted in higher consumer prices (Quinzenberry, 1982).

The devastating effect of drought on the South African population made newspaper headlines in 1995 (Kleponis, 1995, Anonymous, 1995a, Strachan, 1995 and Anonymous, 1995b). According to the World Bank the world faces a growing water crisis as 80 countries representing 40 percent of the world's population are already experiencing chronic water shortages (Kleponis, 1995). This is especially true for the Northern Province of South Africa where low crop yields have left many people starving (Strachan, 1995). The Economist reported that the United Nations World Food Programme and the Food and Agriculture Organisation predict that Southern Africa will soon make a collective appeal for food aid due to crop failures. But drought is a double-edged threat to African farmers: hunger today versus food aid (wheat surpluses from rich countries) will be followed by low prices for the local farmer's crops tomorrow (Anonymous, 1995a).

From a genetic point of view minimal yield loss associated with drought conditions is a very elusive trait. Cultivars that are successfully grown in one dry year might fail in another dry year as duration, timing and severity of drought varies from year to year. Furthermore drought is seldom the only abiotic stress present; it interacts with other abiotic stresses such as heat and high salinity (Ceccarelli and Grando, 1995). Although some plants are sensitive to drought there are

differences in the degree of sensitivity. Strategies for the selection of drought tolerance in breeding programmes have been developed (Vayda, 1994). However, little success has been achieved through these empirical methods. This may be due to poor understanding of the complex phenomenon of drought resistance, lack of reliable non-destructive screening methods (Bansal, Nagaragan and Sukimaran, 1991) and lack of data on the inheritance of stress tolerance in the plant (Hoogendoorn and Arntzen, 1992).

Richards (1995) stated that it will never be possible to overcome the devastating effects of drought, that progress to improve yield during drought will be slow and that gains will be small. There are no well-documented examples of the release of drought tolerant cultivars bred on the basis of a physiological understanding of plant responses to drought. We nevertheless propose a strategy to evaluate certain physiological processes as possible screening methods for drought tolerance in an attempt to improve yield under drought conditions.

In general, like yield, quality and stress tolerance, there are no gene(s) for drought tolerance as such. Rather, there are genes for traits that contribute to drought tolerance (Ludlow, 1993). The traits involved in drought tolerance may be used in the development of a screening method. This approach is similar to conventional breeding, except that the selection is based on certain traits contributing to drought tolerance rather than for drought tolerance itself (Figure 1). Firstly, potential traits contributing to drought tolerance must be identified. The potential traits to be evaluated as possible screening methods for drought tolerance include: drought-related protein synthesis (Van der Mescht *et. al..*, 1992b), changes in free proline concentrations (Verma, Hu and Delauney, 1993), 2,3,5 Triphenyltetrazolium chloride assays (De Ronde, van der Mescht and Cress, 1995), chlorophyll fluorescence (Ögren, 1990), polyamine concentrations (Slocum Kuar-Sawhney and Galston, 1984) and the levels of copper/zinc (Cu/Zn) superoxide dismutase, glutathione reductase, peroxidase, ascorbate peroxidase and catalase (Bowler, Van Montagu and Inzé, 1992). These five enzymes are involved in the primary defense against reactive oxygen intermediates induced by the metabolic disturbance as a result of physiological stresses. Secondly, the screening method must be able to distinguish reliably between sensitive and tolerant cultivars. Thirdly, the value of the screening method must be proven by correlation with results obtained under field conditions. The ultimate test for any characteristic consists of yield trials under target

conditions, in this case drought stress (Hoogendoorn and Arntzen, 1992). Heritability studies can be combined with the studies on drought markers. The procedure to develop a screening method for drought tolerance is summarized in Figure 1.1.

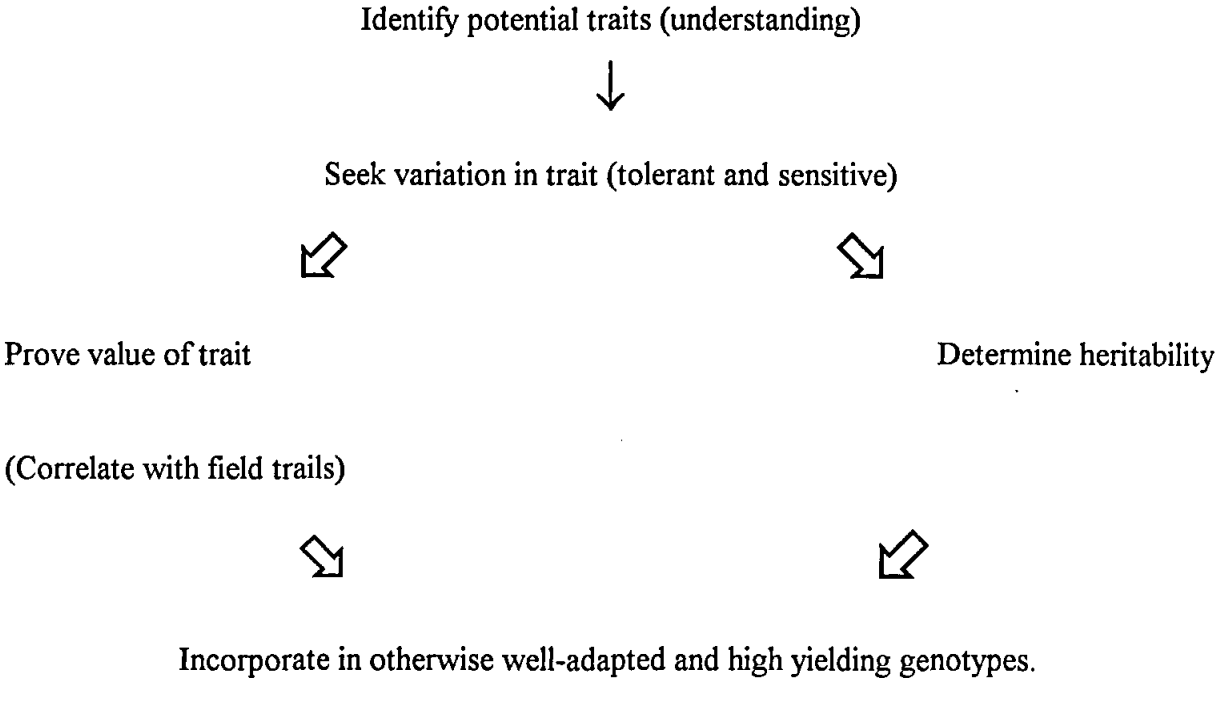


Figure 1.1: Schematic presentation of the procedure to develop a screening method for drought tolerance (Ludlow, 1993)

Although rapid and economical screening methods for drought tolerance have proved to be difficult to develop, such techniques offer great promise for in assisting breeding programmes and incorporating specific drought resistance traits into new varieties. A detailed knowledge of the biochemistry and physiology of the trait may also lead to the identification of genes contributing to drought tolerance. To evaluate the contribution of these specific genes to drought tolerance, the drought-responsive gene of interest must either be transferred into a plant via genetic engineering or isogenic lines differing in this gene must be produced.

The general aim of the project was to identify and characterise the genes, which are involved in drought tolerance in plants, and to transfer such genes to drought sensitive plants. Different approaches were followed with different crops.

1.2. LITERATURE SURVEY

1.2.1. Physiological processes involved

In South Africa, large areas are subjected to poor rainfall distribution and/or low annual rainfall with the result that drought is a major limiting factor to crop production (Mould and Rutherfoord, 1980). Since most plants can only survive a limited period of drought, an understanding of how drought affects their growth, metabolism, development and yield is essential (Bewley, 1979). Weisz, Kaminski and Smilowitz, (1994) suggested that not only limited soil water extraction but also physiological processes related to leaf expansion must contribute to sensitivity to drought.

Besides stomatal closure, the primary effect of drought is the impairment of carbon assimilation. Information on the carbon reduction cycle can be provided by chlorophyll fluorescence as the proton gradient and the redox state of the primary electron acceptor of photosystem II is influenced by the consumption of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide hydrogen phosphate (NADPH) during carbon metabolism (Ögren, 1990). Additionally chlorophyll fluorescence measurements can give a quantitative assessment of inhibition or damage to electron transfer. The misdirection of electrons in the photosystem results in the formation of reactive oxygen species (Bowler *et. al.*, 1992). As drought and other physiological stresses cause oxidative injury, high antioxidant capacity or increased levels of antioxidants can prevent cell damage and may correlate with stress tolerance. However, the mechanisms involved in the minimization of oxidative stress may play a secondary role during drought tolerance and a direct correlation between increased concentrations of an enzyme and drought tolerance may be complicated. Evaluation of changes in the activity of two or more enzymes involved in the reduction of oxidative injury has been suggested (Malan, Greyling and Gressel, 1990). The interaction between the enzymes is of great importance as they have different roles to play during the elevation of oxidative stress e.g. Cu/Zn superoxide dismutase breaks down the superoxide radical to hydrogen peroxide and dioxygen (Van Camp, Willekens, Bowler, Van Montagu, Inzé, Reupoldpopp, Sandermann, and Langebartels, 1994). The hydrogen peroxide is potentially toxic to the cells and is removed by glutathione reductase, ascorbate peroxidase and dehydro ascorbate reductase through the Halliwell-Asada pathway (Bowler *et. al.*, 1992). Optimal enzyme activity may be reduced by a decrease in cell pH. Additionally, (Handa,

Handa, Hasegawa and Bresson, 1986) proposed that proline accumulation could be associated with a change in cytoplasmic pH. This is in agreement with other reports on the physiological functions of proline, namely as a protectant against denaturation of proteins (Schobert and Tschesche, 1978) and in controlling the cell pH, thus reducing acidity (Verbruggen, Villarroel and Van Montagu, 1993). Furthermore, the syntheses of free proline and of polyamines share a biochemical pathway at intermediates, glutamic acid and L-ornithine (Adams and Frank, 1980; Altman, Friedman and Levin, 1982). It is suggested that the role of polyamines be in maintaining the cation-anion balance in the plant cell. The polyamines are protonated at the physiological pH of cells. Thus electrostatic binding of polyamines to negatively charged functional groups of membranes is favored (Slocum *et. al.*, 1984). In binding to the negatively charged phospholipid head groups on membranes, the polyamines influence the stability and permeability characteristics of these membranes, e.g. the loss of chlorophyll from thylakoid membranes is prevented by maintaining membrane integrity, thus stabilizing the photosystem complexes during drought stress (Besford, Richardson, Campos and Tiburcio, 1993).

As a result of these interactions between physiological mechanisms during drought stress it was decided to evaluate stress - related protein synthesis, abscisic acid levels, chlorophyll fluorescence, chlorophyll content, Cu/Zn superoxide dismutase levels, glutathione reductase levels, ascorbate peroxidase levels, free proline concentrations and polyamine titres as possible screening methods for drought tolerance. 2,3,5-Triphenyltetrazolium chloride (TTC) reduction was added to the list at the TTC-assay measures the capability of plant tissue to carry out electron transport (Chen, Shen and Li, 1982).

1.2.2. Screening Methods

1.2.2.1. Stress Related Protein Synthesis

For plants to survive in hot, semi arid conditions, they require the ability to withstand extreme drought and heat stress. Mechanisms to accomplish this include water stress avoidance or drought tolerance. A change in gene expression of tolerant plants results in either alteration of the nucleic acid composition and protein metabolism or both (Valluri, Treat, Newton, Cobb and Soltes, 1988). Changes in protein profiles during drought stress may enhance our understanding of

drought tolerance and may be amenable to genetic manipulation. The few reports showing *de novo* drought stress-related protein synthesis include the plants; maize (Belver and Travis, 1990), barley (Dasgupta and Bewley, 1984), soybean (Sachs and Ho, 1986), pumpkin (Burkhanova, Fedina, Khokhlova, Samokhvalova, Porfirova, Danilova, Levin and Kulaeva, 1988) and slash pine (Valluri *et. al.*, 1988).

Heat shock proteins (HSPs) have been strongly correlated to the development of thermotolerance (Black and Subjeck, 1990). It is widely assumed that the purpose of the heat shock response (HSR) is to protect organisms from the detrimental effects of heat and other stresses (Lindquist, 1986), and in mediating the expression of thermal tolerance to otherwise lethal temperatures (Key, Lin, Ceglarz and Schöffl, 1982). Thermotolerance is defined as the ability of an organism to withstand an otherwise lethal heat treatment when pre-treated with some appropriate non-lethal heat shock (HS) (Nagao, Kimpel, Vierling, and Key, 1986) or an agent which induces a HS-like response (e.g. arsenite) (Key, Nagao, Czarnecka and Gurley, 1987). A pre-conditioning heat treatment can result in increased survival following severe heat treatment (Black and Subjeck, 1990). Thermotolerance appears to be a universal characteristic in all organisms tested (Lindquist and Craig, 1988).

Immediately following an abrupt rise in temperature of 8 to 10°C above the optimum, heat shock proteins (HSPs) are synthesized with a simultaneous decline in the synthesis of the normal complement of cellular proteins (Kimpel and Key, 1985). Plants known to exhibit the heat shock response (HSR) include peas and maize (Vierling, Mishkind, Schmidt and Key, 1986), cotton (Burke, Hatfield, Klein and Muller, 1985) and potatoes (Vratsanos and Rossouw, 1991). The impact of HSPs within organelles is presumed to be associated with their role as molecular chaperones, which are involved in the transport of proteins across organelle membranes (Reading, Halberg and Myers, 1989). Acquisition of thermotolerance in sorghum (Ougham and Stoddart, 1986) and wheat (Krishnan, Nguyen and Burke, 1987) has been reported to be correlated with the high abundance of certain HSPs. In potato, Vratsanos and Rossouw (1991) found that although the low molecular weight heat shock proteins (LMW HSPs) synthesized over 4 hours were different among 3 different cultivars, the amount of high molecular weight heat shock proteins (HMW HSPs) was approximately the same for all cultivars. They stipulated that

genotypic differences among thermotolerant cultivars became apparent only after the HSR had been activated. The daily exposure of cotton cells to a short temperature treatment at 45°C allowed the selection of cells better able to withstand longer exposures to higher temperatures (Trolinder and Shang, 1991).

1.2.2.2. Abscisic Acid

Although the signal detection and the signal transduction pathway are little understood, it is known that an important component of the response is abscisic acid (ABA), which has been called the stress hormone. Its role in stomatal adjustment, for instance, has been known for some time. ABA accumulates in response to cellular desiccation either by its redistribution in the cell or by *de novo* synthesis. Its accumulation has been shown to induce gene expression, and a promoter sequence inducible by ABA has been isolated (Skriver, Olsen, Rogers and Mundy, 1991). Genes inducible by ABA have been termed responsive to abscisic acid (*rab*) genes, and many of these are the same as *lea* genes (Skriver and Mundy, 1990).

Sequence analysis has shown that several of the *lea* and *rab* genes are homologous, and that the conserved domains in the encoded proteins are important in desiccation protection (Dure Iii, Crouch, Harada, Ho, Mundy, Quatrano, Thomas, and Sung, 1989). Conserved domains of other protein products of *rab* genes suggest they can bind to nucleic acids (Mundy and Chua, 1988) and thus may be capable of altering growth and development following stress (Skriver and Mundy, 1990).

Consideration of whether a response is adaptive or desirable depends on its context. For instance, in an ecological context, physiological responses that ensure survival such as stomatal closure would be considered desirable. In an agronomic context, survival alone is not sufficient and maintenance of harvestable yield becomes the desired goal. Over an extended period of drought, stomatal closure will lead to a reduction in yield (Turner, 1979) so the same response in a agronomic context becomes undesirable.

1.2.2.3. Chlorophyll Fluorescence

Photosynthesis is one of the physiological processes in plants, which is severely affected by drought stress. The primary response during drought stress is the increased concentration of abscisic acid which causes the closure of stomatal guard cells to reduce water loss (Bowler *et. al.*, 1992). The rate of photosynthesis declines during drought stress as a result of decreased chloroplast activity, decreasing intercellular CO_2 concentrations and the subsequent impairment of carbon assimilation (Ögren, 1990). What is less well known is which chloroplast activity is the most severely affected by drought (Ögren and Öquist, 1985). However, the solar energy, trapped by photosystem II and photosystem I, is converted to chemical energy through electron transport and carbon assimilation which in turn produce ATP and carbon skeletons for all major metabolic processes (Anderson, Park and Clow, 1997). Chlorophyll fluorescence can provide information on the carbon reduction cycle. The proton gradient and the redox state of the primary electron acceptor of photosystem II (light reaction) is affected by consumption of ATP and NADPH during carbon metabolism (dark reaction). Thus fluorescence yield is influenced by carbon assimilation. On the other hand there are reports on the effect of drought on thylakoïd reactions. Havaux (1992) showed that within the photosynthetic apparatus, photosystem II seems to be heat sensitive, while photosystem I activity, stromal enzymes and the chloroplast envelope are comparatively more thermostable. Changes in leaf water potential and osmotic potential influence the thermal tolerance of photosynthesis (Seemann, Sownston and Berry, 1986). During increased temperatures or sensitivity (caused by drought) the photosystem II reaction centers are blocked followed by a dissociation of antennae pigment protein complexes (Havaux, Ernez and Lannoye, 1988) or a degradation of protein (Dannehl, Herbik and Godde, 1995). This results in a decrease in the amount of photosystem II centers bound to the photosynthetic membrane (Dannehl *et. al.*, 1995).

Chlorophyll *a* fluorescence measurements can give a quantitative assessment of inhibition or damage to electron transfer. The technique is rapid, sensitive, non-destructive, relatively cheap and able to detect injury even before visible symptoms appear (Srinivasan, Takeda and Senboku, 1996). Jefferies (1992) evaluated the effects of drought on chlorophyll fluorescence in potato cultivars, but he did not correlate his findings to variation in drought tolerance or sensitivity.

1.2.2.4. Enzymes from the Antioxidative System in Response to Drought Stress

Drought related responses in plants are of a complex nature and result from genomic re-organization and alterations in gene expression (Edreva, 1992). With the availability of CO_2 for photosynthesis reduced as a result of drought, the misdirecting of electrons in the photosystem leads to the formation of reactive oxygen species (Bowler *et. al.*, 1992). Thus, as drought and other physiological stresses cause oxidative injury, high antioxidant capacity or increased levels of antioxidants can prevent cell damage and may correlate with stress tolerance.

Superoxide dismutase (SOD) is a well described enzymatic antioxidant which breaks down the superoxide radical to hydrogen peroxide and dioxygen (Monk, Fagerstedt and Crawford, 1989); Van Camp, Willekens, H., Bowler, C., Van Montagu, M., Inzé, D., Reupoldpopp, P., Sandermann, H. and Langebartels, 1994). The hydrogen peroxide resulting from this reaction is potentially toxic to cells and is removed by glutathione reductase, dehydro ascorbate reductase and ascorbate peroxidase through the Halliwell-Asada pathway. Glutathione reductase (GR) cooperates with SOD to remove superoxide radicals mainly in chloroplasts but also in the mitochondria and cytoplasm. According to Bowler *et. al.*, (1992) GR has a regulatory function due to the dependence of its activity on the activity on the availability of NADPH. Additionally, this increase in GR enhances nicotinamide adenine dinucleotide phosphate (NADP) availability and electrons can now be accepted from ferredoxin, thereby reducing superoxide formation. Peroxides are also involved in reactions with a number of organic hydro peroxides. The reactions involve the acceptor molecules with simultaneous reduction of the peroxidic substrate namely ascorbate (Larson, 1988). Ascorbate peroxidase activity is mainly found in the chloroplasts (Bowler *et. al.*., 1992).

However, it is possible that mechanisms that reduce oxidative stress may play a secondary role during drought tolerance (Bowler *et. al.*, 1992). This may complicate a direct correlation between the increased concentrations of an enzyme and drought tolerance. Correlations between the simultaneous increase in two or more enzymes involved during the minimization of oxidative injury and known drought tolerance may enhance our understanding of drought tolerance. Malan, Greyling and Gressel (1990) found a correlation between drought tolerance in maize inbreds and

CuZn SOD and GR activities. Increased concentrations of one enzyme alone did not confer drought tolerance.

1.2.2.5. Changes in Free Proline Concentrations and Polyamine Levels During Drought Stress

The accumulation of proline has been reported in many organisms, from bacteria to higher plants in response to environmental stress. The adaptive value of proline accumulation to the plant is still debatable, as the mechanism of action of proline has not yet been fully elucidated (Chrominski, Halls, Weber and Smith, 1989). Additionally the role of proline appears to differ in different species. This leads to differing opinions such as the argument that proline accumulation is beneficial to plants subjected to drought as opposed to the suggestion that proline accumulation is only an indication of damage caused during stress (Van Rensburg and Krüger, 1994a). The possible physiological functions of proline accumulation during drought stress include the maintenance of osmotic potential in response to stress (Bogess, Aspinall and Paleg, 1976), the maintenance of a nitrogen reserve in leaves able to recover after the stress (Tully, Hanson and Nelson, 1979), act as a storage compound for reduced nitrogen and carbon under stress situations (Hsiao, 1973), is a hydroxy-radical scavenger (Verbruggen *et. al.*, 1993), protects against denaturation of proteins (Schobert and Tschesche, 1978), controlling the cell pH thus reducing acidity, has a role in the regulation of cellular redox potentials (Verbruggen *et. al.*, 1993) and is a signal of senescence (Aspinall and Paleg, 1981).

Existing literature indicating the potential value of proline accumulation includes the reported research of Van Rensburg and Krüger (1994a), who showed a correlation between drought tolerance and the leaf water potential level at which proline started to accumulate rapidly in tobacco. Proline accumulation was also positively correlated with drought tolerance in ten barley cultivars (Singh, Aspinall and Paleg, 1972), partial desiccation of celery somatic embryos (Saranga, Rhodes and Janick, 1992) and of two maize cultivars (O'Regan *et. al.*, 1993).

Investigations by Levy (1983) on free proline concentrations in stressed potato tubers showed, paradoxically, a possible correlation between low proline content and drought tolerance in tubers.

Since the metabolic rate is low in storage organs such as tubers, a more active tissue would provide more information on free proline concentrations in potato.

Furthermore, there is increasing interest in the value of polyamines during abiotic stress (Reggiani, Hockkoeppler and Bertani, 1989). The synthesis of free proline and polyamines share a biochemical pathway at intermediates, glutamic - acid and L-ornithine (Adams and Frank, 1980; Altman *et. al.*, 1982).

It is suggested that the role of polyamines is the maintenance of the cation-anion balance in the plant cell. As putrescine, diaminopropane, spermidine and spermine are protonated at the physiological pH of cells, electrostatic binding of polyamines to nucleic acids, negatively charged functional groups of membranes and to proteins is favored (Slocum *et. al.*, 1984). In binding to the negatively charged phospholipid groups on membranes, the polyamines influence the stability and permeability characteristics of these membranes, e.g. the loss of chlorophyll from thylakoid membranes is prevented by maintaining membrane integrity thus stabilizing the photosystem complexes during drought stress (Besford *et. al.*, 1993).

Electrostatic binding of polyamines to nucleic acids is well known. Spermine binds two phosphates on each of the DNA strands, imposing rigidity of structure. Alteration of the interaction between DNA and spermine results in the transformation from the functional B form of DNA to the non-functional Z form. Spermidine is an integral component of some t-RNA's implying a role in the transcription-translation sequence (Galston, 1983). Its possible role may be as a second messenger. Additionally, it is reported that polyamines activate a nuclear protein kinase that phosphorylates non-histone proteins (Galston, 1983). Besford *et. al.* (1993) compared the accumulation of different polyamines during drought stress. They correlated putrescine accumulation in monocotyledons with drought tolerance, while spermine and spermidine accumulation was found in drought tolerant dicotyledons during drought stress.

There are two major pathways of polyamine biosynthesis in plants (Turano and Kramer, 1993). Arginine may be decarboxylated to agmatine, which is then hydrolysed to putrescine via arginine decarboxylase (ADC). Alternatively ornithine may be directly decarboxylated to putrescine by

the enzyme ornithine decarboxylase (ODC). Many studies on ODC and ADC have depended on the release of ^{14}C CO_2 from L-^{14}C ornithine and L-^{14}C arginine and it has been assumed that this could provide a useful measure of activities leading to the formation of putrescine. It was however observed by Birecka *et. al.* (1985) that the total ^{14}C CO_2 production found was much higher than that due to ornithine decarboxylase or arginine decarboxylase activities calculated from labeled putrescine. Thinlayer electrophoresis results in lower activities for ADC and ODC than found with ^{14}C CO_2 (Birecka *et. al.*, 1985). A spectrophotometric method was described for the estimation of ADC (Smith 1979). This is based upon the consecutive oxidation of diamine oxidoreductase through utilization of hydrogen peroxide, which is generated to oxidase guaiacol, in the presence of peroxidase, as a red brown chromogen. It was shown in soybean that the accumulation of polyamines was predominantly due to ADC and not ODC (Turano and Kramer, 1993)

1.2.2.6. 2,3,5-Triphenyltetrazolium Chloride Reduction

Several physiological methods have been developed to measure abiotic stress tolerance in crop plants. These methods include: regrowth (Ishikawa, Robertson and Gustav, 1995), triphenyltetrazolium chloride reduction (TTC) (De Ronde *et. al.*, 1995), vital staining (Chen and Gusta, 1982), protoplasmic streaming (Larcher, 1980), plasmolysis (Palta, Levitt and Stadelmann, 1977), leakage of ions (Sukimaran and Weiser, 1972) and measurement of ultra violet absorbing compounds (Weisz, Kaminski and Smilowitz, 1976). Ishikawa *et. al.* (1995), compared a number of viability assays in an attempt to identify the best ones for estimating freezing, heat and salt tolerance in brome grass. They found that TTC reduction was the most convenient assay whereas regrowth, although time consuming and labor intensive, was the most sensitive and reliable assay.

Although membrane injury as estimated by electrolyte leakage has been found to be unreliable in some cases (Zhang and Scandalios, 1993), TTC tests and conductivity have been successfully used for evaluating heat and cold tolerance in potato. However, TTC reduction was found to be more sensitive than the conductivity test for evaluating heat tolerance (Li, Huner, Toivio - Kinnucan, Chen and Palta, 1981). Vratsanos and Rossouw (1991) also showed a positive correlation between TTC viability assays and heat tolerance in three potato cultivars. De Ronde

and Van der Mescht (1997) expanded the TTC assay to measure drought stress in cotton. A positive correlation between drought tolerance and TTC reduction was shown in six cotton cultivars.

TTC reduction occurs in the mitochondria by the tetrazolium salt accepting electrons from the electron transport chain via the dehydrogenase pathway (Nachlas, Margulies and Seligman, 1960). Plant mitochondria contain a branched mitochondrial electron transport chain where electrons reduce oxygen in a reaction catalyzed by cytochrome oxidase (Whelan, Hugosson, Glaser and Day, 1995). Cytochrome oxidase is at sufficiently high concentration not to be rate -limiting. The limiting enzyme in the sequence of electron transport in this system is known to be succinic dehydrogenase. Succinic dehydrogenase provides plenty of electrons to the electron transport chain and its activity indicates the metabolic state of the organ (Aithal and Ramasarma, 1969). Plants with a tolerance to the environmental stress produce a higher activity of succinic dehydrogenase and have the ability to reduce more of the TTC into formazan.

The nicotinamide adenine dinucleotide phosphate (NADP)- dependent malate dehydrogenase is a key enzyme of the CO_2 fixation pathway. It catalyses the reduction of oxaloacetate to malate by using nicotinamide adenine nucleotide hydrogen phosphate (NAPH) generated by the photosynthetic electron transfer. Malate serves as an intermediate in the tricarboxylic acid (TCA) cycle (Douce and Neuberger, 1989). The exchange of oxaloacetate and malate is a plant specific transport system, which has a high affinity for oxaloacetate but only weakly for malate. The malic dehydrogenase reaction favors malate formation (Douce and 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 motor for guard cells. Alterations in the malate concentration influence the photosynthetic capacity on the one side and stomatal closure on the other (Raschke, 1979). Hedrich *et. al.* (1994), demonstrated that alterations in the $[CO_2]$ of the guard cells which controls stomatal aperture and thus transpiration with respect to the photosynthetic capacity of the leaf modifies 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). It was found that malic acid fluctuation increased with decreasing

PEG water potential below a threshold of -0.1MPA in *Sedum telephium* (Conti and Smirnoff, 1994).

The TTC assay is based on the ability of viable cells to metabolically reduce tetrazolium salts into soluble formazans (Berridge, Tan, McCoy and Wang, 1967). Earlier work of ours had shown that formazan production was relatively lower in stressed leaves of drought sensitive cotton cultivars compared to the leaves of the unstressed control treatment. However, for tolerant cultivars the opposite reaction was observed where the formazan levels were higher in the stress treatment compared to the unstressed control treatment. Similar tendencies were found for drought as well as heat stress (De Ronde *et. al.*, 1995).

This is possible due to the fact that the sensitive clones had inefficient tolerance mechanisms to survive a moderate drought stress and the plant could not adapt to drought stress when a severe stress was applied. The tolerant reaction is the opposite of this. Cultivars had efficient tolerance mechanisms during a moderate stress and also survived better when a severe stress was applied. This resulted in higher formazan production in the stress treatment compared to the control treatment. Thus, the method measures the ability of the plant tissue to adapt to increasing stress conditions (De Ronde *et. al.*, 1995). De Ronde and Van der Mescht (1997) hypothesized that a lower formazan level in the control treatment compared to a stress treatment indicates a drought tolerant reaction. Van der Mescht, De Ronde and Rossouw (1993) showed that drought related protein synthesis is cultivar and organ specific in potato. It is thus important to evaluate the effect of drought stress on different organs.

1.2.3. Enhanced Drought Tolerance in Transgenic Plants Expressing the *Arabidopsis thaliana* Cu/Zn Superoxide Dismutase Gene

All aerobic organisms must possess the means to protect themselves from the toxic effects of reduced oxygen species generated during normal cell metabolic activity or as a result of environmental stresses such as temperature extremes and/or drought. Drought in combination with high light intensities, ambient ozone, sulfur dioxide and some pathogens exacerbates the effect of oxygen radicals. Oxidative injury occurs when oxygen-centered radicals overwhelm the

capacity of cellular antioxidant systems and other oxidants generated within the cell (Sen Gupta, Heinen, Holaday, Burke and Allen, 1993). The mitochondrial electron transport system as well as the electron transport chain of the photosynthetic apparatus within the chloroplasts is well-documented sources of superoxide radicals. Additionally single oxygen can be generated during the transfer of energy from chlorophyll to oxygen. The resulting hydroxyl radicals are among the most reactive species known to chemistry, able to cause lipid peroxidation, the mutation of deoxyribonucleic acid (DNA) and the denaturation of proteins. These molecular reactions in turn have some cellular effects such as membrane damage, loss of organelle function, reduced carbon fixation and electrolyte leakage. The total of cellular effects results in cell death (Bowler *et. al.*, 1992; Scandalios, 1993).

Cells are protected from the deleterious effects of the free oxygen radicals by superoxide dismutase (SOD) which catalyses the initial step in detoxifying activated oxygen species. The superoxide anion radicals are reduced to hydrogen peroxide and molecular oxygen (Zhu and Scandalios, 1994). A positive correlation between enzymes from the antioxidative system and drought tolerance was reported for maize (Malan *et. al.*, 1990), tobacco (Van Rensburg and Krüger, 1994b) and alfalfa (Mc Kersie, Dowley, Harjanto and Leprince, 1996).

As a result, superoxide dismutase (SOD) has become the object of intensive research in physiology, biochemistry and molecular as well as cell biology of plants. The superoxide dismutases are a divergent class of metalloenzymes, which exist as distinct isozymes in different subcellular compartments. The manganese-SOD (Mn-SOD) usually is found within the mitochondrial matrix while the iron-SOD (Fe-SOD) occurs in plastids and the copper/zinc- SOD (Cu/Zn - SOD) is localized in both the cytosol and plastids (Sakamuto, Ohsuga, Wakaura, Mitsukawa, Hibino, Masumura, Sasaki and Tanaka, 1993).

Correlations between elevated SOD activity and stress tolerance suggest that the regulation of SOD levels provides plants with a tolerant mechanism against oxygen toxicity; however, direct proof of this effect is lacking (Perl, Perl-Treves, Galili, Aviv, Shalgi, Malkin and Galum, 1993). A true evaluation of the effects of changing SOD activity in plants might be obtained by genetic engineering (Bowler *et. al.*, 1992). The effect of overproduction of SOD-activity (increased copy

number) or lack of SOD-activity (antisense technology) during drought stress may enhance our understanding of the role of Cu/Zn SOD activity.

Genetic manipulation of SOD in plants was first described for tobacco and tomato. The regenerants overproduced a chloroplastic Cu/Zn SOD derived from petunia. There was no significant difference between either tobacco or tomato plants that produced elevated Cu/Zn SOD and the control plants. The authors concluded that the increased activity of SOD alone in the chloroplasts was not adequate to protect the cells against oxygen toxicity caused by ozone fumigation or the herbicide methyl viologen (Tepperman and Dunsmur, 1990).

Different results were obtained when a chloroplastic Cu/Zn SOD from pea was introduced into tobacco and potato. The transgenic plants were more tolerant when subjected to methyl viologen (paraquat) and the membrane damage measured by electrolyte leakage (Allen, 1995). Additionally tobacco plants that express a chimeric gene that encodes chloroplast-localized Cu/Zn from pea has been shown to be more tolerant to chilling and high light intensity (Sen Gupta *et. al.*, 1993), transgenic tobacco plants that over-expressed mitochondrial Mn SOD as well as a chloroplast-targeted Mn-SOD showed increased resistance to methyl viologen (Bowler *et. al.*, 1991) and transgenic potato plants that expressed tomato Cu/Zn SOD's also have protection form methyl viologen toxicity (Perl *et. al.*, 1993). Transgenic alfalfa (*Medicago sativa*) expressing Mn-SOD were more drought tolerant compared to control plants. A three year field trial showed that yield and survival of transgenic plants were significantly improved, suggesting for the first time that increased tolerance of oxidative stress is also successful in adaption to stressful field environments (Mc Kersie *et. al.*, 1996).

1.2.4. Differential Screening

1.2.4.1. RNA Isolation, cDNA Library Construction and Differential Plaque Cultivarization

There are three techniques commonly employed to investigate differential gene expression. These are differential plaque cultivarization, subtraction cultivarization, and polymerase chain reaction (PCR) differential display. All three techniques detect the increased abundance of messenger

ribonucleic acid (mRNA) transcripts in the treatment of interest, which in this study is the water stress treatment. Differential plaque cultivarization was used in this study, which involves the construction of a complementary dioxyribonucleic acid (cDNA) library of the treatment of interest in a suitable vector and screening the library with labeled cDNA probes to detect differential cultivarization.

Differential plaque cultivarization involves the construction of a cDNA library of the treatment of interest in a suitable vector and screening the library with labeled cDNA probes to detect differential cultivarization. A commonly used vector in this technique is phage λgt10 due to its high transformation efficiency and single *Eco*RI cloning site suitable for selection of recombinants. A high cloning efficiency is required since approximately 2×10^5 recombinants need to be screened to detect low abundance mRNAs (Sambrook, Fritsch, and Maniatis, 1989). Selection of recombinants is facilitated since the *Eco*RI site resides in the repressor (*c*I) gene, which activates the lysogenic growth pathway when plated out on a suitable *Escherichia coli* (*E. coli*) strain. Lysogenic growth results in turbid plaques. When an insert is cloned into the *Eco*RI site the *c*I gene is inactivated and the growth pathway becomes lytic which results in clear plaques. Recombinants can be selected by visualizing the clear plaques that result (Murray, Brammar, and Murray, 1977). The proportion of recombinant phage in the library is further increased on an *E. coli* strain such as NM514 that carries the high frequency lysogeny *(hfl)* mutation. On this strain, phage with an intact *c*I gene lysogenise at such a high frequency that plaque formation is suppressed, while recombinant phage plate normally (Hoyt, Knight, Das, Miller and Echols, 1982).

To detect differences in the abundance of a cDNA species homologous to mRNAs that are differentially expressed, recombinant phage are plated out on *E. coli* NM514 and two replica membrane lifts made of the resulting plaques. Each membrane is probed with labeled cDNA, one from the control treatment and the other from the stress treatment. If a cDNA species is more abundant in the stress probe, it will give a stronger signal when it cultivarizes with the homologous plaque on the replica membrane compared with the replica membrane cultivarized to the control probe.

1.2.4.2. Sequence Analysis of Two of the Drought-related Clones

The paradigm of gene expression is: DNA makes messenger RNA and messenger RNA makes protein. If one wishes to know the function of a gene then one must know the polypeptide for which it codes, since this is the end product. The amino acid sequence of the polypeptide can be derived from the open reading frame of the nucleotide sequence. There are certain gene components, such as the TATA box, promoter regions and introns, which are not part of the mature mRNA molecule, so determining that the open reading frame from the genome sequence is not that straightforward. There are characteristics of the mRNA molecule, such as the start and stop colons, the polyadenylation signals and the lack of introns that enable the open reading frame to be recognized more easily. Since a cDNA library is constructed from mRNA, determining the open reading frame and deriving the amino acid sequence from a cDNA clone is relatively straightforward.

Accessing a large library of sequences that have been derived and stored in computer databases can extend the information contained in the amino acid sequence. Discovering sequence homologies in these databases can offer a quick route to determining the function of a newly sequenced gene. The most widely used algorithm for accessing the nucleotide and amino acid sequence databases is the Basic Local Alignment Search Tool (BLAST) (Altschul, Gish, Miller, Myers and Lipman, 1990).

The BLAST algorithm for searching amino acid sequence homologies is based on a matrix of similarity values between all the possible pairs of amino acids. Identical pairs and pairs with similar characteristics have positive scores, while mismatches have negative scores. The maximal segment pair (MSP) is a score given to identical lengths of the query and subject sequences based on the sum of the matrix values of all pairs in the sequences. The boundaries of the MSP are defined by the maximum MSP value for any length of the two sequences. Comparisons of DNA sequence homologies use the value +5 for identical nucleotide pairs, and -4 for mismatches (Altschul *et. al.*, 1990).

The three dimensional, tertiary structure of a protein is critical to its function. Although there is

a wide range of tertiary structures, this range is based on a remarkably limited variety of secondary structures. This secondary structure is composed of the α-helix, the β-sheet, and the random coil. In a survey of all proteins whose structures were known, about 90% of the amino acid residues were involved in ordered secondary structure, of which more or less equal proportions were in α-helices, β-sheets or random coils (Darby and Creighton, 1993).

The most widely used algorithm for predicting protein secondary structure is that of Chou and Fasman (according to Prevelige and Fasman 1989). The method is based on the correlation between amino acids and the secondary structures in which they are found in proteins of known structure. The secondary structures they investigated were the α-helix, β-sheet, and random coil. This correlation is then used to predict the probability of a particular secondary structure occurring in proteins in which only the amino acid sequence is known. The probability of assuming a particular secondary structure is increased when amino acids that occur preferentially in that structure are contiguous. The Chou-Fasman algorithm therefore calculates a probability of a particular secondary structure based on short lengths of amino acids (Chou and Fasman 1974a, 1974b, 1978a, 1978b).

1.2.4.3. Characterization of Isolated Genes

Drought-related genes have been shown to respond to a range of stimuli, including ABA, turgor loss and osmoticum (for reviews, see Skriver and Mundy, 1990; Bray, 1993; Chandler and Robertson, 1994). It has been suggested, however, that the number of drought related genes that have been isolated might be biased towards ABA-responsive genes because response to ABA has often been the criterion for selection (Gosti, Bertauche, Vartanian and Giraudat, 1995). When the criterion for selection has been a stimulus other than ABA, such as water stress, a large proportion of the isolated genes have not been responsive to exogenously applied ABA (Guerrero, Jones and Mullet, 1990; Yamaguchi-Shinozaki, Koizumi, Urao and Shinozaki, 1992; Gosti *et. al.*, 1995).

Conversely, there can be interaction between the different stimuli. For instance, gene expression is enhanced to a greater extent when ABA is applied exogenously in conjunction with desiccation

than when it is applied alone to unstressed tissue (Cohen and Bray, 1990; Plant, Cohen, Moses and Bray, 1991; Robertson and Chandler, 1992). This observation could be due to the fact that loss of turgor brought about by desiccation leads to a change in the physicochemical properties of the cytosol and in the distribution of ABA within the cell.

The physicochemical properties and the compartmentalization of ABA within the cell is therefore at least as important as absolute levels of ABA (Hartung and Slovik, 1991). It has also been shown that the interaction between different stimuli can be mediated by different regulatory elements within the same promoter, each element responding to a different stimulus and acting synergistically to enhance gene expression (Shen and Ho, 1995).

Although there have been studies that have isolated genes whose expression is enhanced in response to stress, very few of them have investigated the expression in genetically related plants that differ in their stress tolerance. If a response were adaptive, then the gene expression that induces this response would be different between drought sensitive and drought tolerant genotypes.

1.2.4.4. Two-Cultivar Analysis of Water-Stress Induced Genes from Maize

There is a great need for general methods to characterize the proteins that contemporary biology makes available. The list of such proteins needing further characterization is growing and includes proteins already known to be important for specific cellular functions, mutant proteins identified *in vivo* or made *in vitro*, and very large numbers of protein being identified by genome projects. Additionally workers involved in subtraction library studies need methods to help determine functions of proteins which have been isolated using this technique. This is particularly true in studies in which little is know about the genes induced (I.E. studies on the response of plants to environmental stress).

The recent success of two-cultivar systems is due to the fact that proteins that touch one another carry out many cellular functions. For example, the complex process of transcription initiation requires the ordered assembly of numerous interacting transcription factors with RNA polymerase

and ancillary proteins, into a protein machine that initiates transcription (Guarente, 1996; Tjian and Maniatis, 1994). This machine can be viewed as a network of interacting proteins, as can the machines that control other processes, such as DNA replication, and the cell cycle. A full understanding of these processes will require knowledge of, not only the protein (parts) that make up each machine, but also of the topological relationships (connections) that individual parts make with one another.

Likewise, a full understanding of the function of any new protein will require knowledge on the interactions it makes with previously identified proteins. Currently, most new proteins are being identified by large scale sequencing projects. For many of these new proteins the sequence alone sheds little or no light on their function if that function is not already known.

Two-cultivar systems have been used to probe the function of new proteins ever since they were developed (Chien, Bartel, Sternglanz. and Fields, 1991; Fields and Song, 1989). The first application of two-cultivar methods to probe protein function, was to examine the interactions between proteins isolated by two cultivar methods and relatively small numbers of test protein (see for example, Durfee, Becherer, Chen, Yeh, Yang, Kilburn, Lee, and Elledge, 1993; Gyuris, Golemis, Chertkov and Brent, 1993; Harper, Adami, Wei, Keyomarsi and Elledge, 1993; Zervos, Gyuris and Brent, 1993), but this use quickly spread to the analysis of many other proteins (Choi, Satterberg, Lyons and Elion, 1994; Kranz, Satterberg, Lyons and Elion, 1994; Marcus, Polverino, Barr and Wigler, 1994; Printen and Sprague, 1994; Van Aelst, Barr, Marcus, Polverino and Wigler, 1993; Yuan , Stroke and Fields, 1993). In anticipation of the utility of applying these methods to larger sets, researchers have begun devising ways to do so.

1.2.4.4.1. Principle of the two-cultivar assay

The yeast two-cultivar assay is based on the fact that many eukaryotic *trans*-acting transcriptional regulators are composed of physically separable, functionally independent domains. Such regulators often contain a DNA-binding domain (DNA-BD) that binds to a specific promoter sequence and an activation domain (AD) that directs the RNA polymerase II complex to transcribe the gene downstream of the DNA-binding site (Keegan, Gill and Ptashne., 1986; Hope

and Struhl, 1986; Ma and Ptashne, 1987). Both domains are required to activate a gene and, normally (as in the case of the native *E. coli* LexA protein), the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD peptides do not directly interact with each other and thus cannot activate the responsive genes (Ma and Ptashne, 1988; Brent and Ptashne, 1985). However, if the DNA-BD and AD can be brought into close physical proximity in the promoter region, the transcriptional activation function will be restored. In principle, any AD can be paired with any DNA-BD to activate transcription, with the DNA-BD providing the gene specificity (Brent and Ptashne, 1985).

In the LexA Two-Cultivar System, the DNA-BD is provided by the entire prokaryotic LexA protein, which normally functions as a repressor of SOS genes in *E. coli* when it binds to LexA operators (Ebina, Takahara, Kishi and Nakazawa, 1983). (With the promoters used in the two-cultivar system the LexA protein does not act as a repressor.) The AD is an 88-residue acidic *E. coli* peptide (B42) that activates transcription in yeast (Ma and Ptashne, 1987). Two different cloning vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other, and the recombinant cultivar proteins are co-expressed in yeast. An interaction between a target protein (fused to the DNA-BD) and a library-encoded protein (fused to the AD) creates a novel transcriptional activator with binding affinity for LexA operators (Gyuris *et. al.*, 1993). This factor then activates reporter genes having upstream LexA operators and this makes the protein-protein interaction phenotypically detectable. If the two cultivar proteins do not interact with each other, the reporter genes will not be transcribed.

CHAPTER 2

MATERIALS AND METHODS

2.1. PLANT MATERIAL

2.1.1. Potatoes

The potato cultivars with known drought tolerance or susceptibility (Table 2.1) were grown in a greenhouse under conditions as previously described by Van der Mescht, De Ronde and Rossouw, (1992a). Drought shock was induced three weeks after sprout emergence by the withholding of water. The leaf on the third apical node was harvested weekly from drought stressed and non-stressed control plants. The procedure continued for approximately 29 days at which time a lethal drought shock seemed to be induced. Leaf samples were freeze dried immediately after harvesting. Three replicates were analyzed.

2.1.2. Cotton

The cotton cultivars (Table 2.2) were grown in a greenhouse at 29°C/15°C day/night temperatures and were watered three times a week. Drought shock was induced two weeks after emergence by the withholding of water in the greenhouse at 29°C/15°C day/night temperatures. Heat shock was induced two weeks after emergence by growing well-watered plants in a growth cabinet at 40°C/29°C day/night temperatures. Combination stress was induced two weeks after emergence by growing drought treated plants in a growth cabinet at 40°C/29°C day/night temperatures.

Leaf tissue from the apical leaf on the last vegetative branch before the first fruiting branch was harvested daily from stressed and well-watered control plants. Leaf samples were freeze dried immediately after harvesting. The procedure continued for one week after which time leaf tissue was not available. Three replicates were measured.

Table 2.1: The drought response and growth period of 12 potato cultivars. Drought tolerance is defined in terms of yield reduction under dryland conditions (Van der Mescht *et. al.*, 1998a)

Cultivar	Growth period *	Drought response
Raritan	short	sensitive
Vanderplank	short	sensitive
Devlin	short	tolerant
Aviva	short	tolerant
Sebago	medium	sensitive
Ono	medium	sensitive
Darius	medium	tolerant
Baraka	medium	tolerant
Bravo	long	sensitive
Kimberley Choice	long	sensitive
Hoëvelder	long	tolerant
Late Harvest	long	tolerant

* Growth period is measured from emergence to haulm dieback.

Short growth period : ± 80- 90 days

Medium growth period : ± 90-100 days

Long growth period : ± 100-130 days

Table 2.2: The drought and heat response of cotton cultivars according to TTC assays (De Ronde, Van der Mescht, 1997)

Cultivar	Heat response	Drought response
Delta Pine Acala 90	Tolerant	Tolerant
Acala 1517-88	Tolerant	Tolerant
Sicala	Sensitive	Sensitive
Molopo	Tolerant	Sensitive
OR19	Sensitive	Sensitive
Alpha	Tolerant	Tolerant

2.1.3. Tobacco

Seven tobacco cultivars with varying tolerance to drought were used in this study (Table 2.3). The tobacco seeds were sown in seed-trays. After six weeks the seedlings were planted in plastic pots containing a soil mixture of loam: sand: vermiculite (5:2:2). The pots had a diameter of 25 cm and were 30 cm deep. The plants were grown in a greenhouse at 29°C/22°C day/night temperature and watered every day with 100ml tap water.

Drought shock was induced eight weeks after emergence by withholding water. The leaf on the fourth apical node was harvested weekly from drought stressed and watered control plants. Leaf samples were freeze dried immediately after harvesting. The procedure continued for three weeks at which time a lethal drought shock was induced. Six replicates were measured (except for chlorophyll fluorescence where four replicates were used).

Table 2.3: The drought response of tobacco cultivars according to the breeders. (Dr. Van Heerden, Tobacco and Cotton Research Institute)

Cultivar	Type of tobacco	Breeders comment
Elsoma	Oriental	Tolerant
Basma	Oriental	Sensitive
Pobeda	Oriental	Strong grower, frequently used in breeding program
Domkrag	Air dry	Morphologically adapted to drought
CDL 28	Air dry	Sensitive
CDL 3	Air dry	Good quality, planted by farmers
TL33	Oven dry	Similar to CDL 3

2.1.4. Maize

The maize cultivars chosen for the study on gene expression during water stress were; SR52, M37W and M162W and PAN473, P213 and K64R 22. Maize plants were grown in a 1:1 mix of sieved compost and sand to which approximately 20 g l^{-1} of 2:3:2/N:P:K was added. A single mix of soil was used for the experiment. The soil was contained in vertical tubes of PVC, each 10 cm in diameter and 60 cm in depth and cut longitudinally with the two halves reattached with masking tape. Plants were grown in a greenhouse (29°C/15°C day/night) until the onset of pollen shed and were watered three times a week.

The inbred lines chosen for the 2,3,5- triphenyltetrazolium chloride reduction assays were similar to the lines chosen by Malan *et. al.* (1990) (Table 2.4). The inbred lines were evaluated at the ten leaf stages as well as at the onset of pollen shed.

Table 2.4: Summary of maize inbred lines and the drought response according to K^+ leakage (Malan *et. al.*, 1990).

Inbred lines	**Drought rating according to Malan *et. al.*, 1990.**
A281	tolerant
A272	Unknown
M37W	1 (tolerant)
P570	2
21A-6	3
P1	4
I137TN	5
M162W	6
G556-B	7
B73	8
A441-5	9
F2834T	10
Mo17	11
K64R-22	12

2.2. SCREENING METHODS

2.2.1. Drought Related Protein Synthesis: Potato and cotton

Osmotic stress measurements were performed on leaf discs (0.02 g) or tuber slices (0.02 g) that were pre-incubated for 4 h at 25°C in 300 µl 0.2 M sodium hydrogen maleate buffer (pH 6.0) (McKenzie, 1969). Osmotic stress was induced by substituting the pre-incubation medium with a sodium hydrogen maleate buffer containing 0.25 M (-0.62 MPa), 0.5 M (-1.24 MPa) or 1.0 M (-2.48 MPa) mannitol followed by incubation for 3 h at 25°C. The controls did not contain mannitol. Newly synthesized proteins were labeled by the addition of ^{14}C-protein hydrolysate (10 µCi) at the start of the 3 h stress period.

2.2.2. Heat Shock Protein Synthesis: Cotton

Twenty micrograms of leaf tissue from each of six cultivars, Selati, Nik 2, Letaba, Delta Pine Acala 90, Acala 1517-70 and Acala OR3, was pre-incubated at 29°C for 3 hours to overcome the wounding shock and then stressed either at 37°C, 40°C or 43°C for 3 hours. Newly synthesized proteins were labeled during the stress period by the addition of 10µCi ^{14}C protein hydrolysate to the incubation medium.

2.2.3. Protein Extraction and Autoradiography: Potato, cotton and tobacco

De novo polypeptides synthesized during osmotic stress or heat treatments were labeled with 10 µCi ^{14}C-protein hydrolysate. Proteins were extracted, precipitated and fractionated using 12.5% sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) (Van der Mescht *et. al.*, 1992). Gels were fixed and stained in a basic solution containing Coomassie blue and formaldehyde (Steck, Leuthard and Bürk, 1980). The dried gels were exposed to ß-max X-ray films (Amersham) for 6 weeks at -70°C.

2.2.4. Protein-DNA binding study: Potato and cotton

Protein-DNA binding was performed as outlined in Lindeque, Van der Mescht, Slabbert and Henn, (1991). Extracted proteins from leaf tissue were fractionated on 12.5% SDS PAGE and electro-eluted onto Hybond C nitrocellulose membranes. Total DNA was extracted and digested with EcoRI. The multi prime labeling system (Amersham) was used to incorporate phosphorus 2'-deoxy-cytidine-5'-triphosphate (^{32}P-dCTP) into the digested DNA. The labeled DNA was incubated with the nitrocellulose membranes. The membranes were subsequently washed, dried and exposed to X-ray films for 16 hours.

2.2.5. Efficiency of Protein Synthesis: Potato and cotton

The uptake and incorporation of the radioactive precursor was monitored by scintillation counts as described by Valluri *et. al.* (1988). ^{14}C-protein hydrolysate was used instead of ^{35}S-methionine as the drought related polypeptides had been visualized with ^{14}C-protein hydrolysate in a previous study (Van der Mescht *et. al.*, 1992). The efficiency of protein synthesis was calculated as the amount of label incorporated expressed as a percentage of label uptake.

2.2.6. Incorporation of ^{14}C-proline: Tobacco

Leaf samples of the tobacco cultivars were cut into small pieces and pre-incubated for 3 h. The drought stress was applied as described. When the stress treatment was started, 20 mCi ^{14}C proline in different concentrations of mannitol (0.25 M and 0.5 M) were added. The proteins were extracted and fractionated by SDS-PAGE. The radioactive gels were dried and exposed to ß-max-film (Amersham) for 6 weeks at -70°C.

2.2.7. Water Gradient Study: Tobacco

A method based on that of Johnson, Rumbaugh, Willardson, Asay, Rinehart and Aurasteh, (1982) was used in this study. Due to the large surface of tobacco leaves a water sprinkler system was

inappropriate. It had therefore been decided that the plants would be watered in a gradient by hand. Eight-week-old tobacco plants were planted one plant per pot and five plants per row, in a greenhouse under optimum environmental conditions. Five replicates of each cultivar were randomly placed in the greenhouse. The five pots per row received 100 ml, 80 ml, 60 ml, 40 ml and 20 ml water respectively.

2.2.8. Soil Water Potential and Dry Weight Measurement: Maize

The control plants were watered daily to run-off throughout the experiment. The water-stressed plants were watered daily to run-off for the first fifteen days from planting, after which they received no water at all. All plants were harvested 30 days after planting. Ten of the 15 tubes per cultivar per treatment were used to measure leaf and root dry weights, and five tubes were used to determine soil water potential. The plants were grown in a greenhouse where day temperatures varied between 23°C and 35 °C and night temperatures between 12°C and 20°C. The relative humidity varied between 29% and 53%, and PAR was between 600 and 950 μmol m^{-2} s^{-1}.

On harvesting, the aboveground portion of the plants was removed. The tube was then laid on its side, the masking tape cut, and one half of the tube removed. The soil profile was then sectioned into three equal twenty-centimetre depths and the bulk of the roots removed from each section by washing over a sieve. The roots and aboveground portions of the plants were dried in an oven at 80°C for 48 h., weighed separately, and expressed as dry weight of whole plant, and dry weight of root at different soil depths. Percentage water content on a dry weight basis of the separate soil sections in the additional tubes was calculated from the weights before and after drying in an oven at 80°C for 48 h. Soil water potentials were then calculated from a curve of percentage soil water content against soil water potential constructed using a pressure plate apparatus.

2.2.9. Water Relations: Maize

A separate set of plants was used for the physiological measurements of water status and proline and ABA levels. The plants were divided into two treatments; control and water-stress. In the water-stress treatment watering was stopped 44 days after planting. When the plants began to wilt

after a further eight days, watering was resumed until full recovery after 13 days. The controls were watered normally throughout. The pots were weighed daily and the dry weight determined at the end of the experiment to calculate percentage water content of the soil. Bulk soil water potentials were then calculated from the curve of percentage soil water content against soil water potential.

Various measurements were done before, during, and on recovery from the water stress on plants in both treatments. The measurements were of transpiration rate, diffusive resistance, relative water content of the leaf, and leaf proline and ABA concentrations. Transpiration rate and diffusive resistance were measured using a Li-Cor LI-1600 steady state parameter. Measurements were done at the same time each day, at mid morning, on the two youngest fully expanded leaves of a plant. These were the sixth and seventh leaves to emerge. Measurements were done on both sides of the leaf. To estimate transpiration rate, the values of both surfaces were added. The data for diffusive resistance are presented for the abaxial surface only. The mean of these values was calculated for each plant. Each day, five separate plants per cultivar per treatment were measured in this way, and the mean of the means calculated.

Relative water content was measured by the method of Barrs and Weatherley (1962). Relative water content was calculated by the formula ((fresh weight-dry weight)/(turgid weight-dry weight)) x100. The mean of these values per plant was calculated, and the mean of the means of five plants per treatment per day calculated.

2.2.10. Proline and Abscisic Acid: Maize

For determining proline and ABA concentrations, the sixth and seventh leaves to emerge were excised, the midrib removed, and the two halves of the lamina weighed, placed in liquid nitrogen and stored separately at -70°C. The two halves were used to determine the proline and ABA concentrations.

Proline concentration was determined by homogenising the plant material in an extraction medium of 3% aqueous sulfosalicylic acid and measuring the absorbence at 520 nm by the ninhydrin

colorimetric procedure (Singh, Paleg and Aspinall, 1973). Measurements were done on five plants per cultivar per treatment on six different days. ABA concentration was determined by the method of Hubick and Reid (1980).

2.2.11. Chlorophyll Fluorescence: Potato, cotton and tobacco

Chlorophyll fluorescence measurements were taken weekly using a pulse - amplitude modulated fluorometer (PAM 101-103; H. Walz, Effeltrich, F.R.G. attached to a strip chart recorder (Pederson). Dark-adapted leaves, achieved by covering the leaf with a black plastic bag for 20 minutes, were subjected to various light intensities. The minimal fluorescence level (F0), was taken as the average signal during the first 3 seconds after the measuring light of 3 000 microeinsteins m^{-2} sec^{-2} was switched on. A saturation pulse of 5 000 microeinsteins m^{-2}. sec^{-2} was applied for 3 seconds to induce the maximal fluorescence level (Fm). This was followed by a light intensity of 1 000 microeinsteins m^{-2}.sec^{-2} until the fluorescence curve stabilised (Van der Mescht, De Ronde, Van der Merwe, Laurie, Bester and Wentzel, 1997).

2.2.12. Leaf Water Potential: Potato and cotton

Leaf water potential was monitored at weekly intervals of all plants subjected to drought and the respective controls to monitor the drought stress. A pressure chamber, PMS-instrument, Oregon, U.S.A. was used for measurements (Van Rensburg and Krüger, 1994b).

2.2.13 Cu/Zn Superoxide Dismutase, Glutathione Reductase and Ascorbate Peroxidase Levels: Potato and cotton

Enzyme extractions were performed as described by Malan *et. al.* (1990) with minor modifications. Leaf tissue (100 mg) was homogenised in 2,5 ml 0,1M potassium phosphate extraction buffer (pH 7,5) containing 0,1 mM EDTA, 200 mg polyvinylpyrrolidone and 1% w/v bovine serum albumin. The β-mercaptoethanol was omitted as this result in the inhibition of glutathione reductase (GR) activity (Mahan and Burke, 1987). Extracts were centrifuged at

13000g for 30 minutes. SOD and GR activity in the supernatant were spectrophotometrically determined. The level of GR activity was measured by following the oxidation of NADPH spectrophotometrically at 340 nm (Carlberb and Mannervik, 1985). SOD was calculated after measuring the inhibition of nitrate formation from hydroxyl ammonium chloride oxidation at 530 nm (Elstner and Heupel, 1976). Enzyme activities were expressed as changes in absorbance min^{-1} g^{-1} dry weight. Ascorbate peroxidase activity was measured at 265 nm (Dalton, Russell, Hanus, Pascoe and Evans, 1986). The procedure is based on the rate of decrease in absorbance of ascorbate during ascorbate peroxidation.

2.2.14. Free Proline Determination: Potato, cotton and tobacco

The method of Bates, Waldren and Teare (1973) was used with minor modifications. Freeze-dried leaf samples (0,1g) was homogenized in 10 ml of a 3 % sulfosalicylic acid solution. The homogenate was filtered through Whatman # 2 filter paper using a buchner funnel and vacuum pump. The reaction mixture containing 2 ml filtrate, 2 ml acid ninhydrin and 2 ml glacial acetic acid was incubated in test tubes for 1 hour at 100°C. The reaction was terminated on ice. Free proline was extracted by the addition of 4 ml toluene. The reaction mixture was mixed vigorously with a vortex for approximately 15 seconds and allowed to warm to room temperature. Absorbance of the chromophore containing toluene phase was read at 520 nm. The concentration of free proline was calculated from a standard curve using the following equation: [(µg proline/ml x ml toluene)/115,5 µg/µmole]/[(g sample)/5] = µmoles proline/g dry weight.

2.2.15. Polyamine Analysis: Potato, cotton and tobacco

The HPLC-method as developed by Flores and Galston (1982) was used with minor modifications, as the concentration of different polyamines can be determined at the same time. Polyamines were extracted from 0.1 g freeze-dried leaves at 4°C. Samples were homogenized in 5ml of 5% perchloric acid and incubated for 1 hour on ice. After incubation, the samples were centrifuged at 14 000 r.p.m. for 20 minutes at 4°C. An aliquot of 500µl of the supernatant was added to 1 ml 2 M sodium hydroxide and 10 µl benzoyl chloride. The mixture was incubated for 20 minutes at room temperature, 2 ml of saturated NaCl was added and the benzoylated

polyamines were extracted in 2 ml diethyl ether. The mixture was centrifuged at 14 000 r.p.m. at 4°C for 5 minutes and 1 ml of the ether phase was collected, evaporated to dryness and redissolved in 1 ml methanol. The samples were HPLC analysed at a flow rate of 1ml/minute. Separation was achieved using a step gradient elution in a methanol: water solvent system and a high resolution reversed phase C18 column, (250 mm x 4.6 mm id) packed with 5μm size silica particles. The eluate was monitored by UV detection at an absorbance wavelength of 254nm to determine the titres of putrescine, diaminopropane, spermidine, spermine and agmatine.

2.2.16. Analysis of Polyamine Biosynthetic Enzymes

2.2.16.1. Enzyme Assays for ADC and ODC: Cotton

Modifications of the assay of Beranger-Novat, Monin and Martin-Tanguy (1994) and Altman, Friedman and Levin (1982) were followed. Two hundred μl extracted enzyme was mixed with 300 μl of K_2HPO_4 10 mM pH7.2, EDTA 20 mM, pyridoxal phosphate 1mM, DTT 0.1 mM and 100 μl 59 mM ornithine (ODC assay) or 20 mM arginine (ADC assay). The reaction was incubated for 1h at 36°C. Termination occurred with the injection of 0.2 ml 8% (v/v) $HCLO_4$ into the reaction mixture.

2.2.16.2. Method for Estimation for ADC and ODC: Cotton

SDS PAGE (Flores, Young and Galston, 1985), thin layer chromatography (Galiba, Kocsy, Kaur-Sawhney, Sutka and Galston, 1993), thin layer electrophoresis (Beranger-Novat, Monin and Martin-Tanguy, 1994), spectrophotometric method (Smith, 1979) and ^{14}C release (Altman *et. al.*, 1982) were tested. Aliquots of 10 μl were analysed on TLC precoated 20 cm^2 silica 60 plates. The TLC plates were run in acetic acid/ pyridine/ water (5:1:94) (Beranger-Novat *et. al.*, 1994). Unlabelled agmatine and putrescine were also spotted on the plates and developed with ninhydrin (5% w/v in ethanol). The silica spots were scraped off, redissolved and measured spectrophotometrically. Enzyme analyses were performed as described above and spectrophotometric measurements done at 430 nm. The ADC and ODC levels were calculated per μg protein.

2.2.17. 2,3,5-Triphenyltetrazolium Chloride Reduction: Potato, cotton, tobacco and maize

The accumulation of formazan was measured as described by Chen *et. al.* (1982) with minor modifications. Leave discs (7 mm in diameter) or tuber slices (4 mm in diameter with a 2 mm width) from fresh tubers of the control treatments were subjected to a control treatment of three hours in 3 ml of 0.2 M sodium phosphate buffer (McKenzie, 1969) and a moderate stress of three hours in 3 ml 0.5 M mannitol (-1,24 MPa) as an osmoticum (Van der Mescht *et. al.*, 1992) for acclimation, before incubation of both samples in 3 ml 1.0 M mannitol .(-2,48 MPa) solution and sampled over a period of 120 minutes. The experiment was performed at 29°C. The leaf discs were submerged in 3 ml of 0.8 % (w/v) TTC solution in 0,2 M sodium hydrogen maleate buffer, pH 6.9 (De Ronde *et. al.*, 1995). The moderate heat stress was induced by incubating the stress treatment at 37°C for 3 hours before the lethal stress was induced at 45°C (Zhang *et. al.*, 1993).

The discs were vacuum infiltrated for 5 minutes to ensure solution penetration into the tissue prior to a 18 hour incubation at 29°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 till dry and resuspended in 3 ml 95% ethanol when cooled. The formazan accumulation was measured spectrophotometrically at 485 mm as described by De Ronde, Van der Mescht and Cress (1995). Five replicates were measured.

2.2.18. Succinic Dehydrogenase and Malic Dehydrogenase Enzyme Assays: Cotton

The freeze-dried leaf tissue of drought treated cotton plants (0.01 g) was homogenized with liquid nitrogen and 1 ml extraction buffer containing 0.1% PVPP and 0.05 M PO_4 buffer (Kavi Kishor and Mehta, 1988). The procedure of Owen and Freer (1970) was followed with minor modifications for the assay of succinate dehydrogenase (SDH). The test cuvette contained 0.02 ml 2.5 mM DCIP (terminal electron acceptor), 0.1 ml 1 mM KCN (inhibitor of the terminal oxidase), 0.2 ml 20 mM disodium succinate (substrate) and 0.65 ml 0.1 M PO_4 buffer (pH 7.2).

Ten µl of the sample extract was added. The reaction was started with the addition of 0.01 ml PMS 15 mg/ml (intermedium electron acceptor). The decrease in absorbance at 600 nm was recorded over one minute.

Malate dehydrogenase (MDH) activity was assayed by following the decrease of absorbance at 340 nm at 30°C over one minute (Issakidis, Miginiac-Maslow, Decottignies, Jacquot, Cretin and Gadal, 1992). The test cuvette contained 750 µM oxaloacetate, 100 mM Tris/HCl pH 7.9 and 140 µM NADH. The reaction was started with the addition of 10 µl sample extract.

2.3. TRANSFORMATION: POTATO

2.3.1. Maintenance of *In Vitro* Plantlets

Aviva plantlets were obtained from the Potato gene bank (ARC-Roodeplaat) and were multiplied on Murashige and Skoog nutrient medium (MS stocks 1-6, 20 g/l sucrose, 7.5 g/l agar, pH 5.8) in large bottles. Plant cultures were incubated in a growth room set at 26°C and a photoperiod of 16 hours light/8 hours dark.

2.3.2. Leaf Disc Regeneration

Leaves were excised from 4-5 week old *in vitro* plantlets, the apical and basal 3 mm were cut off, and the leaves were placed abaxial side down on the medium which had been poured into petri dishes. Twenty-five leaves were used, leaf explants were subcultured onto fresh medium once a week and incubated in a growth room set at 26°C with a photoperiod of 16 hours light/8 hours dark. The RIAT two-step regeneration procedure was used.

2.3.3. Kanamycin Tolerance Experiments

In order to determine the optimal kanamycin concentration for selection of transformed cells, Aviva leaf explants were incubated on regeneration media containing various levels of kanamycin (0 mg/l; 25 mg/l; 50 mg/l; 75 mg/l and 100 mg/l). Regeneration on medium containing 250 ml/l cefotaxime (the antibiotic added in order to control the *Agrobacterium tumefaciens* growth after transformation) was also evaluated. Results were taken after seven weeks.

2.3.4. Cloning of *Arabidopsis thaliana* Cu/Zn SOD cDNA Into Transformation Vectors

The plasmid pcSODRH consists of a 788-bp cDNA clone of a cytosolic Cu/Zn SOD from *Arabidopsis thaliana* in the Eco R1 site of p Bluescript (SK^+). The insert consists of a full coding

sequence and 112 bp 5' and 206 bp 3' - untranslated region + 14 bases of poly A tail as previously described by Hinges and Slusarenko (1992). The pcSODRH was first restricted with *Sac I* and, after precipitation with *Eco RV* to yield the SOD insert (0.8kb). The pBI 221 plasmid (5.7-kb) was first restricted with the *Sac I* restriction enzyme and then with *Sma I* to yield the pBI 221 Vector (3.8kb) (Figure 2.1).

After the ligation (1 insert: 1 vector), a transformation experiment was performed according to Chung and Miller (1988). The successful transformation of DH5∝ yielded ampicillin resistant colonies. Plasmid DNA was extracted using the JAT preparation. Additionally a *Pst I* digestion of the uncut pBI 221 and transformed DH5∝ cells yielded two bands of 1.6 kb and 2.8 kb. This confirmed positively transformed DH5∝ cells with the SOD gene as the pBI SOD plasmid (4,6 kb) was first restricted with *Sac* I and after precipitation with *Hind* III to yield the SOD insert (1,6 kb). The pBI 121 plasmid was first restricted with the *Sac* I restriction enzyme and then with *Hind* III to yield the pBI 121 vector (10.3 kb). Ligation and transformation yielded the pBI 121 SOD Vector (Figure 2.2).

2.3.5. Growth of *Agrobacterium tumefaciens*

An aliquot of frozen ***Agrobacterium tumefaciens*** ***(A. tumefaciens)*** LBA 4404 cells, into which the pSOD plasmid had been inserted by triparental mating (Armitage, 1988) was grown in YM medium (GIBCO BRL) supplemented with 50 µg/ml kanamycin and 100 µg/ml rifampicin on a shaker at 28°C for 36 hrs.
Before plant transformation, the bacterial cells were pelleted by centrifugation (3300 rpm, 25 min, 4°C) and resuspended in YM medium only.

2.3.6. Leaf Disk Transformation and Regeneration.

Potato leaf disks were pre-incubated on MS plates for 48 hours. Following this, leaves were immersed in *A. tumefaciens* cell suspensions for various time periods, blotted on sterile filter paper and replated onto the MS plates for two days of cocultivation, as had previously been determined for potatoes (Murray *et. al.*, 1998).

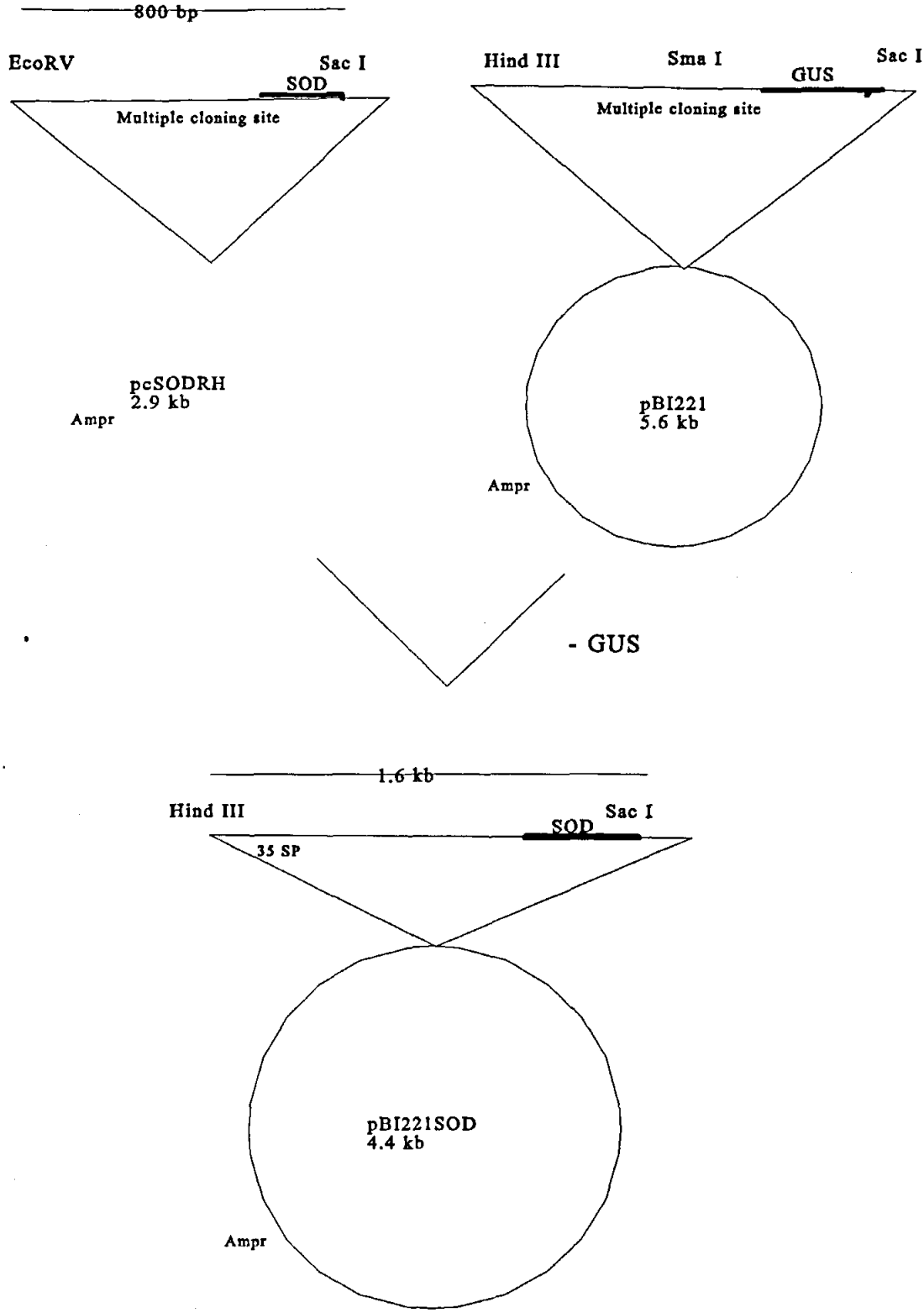


Figure 2.1: Schematic representation of the ligation of the insert (800bp), from the pcSODRH plasmid, with the vector (3.8bp), from the pBI 221 plasmid, to produce the pBI 221 SOD plasmid.

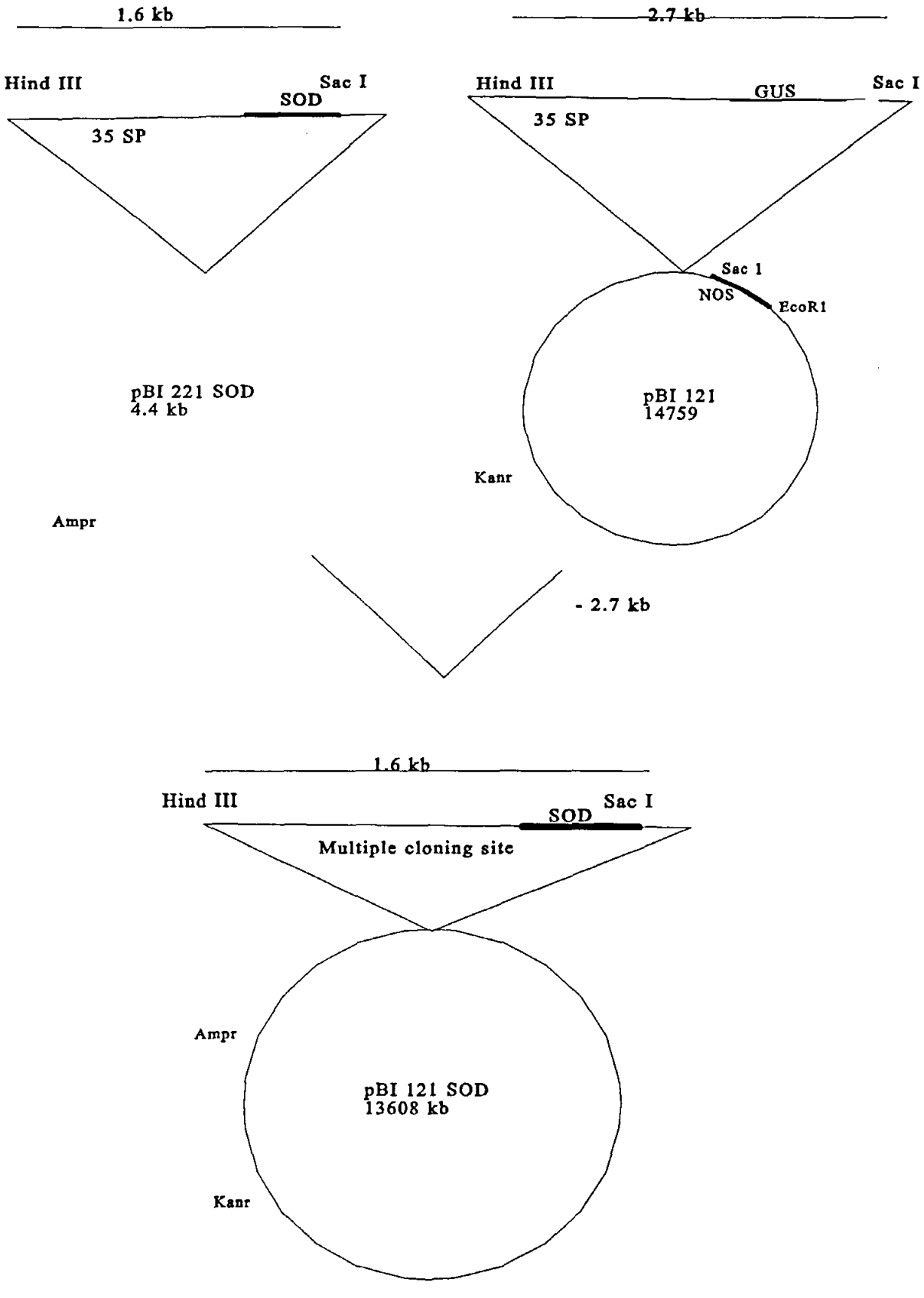


Figure 2.2: Schematic representation of the ligation of the insert (1.6 kb), from the pBI 221 plasmid, with the vector (12 kb), from the pBI 121 plasmid, to produce the pBI 121 SOD plasmid.

Following co-cultivation, explants were transferred to the RIAT two-step indirect regeneration medium. Leaf explants were first incubated on RIAT medium until callus production could be seen on the cut edges of the leaf. Explants were subsequently transferred to RIAT medium with the auxin component (NAA) removed (designated RIAT-).

2.3.7. Molecular Confirmation of Transformed Potatoes

DNA was extracted from seven putative transgenic tissue culture Aviva plantlets containing the SOD gene as well as a control Aviva plant. The method of Ish-Horowicz and Burke (1981) was used for the isolation of the SOD plasmid DNA. The DNA was quantified with the use of a fluorometer. Electrophoresis of isolated DNA was performed on a 1% agarose gel. In order to verify that transgenes had been integrated into the potato genome, PCR analysis was performed using specific primers. The SOD and nptII primers were used. The PCR products were determined on a 1% agarose gel and visualised with ethidium bromide.

Southern blot analysis was performed with SOD as well as ntpII probes which were DIG labelled according to the manufacturers procedure using Boehringer Mannheim's DIG labelling and detection kit. The DNA was digested with HindIII and EcoRI.

2.3.8. Hardening off and Drought Stress of Transformed Plants

Four transgenic (containing the SOD gene) and one control (untransformed) tissue culture Aviva plantlets were hardened off (Murray *et. al.*, 1998). The potatoes were grown in a greenhouse under conditions as previously described by Van der Mescht *et. al.* (1992). Drought shock was induced six weeks after introduction to the greenhouse by withholding water. The leaf on the third apical node was harvested weekly from drought stressed and non-stressed control plants. Three replicates were harvested. Leaf samples were freeze dried immediately after harvesting. The procedure continued for six weeks at which time a lethal drought shock was induced to the transgenic plants. The control (untransformed) plants died after four weeks without water.

2.4. DIFFERENTIAL SCREENING: MAIZE

2.4.1. RNA Isolations and cDNA Synthesis

Maize plants were grown until the onset of pollen maturation when they were divided into two treatments, control and water stress. In the control treatment the plants were watered normally, and in the water stress treatment water was withheld and the plants subjected to a drying period lasting ten days. This method was used to imitate as much as possible the conditions that would prevail in the field. At the point in the stress period when the plants were judged to be at maximum stress, that is when the leaves were still rolled the next morning, leaf samples were removed from the plants. The first two fully expanded leaves were used per plant, and five plants per treatment. The midrib was excised, and only the lamina used for RNA extraction. All leaf samples were pooled and subdivided into ten-gram subsamples, frozen in liquid nitrogen and stored at -70°C. Leaf samples were removed from both control and water stressed plants at the same time.

Total RNA was extracted from each 10 g subsample by the phenol/sodium dodecyl sulphate (SDS) method, followed by two LiCl precipitations to remove contaminating DNA (Chirgwin, Przbyla, MacDonald and Rutter, 1979; Ausubel, Brent, Kingston, Moore, Seidman, Smith and Struhl, 1989). Ten µl total RNA was diluted in one ml water and the absorbance were measured at 230 nm, 260 nm, 280 nm and 320 nm using water as a blank to estimate quantity and purity of poly $(A)^+$ RNA. One µg total RNA was run on a 1% formaldehyde denaturing gel to determine the integrity of the RNA.

Poly adenosine ribonucleic acid (poly $(A)^+$ RNA) was isolated from the total RNA following the method of Ausubel *et. al.* (1989). Two µl of poly $(A)^+$ RNA was diluted in 100 µl TE buffer and the absorbence at 230 nm, 260 nm, 280 nm and 320 nm measured using TE buffer as a blank to estimate quantity and purity. One µg of the poly $(A)^+$ RNA was run on a 1% formaldehyde denaturing gel to determine the integrity.

cDNA was synthesised using the Amersham cDNA Synthesis System Plus (RPN 1256) following the protocol supplied with the kit. To estimate the quantity, 1 µl cDNA solution was diluted in 2 ml 1 x TNE buffer/Hoechst 33258 (bisbenzimid) and read on a Hoefer TKO 100 fluorometer. One hundred ng calf thymus DNA was used as a standard. Five hundred ng of the cDNA was run on a 1% agarose gel to determine the size range.

2.4.2. cDNA Probe Synthesis and Plaque isolation

cDNA from the water stress treatment only, of both cultivars PAN473 and SR52, was cloned into the EcoR1 site of bacteriophage λgt10 using the Amersham cDNA Cloning System (RPN 1257) following the protocol supplied with the kit. One µg cDNA was blunt-end ligated to adapters containing one blunt end and one *Eco* RI end, converting the cDNA to *Eco* RI ends. The adapters lacked 5' phosphates so that self-ligation of adapters could not occur.

Separation of unreacted adapters and size enrichment of cDNA >500 bp was done by column purification. The "adapted" cDNA ends were then phosphorylated by incubation with 40 units T4 polynucleotide kinase in a reaction volume of 400 µl at 37°C for 30 minutes. The reaction mix was extracted twice with an equal volume of phenol:chloroform 1:1 and twice with an equal volume chloroform:isoamyl alcohol 24:1. The cDNA was ethanol precipitated with sodium acetate at -20°C overnight. The concentration of cDNA was estimated by diluting a 2 µl subsample in 2 ml 1 x TNE buffer/Hoechst 33258 (bisbenzimid) and reading on a Hoefer TKO 100 fluorometer. One-hundred ng calf thymus DNA was used as a standard.

The cDNA was then ligated to λgt10 vector arms using 2.5 units T4 DNA ligase in a reaction volume of 10 µl and incubating at 15°C for 16 hours. The reaction was optimised for the quantity of insert cDNA by doing three different ligation reactions with 50 ng, 100 ng and 150 ng cDNA. In addition, *Eco*RI-ended, dephosphorylated λgt10 arms were ligated without any insert as a control reaction to estimate the level of background self-ligation. The arms should not self-ligate since they have been dephosphorylated. The λgt10 was then packaged and stored in 500 µl SM buffer at 4°C.

Escherichia coli strain NM514 was infected with the packaged phage plated out on L-agar plates, and the titre in plaque forming units (pfu) μg λgt10 arms^{-1} counted. Individual plaques were cored out and stored in 500 μl SM buffer. The insert was amplified by PCR using primers complementary to the *Eco*R1 site flanking regions of λgt10. The total reaction mix was run on a 1% agarose gel to determine the size range of the inserts and to confirm that insertion had taken place successfully. The λgt10 water stress library of each maize cultivar was plated out at a density of less than 1 000 plaques per plate. Replica membrane lifts were made of each plate using Amersham Hybond N+ nylon membranes.

Radio labelled probes were generated from the cDNA of both the control and water stress treatments of both maize cultivars. Ten μg of cDNA was boiled for 2 minutes with random hexanucleotides in reaction buffer (Boehringer Mannheim) and snap cooled on ice. 2'-Deoxy-adenosine-5'-triphosphate (dATP), 2'-deoxy-guanosine-5'-triphosphate (dGTP) and 2'-deoxy-thymidine-5'-triphosphate (dTTP) were added to a final concentration of 20 μM with 250 μCi [^{32}P]-labelled 2'-deoxy-cytidine-5'-triphosphate dCTP. Fifty units of Klenow enzyme (Boehringer Mannheim) were added and the reaction volume made up to 500 μl and incubated at 37°C for one hour. The reaction mix was passed through a Sephadex G50 column with TE buffer as carrier in 200 μl aliquots to separate out the unincorporated [^{32}P]-labelled dCTP. The radioactivity of each aliquot was measured by Cerenkov counting on a LKB1219 Rackbeta scintillation counter and the aliquots containing the incorporated radiolabel were pooled and used for cultivarization. The percentage incorporation could be calculated from: (counts per minute (cpm) of the aliquots containing the incorporated radiolabel/total cpm) x 100.

Probe cultivarization to replica membranes was performed overnight at 68°C. The one replica membrane was probed with radiolabelled cDNA from the control treatment, and the other with radiolabelled cDNA from the water stress treatment. Those plaques producing a stronger signal on the autoradiograph of the water stress probe compared with the control probe represent those mRNAs that are in greater abundance in the water stress treatment. These plaques were located on the original plate by aligning the autoradiograph with the marks, and then aligning the signal spot with the original plaque. The plaque was cored out and stored in 500 μl sulphate magnesium (SM) buffer at 4°C. A second round of differential screening was done on these isolated plaques

to confirm the first differential screening and to remove any false positives. Replica membrane lifts were made of each plate and probed with [^{32}P]-labelled cDNA from the control and water stress treatments. Those plaques producing a stronger signal on the autoradiograph of the water stress probe were cored out and stored in 500 µl SM buffer at 4°C.

To determine the number of different species of cDNA insert represented by the isolated plaques, each plaque isolate was used to probe all the positives. Probe was synthesised from the isolated λgt10 clones by PCR using primers complementary to the flanking sequences of the *Eco*RI insertion site in λgt10. A radio labelled probe was synthesised from the redissolved DNA.

2.4.3. Sequence Analysis of Two of the Drought-related Clones

2.4.3.1. Clone Selection

Several of the isolated clones cross cultivarized. It is possible that these clones were not identical, but only shared sequence homologies. In order to confirm their identity, the insert from each clone was amplified by PCR and run on a gel. The longest fragment within each cDNA species was sequenced fully, and the remaining clones within each species sequenced at the 5' end only and compared to the fully sequenced clone. This was done with the two cDNA species that contained the most individual clones. The remaining seven clones were sequenced once only at their 5' ends. Due to time considerations, only those clones isolated from maize cultivar PAN473 were sequenced. Clones *rws7* and *rws16* were sequenced along their entire length by primer walking.

2.4.3.2. Sequencing

Sequencing was done using the Sequenase sequencing kit (Amersham). The cDNA insert in each clone was amplified straight from the phage by PCR using forward and reverse primers complementary to the flanking sequence of the *Eco*R1 site in phage λ gt10. The whole sequence was read by primer walking. A new primer complementary to a 16-mer section of the previous run, at least 50 bp from the last base, was designed. Synthesis of the primer was done at the Department of Molecular Virology, University of Natal Medical School, Durban. Once the sequence was read all the way to the poly(A) tract, a reverse primer could then be designed and

synthesised to read the reverse sequence. The sequence was read in both directions for confirmation.

2.4.3.3. Sequence Analysis

Nucleotide and amino acid sequence analysis was done using the computer program Genepro, and the database search for homologous amino acid sequences was done by using the BLAST search tool (Altschul *et. al.*, 1990).

2.4.4. Characterisation of Isolated Genes

2.4.4.1. RNA Slot Blots

Thirty µl (3 µg) poly(A)+ RNA was incubated at 65°C for five minutes in three volumes of denaturing solution. The solution was snap cooled on ice and one volume of 20 x SSC added. Each sample (total volume 240 µl) was blotted onto a Hybond-N+ nylon membrane, prewetted with 10 x SSC, using a commercial slot blot filtration manifold. The RNA was fixed to the membranes by baking at 80°C for two hours.

2.4.4.2. Probe Synthesis and cultivarization

Radio labelled probe was synthesised from 100 ng of the PCR product of the λgt10 clone by random-primed labelling with Klenow enzyme as previously described. Radio labelled probe was synthesised from *rws7*, *rws16* and *rws5* to a specific activity greater than 1 x 10^6 cpm μg^{-1}. The membranes were cultivarized with [^{32}P]-labelled probes of *rws7*, *rws16* and *rws5*, washed, and exposed to X-ray film at -70°C for four to seven days.

2.4.4.3. Poly(A)+ RNA Quantification

The density of the slots on the autoradiographs was determined by the absorbence using a Beckman DU-70 spectrophotometer on the gel scan function. The spectrophotometer was calibrated by using the background of the autoradiograph as the blank. Relative quantities of the

RNA in each sample could be calculated from the absorbence readings. The value of the control band at day 0 was designated as 1 (arbitrary units), so that all other bands were multiples of this.

2.4.4.4. *In situ* cultivarization

In situ cultivarization was done using a combination of the protocols described by McKhann and Hirsch (1993), Ausubel *et. al.* (1989) and the Boehringer Mannheim protocol supplied with the DIG labelling and detection kit. Unless otherwise stated, all steps were done at room temperature.

Samples were fixed in a formaldehyde acetic acid buffer (FAA) for 24 hours, after which they progressed through the following dehydration and embedding steps, two hours per step: 50% ethanol (x 2), 75% ethanol, 100% ethanol, 75% ethanol/25% xylene, 50% ethanol/50% xylene, 25% ethanol/75% xylene, 100% xylene, 50% xylene/50% liquid paraffin, 100% liquid paraffin and 50% liquid paraffin/50% histosec wax molten at 60°C. The samples were then infiltrated with Histosec wax at 60°C for 24 hours. The samples were mounted in molten wax blocks, allowed to cool and solidify at room temperature, and sectioned on a rotary microtome at 20 µm. The section ribbons were affixed to glass slides with a thin film of Haupt's adhesive to which a few drops of 4% formalin was added. They were warmed at 40-50°C for one hour and dried overnight.

In preparation for digoxigenin (DIG) labelling, the slides were dewaxed and rehydrated by progression through the following rehydration series, two minutes per step: 100% xylene (x 3), and 100%, 95%, 70%, and 50% ethanol. To denature proteins and nick the DNA, the slides were acid washed with 0.2 M HCl for 20 minutes, then rinsed twice in 2 x SSC for five minutes at 70°C, followed by water twice, five minutes per wash.

To reduce background by non-specific binding, an acetic anhydride step was done. Slides were dipped in 100 mM triethanolamine in water for five minutes, after which acetic anhydride was added to a concentration of 0.25%, and incubated for ten minutes. The slides were rinsed twice in 2 x SSC for five minutes, then dehydrated by progression through an ethanol series, 30%, 70%, 95% and 100%, two minutes per step. Slides were air dried under vacuum for at least 1 hour.

Labelling and detection with DIG was done using the Boehringer Mannheim protocol supplied with the labelling and detection kit. Template DNA was synthesised by PCR from the phage as previously described. One microgram of template cDNA was labelled using random primers. After ethanol precipitation with LiCl, the pellet was resuspended in 50 µl TE buffer. The DIG-labelled probe was diluted 1:50 in cultivarization buffer and 200 µl added to each slide. The slide was covered with a baked cover slip and incubated overnight at 42°C. The colour precipitate was allowed to develop overnight.

All of the DIG detection steps were done in small plastic containers with sealable lids to prevent the slides from desiccating. DIG-labelled probe was synthesized from the three isolated clones. Cultivarization was done with leaf and root sections from control and water stressed plants of both maize cultivars. Two control cultivarization were also done. A negative control was done by probing with labelled λgt10 DNA to determine the extent of non-specific binding. A positive control was done by probing with total cDNA to determine that the reaction was working.

2.4.5. Two-cultivar Analysis of Water-stress Induced Genes

In order to clone the water stress relate cDNA clones into the expression vectors it was necessary to synthesise a set of linkers which would allow the cDNA's to be clones in all three reading frames. A set of three Xho 1 linkers with the following sequences were synthesised;

1. 5'-TCG AGA AAG AGA G-3'
   3'-CT TTC TCT C-5'
2. 5'-TCG AGA AAG AGA GA-3'
   3'-CT TTC TCT CT-5'
3. 5'-TCG AGA AAG AGA GAA-3'
   3'-CT TTC TCT CTT-5'

The two cDNA clones rws7 and rws16 which previously had been sequenced were cloned into the Xho I restriction sites of both the pB42AD (activation domain) and the pLexA (binding domain) plasmids in the correct reading frame. Thus each cDNA could be tested as a binding domain and an activating domain. The cDNA clones were first ligated to the Xhol linkers and the

cloned into each vector (Figure 2.3). This created a set of clones in both orientations. See Figure 2.3 below for a detail map of the two vectors.

The DNA-BD is the entire LexA Protein, including the LexA DNA-binding domain and the dimerization domain. The Two reporter genes are separate constructs: *lacZ* is located on the p8op-lacZ reporter plasmid, and *LEU2* is integrated in the EGY48 genome. AD is an 88-residue acidic peptic with transcriptional activation function. These functions are located on three plasmids that are transformed into the yeast train EGY48. The clones were then tested using the Matchmaker LexA Two-Cultivar System to look for interactions. Figure 2.4 outlines a standard interaction test between two known proteins or polypeptides.

The yeast strain Egy48 was first transformed with the p8op-lacZ plasmid, which integrated into the yeast chromosome and then co-transformed with the rws7 pLexA and rws16 pB42AD plasmids. The transformed cells were then plated onto selective culture media (SD/ -His/ -Trp/ -Ura) to select to all co-transformants (Figure 2.4).

Five of the colonies, which grew on the selective media, were plated onto the media listed below to test for interactions.

1. SD/ -His/ -Trp/ -Ura +X-Gal + BU salts
2. SD/ -His/ -Leu/ -Trp/ -Ura/ +X-Gal + BU salts
3. SD/Gal/Raf/ -His/ -Trp/ -Ura/ +X-Gal + BU salts
4. SD/Gal/Raf/ -His/ -Leu/ -Trp/ -Ura/ +X-Gal + BU salts

The colonies gave the result that indicated that there was no interaction.

1. White colonies;
2. no growth;
3. white colonies;
4. no growth

This result was confirmed and the orientation of the inserts was determined by restriction analysis to be in correct orientation.

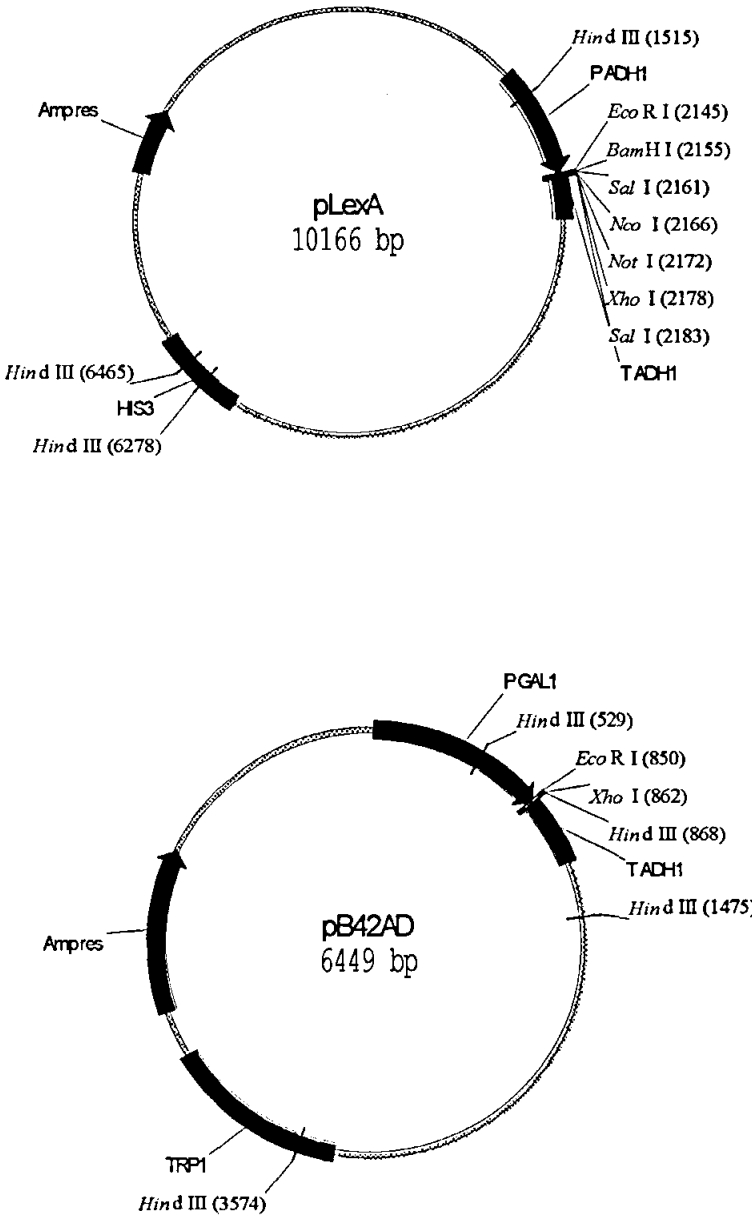


Figure 2.3: A detailed map of the vectors pB42AD and pLexA

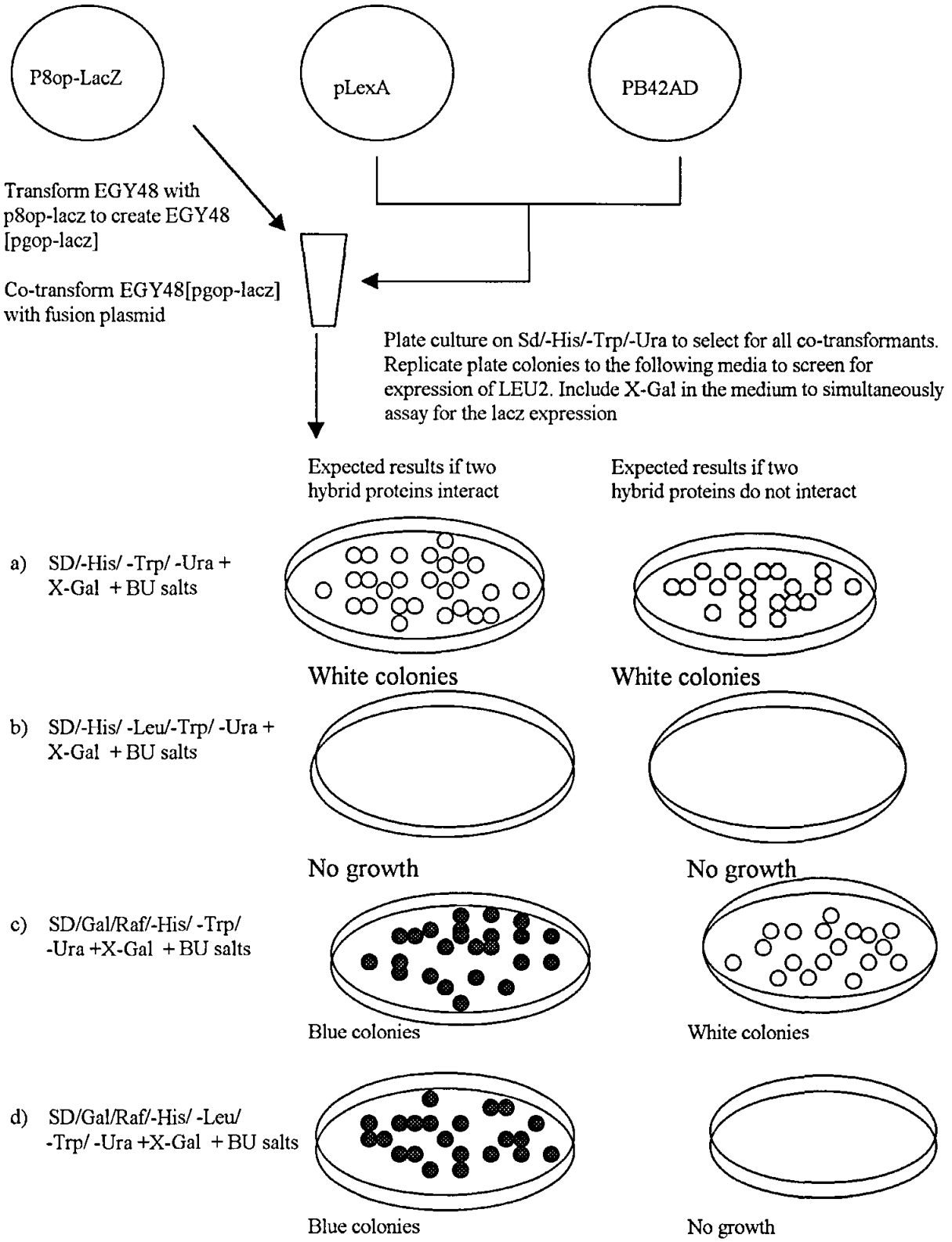


Figure 2.4 : Using the MATCHMAKER LexA Two - cultivar system to test for an interaction between two known proteins.

2.5. TRANSFORMATION: MAIZE

2.5.1. Plant Material

Seed of *Zea mays* L. (Line M162W) was obtained from ARC Grain Crops Institute (Potchefstroom). The seeds were planted in large pots in a greenhouse compartment set at 28°C (day temperature) and 16°C (night temperature). The ears were hand-pollinated under controlled conditions, by collecting fresh pollen each day in the early mornings in paper bags. The young ears were then individually covered with a paper bag with fresh collected pollen and enclosed with a rubber band. After 10 -14 days of hand-pollination, the young cobs were harvested from which immature embryo's (1- 1,5 mm in length) could be dissected.

2.5.2. Surface Sterilisation of Young Cobs

Leaves were removed just before cobs were surface sterilised with 3.5% NaOCl (m/v). Cobs were surface sterilised by agitating them for 10 minutes in this solution after which they were thoroughly rinsed with sterile distilled water. Cobs were sprayed with 98% ethanol (v/v) before embryos were dissected from kernels.

2.5.3. Initiation of Embryogenic Tissue

Immature embryo's (1-2 mm in length) were aseptically removed from the kernels after which they were placed with their axis facing down onto the medium. They were cultured in petri dishes, 90 mm in diameter, containing 25 ml of regeneration medium. Immature embryos were cultured under low light conditions in a growth room set at 26°C. Embryogenic calli were obtained after two to three weeks and were sub cultured onto freshly prepared MS-medium for further development.

2.5.4. Prepare Embryogenic Tissue for Bombardment

Three days after inoculation onto regeneration medium, the immature embryos were removed and used for particle bombardment. The immature embryos were placed on a high osmotic-medium for 4-6 hours prior to bombardment. This medium consisted of MS-basal medium, 0.2 M Sorbitol and 8 g/l Agar.

2.5.5. Phosphinotricin Selective Medium

Bombarded embryos were kept on the high osmotic medium for 12 hours after which they were transferred to the selective phosphinotricin (PPT) medium. These cultures were incubated under low light conditions in a growth room set at 28°C. PPT, a glutamate analogue has been used widely in plant tissue cultures as a selective agent (De Block, Botterman. Van de Wiele, Dockx, Thoen, Gossele, Movva, Thompson, Van Montagu and Leemans, 1987) Maize transformants have also successfully been recovered by using Basta, a commercial formulation of glufosinate, which is the ammonium salt of PPT (Fromm, Morrish, Armstrong, Williams, Thomas, and Klein, 1990) Inert ingredients present in the commercial formulations do not have a detrimental effect on the growth and health of maize embryogenic calli at the various levels required for selections.

PPT (Ignite) was used during this experimental trial to select transgenic maize plantlets. The Ignite selective range was 0, 0,3, 1, 2 and 3 mg/l. L-proline was not added to the selective medium. Embryogenic tissue was transferred gradually from the lowest to the highest concentration level of Ignite.

2.5.6. Particle Gun Bombardment

2.5.6.1. Coating of Particles

Tungsten or gold particles were prepared on the day of bombardment by weighing 60 mg of particles. One ml Ethanol (70-100%) was added to the particles after which they were vigorously mixed. They were soaked for 15 minutes at room temperature after which they were centrifuged for 5 minutes at 15 000 rpm. The supernatant was decanted and particles were washed three

times with sterile distilled water. Particles were resuspended in 1ml of a 50% Glycerol solution. Enough particles per tube were prepared for 3-6 bombardments. Micro projectiles were aliquoted in 25 µl quantities and needed to be mixed continuously.

2.5.6.2. DNA Precipitation

DNA precipitation was done by mixing 25 µl tungsten or gold particles with 5 µl DNA plasmid (pACH25), 25 µl $CaCl_2$ and 10 µl Spermidine. The sequence of these chemicals is very important. Everything was mixed well (vortex) and placed on ice for 5 minutes to allow good precipitation. Forty µl of the supernatant was discarded after 2µl quantities were aliquoted onto the grid of a swinney, immediately before bombardment. The DNA was not used longer than 15 minutes after precipitation.

2.5.7. Manipulation of the Superoxide Dismutase Gene for Transformation

In this study, the ligation and transformation studies of pAHC25 with the *Arabidopsis thaliana* Cu/Zn superoxide dismutase (SOD) gene, from the pcSODRH plasmid, is represented. The plasmid pcSODRH consists of a 788 bp cDNA clone of a cytosolic SOD from *Arabidopsis thaliana* in the *EcoRI* site of pBluescript (SK^+).

The plasmid pAHC25 contains both a selectable marker (*bar*) and a scorable marker (ß glucoronidase (GUS)), each under the transcriptional control of a separate *Ubi-1* promoter. The maize *Ubi-1* promoter has a very high activity in monocotyledons (Christensen *et. al.*, 1992). The bar gene encodes for a phosphinotricin acyltransferase, which acetylates the NH_2 - terminal group of phosphinotricin, abolishing its herbicidal activity.

Plasmid DNA were isolated (maxi preparations) from *E.coli*, containing pcSODRH and pAHC25 separately, grown with the use of overnight cultures using Luria-Bertani (LB) broth (Sambrook *et. al.*, 1989). The method of Ish-Horowicz and Burke (1981) was used for the isolation of the plasmid DNA. The plasmid DNA was quantified using a fluorometer. Horizontal 1% agarose gels

were used to determine the purity of the extracted DNA. The pcSODRH was restricted with *EcoR1* to splice the SOD gene from the plasmid after which the SOD fragment was identified and isolated from a 1.5% LMP agarose gel. The fragment was purified by means of Gelase treatment and consequently treated with Klenow enzyme to create blunt ends on both sides of the DNA fragment.

The plasmid pAHC25 was restricted with *Sma1* to create a linear fragment and isolated from a 1.5% low melting point (LMP) agarose gel and treated with alkaline phosphatase to eliminate the probability of circulation. Ligation of the SOD insert and pAHC25 was performed. Mini extractions were done on the colonies to obtain DNA to be subjected to polymerase chain reaction (PCR) analyses. DNA of the colonies as well as various controls together with DNA specific primers was used in the PCR reaction. The PCR products were visualised on a 1% agarose gel stained with ethidium bromide. To confirm furthermore the cloning of the SOD, DNA was extracted from the positive colonies. The DNA was subjected to enzyme restriction digestion of *Sal 1* and *Pst 1*. The digested samples were visualised on a 1% agarose gel.

2.5.8. Transformation of the Maize with the Plasmid pAHC25SOD

After bombardment and growth on high osmoticum medium the calli were put on MS medium supplemented with 0.3 mg/l PPT. The calli developed into somatic embryos that gave rise to the development of the plantlets. The plantlets were transferred to fresh MS medium every 2 weeks supplemented with a increasing range of PPT concentrations. The concentrations of the PPT were 1 mg/l, 2 mg/l and 3 mg/l. Calli were submitted to GUS gene detection by submersion in GUS extraction buffer supplemented with X-GLUC, for 12-16 hours.

2.5.9. Confirmation of Transformation of pAHC25SOD Into Maize Calli

In order to be able to confirm the transformation of the pAHC25SOD into maize we collected leaf material from as much plantlets as possible. DNA extractions according to Edwards, Johnstone and Thompson (1991) were executed on all the samples. The DNA was subjected to PCR analyses.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. POTATOES

3.1.1. Screening methods

3.1.1.1. *Drought Related Protein Synthesis*

Although the uptake of labelled amino acids was significantly higher in potato tubers than in leaves, the incorporation of amino acids and efficiency of protein synthesis were much higher in leaves. This could be explained by the fact that the rate of protein synthesis in storage organs, such as tubers, is much lower than in leaves. All reports concur that protein synthesis is reduced during drought stress (Bewley, Larsen and Papp, 1983; Valluri *et. al.*, 1988).

During analysis of the incorporation of labelled amino acids into leaf proteins, significantly more labelled amino acids were incorporated into proteins when samples were subjected to very mild water stress (-0.62 MPa). When water stress was increased to -2.48 MPa a significant decrease in incorporation was observed. A similar response was observed during analysis of the efficiency of leaf protein synthesis. This phenomenon during increasing drought stress has never before been reported in the literature, although similar responses have been observed during increasing heat stress in soybean seedlings (Key, Lin, Ceglarz and Scöffl, 1982) and sorghum seedlings (Frova, Taramina and Ottaviano, 1991). Interaction between treatment and organs showed that potato leaves subjected to heat stress followed a response similar to that of osmotic stress. However, unlike leaves, in tubers the efficiency of protein synthesis was not altered by increasing osmotic stress during mild to moderate osmotic stress (-0.62 MPa and -1.24 MPa) (Table 3.1).

The incorporation of radioactive amino acids into proteins synthesised during shock treatments is a sensitive method for identifying newly synthesised polypeptides. During previous experiments to observe the *de novo* protein synthesis, decreasing water potentials were used to simulate drought (Turner and Jones, 1980). Leaf drought-related polypeptides have been found to be cultivar specific. The only exception was a polypeptide with an apparent molecular weight of 68

kDa, which was newly synthesised in Late Harvest and which showed a concentration increase in R100 and Pimpernel (Van der Mescht *et. al.*, 1992b). Although the molecular weight of the polypeptide was similar in the three cultivars, it is not yet known whether this is the same polypeptide occurring in all three cultivars. Immunological tests should be done to investigate this possibility (Table 3.2).

When the effect of osmotic stress on peeled tuber slices was examined, it was noticed that the drought stress response was also cultivar specific. Drought related polypeptides were not observed in Late Harvest. Similarities among drought related polypeptides synthesized in the sensitive cultivars, R100 and Pimpernel, include a newly synthesized polypeptide with an apparent molecular weight 69 kDa as well as an increase in the concentration of a polypeptide with molecular weight 110 kDa. Contrary to the leaf disc experiments, most drought related polypeptides in tubers were synthesized in the drought sensitive cultivar Pimpernel. To date, drought related proteins in tubers have not yet been documented in the literature.

Table 3.1: Uptake and incorporation of ^{14}C-protein hydrolysate into leaf discs and tuber slices subjected to decreasing water potentials.

Water Potential Mpa	**Uptake cpm. g.fw^{-1}x10^{-6}**		**Incorporation cpm. g.fw^{-1}x10^{-6}**		**Efficiency^{z} %**	
	Tubers	**Leaves**	**Tubers**	**Leaves**	**Tubers**	**Leaves**
0	64.5^{a}	45.6^{b}	5.2^{c}	12.3^{b}	8.3^{c}	27.9^{b}
-0.62	50.0^{ab}	48.7^{ab}	4.4^{cd}	18.4^{a}	8.7^{c}	38.6^{a}
-1.24	45.9^{ab}	42.1^{bc}	5.5^{c}	12.3^{b}	12.2^{c}	31.0^{b}
-2.48	58.3^{ab}	24.9^{c}	6.3^{c}	2.2^{d}	10.9^{d}	9.2

^{z} Efficiency of protein synthesis is calculated using label incorporation as a percentage of label uptake.

* Numbers followed by different letters differ significantly on the 5% level (P=0,05).

Table 3.2: Drought related protein synthesized in leaf segments and tuber slices from three potato cultivars

Drought-related proteins KDa	Cultivars					
	Late Harvest		R100		Pimpernel	
	Leaves[z]	Tubers	Leaves[z]	Tubers	Leaves[z]	Tubers
110				*		*
100						*
95						+
92	*					+
91	*					
82			+			
76	+					
70			*			
69				+		+
68	+		*		*	
60	*					
56	*					
48						+
47			*			
45	*					
43			*			
34	+			*		
32	+		*			
28	*					
25	+					
22	*					

\+ newly synthesised polypeptides

* polypeptides with increased concentrations

z Drought-related protein synthesis in leaves as published by Van der Mescht *et. al., 1992.*

A comparison between the molecular weights of drought related polypeptides obtained from leaves and tubers showed that a polypeptide of 32 kDa was newly synthesized in the leaves of Late Harvest while there was an increase in concentration of a polypeptide of similar molecular weight in R100 tubers. An increase in the level of a polypeptide of 92 kDa was observed in leaf segments of Late Harvest while a similar polypeptide was newly synthesized in Pimpernel tubers. It would, then, appear that drought related polypeptide synthesis is not only cultivar specific but also organ specific.

It is possible that drought-related polypeptides may have a regulatory function. As such they may attach to DNA during drought shock (Zuckerkandl and Villet, 1988). A protein (38 kDa) bound DNA in drought-stressed plants and osmotically stressed leaf segments. Binding was not visible in the control samples of the osmotic stress experiments. This would suggest that the polypeptide's ability to bind DNA is drought or osmotic stress specific, as such a polypeptide was not evident during the *de novo* polypeptide synthesis study (Figure 3.1).

3.1.1.2. Chlorophyll fluorescence as a measure of drought tolerance

Drought is considered as one of the most serious constraints during potato production. During drought stress the rate of photosynthesis declines as a result of decreasing intercellular CO_2 concentrations. Much information on photosynthetic processes can be obtained from chlorophyll fluorescence. In the work presented here twelve potato cultivars differing in their drought response and representing three growth periods, short, medium and long, were subjected to drought by withholding water. Chlorophyll fluorescence parameters were measured at weekly intervals in drought stressed and well-watered controls. Additionally the levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll content and the ratio between chlorophyll *a* and *b* were determined.

Results showed that cultivar differences following the drought stress could be measured using these chlorophyll fluorescence parameters (Table 3.3). Lower minimum fluorescence (Fo) values may be an indication of drought tolerance, thus it was expected that the drought tolerant cultivars should have lower Fo values during drought stress when compared to the sensitive cultivars.

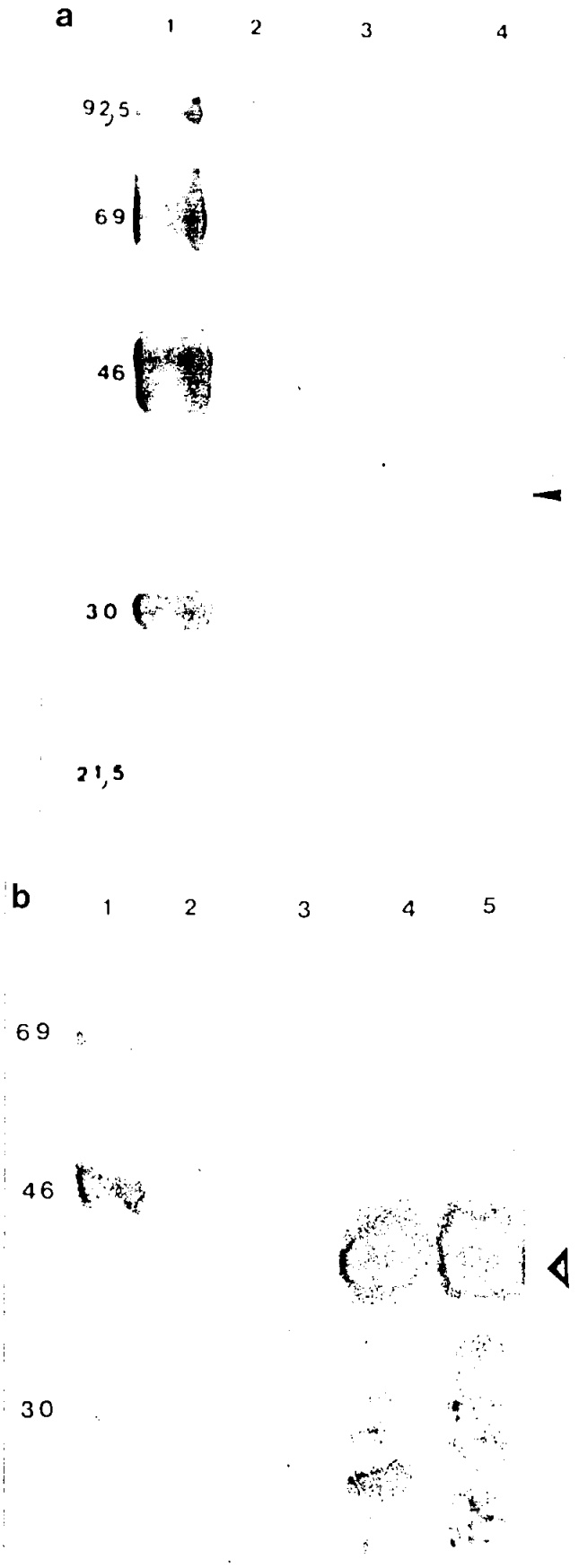


Figure 3.1: Polypeptide's ability to bind potato DNA (a) drought stress (b) osmotic stress

Table 3.3: Drought tolerance as estimated by chlorophyll fluorescence and chlorophyll *a* and *b* levels

Growth period	Cultivar	Known tolerance	Fo	Fm	Fv/Fm	Chl.*a*	Chl.*b*	Total Chl.
(a) Short season	Raritan	s	-	-	-	-	-	-
	Vanderplank	s			-	-	-	-
	Devlin	t	+	+	+	+	+	+
	Aviva	t	+	+	+	+	+	+
(b) Medium season	Sebago	s	-	-		+	+	-
	Ono	s	+	+	-	-	-	+
	Darius	t	-	-	-	-	-	-
	Baraka	t	+		+	-	-	-
(c) Long season	Bravo	s		-	-	-	-	-
	Kimberley Choice	s		+	+	+	-	-
	Hoëvelder	t	-		-	+	-	+
	Late Harvest	t	+	+	+	+	-	-

\+ = test positive for tolerance

\- = test negative for tolerance

Chl = chlorophyll

s = sensitive

t = tolerance

The cultivar with the highest Fo for the stress treatment after two weeks without water was Darius followed by Sebago, Hoëvelder and Raritan. The photochemical maximum (Fm) values are an indication of the ability of the plant to absorb light. The number of weeks which maximum values is reached before the subsequent decline is of importance. Devlin, Aviva, Late Harvest and Kimberley Choice, which reached peak values after four weeks without water, reacted as drought tolerant. The cultivars Raritan and Sebago, which reached peak values after two weeks without water, reacted as drought sensitive. Stress can affect the function of PSII and this reflects on the maximum quantum efficiency of photosystem II (Fv/Fm) as a decrease. After four weeks without water cultivars Vanderplank, Devlin, Aviva, Kimberley Choice and Late Harvest had Fv/Fm values higher than 0.70, indicated tolerance reactions. Darius, Hoëvelder and Raritan showed the lowest Fv/Fm values over time, indicated sensitive reactions.

Chlorophyll *b* is biosynthetically derived from chlorophyll *a* and may play an important role in the reorganization of photosystems during adaptation to light quality and intensity. As a result the loss of chlorophyll *a* and/or *b* will have a negative effect on the efficiency of photosynthesis. The results after four weeks without water were used as an indication of drought tolerance as this gave the best correlation to field trials. A significant decrease indicated drought sensitivity while non-significant difference were an indication of drought tolerance (Table 3.3).

There was a positive correlation between drought tolerance and chlorophyll fluorescence in cultivars with a short growth period (SGP)(Table 3.3 a). The drought tolerance of cultivars, Devlin and Aviva, were consistently indicated by positive chlorophyll fluorescence tests and chlorophyll levels, while the drought sensitive cultivars, Raritan and Vanderplank, consistently tested negative for all tested parameters.

When the medium growers (MG) were evaluated (Table 3.3 b), it was found that the sensitive cultivar Sebago gave a sensitive reaction with parameters Fo, Fm and total chlorophyll content; the sensitive cultivar Ono gave sensitive reactions with the fluorescence parameter Fv/Fm as well as chlorophyll *a* and chlorophyll *b* content. The drought tolerant cultivar Darius had no tolerant reactions while the drought tolerant cultivar Baraka had Fo and Fv/Fm indicating tolerance.

When the cultivars with a long growth period (LGP) were evaluated (Table 3.3 c) the sensitive cultivar Bravo tested sensitive with all parameters; the sensitive cultivar Kimberley Choice showed a sensitive phenotype for both chlorophyll *b* and total chlorophyll content. The drought tolerant cultivar Hoëvelder was tolerant when judged on the basis of chlorophyll *a* and total chlorophyll measurement while the tolerant cultivar, Late Harvest, showed a tolerant reaction for Fo, Fm and Fv/Fm.

From these results we can conclude that chlorophyll fluorescence can only be used as a measure of drought tolerance in potato cultivars with a short growth period (Table 3.3 a).

Stomatal closure limits photosynthesis leading to reduced assimilate production (Bowler *et. al.*, 1992). Information on the carbon reduction cycle can be provided by chlorophyll fluorescence as the proton gradient and the redox state of the primary electron acceptor of photosystem II is influenced by the consumption of ATP and NADPH during carbon metabolism (Anderson *et. al.*, 1997). Additionally chlorophyll fluorescence measurements can give a quantitative assessment of inhibition or damage to electron transfer. In our present study the twelve potato cultivars were grouped into three groups namely a tolerant group which tested positive for all fluorescence parameters (two cultivars), an intermediate group (six cultivars) and a sensitive group which tested negative (or neutral) for all fluorescence parameters (four cultivars).

In conclusion it was found that with the chlorophyll fluorescence parameters used the cultivars Aviva (SGP), Devlin (SGP) and Late Harvest (SGP) were drought tolerant while Darius (MGP), Raritan (SGP), Vanderplank (SGP) and Bravo (LGP) were drought sensitive. Intermediate reactions were observed for Sebago (MGP), Ono (MGP), Baraka (MGP), Kimberley Choice (LGP) and Hoëvelder (LGP). When the results of chlorophyll fluorescence as well as chlorophyll levels were compared drought tolerance as indicated in Table 3.3 a perfect correlation was found between drought tolerance and fluorescence results for the cultivars with a short growth period. It is suggested to test a larger sample of cultivars with a short growth period to evaluate the possibility using chlorophyll fluorescence parameters to test such cultivars for drought tolerance.

3.1.1.3. Cu/Zn Superoxide Dismutase, Glutathione Reductase and Ascorbate Peroxidase levels during Drought Stress

The misdirection of electrons in the photosystems results in the formation of reactive oxygen species (Bowler *et. al.,* 1992). As drought and other physiological stresses cause oxidative injury, high antioxidant capacity or increased levels of antioxidants can prevent cell damage and may correlate with stress tolerance. However, the mechanisms involved in the minimization of oxidative stress may play a secondary role during drought tolerance and a direct correlation between increased concentrations of an enzyme and drought tolerance may be masked.

The interaction between the enzymes if of great importance as they play a role in different parts of the mechanism involved during the elevation of oxidative stress e.g. CuZn superoxide dismutase breaks down the superoxide radical to hydrogen peroxide and dioxygen (Van Camp *et. al.,* 1994). The hydrogen peroxide is potentially toxic to the cells and is removed by glutathione reductase, ascorbate peroxidase and dehydro-ascorbate reductase through the Halliwell - Asada pathway (Monk, Fagerstedt and Crawford, 1989). The levels of Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase were evaluated during drought stress in twelve potato cultivars. Differences in glutathione reductase and ascorbate peroxidase activities could not be correlated with drought tolerance (data not shown). The levels of glutathione reductase in stressed potato cultivars were consistently lower compared to control treatments. The levels of ascorbate peroxidase activity were generally higher in stressed plants compared to control plants.

When drought stressed plants were compared to well watered control plants it was observed that the ability to maintain adequate or decreased concentrations of Cu/Zn superoxide dismutase correlated with drought tolerance under field conditions (Table 3.4). This may be due to the fact that drought tolerance is defined in terms of yield reduction and that we are selecting for plants avoiding drought by tuberization rather than for tolerance. The ability of potatoes to maintain adequate levels of superoxide dismutase activity seemed more important than an increase in enzyme activity (Table 3.4).

Table 3.4: The mean values (n=3) of superoxide dismutase levels in drought stressed (two weeks without water) and non-stressed control potato plants. The LSD (Bonferroni) = 2,623

Growth period	Cultivar		Control treatment	Stress treatment	Significance ($\alpha = 0,05$)
Short season	Raritan	s	20.831	36.205	Significant increase
	Vanderplank	s	32.484	34.216	Non significant
	Devlin	t	24.125	35.762	Significant increase
	Aviva	t	13.597	13.043	Non significant
Medium season	Sebago	s	29.371	35.773	Significant increase
	Ono	s	14.418	35.099	Significant increase
	Darius	t	35.086	25.960	Significant decrease
	Baraka	t	34.412	33.451	Non significant
Long season	Bravo	s	15.949	34.772	Significant increase
	Kimberley Choice	s	27.418	36.211	Significant increase
	Hoëvelder	t	36.404	38.057	Non significant
	Late Harvest	t	37.285	37.577	Non significant

s = sensitive

t = tolerance

3.1.1.4. The Effect of Drought on Proline and Polyamine Levels

Optimal enzyme activity may be reduced by a decrease in cell pH. Additionally Handa, Handa, Hasegawa and Bressan (1986) proposed that proline accumulation could be associated with a change in cytoplasmic pH. This is in agreement with other reports on the physiological functions of proline namely a protectant against denaturation of proteins (Schobert and Tschesche, 1978) and controlling the cell pH thus reducing the acidity (Verbruggen, Villarroel and Van Montagu, 1993). In the present study it was found that proline accumulation is a function of growth period (Table 3.5). Drought tolerant cultivars with a short growth period accumulated the highest levels of proline two weeks after water was withheld, cultivars with a medium growth period accumulated most proline three weeks without water while cultivars with a long growth period accumulated most proline after four weeks without water.

It is possible that potatoes with a short growth period avoid drought by early tuberization. As drought is defined in terms of yield reduction, these cultivars are termed drought tolerant. The cultivars able to sustain vegetative growth longer take longer to accumulate maximum proline (Table 3.5).

Furthermore the synthesis of free proline and polyamines share a biochemical pathway at intermediates, glutamic acid and L-ornithine (Adams and Frank, 1980; Altman, Friedman and Levin, 1982). It is suggested that the role of polyamines be in maintaining the cation-anion balance in the plant cell. The polyamines are protonated at the physiological pH of cells, thus electrostatic binding of polyamines to negatively charged functional groups of membranes is preferred (Slocum, Kuar-Sawhney and Galston, 1984). In binding to the negatively charged phospholipid head groups on membranes, the polyamines influence the stability and permeability characteristics of these membranes, e.g. the loss of chlorophyll from thylakoid membranes is prevented by maintaining membrane integrity thus stabilizing the photosystem complexes during drought stress (Besford, Richardson, Campos and Tiburcio, 1993). Polyamines also stabilize other double-helix structures such as stems and loops in messenger RNA (mRNA) and ribosomal RNA (rRNA) and polyamines stabilize transfer RNA (tRNA) by binding to specific sites. It is suggested that these interactions may be the basis for their effects on DNA, RNA and protein synthesis.

Table 3.5: Proline accumulation calculated at weekly intervals for 12 potato cultivars and expressed as a percentage of total proline accumulation

Growth period	Cultivar		Week 1	Week 2	Week 3	Week 4
Short season	Raritan	s	10.0%	24.3%	15.0%	50.7%
	Vanderplank	s	5.5%	30.3%	36.8%	27.4%
	Devlin	t	7.1%	35.9%	25.7%	31.3%
	Aviva	t	10.7%	34.5%	28.0%	26.8%
Medium season	Sebago	s	14.3%	34.2%	21.4%	30.1%
	Ono	s	3.3%	30.8%	32.7%	33.2%
	Darius	t	11.9%	26.5%	37.0%	24.6%
	Baraka	t	3.8%	25.1%	39.2%	31.9%
Long season	Bravo	s	8.9%	25.9%	28.4%	36.8%
	Kimberley Choice	s	7.7%	27.8%	34.1%	30.4%3
	Hoëvelder	t	11.0%	28.1%	26.3%	34.6%
	Late Harvest	t	14.0%	21.7%	29.5%	34.8%

s = sensitive

t = tolerance

Additionally polyamines are involved in free radical scavenging (Tiburcio, Campos, Figueras and Besford, 1993). Drolet, Dambroff, Legge and Thompson (1986) have shown that both chemically and enzymatically generated superoxide radicals were scavenged by polyamines. The polyamines were not simply inhibiting enzyme activity as they also had the capacity to scavenge superoxide radicals, which were generated photochemically. The author concludes that the actual mechanisms by which polyamines act as free radical scavengers have not been resolved. In our present study it was found that the most abundant polyamine was spermine. In this study, the synthesis of spermine, agmatine and spermidine proved to be cultivar and age dependent; however, the synthesis of spermine, after four weeks without water, showed a correlation with drought tolerance in potato (Table 3.6). This is in agreements with results obtained by Aziz and Larher (1995) on rape leaf discs subjected to osmotic stress that found that the most abundant polyamine was spermidine. It is suggested to determine the levels of polyamines during drought stress in potato using growing tissue e.g. root tips instead of mature leaves as it is possible that the polyamine titres in mature tissue are probably too low to detect (Pfosser, Königshöfer and Kandeler, 1990) (Table 3.6).

3.1.1.5. 2,3,5-Triphenyltetrazolium Chloride Reduction as a Measurement of Drought Tolerance

Although potato yield is extremely sensitive to drought and heat stress, there are variations in the degree to which cultivars are affected by these stresses. Strategies for the selection of tolerant cultivars in a breeding programme can therefore be developed. The 2,3,5-triphenyltetrazolium-chloride viability assay was evaluated to estimate drought and heat tolerance of leaves and tubers of 12 potato cultivars, which differ in their response to drought. Drought was simulated by floating leaf discs and tuber slices in a 0,5M mannitol (-1,24mPa) solution. After the drought acclimation treatment the leaf discs were subjected to a lethal drought stress by exposure to an osmotic potential of -2,48MPa. The viability of the leaf discs after the drought treatment was estimated by spectrophotometrically measuring the formazan concentration at 485 nm. Lower absorbance values in the control treatment compared to the stress treatment, indicated a tolerant reaction.

Table 3.6: Spermine concentrations calculated at weekly intervals during drought stress in potato cultivars

Growth period	Cultivar		Treatment	Week 1	Week 2	Week 3	Week 4
Short season	Raritan	s	control	-	0.05±0.01	-	-
			stress	-	0.06±0.02	-	-
	Vanderplank	s	control	2.15±2.56	1.09±1.33	3.31±1.73	-
			stress	0.40±0.73	-	0.07±0.73	-
	Devlin	t	control	-	0.20±0.15	0.68±0.40	0.19±0.26
			stress	0.13±0.01	0.30±0.22	0.26±0.34	0.42±0.65
	Aviva	t	control	1.67±2.44	0.050±.01	0.06±0.02	0.08±0.02
			stress	0.09±0.04	0.060±0.01	0.41±0.59	0.08±0.03
				-			
Medium season	Sebago	s	control	-	-	-	-
			stress	-	-	-	-
	Ono	s	control	0.90±0.86	0.69±0.11	0.76±0.21	0.70±0.15
			stress	0.58±0.22	0.60±0.49	0.83±0.25	0.72±0.10

Growth period	Cultivar		Treatment	Week 1	Week 2	Week 3	Week 4
	Darius	t	control	-	0.28±0.22	0.21±0.07	-
			stress	-	0.09±0.08	0.24±0.03	0.26±0.094
	Baraka	t	control	0.24±0.33	0.19±0.16	0.09±0.04	0.61±0.83
			stress	0.05±0.02	0.54±0.69	0.07±0.01	0.26±0.05
Long season	Bravo	s	control	0.06±0.03	0.05±0.01	-	-
			stress	0.05±0.01	0.05±0.01	-	-
	Kimberley Choice	s	control	-	-	-	-
			stress	-	-	-	-
	Hoëvelder	t	control	0.42±0.22	1.19±1.50	0.63±1.13	0.57±0.06
			stress	0.58±0.31	0.60±1.17	2.00±2.34	0.59±0.06
	Late Harvest	t	control	1.63±2.73	0.05±0.01	0.05±0.01	0.05±0.03
			stress	3.61±1.15	0.05±0.01	-	0.05±0.02

s = sensitive

t = tolerance

Information about the correlation between heat and drought tolerance is of great economic value. It may be used to determine the best locality for a specific cultivar as well as for cultivar improvement in a breeding programme. The data in Table 3.7 is an indication of the correlation between heat and drought tolerance. When leaves were evaluated a negative correlation was found in the cultivars Vanderplank, Darius, Baraka and Late Harvest. The cultivars Vanderplank and Darius were relatively drought tolerant and heat sensitive while the cultivars Baraka and Late Harvest were relatively heat tolerant and drought sensitive.

In the case of tubers it was found that the cultivar Darius was relatively drought tolerant and heat sensitive while Late Harvest and Hoëvelder were relatively heat tolerant and drought sensitive (Table 3.7). Results of Li *et. al.* (1981) showed a negative correlation between heat and cold tolerance in potato using TTC-reduction. It is possible that heat and drought tolerance is mutually exclusive in potato.

We conclude that heat and drought tolerance is organ and cultivar specific. It is suggested to use leaves in all future experiments. Preceding our results on tubers, that showed a hypersensitive reaction to drought, Shimshi and Susnoschi (1985) found a linear relationship between reduction in tuber yield and reduced soil moisture content. Vayda (1994) suggests that this relationship disguise a complex set of responses. A reduction in photosynthetic efficiency may play a major role in yield loss during water stress as it influences carbon assimilation (Ögren, 1990). According to Walter (1989) stress responses during phenylpropanoid biosynthesis are preferentially expressed in order of heat shock, fungal elicitor and ultra violet light.

Thus, it is possible that heat plays a dominant role when plants are subjected to both heat and drought stress. This is in agreement with the visual observations of breeders and this interaction complicated selection for drought tolerance (H.J. Vorster, personal communication). Steyn, Du Plessis, Fourie and Hammes (1989), subjected three of the cultivars (Vanderplank, Hoëvelder and Late Harvest) used in this study, to differential water treatments in rain shelter trials during the autumn and spring seasons. Results showed that the effect of drought on yield was more detrimental in spring plantings compared to autumn plantings. According to Steyn *et. al.* (1989), higher temperatures during spring trials may aggravate the effect of water stress.

Table 3.7: Summary of the drought- and heat response in potato leaves and tubers. Cultivars were indexed from the most viable to the least viable on a scale from 1-12

Growth period	Cultivar		Drought tolerance rating		Heat tolerance rating	
			Leaves	Tubers	Leaves	Tubers
Short season	Raritan	s	9	9	6	9
	Vanderplank	s	3	3	12	2
	Devlin	t	8	11	7	8
	Aviva	t	10	12	9	12
Medium season	Sebago	s	7	4	11	7
	Ono	s	2	10	5	10
	Darius	t	1	5	10	11
	Baraka	t	11	6	2	6
Long season	Bravo	s	4	2	1	1
	Kimberley Choice	s	6	10	4	5
	Hoëvelder	t	5	7	8	3
	Late Harvest	t	12	6	3	4

s = sensitive

t = tolerance

When our data was compared to the data from the rainshelter trial disagreement was found between results from the TTC-reduction and the rainshelter trials. For example, Late Harvest was drought tolerant according to the results from the rain shelter trials but tested sensitive with the viability assay while Vanderplank was drought sensitive according to the results from the rainshelter trials and tolerant according to the TTC-reduction experiments. Hoëvelder showed sensitivity to drought but had a high yield potential according to the rainshelter trials but with the viability assay it tested more tolerant than Late Harvest.

In field studies, cultivar assessment is complicated by the interaction between heat and drought stress (Vayda, 1994), thus the results from the rainshelter trials were compared to heat tolerance as estimated by TTC-reduction in potato leaves. The comparison resulted in a positive correlation with Vanderplank and Hoëvelder testing sensitive and Late Harvest tolerant. Thus, viability as an indicator of heat- (Schaff, Clayberg and Milliken, 1987) and drought tolerance can be tested under uniform laboratory conditions and in much less time than yield trials. Additionally, osmotic substrates such as mannitol provide controlled water potentials and offer the opportunity to bypass many uncertainties in field studies (Parmar and Moore, 1968).

3.1.1.6. Summary

Table 3.8 summarized the physiological reactions of the 12 potato cultivars tested to drought stress, using ten different parameters. From the presented data it is evident that the most promising physiological parameters to use as a screening method for drought tolerance are the levels of Cu/Zn SOD activity, free proline accumulation and spermine levels. Cu/Zn SOD, proline and polyamines act as free radical scavengers during oxidative stress. It is suggested that these three parameters must be used in future when screening for drought tolerance in potato. With the use of these three parameters it can thus be concluded that the cultivars Aviva, Darius, Baraka, Late Harvest and Hoëvelder are tolerant and Raritan, Sebago and Kimberley Choice are sensitive. The Cu/Zn SOD gene was identified in playing a dominant role in the tolerance mechanism in potatoes.

Table 3.8: Summary of the physiological reactions of 12 potato cultivars to drought stress

Growth period	Cultivar	Cu/Zn SOD	Free Proline	Spermine	Fo	Fv/Fm	Fm	Chl a	Chl b	Total Chl	TTC
Short season	Raritan	-	-	-	-	-	-	-	-	-	+
	Vanderplank	+	-	-	0	+	0	-	-	-	+
	Devlin	-	+	+	+	+	+	+	+	+	-
	Aviva	+	+	+	+	+	+	+	+	+	-
Medium season	Sebago	-	-	-	-	0	-	+	+	-	-
	Ono	-	-	+	+	+	+	-	-	+	+
	Darius	+	+	+	-	-	-	-	-	-	+
	Baraka	+	+	+	+	+	0	-	-	-	-
Long season	Bravo	-	+	-	0	+	-	-	-	-	-
	Kimberley Choice	-	-	-	0	+	+	+	-	-	-
	Hoëvelder	+	+	+	-	0	0	+	-	+	-
	Late Harvest	+	+	+	+	+	+	+	-	-	-

\+ tolerant reaction

\- sensitive reaction

0 no reaction

s sensitive

t tolerance

3.1.2. Transformation in an Attempt to Enhance Drought Tolerance

It is evident that the most promising physiological parameter to use as a screening method for drought tolerance is the level of Cu/Zn superoxide dismutase (SOD) activity (Table 3.4). This result is supported by results on spermine levels during drought stress, which correlated with drought tolerance (Table 3.6). Both Cu/Zn SOD and polyamines act as free radical scavengers during oxidative stress (Van Camp *et. al.*, 1994; Drolet *et. al.*, 1986). The final test of the value of the Cu/Zn SOD was to evaluate the contribution of this gene to drought tolerance by the transformation of a well-adapted and high yielding genotype. It was shown that the potato cultivar Aviva had only half the Cu/Zn SOD activity when compared to eleven other cultivars differing in growth period and drought tolerance. Aviva was consequently transformed with a cytosolic Cu/Zn SOD gene from *A. thaliana* using *Agrobacterium* mediated gene transformation. Four transgenic potato lines were identified with molecular techniques and evaluated for drought tolerance in the greenhouse.

3.1.2.1. Cu/Zn SOD Activity of Transformants

Van der Mescht, De Ronde and Rossouw (1998a) have shown a correlation between increased Cu/Zn SOD activity during drought stress and drought sensitivity. These results from the present study are in agreement with this observation. Although there was a slight increase in enzyme activity in the four transformed lines when they were watered, the Cu/Zn SOD activity under drought stressed conditions showed either a non-significant response or a significant decrease when compared to non-stressed plants (Table 3.9).

Additionally it was found that the transformed lines SOD1 and SOD2 could withstand drought in greenhouse for two weeks longer, than the untransformed plants and one week longer than the transformed lines SOD6 and SOD7. High levels of Cu/Zn SOD activity in transgenic tobacco according to Tepperman and Dunsmur (1990) did not confer tolerance to oxidative stress while a small increase in Cu/Zn SOD activity was able to provide resistance against methyl viologen in human and mouse cells. It is possible that glutathione reductase activity and ascorbate peroxidase activity were the limiting factors when the SOD activity was increased to very high levels.

Table 3.9: Cu/Zn Superoxide dismutase levels during drought stress compared to control conditions in untransformed Aviva plants as well as four transformed Aviva lines. Enzyme activity was measured in unit/gram dry weight.

Cultivar	Treatment	Week 1*		Week 2		Week 3		Week 4		Week 5	
Aviva (untransformed)	control	1.981	NS	1.619	NS	2.070	*↓	lethal stress			
	stress	1.711		1.502		1.673					
SOD 1	control	2.179	NS	2.066	NS	1.161	NS	2.179	*↓	2.024	*↓
	stress	2.170		2.117		2.003		1.782		1.265	
SOD 2	control	2.135	NS	2.370	*↓	1.963	NS	2.534	*↓	2.179	*↓
	stress	2.153		1.908		1.725		1.773		1.916	
SOD 6	control	2.345	NS	2.198	NS	2.216	NS	2.281	NS	lethal stress	
	stress	2.117		2.034		2.324		2.435			
SOD 7	control	2.948	NS	2.884	*↓	2.525	*↓	2.613	NS	lethal stress	
	stress	2.904		2.542		2.234		2.543			

* = Weeks without water; *↓ = Significant decrease; NS = not significant

3.1.2.2. 2,3,5-Triphenyltetrazolium Chloride Reduction Assays

This result was confirmed by the results from the 2,3,5-triphenyltetrazolium chloride reduction assays, which showed that the four transformed lines were more drought tolerant than the untransformed Aviva plants (Figure 3.2). This is in agreement with visual observations in the greenhouse where the transformed plants were able to withstand drought two weeks longer before a lethal stress was induced compared to untransformed plants.

3.1.2.3. Conclusion

In view of the current data it seems reasonable to conclude that SOD plays a significant role in protecting living cells against the toxicity and mutagenicity of active oxygen species by virtue of its capacity to savage the superoxide radical. Whether SOD has other biological functions remains an open question (Scandalios, 1993). Additionally, little is currently known as to how the genome reacts to oxidative insult and mobilizes a response to it. Such information is interesting itself, but it is also essential for any future attempt to raise tolerance to environmental oxidative stress in organisms and to reduce cellular damage by active oxygen. To understand these mechanisms it is necessary to identify the responsive genes and to understand their structure, regulation and expression (Scandalios, 1993).

According to Scandalios (1993) future research should include the identification and characterization of cis-acting elements and trans-acting factors involved in SOD gene regulation and expression. This research will provide some depth in our understanding of the entire signal transduction pathway during oxidative stress and will enhance our efforts towards engineering organisms to better cope with oxidative insult.

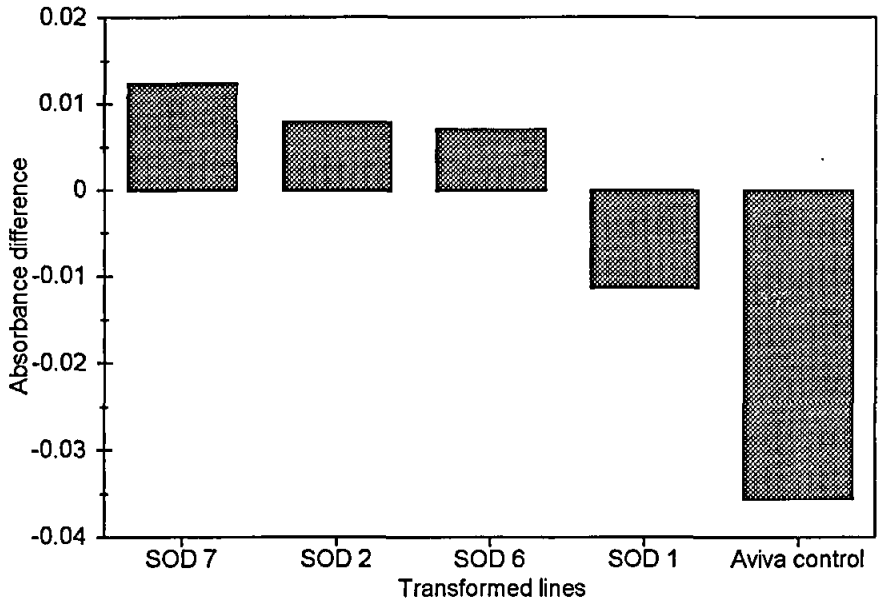


Figure 3.2: The mean difference between the stress and control treatments in leaf discs presented in a histogram. The positive values indicate a drought tolerant reaction while the negative values indicate a sensitive reaction to drought when measured by TTC-reduction.

3.2. COTTON

3.2.1. Screening methods

3.2.1.1. Drought Related Protein Synthesis

The uptake and incorporation of amino acids determined by scintillation counts according to Valluri *et. al.* (1988) shown that the efficiency of protein synthesis decreased with increasing concentrations of mannitol. Drought related protein synthesis during osmotic stress was found in the cultivars Nik 2, Delta Pine Acala, Acala 1517-70 and Acala OR3, while drought related proteins were not observed in cultivars Selati and Letaba (Table 3.10). It would appear that drought related protein synthesis was cultivar specific.

3.2.1.2. Analysis of Heat Tolerance in Cotton

3.2.1.2.1. Heat Related Protein Synthesis

The analysis of heat tolerance is based on the overall response of a plant when subjected to high temperatures. A precise evaluation can be difficult to obtain because heat tolerance has a low heritability and repeatability (Blum, 1986). To avoid this difficulty, a number of physiological tests have been proposed.

Changes in protein synthesis in leaf tissue of six cotton cultivars were studied by comparing the heat shock protein patterns synthesized at different heat treatments. Immediately following a direct shift in temperature of 8° to 16°C above the optimum growing temperature of 29°C, the synthesis of the normal protein complement declined and a new set of proteins were induced. Leaf tissue responded to thermotolerance treatments by synthesizing a specific set of proteins for each treatment and cultivar (Table 3.11), which differed from profiles observed with a direct shift to high temperatures (Table 3.12). Letaba and Delta Pine Acala 90 were the only cultivars which synthesized any HSPs when directly shift to 45°C, whereas all the cultivars synthesized HSPs when pre-treated before subjected to 45°C as was shown in the experiment on thermotolerance (Table 3.11 and 3.12). The different temperatures also affected the strength and pattern of HSP synthesis. Some HSPs were only detected at certain temperatures (Table 3.12).

Table 3.10: Summary of newly synthesized drought-related polypeptides in six cotton cultivars, subjected to 18 hours of osmotic stress (0.25M mannitol), when compared to the control (0M mannitol) treatment

Drought related proteins (kDa)	CULTIVARS					
	Selati	**Nik 2**	**Letaba**	**Delta Pine Acala 90**	**Acala 1517-70**	**Acala OR 3**
63						-
45		+		+	+	+
44						*
42		*		*	*	*
38				-		-
31						-
30					*	*
25				-		-
24					*	*

* = Newly synthesized

\+ = increased concentration

\- = decreased concentration

Table 3.11. Summary of newly synthesized heat-related polypeptides in six cotton cultivars, Selati, Nik 2, Letaba, Delta Pine Acala-90, Acala 1517-70 and Acala OR3, subjected to different thermotolerance treatments, when compared to the control treatment (29°C)

HSP (kDa)	CULTIVARS																							
	Selati				Nik 2				Letaba				Delta Pine Acala 90				Acala 1517-70				Acala OR 3			
	TREATMENT																							
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
115		+									*							*	*					
89		*									+			*				*	*		*	*	*	
75	*	*	*		+	+							+	+	+	+	+	+	+		*	*	*	
72					+	+	+				+	+	+	+	+	+								
60	+				+				+				+				+				+			
51	*	*																						
41									*															
38					*	*	*																	
37					*	*	*																	

1. Gradual 3°C per hour increase in temperature from 28°C up to 45°C.
2. Pre-treatment at 29°C for 3 hours, followed by 3 hours at 40°C, prior to a 2 hour exposure at 45°C.
3. Pre-treatment at 29°C for 3 hours, followed by a 15 minute treatment at 45°C, followed by 2 hours at 29°C prior to a 2 hour exposure at 45°C.
4. Pre-treatment at 29°C for 3 hours, followed by a 3 hour exposure at 45°C.

* Newly synthesized polypeptides

\+ Polypeptides with increased concentration

Table 3.12. Summary of newly synthesized heat related polypeptides of six cotton cultivars subjected to 3 hours at 37°, 40°, 43° or 45°C when compared to the control treatment (29°C)

HSP (kDa)	Cultivars																							
	Selati				Nik 2				Letaba				Delta Pine Acala 90				Acala 1517-70				Acala OR 3			
	Temperature (°C)																							
	37	40	43	45	37	40	43	45	37	40	43	45	37	40	43	45	37	40	43	45	37	40	43	45
150	+	+	+																					
100										*	*													
94	+	+	+						*	*	*				*									
89															*				*			*	*	
86	+	+	+														+	+						
75																+								
72	+	+	+		+	+	+					+			+	+		+	+			+	+	
60	+	+	+		+	+	+		+	+	+		+	+	+			+	+			+	+	
51			*																					
48	+	+																						
43					+	+																		
41									*	*	*								*					
39	+	+			*	*																		
37	+	+			*	*																		

* = Newly synthesized polypeptides

\+ = Polypeptides with increased concentration

Table 3.13. Summary of newly synthesized heat-related polypeptides in six cotton cultivars, Selati, Nik 2, Letaba, Delta Pine Acala-90, Acala 1517-70 and Acala OR3, subjected to 3, 9 or 15 hours at 40°C and 43°C, when compared to the control (29°C) treatment

HSP (kDa)	Cultivars																																	
	Selati						Nik 2						Letaba						Delta Pine Acala 90						Acala 1517-70						Acala OR 3					
	Temperature (°C) , Time (h)																																	
	40°			43°			40°			43°			40°			43°			40°			43°			40°			43°			40°			43°		
	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15	3	9	15
150	+			+																																
100													*	*	*	*	*	*																		
94	+			+									*	*	*	*	*	*		*		*														
89																						*						*			*			*	*	*
86	+	+	+	+	+						+	+													+	+	+									
72	+	+	+	+	+		+	+	+	+				+	+		+	+		+	+	+	+		+			+			+	+	+	+	+	+
60	+	+	+	+	+		+	+	+	+		+	+			+			+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+
51				*																																
48	+																																			
43							+	+	+																											
41													*	*	*	*	*	*										*								
39	+		+		+		*																													
37	+	+	+		+		*				*	*															*			*						
35																				*	*		*													
32																																				
23		+	+		+								.											.											.	

* = Newly synthesized polypeptides; + = Polypeptides with increased concentration

It was found that there were minor differences in the heat shock proteins profile observed in the prolonged heat treatment when compared with the direct shift treatment profiles (Table 3.13). Proteins synthesized during the prolonged treatment not found in previous studies were 35kDa, 37kDa and 23kDa in Delta Pine Acala 90, Acala 1517-70 and Selati respectively. Molecular differences were found between the cotton cultivars tested and the HSP patterns differed between heat treatments. A minor difference in the HSP profile was observed in the prolonged heat treatment when compared to the direct shift treatment. These protein profiles however, differed from thermotolerance patterns. It can be conclude that Selati was unstable under the different heat treatments, while the HSP profiles were more stable in Nik 2, Letaba and Delta Pine Acala 90.

3.2.1.2.2. Efficiency of protein synthesis

The effect of temperature on the relative incorporation of ^{14}C-protein hydrolysate into the proteins of cotton leaf tissue was compared for different heat treatments (Table 3.14). The performances of Acala 1517-70 and Selati were very similar with a decrease in efficiency from 37°C to 40°C and 43°C, which indicated a similar response to heat shock. Acala OR3 displayed adaptability to 40°C, as the efficiency of protein synthesis increased from 37°C to 40°C in this cultivar. Nik 2 showed the minimum variance between the different treatments, as no significant differences in the efficiency of protein synthesis between the different heat treatments was observed which indicated that its protein synthesis is relatively stable during changes in temperature.

In the work shown here it was found that the amount of label incorporated into protein increased from control treatment (29°) to a moderate stress (37°) and then decreased during a more severe stress (40° and 43°)(Table 3.14). There was a significant difference between the 37°C treatment and the rest of the treatments. This trend was also reflected in the corresponding lanes on SDS PAGE. It can be explained in that the first HSPs were synthesized at 37°C, while the normal complement of proteins was also being synthesized. At 40°C the normal protein synthesis declined and reaches a minimum at 43°C. This could be one of the factors leading to an increase in efficiency at 37°C. It can also be an indication that the cultivars are adapted to at least 37°C. These results are comparable with finding of Frova, Taramino and Ottaviano (1991), in sorghum

Table 3.14. Percentage incorporation of ^{14}C-protein hydrolysate into the proteins of cotton leaf tissue of six cotton cultivars Incorporation is expressed as a percent of label uptake

Cultivar	Temperature °C	Uptake $cpmg^{-1} \times 10^{-3}$	Incorporation $cpmg^{-1} \times 10^{-3}$	Incorporation %
a) Selati	29	106.88	12.25	11.46a*
	37	56.53	10.62	18.79b
	40	52.94	4.93	9.31a
	43	67.10	7.49	11.16a
b) Nik 2	29	123.27	14.68	11.9a
	37	61.28	9.28	15.14a
	40	78.31	8.13	10.38a
	43	63.98	7.57	11.83a
c) Letaba	29	108.44	14.08	12.98ab
	37	56.28	9.28	16.49a
	40	70.18	8.11	11.56ab
	43	89.07	9.33	10.48b
d) Delta Pine Acala 90	29	137.43	18.52	13.47a
	37	62.56	14.29	22.84b
	40	75.62	9.91	13.11a
	43	69.98	4.75	6.79c
e) Acala 1517-70	29	177.42	19.04	10.73a
	37	47.61	9.08	19.08b
	40	61.53	7.81	12.69a
	43	81.54	6.05	7.42a
f) Acala OR3	29	55.74	5.05	9.06ac
	37	63.25	9.20	14.55ab
	40	74.59	11.12	14.90b
	43	73.47	5.81	7.91c

* indication of significant difference (p=0.05).

with ^{35}S -methionine and the findings in soybean (Key *et. al.*, 1982) and *Gladiolus* (Ginzburg and Solomon, 1986) with ^{3}H - leucine.

3.2.1.2.3. DNA Binding

DNA-protein binding has been studied because of its involvement in precise regulatory effects (Zuckerkandl and Villet, 1988). The protein-DNA binding profiles and intensity of cultivarization of cotton plants tested in our study differed between cultivars and between DNA from different cultivars used as probes, although no substantial difference using a specific probe was observed between shock and control treatment within a cultivar (data not shown). There were, however, no differences in proteins which bound DNA between shock and control treatment of heat shocked cotton leaves. This indicates that high molecular weight (HMW) HSPs probably do not have a regulatory function.

It was previously found that binding occurs with polypeptides of low molecular weight (Echols, 1986) and our protein study only showed high molecular weight HSPs. It is proposed that heat shock proteins in cotton may have a structural function as molecular chaperones. Molecular chaperones are known to be associated with the heat shock response (HSR) or constitutively expressed HSPs (Nover and Scharf, 1984). No binding was observed when Letaba and Acala OR3's DNA were used as probes. The most intense cultivarization was observed with protein from Letaba cultivarized with ^{32}P-labelled DNA from Acala 1517-70. The tendency of Letaba to show a higher binding ability could be an indication of stress tolerance as has been found in osmotic stress (Van der Mescht *et. al*, 1992a).

3.2.1.2.4. Viability assay

The response of cotton leaves to heat stress was investigated using 2,3,5 -triphenyltetrazolium chloride in a viability assay, comparing absorbances at 485nm of control (29°C) and acclimated (40°C) plants (Table 3.15). Vratsanos and Rossouw (1986) found that in heat tolerant potato cultivars, heat killing time increased to a greater extent after acclimation than in heat-sensitive cultivars. We adapted their hypothesis and found that when the absorbance values of the control

treatment of the cotton plants were lower than the stress treatment, it indicates that the cultivar reacted like a heat tolerant cultivar. This is in contrast with a heat sensitive cultivar, where the absorbance values of the stress treatment were lower than the control treatment. The difference between the mean absorbances at 29°C and 40°C ranked the cultivars in a series with Letaba the most heat tolerant and Selati the most heat sensitive (Table 3.15). The smaller the difference between the treatments in the mean absorbance, the more convergent were the two treatments. Results indicated that this assay could be used as a measurement of heat tolerance in cotton.

Table 3.15. Rating of the six cotton cultivars using the difference between mean absorbance at 29°C and mean absorbance at 40°C

Cultivar	**TTC rating**
Letaba	1 (heat tolerant)
Delta Pine Acala -90	2
Nik 2	3
Acala 1517-70	4
Acala OR3	5
Selati	6 (heat sensitive)

3.2.1.2.5. Conclusion

From the summary of all the results using the different tests described (Table 16), we conclude that Letaba performed as a relatively heat tolerant cultivar. This assumption can be supported by the finding that Letaba synthesized HSPs after a 3-hour shift from 29°C to 45°C (De Ronde, Van der Mescht, Cress, 1993). Nik 2 was the most stable cultivar over the range of heat treatments. Acala 1517-70 and Selati performed as heat sensitive cultivars. Acala OR3 might have an alternative mechanism to tolerate heat stress up to 40°C for a short time, but it cannot tolerate

much higher temperatures or prolonged heat stress. Yield trials in hot and dry areas correlate with our findings.

Table 3.16. Predicted expression of relative heat sensitivity and heat tolerance of different cotton cultivars based on experimental results

	Cultivars					
Experiment	**Selati**	**Nik 2**	**Letaba**	**Delta Pine Acala 90**	**Acala 1517-70**	**Acala OR3**
Autoradiograph	unstable	stable		stable		stable
Efficiency	sensitive	adapted at 43°C stable			sensitive	adapted at 40°C sensitive at 43°C
TTC assay	sensitive	tolerant	tolerant	tolerant	sensitive	avoidance mechanism
Protein-DNA binding			tolerant			

3.2.1.3. Chlorophyll Fluorescence as Measure of Drought and Heat Tolerance

Chlorophyll fluorescence parameters were measured at weekly intervals in heat stressed and drought stressed cotton plants. The parameters used were maximum quantum of photosystem II (Fv/Fm), photochemical maximum (Fm), minimal fluorescence (Fo) and quenching (qQ). The ratio Fv/Fm indicated the photochemical capacity of the photo system II (PS II). A decrease in this ratio is thus a sign of photoinhibition. Results obtained indicated that cotton cultivars could be screened for drought tolerance using Fv/Fm as a parameter (Table 3.17). The cultivars Alpha, Acala 1517-88 and Delta Pine Acala 90 responded with an increasing activity with regard to the

control. This was in contrast with the other cultivars, which demonstrated increasing photoinhibition. This parameter correlated thus perfectly with drought tolerance. Photochemical maximum is an indication of the plant's ability to absorb light, thus the higher Fm values, the more viable the plant. The cultivar Molopo displayed low Fm values, which can be an indication of drought sensitivity. This was in contrast with the other cultivars, which displayed high Fm values (Table 3.17).

The qQ values are strongly influenced by the utilization of ATP during photosynthesis and depend on the metabolic activity of the plant. Higher qQ values implied thus higher metabolic activity. Four of the cultivars responded with increasing qQ activity with regard to the control, indicating drought tolerance (Table 3.17). When the results of chlorophyll fluorescence tests were compared (Table 3.17), the drought tolerant cultivars (Acala 1517-88, Alpha and Delta Pine Acala 90) tested positively for all fluorescence parameters measured, while the sensitive cultivars (Molopo and Sicala) tested negative for two of the fluorescence parameters.

Table 3.17: Drought tolerance as estimated by chlorophyll fluorescence

Known drought tolerance	**Cultivar**	**Fm**	**qQ**	**Fv/Fm**
T	Alpha	+	+	+
T	Acala 1517-88	+	+	+
T	Delta Pine Acala 90	+	+	+
S	OR 19	+	-	+
S	Sicala	+	-	-
S	Molopo	-	+	-

+ = drought tolerant; - = drought sensitive

t = tolerant cultivar; s = sensitive cultivar

Results showed that cultivar differences following the heat stress could be measured using chlorophyll fluorescence. The Fv/Fm ratios correlated perfectly with heat tolerance, with the cultivars Molopo, Acala 1517-88 and Delta Pine Acala 90 demonstrating increasing ratios with regards to the control (Table 3.18). When the results of different chlorophyll fluorescence parameters (Fo, Fm and Fv/Fm) were examined with respect to heat tolerance, the heat tolerant cultivars (Molopo and Acala 1517-88) tested positive for 2 of the parameters measured, while the heat sensitive cultivar (Sicala) tested negative for all parameters measured (Table 3.18).

Although the Fv/Fm parameter proved valuable, it can be concluded that the combined use of three parameters of chlorophyll fluorescence proved more reliable. It is thus recommended that more than one chlorophyll fluorescence parameter be used as a screening method. From these results we can conclude that photochemical efficiency correlates the best with drought and heat tolerance in cotton.

The results obtained with the chlorophyll a levels indicated that very stable difference in levels are reached between stressed and the control treatments after one and two weeks without water. No significant difference was obtained between the stress and the control treatments for five of the cultivars. Sicala is the only cultivar that showed a significant difference, with the chlorophyll a levels decreasing after a stress duration of one week.

The chlorophyll b levels determined after a drought stress period demonstrated that the average chlorophyll b levels in cotton stayed almost the same for up to two weeks without water (2.7). At three weeks without water the levels decrease in all the cultivars (average 1.8). It was observed that the chlorophyll a levels are much higher than the chlorophyll b levels in all the cultivars tested. Chlorophyll a and b levels could not be correlated to drought tolerance, apart from the decreasing tendency with Sicala (chlorophyll a levels) which may be an indication of drought sensitivity.

Table 3.18: Heat tolerance as estimated by chlorophyll fluorescence

Known heat tolerance	Cultivar	Fo	Fm	Fv/Fm
T	Molopo	+	-	+
T	Acala 1517-88	+	+	+
I	Delta Pine Acala 90	-	-	+
I	Alpha	-	+	-
S	OR 19	+	+	-
S	Sicala	-	-	-

+ = heat tolerant

- = heat sensitive

t = tolerant cultivar

s = sensitive cultivar

3.2.1.4. Enzymes from the Antioxidative System in Response to Drought and Heat Stress

The Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase levels in cotton were measured after cotton plants were subjected to a drought or heat stress treatment. The results obtained from the Cu/Zn superoxide dismutase experiments on plants subjected to a drought stress demonstrated an increase in the activity of the stress treated plants with regard to the control treated plants in the cultivars Alpha, OR 19 and Acala 1517-88. This was in contrast with the cultivars, Delta Pine Acala 90, Sicala and Molopo, which demonstrated a decrease in the activity with regard to the control treatment. When subjected to a heat stress, all the cultivars recorded lower activities for the stressed treatment than for the control treatment. This can be an indication that the cultivars are more heat sensitive than drought sensitive. These enzyme

activities correlated to the drought response as observed with the TTC viability assays during drought and heat (Table 3.19 and 3.20).

Peroxidase levels evaluated during heat tolerance resulted in increasing peroxidase levels which correlated with the tolerance ratings obtained with the TTC assays during a heat stress (Table 3.20). The cultivars Molopo, Acala 1517-88 and Alpha responded with an increasing peroxidase activity with regard to the control treatment. The peroxidase levels observed during a drought treatment did not correlate to known drought responses (data not shown). When the cultivars were subjected to a heat stress the ascorbate peroxidase activities increased in the cultivars Acala 1517-88 and Delta Pine Acala 90 with regard to the control treatment. The other cultivars showed a decreasing tendency (Table 3.20).

The interaction between the enzymes is of great importance as they play a role in different parts of the antioxidase pathway during the elevation of oxidative stress. The tolerant cultivars have the ability to increase their enzyme levels after a heat treatment for two of the three enzymes tested. This is in contrast with the sensitive cultivars which do not have this ability and shown a decreasing tendency for two out of three enzymes (Table 3.20).

In conclusion, the enzymes in the antioxidase pathway proved valuable as a screening method for drought and heat tolerance. The best correlation was observed with SOD. However the best results will be obtained in comparing all the enzymes involved in the antioxidase pathway.

3.2.1.5. Leaf Water Potential

The results obtained from leaf water potential measurements showed no significant differences among any of the cultivars tested. The cultivar Alpha however displayed evidence of a mechanism to maintain a lower variance in the leaf water potential than the other cultivars.

Table 3.19: Comparison of the response of the different cultivars under drought stress to the Cu/Zn superoxide dismutase enzyme activity and the TTC viability assay

Cultivar	Cu/Zn superoxide dismutase	TTC response
Alpha	+	+
Acala 1517-88	+	+
Delta Pine Acala 90	-	+
OR 19	+	-
Sicala	-	-
Molopo	-	-

\+ = tolerant reaction; - = sensitive reaction

Table 3.20: Summary of the response of the different cultivars under heat stress to the enzymes from the antioxidative system compared to a TTC rating of heat tolerance

Cultivar	(TTC rating)	Cu/Zn superoxide dismutase	Peroxidase	Glutathione reductase
Molopo	T	+	+	-
Acala 1517-88	T	-	+	+
Delta Pine Acala 90	I	+	-	+
Alpha	I	-	+	-
OR 19	S	-	-	-
Sicala	S	-	-	+

* + = tolerant reaction; - = sensitive reaction

3.2.1.6. The Effect of Drought and Heat on Proline Levels

All the cotton cultivars accumulated free proline during a drought treatment compared to the control treatment, but with a definite genotypical difference. Van Heerden and De Villiers (1996) observed a higher proline accumulation during drought stress in drought tolerant spring wheat cultivars, than in the more sensitive cultivars. Thus, we postulated that the cultivars Sicala, Acala 1517-88, and Alpha, which accumulated more proline under drought conditions than the cultivars Molopo, Delta Pine Acala 90 and OR19, responded as drought tolerant cultivars. This correlated with a previous study (De Ronde and Van der Mescht, 1997) where the cultivars Alpha and Acala 1517-88 proved to be the more tolerant cultivars and Molopo and OR19 the more sensitive cultivars (Table 3.21).

The percentage of total free proline during a drought stress is summarized in Table 3.22. Proline concentrations peaked significantly in all the cultivars tested at 11 days without water. The decline at 15 days is probably due to reduced protein synthesis and any peak thereafter will be due to protein degradation. This supports the hypothesis of Becker and Fock (1986) of inhibition of protein synthesis and protein degradation with severe stress. The critical time for measuring accumulated proline for drought treated cotton is thus 11 days without water.

There are a number of variants that play a role in the establishment of the physiological mechanisms, which equip plants for life. In looking at a plant's response to high temperature stress, it proved difficult to separate the interrelationship between the effects of high temperature and that of water deficits. Plant resistance to cold, heat and water stress is interrelated. The extent of proline accumulation is affected by the rate of imposition of stress, stress precondition, organ type and age as well as genetic variation within and between species.

Although a heat treatment did not result in any meaningful accumulation of free proline, combination experiments exhibited an increase in proline concentrations in five cultivars. It was found that Sicala, which did not display a meaningful increase in free-proline concentration, is heat sensitive, in contrast with Alpha, which is heat tolerant. This could be explained by the activation of heat stress mechanisms before drought stress mechanisms. This correlated with a previous

study (De Ronde and Van der Mescht, 1997) where the cultivars Alpha and Delta Pine Acala 90 proved to be the more tolerant cultivars and Molopo and Acala 1517-88 88 the more sensitive cultivars for a combination of drought and heat stress (Table 3.21).

Table 3.23 summarizes the percentage of proline accumulation during a combined stress. It was observed that the critical stage for measuring proline is earlier with a combined stress than with a drought stress alone. These results indicate that proline accumulation is affected by the rate of imposition of stress, stress applied, as well as genetic variation within species, which support previous findings of Naidu, Paleg, Aspinall, Jennings and Jones (1990).

Table 3.21: Comparison of ranked sensitivities to (a) drought stress and (b) combined heat and drought stress in six cotton cultivars, as measured by proline accumulation and a TTC viability assay

Cultivar	**Drought stress**		**Combination stress**	
	Proline rating	**TTC rating**	**Proline rating**	**TTC rating**
Alpha	3	1	2	2
Acala 1517-88	2	2	4	5
Delta Pine Acala 90	5	3	3	1
OR 19	6	4	1	6
Sicala	1	5	6	3
Molopo	4	6	5	4

Table 3.22: The effect of drought stress on proline accumulation in six cotton cultivars (expressed as a percentage of the respective total proline accumulation)

Known tolerance	Cultivars	Days after onset of drought stress			
		4	7	11	15
S	Sicala	10.73%+/-0.96[a]	15.97%+/-1.75[b]	48.28%+/-2.89[c]	25.02%+/-1.50[d]
T	Acala 1517-88	19.86%+/-2.38[a]	26.19%+/-1.57[b]	31.45%+/-3.50[c]	22.50%+/-1.57[d]
T	Alpha	21.85%+/-0.87[a]	13.29%+/-0.93[b]	40.79%+/-0.40[c]	24.07%+/-1.58[a]
S	Molopo	17.79%+/-1.24[a]	20.83%+/-1.66[a]	47.90%+/-8.14[b]	13.48%+/-1.61[c]
T	Delta Pine Acala 90	3.97%+/-1.07[a]	9.13%+/-1.55[b]	44.93%+/-2.69[c]	41.97%+/-3.77[c]
S	OR 19	17.71%+/-1.41[a]	6.54%+/-0.32[b]	34.19%+/-9.57[c]	41.56%+/-2.49[c]

Table 3.23: The effect of a combination of drought and heat stress on proline accumulation in six cotton cultivars (expressed as a percentage of the respective total proline accumulation)

Known tolerance	Cultivars	Days after onset of drought and heat stress			
		4	7	11	15
S	OR19	17.51%+/-0.87[a]	23.15%+/-3.24[b]	28.06%+/-1.68[b]	31.28%+/-3.75[b]
T	Alpha	11.86%+/-0.23[a]	21.98%+/-0.21[b]	30.90%+/-2.78[c]	35.26%+/-1.76[c]
T	Delta Pine Acala 90	4.72%+/-2.83[a]	30.23%+/-0.30[b]	62.82%+/-3.76[c]	2.23%+/-0.73[a]
S	Acala 1517-88	6.41%+/-3.01[a]	31.24%+/-0.31[b]	61.12%+/-3.66[c]	1.23%+/-0.07[d]
I	Molopo	11.85%+/-0.71[a]	11.75%+/-1.76[a]	33.61%+/-6.38[c]	42.79%+/-12.8[c]
I	Sicala	17.15%+/-1.33[a]	42.56%+/-16.3[b]	38.64%+/-6.07[b]	1.65%+/-0.01[d]

The data presented here indicate that there are differences in the proline level during a drought stress and those found during a combination of drought and heat stress. This can possibly be the result of different mechanisms for drought and heat stress. It appears as if the tolerance or sensitivity of cotton cultivars can be differentiated with the use of a free proline analysis. We also observed that there are differences in the proline level among different cultivars. These results have potential for application in breeding programmes.

3.2.1.7. The Effect of Drought and Heat on Polyamine Levels

When cells are stimulated to grow and divide, the synthesis of polyamines is rapidly induced. In eukaryotic cells ODC catalyses the initial step in the pathway (Heby and Person, 1990). It has been claimed that ODC plays a less important role in mung beans and that ADC is of greater significance (Altman *et. al.*, 1982). Turano and Kramer (1993) also observed in that the ADC activities were higher than the ODC activities in soybean under ammonia stress.

Changes in polyamine levels induced by drought or heat stress indicated that cotton plants have the capacity to increase their polyamine levels, especially those of spermidine and agmatine. The spermidine levels were mostly lower than those of the agmatine. The synthesis of the polyamines was found to be dependent on the cultivar, the age of the plant as well as the stress administered. Polyamine analysis *per se* could not be correlated to either drought or heat stress.

It was observed in six cotton cultivars tested for their ADC and ODC levels over a period of three weeks without water, that the enzyme levels differ over time. The ODC activities were found to be higher in cotton than the ADC activities (Table 3.24). This is in contradiction to Altman *et. al.* (1982)'s finding that cotton has a higher ADC activity than ODC activity in non-stress situations. It may be possible that cotton prefers the ODC pathway to polyamine synthesis under drought stress situations.

When the cultivars are ranked according to the amount of enzyme increase, the cultivars with the highest increase (Molopo, OR19 and Sicala) corresponded to those having the greatest drought sensitivity. The cultivars with the lowest amount of enzyme increase (Acala 1517-88, Alpha and

Delta Pine Acala 90) corresponded to drought tolerant cultivars. This shows that cotton accumulates more polyamine enzymes as a result of a drought stress. The extent of this accumulation is greater in sensitive cultivars (Table 3.24).

Table 3.24: Summary of the ADC and ODC levels observed in six cotton cultivars tested over a period of 3 weeks subjected to a drought stress.*significant difference

TTC rating	Cultivar	S-C ADC	Ranking ADC	Ranking ODC	S-C ODC
T	Alpha	0.066	4	5	0.126
T	Acala 1517-88	-0.263	6	6	-0.243*
T	Delta Pine Acala 90	0.036	5	4	0.286
S	OR19	0.206	2	2	0.426
S	Sicala	0.08	3	3	0.376
S	Molopo	0.336	1	1	0.790*

1 = maximum increase, indication of sensitivity

6 = minimum increase, indication of tolerance

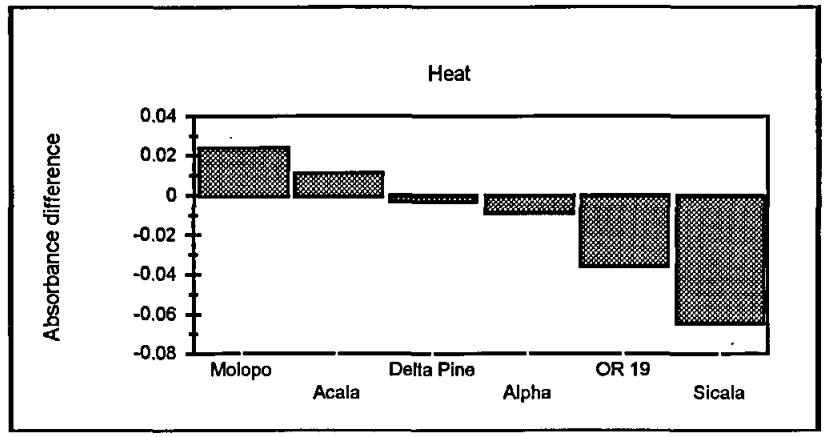
3.2.1.8. 2,3,5-Triphenyltetrazolium Chloride Viability Assay

Extreme temperatures can drastically inhibit photosynthesis by disrupting the electron transport system. To quantify the plant injury response and to be able to express differences between cultivars, much research has been devoted to finding absolute measures of plant injury resulting from temperature stress. These methods included electrolyte leakage (Chen *et. al.*, 1982) and accumulation of formazan (Krishnan *et. al.*, 1989). When leaf tissue is injured by high temperature, membrane permeability is increased and electrolytes diffuse out of the cells (Towill and Mazur, 1974). This allows the assessment of relative heat damage by measuring the capability of plant tissue to carry out electron transport with a TTC viability assay.

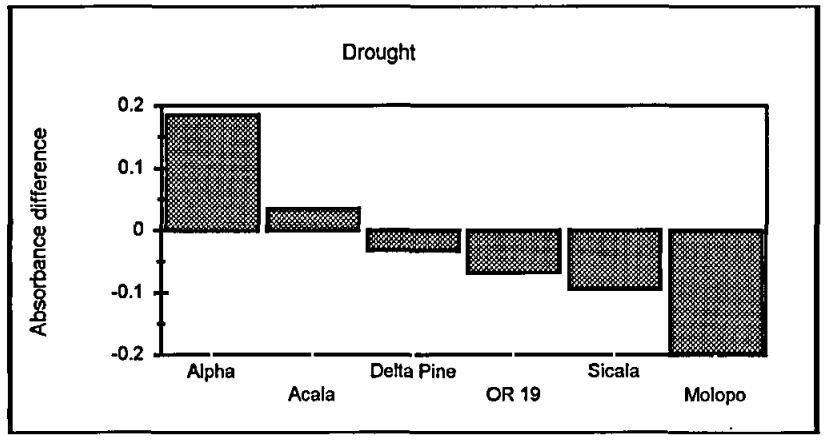
TTC reduction occurs in the mitochondria by the tetrazolium salt accepting electrons from the electron transport chain via the dehydrogenase pathway (Nachlas *et. al.*, 1993). The limiting enzyme in the sequence of electron transport in this system is known to be succinic dehydrogenase. The NADP- dependent malate dehydrogenase is a key enzyme of the CO_2 fixation pathway.

Vratsanos and Rossouw (1991) found that in heat tolerant potato cultivars, heat killing time increased to a greater extent after acclimation than in heat-sensitive cultivars. De Ronde *et. al.* (1995) hypothesized that formazan production would be lower in the stress treatment than in the control treatment in sensitive cultivars. This would be due to the fact that the moderate stress treatment would be experienced as a severe stress in sensitive cultivars and the plant could not adapt to drought stress and subsequently die when a lethal stress was applied. The tolerant cultivars, on the other hand, would have the ability to withstand an otherwise lethal stress when pre-treated with a moderate stress. This would result in a higher formazan production in the stress treatment compared to the control. The cotton plants were evaluated for heat tolerance and were ranked according to the difference between the mean absorbance of the control and the stress treatments. This listed the cultivars in a series with Molopo as the most heat tolerant and Sicala as the most heat sensitive (Figure 3.3a).

a



b



c

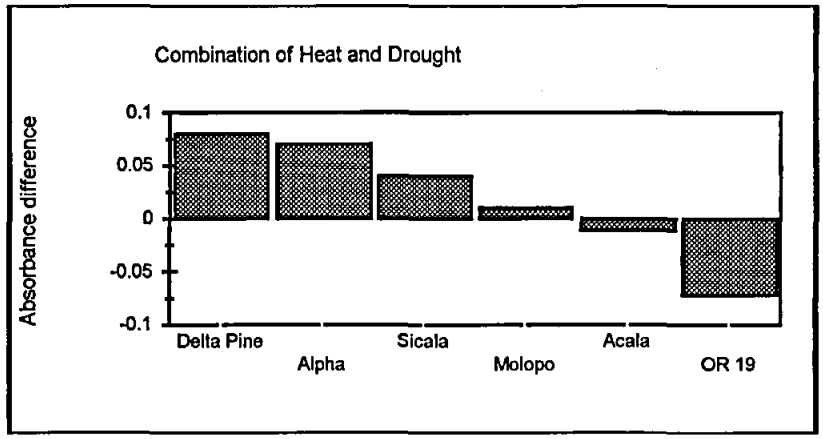


Figure 3.3: A summary of triphenyltetrazolium chloride assay in heat (a), drought (b) and combination (c) stressed cotton cultivars, using the difference between the stress treatment and the control treatment mean as a parameter.

This method of evaluating heat shock in cotton plants has been modified to predict drought tolerance. After drought acclimation, leaf discs were subjected to simulated drought stress by exposure to osmotic potentials. The cultivars were ranked accordingly. This listed the cultivars in a series with Alpha as the most drought - tolerant and Molopo as the most drought - sensitive (Figure 3.3 b). Drought simulation in the laboratory can differ from field conditions since in the latter heat stress cannot be eliminated. A study to determine the combined effect of heat and drought stress in the laboratory, ranked the cultivars with Delta Pine Acala 90 as the most tolerant and OR19 as the most sensitive (Figure 3.3 c).

The lower absorbance values obtained with the heat treatment than during a drought treatment, can be an indication of heat sensitivity. This correlates with lower Cu/Zn superoxide dismutase levels during a heat treatment. The correlation between heat and drought can be either positive or negative. Thus, a heat-tolerant cultivar, although drought sensitive, may give high yields in dry and hot areas. In contrast, a drought-tolerant but heat-sensitive cultivar could give a lower yield in a hot area. Such cultivars might then be incorrectly selected according to their tolerance to drought stress. A stress index can thus be established to distinguish between plant responses to heat and drought.

Succinic dehydrogenase (SDH) provides plenty of electrons to the electron transport chain via the dehydrogenase pathway, where the tetrazolium salt accepts the electrons and reduces TTC to formazan. Thus, a higher SDH activity can result in more formazan reduction. Whereas Sicala demonstrated an increase in SDH activity from one week of drought stress, Delta Pine Acala 90 showed an increase in the activity only after two weeks of drought stress. A summary of the difference between the stress treatment mean and the control treatment mean listed the cultivars with Sicala having the highest SDH activity increase and Delta Pine Acala 90 with the lowest activity increase (Table 3.25).

Malate dehydrogenase (MDH) catalyses the reduction of oxaloacetate to malate by using NAPH generated by photosynthetic electron transfer and plays a role over stomatal closure. All the cotton cultivars except Sicala, responded to a drought stress with an increase in the MDH activity. A summary of the difference between the stress treatment mean and the control treatments mean

listed the cultivars with Acala 1517-88 having the highest MDH activity increase and OR19 having the lowest activity increase (Table 3.25).

The interaction between the enzymes if of great importance as they play a role in different parts of the electron transfer system. It is shown that there is a correlation between the TTC ranking of drought stressed cotton leaves (De Ronde *et. al.*, 1997) and the enzyme activity of the cultivars tested. The cultivars found to be tolerant (Alpha, Acala 1517-88 and Delta Pine Acala 90), reacted to both SDH and MDH assays with an increase in activity. The drought sensitive cultivars (OR19, Sicala and Molopo) experienced an increase in either one enzyme activity or none. We postulated that a cultivar needs both enzymes to increase in activity to be tolerant to drought.

Table 3.25: Summary of the effect of succinic dehydrogenase and malate dehydrogenase on cotton plants subjected to a drought stress

TTC rating	Cultivars	Succinic dehydrogenase	Malate dehydrogenase
T	Alpha	increase after 2 weeks	increase after 2 weeks
T	Acala 1517-88	increase after 2 weeks	increase from week 1
T	Delta Pine Acala 90	increase after 2 weeks	increase from week 1
S	OR 19	decrease after 2 weeks	very low activity
S	Sicala	increase from week 1	decrease from week 1
S	Molopo	decrease after 2 weeks	increase from week 1

3.2.1.9. Summary

The best screening methods for drought stress are summarized in Table 3.26. From the presented data it is evident that the most promising physiological traits to use as a screening method for drought tolerance are chlorophyll fluorescence levels (Fm, qQ and Fv/Fm), TTC viability assay (including ODC/SDH levels) and ADC/MDH levels.

It can be concluded that the cultivars; Alpha, Acala 1517-88 are drought tolerant and the cultivars; Sicala and Molopo are drought sensitive.

The best screening methods for heat stress are summarized in Table 3.27. From the presented data it can be concluded that the best screening traits for heat tolerance are the antioxidative enzymes (Cu/Zn SOD, peroxidase and glutathione reductase) and the TTC viability assay. It can be concluded that the cultivars Molopo, Acala 1517-88 and Delta Pine Acala 90 are tolerant to heat stress and the cultivars Sicala, OR19 and Alpha are sensitive to heat stress.

It was not possible from the presented results to identify specific genes involved in drought or heat tolerance for cotton.

Table 3.26: Summary of the physiological reactions of 6 cotton cultivars to drought stress

Known tolerance	Cultivars (ranked according to TTC)	Chlorophyll fluorescence	Cu/Zn SOD	Proline	ODC, ADC	SDH, MDH	TTC
T	Alpha	+	+	+	+	+	+
T	Acala 1517-88	+	+	+	+	+	+
T	Delta Pine Acala 90	+	-	-	+	+	(+)
S	OR19	(+)	+	-	-	-	-
S	Sicala	-	-	+	-	-	-
S	Molopo	-	-	-	-	-	-

\+ = tolerant reaction

\- = sensitive reaction

(+) = intermediate reaction

Table 3.27: Summary of the physiological reactions of six cotton cultivars to heat stress

Known tolerance	Cultivars (Ranked according to TTC)	Chlorophyll fluorescence	Antioxidative enzymes	TTC
T	Molopo	(+)	+	+
T	Acala 1517-88	+	+	+
I	Delta Pine Acala 90	-	+	(+)
I	Alpha	(-)	-	(-)
S	OR19	(+)	-	-
S	Sicala	-	-	-

\+ = tolerant reaction

\- = sensitive reaction

(+) = intermediate reaction

3.2.2. Heritability and Combining Ability Studies in Cotton

A diallel cross system was performed on the six cotton cultivars with a 62% survival rate. Problems arising included no seed formation, deformation and sterility of seed, as well as mortality of seedlings. It was found that the TTC viability assay correlates with the field trials of drought tolerance (De Ronde *et. al.*, 1997). Thus, it was decided to test all the crosses with a TTC assay in order to determine their heritability and combining ability. The stress minus control values of all the crosses in the diallel was used in measuring the combining abilities of the six cultivars.

According to Griffing method I (Griffing, 1956), the general combining ability (GCA) result differs significantly ($p=0.004$) between the additive effects of the parents in a drought situation. As expected the specific combining ability (SCA) was non-significant ($p=0.26$), indicating no real difference between the specific effects of the parents involved with a drought stress. With regarding to the heat treatment, the GCA was significantly different ($p=0.0021$) and the SCA non-significant.

The cultivars with the highest tolerance ratings for drought stress, Alpha and Acala 1517-88 showed a high combining ability for drought stress. The same tendency was found for the tolerant cultivars for a heat stress, Molopo and Acala 1517-88. The sensitive cultivars, Molopo (drought stress) and OR19 (heat stress) have very low combining abilities (Table 3.28).

Heritability expresses the proportion of the total phenotypic variable in a sample attributable to genetic variance. The heritability observed for drought was $h^2 = 0.30$ and for the heat $h^2 = 0.33$. This indicated that the TTC viability assay can be used in a cotton-breeding programme as the differences between TTC values is heritable.

Table 3.28: Summary of the combining abilities of six cotton cultivars compared with their tolerance ratings

Combining ability drought	**TTC tolerance rating drought**	**Combining ability heat**	**TTC tolerance rating heat**
Acala 1517-88 (0.0028)	Alpha	Molopo (-0.0004)	Molopo
Alpha (0.0007)	Acala 1517-88	Acala 1517-88 (-0.0008)	Acala 1517-88
Sicala (-0.0002)	Delta Pine Acala 90	Delta Pine Acala 90 (-0.0014)	Delta Pine Acala 90
OR19 (-0.0003)	OR19	Alpha (-0.0018)	Alpha
Delta Pine Acala 90 (-0.0003)	Sicala	Sicala (-0.0018)	OR19
Molopo (-0.0015)	Molopo	OR19 (-0.0029)	Sicala

3.3. TOBACCO

3.3.1. Screening methods

3.3.1.1. Water Gradient Study as a Screening Method for Drought Tolerance

The highest leaf dry weight in all treatments was measured in Elsoma, which confirms this cultivar's ability to grow under drought conditions and therefore its drought tolerance. Domkrag can also be classified as drought tolerant and Pobeda, CDL 28 and TL 33 as sensitive, when leaf dry weight is considered. This result correlates with the results obtained during the proline studies.

According to Taylor and Klepper (1973) deep-rooted plants survive drought better than shallow-rooted plants, because more water is accessible to deep root systems. When the root fresh weight was considered no correlation between the drought tolerance and root fresh weight was found. Root fresh weight is not a true indication of true root biomass of plants, due to the possible difference in water content, therefore, the root dry weight was determined. A gradual increase in dry root weight occurred in all the cultivars tested. Elsoma produced the highest biomass, which indicates greater drought tolerance.

The root-leaf interaction can be used as a possible indication of drought tolerance. TL 33, CDL 3 and Pobeda developed the highest root:leaf ratios with mild to severe stress. Elsoma, CDL 28 and Basma developed the highest ratio with a mild water stress. All cultivars (except TL 33) showed a lower ratio with no water stress, which may indicate that deeper rooting was not induced. From this study it is therefore concluded that deeper rooting might be seen as a defense mechanism to overcome drought stress (Table 3.30).

3.3.1.2. C^{14} - Proline Incorporation

Results indicated that this method could not be used as a screening method, as no correlation could be found between incorporation of C^{14} proline and drought tolerance.

3.3.1.3. Chlorophyll Fluorescence as a Possible Screening Method

The measurement of chlorophyll fluorescence is a technique that allows the quantitative assessment of a plant's response to a wide range of environmental stresses such as heat, cold, water stress (Wilson and Greaves, 1992; Havaux *et. al.*, 1988) and salinity (Belkhodja *et. al.*, 1994). A decrease in the photochemical quenching (qP) may be the effect of limitation in the dark reaction, as a result of the inhibition of the Calvin cycle which influence electron transport negative.

During this study Basma and CDL 3 showed a significant decrease in photochemical quenching early in the study and can therefore be classified as sensitive. The other cultivars showed a decrease in photochemical quenching during the last week only and can therefore be considered to be more tolerant (Table 3.31). Leaf dehydration influences qP by inhibition of photosynthetic carbon assimilation, which might be caused by inactivation of the carbon-reduction cycle (Havaux *et. al.*, 1988).

In the more sensitive cultivars (Basma and CDL 3), this decrease in qP was seen during the first two weeks of the stress, but with other cultivars during the last week of the stress. Furthermore, this inhibition also causes dysfunctioning of the thylakoids, which was visualized in the decrease of non-photochemical quenching in the control values after the second week in all the cultivars. These results are in agreement with work done by Krause and Somersalo (1989).

According to Ögren (1990) the activity of the carbon reduction cycle may be especially sensitive to drought stress. This would be reflected in the Fv/Fm ratio. This ratio was therefore used to assess and classify the cultivars according to their tolerance to severe drought stress. Pobeda, Basma and CDL 3 showed a significant decrease in the Fv/Fm ratio; TL 33, Domkrag and CDL 28 showed no or almost no change in the Fv/Fm ratio while Elsoma displayed a significant increase in the Fv/Fm ratio which can reflect an ability to overcome this stress period (Table 3.31).

Table 3.30: Water Gradient study as a screening method for drought tolerance

Known tolerance	Cultivars	Leaf dry weight	Root:Leaf
T	Elsoma	+	+
T	Domkrag	+	-
S	Pobeda	-	-
S	CDL 28	-	+
S	TL33	-	-
I	Basma		+
S	CDL 3		-

\+ = tolerant reaction

\- = sensitive reaction

Table 3.31: Chlorophyll fluorescence as a possible screening method

Known tolerance	Cultivars	Photochemical quenching	Fv/Fm
T	Elsoma	+	+
T	Domkrag	+	(+)
S	Pobeda	+	-
S	CDL 28	+	(+)
S	TL33	(+)	(+)
I	Basma	-	-
S	CDL 3	-	-

\+ = tolerant

(+), (-) = intermediate

\- = sensitive

3.3.1.4. Proline Accumulation

The accumulation of proline during stress conditions has been well documented in the literature and therefore the free proline concentration during stress and control conditions was examined. By examining the free proline accumulation the cultivars were arranged according to proline accumulated after two weeks of drought stress. The accumulation after two weeks was chosen, because the tobacco plants were severely but not lethally stressed and therefore able to recover on re-watering. Elsoma, the drought tolerant cultivar exhibited the highest concentration of free proline, while TL 33 and Basma, known as sensitive cultivars accumulated the least proline. This consistency led to the assumption that there is a correlation between quantity of proline accumulation and sensitivity or tolerance. Accordingly, CDL 28 was classified as more drought tolerant than Pobeda.

Therefore, it is concluded that the accumulation of proline is not merely an incidental result of other stress-induced changes in metabolism but provides an adaptive advantage to the plants. Although different opinions on the accumulation of proline have been documented in the literature, these results are in accordance with work done by Aloni, Rosenshtein (1984) on tomato varieties. They suggested that proline accumulation plays a role in the adaptive response of tomato plants to water deficiency by serving as a substrate for post-stress recovery. By using proline accumulation after two weeks of drought stress the tobacco plants were ranked according to their drought tolerance (Table 3.32).

3.3.1.5. Polyamine Titres during a Drought Treatment

In all the cultivars, except Elsoma the Putrescine (Put) concentration is equal or higher in the stress than in the control treatment. In the literature the accumulation of Put resulting from *de novo* synthesis of arginine decarboxylase is well documented. However in this report we report that in tobacco leaves no such reaction was seen. These results are in agreement with work done by Tiburcio, Campos, Figueras and Besford (1986), who reported a decrease in Put titres in dicotyledonous leaves, while Spermidine (Spd) and Spermine (Spm) titres increased. In this study a major increase in the Spd titres with a minor increase in Spm titres can be observed (Figure 3.4).

Table 3.32: Ranking of free proline during drought stress

Known tolerance	Cultivars	Free –proline ranking
T	Elsoma	1
T	Domkrag	2
S	Pobeda	7
S	CDL 28	3
S	TL33	6
I	Basma	5
S	CDL 3	4

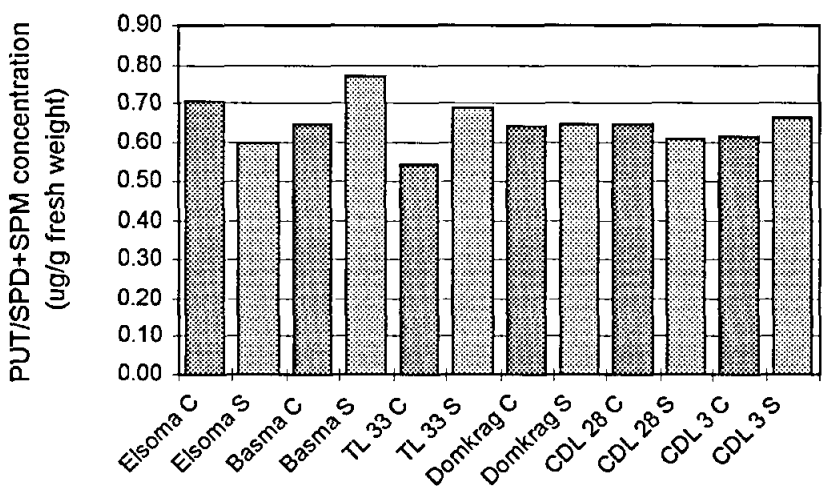


Figure 3.4: Diamine/polyamine ratio at 3 weeks without water

C = control treatment;

S = drought stress treatment

Tiburcio *et. al.* (1986) who suggested that a decrease in the Put/Spd+Spm ratio was advantageous to plants. This would indicate that the diamines are converted effectively to polyamines. They suggested that the higher polyamines (Spd and Spm) may play an important role in DNA synthesis and mitosis since they are essential for cell division and that high concentrations of endogenous Put appear to be toxic to certain cells. This phenomenon was confirmed in our study, especially with regard to the tolerant cultivars (Elsoma and CDL 28) which gave a decrease in the diamine/polyamine ratio (Figure 3.4). Therefore the accumulation of the polyamine Spd might be beneficial to tobacco plants during drought stress.

3.3.1.6. 2,3,5 Triphenyltetrazolium Chloride Viability Assay

The ability of viable cells to reduce tetrazolium salt has been reported in a variety of plants subjected to various stresses. A negative correlation between formazan production and drought stress was observed (data not shown). A possible correlation between heat tolerance and field performance under high temperature conditions was previously found (Chen *et. al.*, 1982; Martineau, Specht, Williams and Sullivan, 1979; Sullivan and Ross, 1979). A number of variants play a role in the physiological mechanism of plants, with the resistance to cold, heat and drought interrelated. It was found in potatoes that drought and heat tolerance was negative correlated (Van der Mescht, De Ronde, Van der Merwe, Daniels and Rossouw, 1998b; Li *et. al.*, 1981). It is thus suggested that the effect of a heat treatment must be investigated as this may explain the negative correlation observed.

3.3.1.7. Conclusion

All these techniques provided an important insight into the drought responses of the tobacco plant. The best screening methods for drought tolerance in tobacco proved to be free proline and polyamine analysis (Summarized in Table 3.33). As the synthesis of free proline share a biochemical pathway with polyamine at the intermediate glutamic acid and L-ornithine (Adams and Frank, 1980; Altman *et. al.*, 1982), it can be postulated that this combined pathway plays a dominant role in the mechanism contributing to drought tolerance in tobacco. From the presented data it is evident that cultivars Elsoma, Domkrag and CDL 28 performed as drought tolerant and

cultivars TL33, Basma and CDL 33 as drought sensitive. As the combined pathway of proline and polyamine work together in sustaining drought tolerance in tobacco, it was not possible to identify a single gene or genes contributing to drought tolerance.

Table 3.33: Summary of the physiological reaction of tobacco cultivars to drought stress

Cultivars	Water gradient	Chlorophyll	Free proline	Polyamine	TTC
Elsoma (T)	+	+	+	+	-
Domkrag (T)	(+)	+	+	+	-
Pobeda (S)	-	(+)	-		+
CDL 28 (S)	(+)	+	+	+	(+)
TL33 (S)	-	(+)	-	-	+
Basma (I)	(+)	-	(-)	-	(-)
CDL 3 (S)	(-)	-	(+)	-	-

+ = tolerant reaction

(+), (-) = intermediate reaction

- = sensitive reaction

T = tolerant

S = sensitive

I = intermediate

3.4. MAIZE

3.4.1. Screening Methods

3.4.1.1. Triphenyltetrazolium Chloride Reduction Assay on Inbred Lines

The hypothesis behind the triphenyltetrazolium chloride reduction assay (TTC) is that sensitive cultivars will have a lower formazan production during the stress treatment than during the control treatment. This is due to the fact that a moderate stress treatment is experienced as a severe stress and the plant cannot adapt to drought stress. Tolerant cultivars on the contrary will have the ability to adapt an otherwise lethal stress when pretreated with a moderate stress. This will result in higher formazan production in the stress treatment compared to the control.

When inbred lines were evaluated at the onset of pollination in the summer growth season, the absorbance values of the drought stressed plants showed that eight of the inbred lines performed better over time since the absorbance values of the stress treatments were higher than in the control treatment. This response indicated that these cultivars were drought tolerant with F2834T giving the best result (Figure 3.5 a). The formazan absorbance values of the other six inbred lines were lower in the stress treatments compared to the control treatments, which indicates that these lines are sensitive to drought stress. The inbred lines were also evaluated at the ten-leaf stage and the lines were ranked according to their drought tolerance in Figure 3.5 b. The developmental stage of the inbred lines has a definite effect on the drought tolerance rating according to TTC-reduction (Table 3.34).

When the plants were tested in the winter, it was found that there was a difference in the ranking of the inbred lines, which might indicate that the off season is not a good time to conduct these tests.

It is possible that in some cases the inbred lines performed differently in the laboratory tests than in the field, due to the fact that the plants tolerance to drought and heat differs. Since heat plays a dominant role in this interaction, the reaction of the lines to heat stress was also tested. The stress index is presented in Table 3.34.

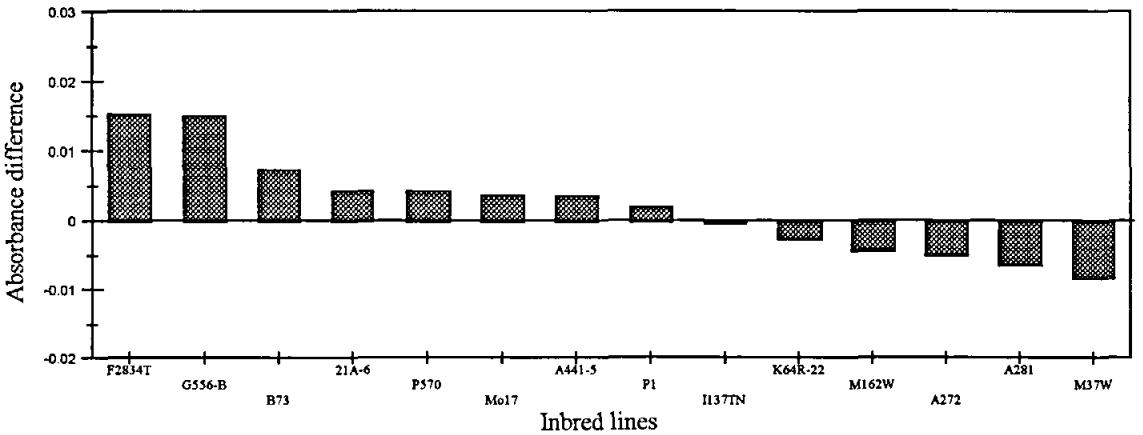


Figure 3.5 a: A summary of a triphenyltetrazolium chloride assay in drought stressed maize inbred lines at the onset of pollination (old), using the difference between the stress treatment mean and the control treatment mean as a parameter.

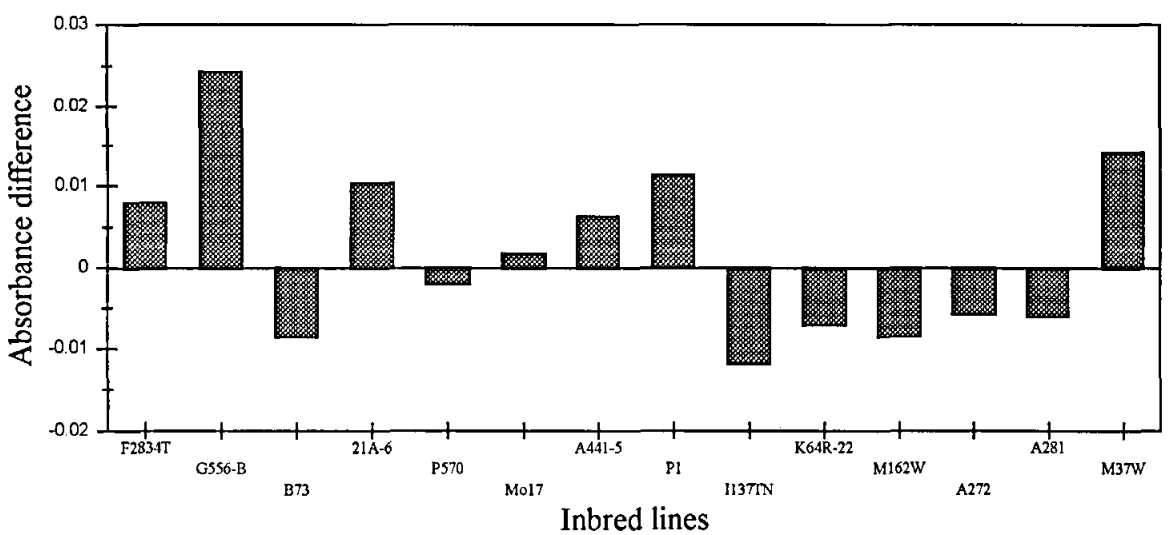


Figure 3.5 b: A summary of a triphenyltetrazolium chloride assay in drought stressed maize inbred lines at the ten leaf stage (young), using the difference between the stress treatment mean and the control treatment mean as a parameter.

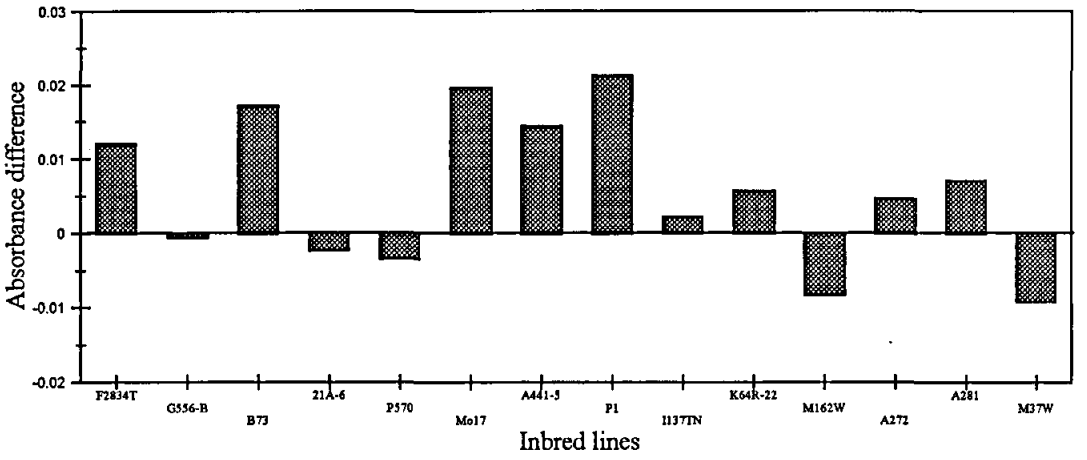


Figure 3.5 c: A summary of a triphenyltetrazolium chloride assay in heat stressed maize inbred lines at the onset of pollination (old), using the difference between the stress treatment mean and the control treatment mean as a parameter.

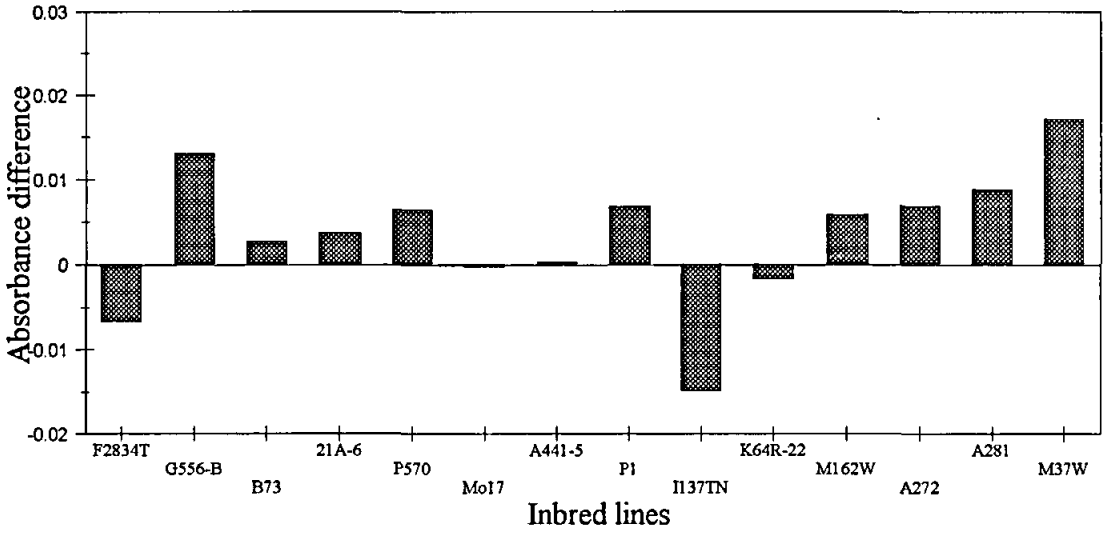


Figure 3.5 d: A summary of a triphenyltetrazolium chloride assay in heat stressed maize inbred lines at the ten leaf stage (young), using the difference between the stress treatment mean and the control treatment mean as a parameter.

Table 3.34. Summary of drought and heat tolerance of fourteen maize inbred lines at different growth stages during the summer growth season as calculated according to TTC reduction tests

Cultivar	Drought (old)	Drought (young)	Heat (old)	Heat (young)
F2834T	1	5	5	13
G556-B	2	1	10	2
B73	3	13	3	9
21A-6	4	4	11	8
P570	5	8	12	6
Mo17	6	7	2	11
A441-5	7	6	4	10
P1	8	3	1	5
I137TN	9	14	9	14
K64R-22	10	10	7	12
M162W	11	12	13	7
A272	12	9	8	4
A281	13	11	6	3
M37W	14	2	14	1

When inbred lines were evaluated, for heat tolerance at pollen shed, the results showed that nine of the inbred lines reacted as heat tolerant while only five reacted as sensitive (Figure 3.5 c). The inbred lines were also evaluated at the ten-leaf stage. Results showed that only two of the inbred lines were sensitive to heat, three were intermediate and the rest of the lines reacted as heat tolerant (Figure 3.5 d). The developmental stages of the inbred lines also have a definite effect on the heat tolerance rating according to TTC-reduction. Nine (old) and ten (young) lines reacted as heat tolerant, in contrast with eight (old) and seven (young) lines that performed as drought tolerant. From the results it is clear that more lines are tolerant to heat than to drought stress.

The correlation between heat and drought can be either positive or negative. Thus, a heat tolerant line, although being drought sensitive, may survive in dry and hot areas. A drought tolerant but heat sensitive line on the other hand, may show lower survival rates in hot areas. Such lines will then be incorrectly selected or rejected as parents in breeding programmes. The correlation between heat - and drought tolerance is summarized in Table 3.34. The economic benefit of being able to distinguish between plant responses to different environmental stresses, in a breeding programme in very high. By using this assay as a metabolic indicator, a stress index can be established

3.4.1.2. Physiological Differences Between Drought Resistant and Drought Sensitive Maize Cultivars in Response to Water Stress

An aspect that has to be considered in applying water stress is that some of the responses may be due to cellular injury resulting from shock. Sudden imposition of stress, such as immersion of leaf discs in PEG solution, can cause damage to cellular constituents which is not related to the plants ability to withstand water stress, whereas the more realistic gradual imposition of stress may lead to adaptation and the ability to withstand even greater stress (Leone, Costa, Tucci and Grillo, 1994).

In this study the application of water stress was simulated by growing plants in pots containing soil and withholding water, thus allowing gradual adaptation to soil drying to reduce the effects of tissue damage from shock. Genotypic differences occur in the growth response of maize (*Zea*

mays L.) to water stress (Hall, Lemcoff and Trapani, 1981; Lorens, Bennet. and Loggale, 1987a and b; Sobrado, 1990). Although genotypic differences in response to water stress have also been identified for a range of morphological and physiological characteristics, including root development (Hurd 1974), stomatal activity (Beadle, Stevenson, Neumann, Thurtel. and King, 1973; Beardsell and Cohen 1975; Ackerson, Krieg and Sung, 1979; Quarrie 1980; Ackerson 1983), osmotic adjustment (Ackerson *et. al.* 1979), ABA (Beardsell and Cohen 1975; Quarrie 1980; Ackerson 1983) and proline levels (Blum and Ebercon 1976; Hanson, Nelsen and Everson,. 1977; Quarrie 1980; Thakur and Rai 1981), it is uncertain which characteristics are important in maintaining growth under conditions of water stress.

Physiological measurements were done on the two cultivars chosen for study (PAN473 and SR52) to establish that there is indeed a physiological basis to their differing response to water stress. Comparisons were made with respect to root and leaf growth, stomatal activity, water relations, ABA and proline levels. The maize plants were water stressed by soil drying in preference to other methods, since this would more accurately reflect what happens in the field.

In the control treatment the mean whole plant dry weight of the sensitive cultivar was greater (14.6 g ±0.58 standard error) than that of the resistant cultivar (13.2 g ±0.53), though the difference was not significant (Student's *t*-test, t=1.94, p=0.068). In the water-stress treatment the mean whole plant dry weight of the resistant cultivar was significantly greater (7.2 g ±0.33) than that of the sensitive cultivar (6.0 g ±0.30) (t=2.71, p=0.014). There was a greater reduction in the stress treatment of mean total dry weight of the sensitive cultivar (41% of the control treatment) than the resistant cultivar (55% of the water-stress treatment). There was no significant difference between the cultivars or the treatments in the root:shoot ratios, which varied between 0.10 and 0.11. In the control treatment, the root dry weight of the resistant cultivar was 1.33 g ±0.052 and the sensitive cultivar 1.47 g ±0.070. In the water-stress treatment the root dry weight of the resistant cultivar was 0.79 g ±0.050 and the sensitive cultivar 0.63 g ±0.045. The growth measurements confirm that there was a difference between the cultivars in their sensitivity to drought, and that PAN473 is indeed more drought resistant than SR52. Under conditions of optimal water availability the growth rate and grain yield of the more sensitive cultivar was higher than that of the more resistant cultivar, but under conditions of severe water stress the positions

were reversed and the resistant cultivar had a significantly higher growth rate and grain yield. This inverse correlation between growth rate and resistance to stress has been widely observed amongst plant species. Plants with a high potential growth rate or yield under optimal conditions are less able to withstand environmental stress compared with their slower growing relatives (Chapin, Bloom, Field and Waring, 1987). It appears that the adaptations required for tolerating stress conditions limit growth rate under optimal conditions.

The differences between the cultivars in the various measurements are inherent in the plants and not due to differences in soil water availability since there was no difference between the cultivars in their soil water potentials (Table 3.35). This is important because a plant transpiring at a faster rate will dry out the soil in the pot and will therefore be exposed to a greater level of drought stress than a plant transpiring at a slower rate. The water content of the soil was maintained by watering each pot to a constant recorded weight. The plants in the control treatment and those in the drought stress treatment up to the point of the drying cycle were therefore exposed to the same conditions of soil water potential.

The relative water content of the sensitive cultivar was lower than those of the resistant cultivar in the stress treatment (Figure 3.6) even though the transpiration rate of the sensitive cultivar was lower (Figure 3.7). This indicated that the sensitive cultivar couldn't replace water lost to transpiration under stress conditions as well as the resistant cultivar. This may be accounted for at least partly by the fact that the resistant cultivar has a greater proportion of its roots in the lower soil layer where the soil water potential was higher (Figure 3.8). Its roots were thus able to extract more water from the soil. An increased effectiveness in supplying water to the leaves of roots deeper in the soil profile when the upper layers have dried has been noted by other researchers (Sharp and Davies 1985). Similar genotypic differences have been found for wheat where cultivars with greater stress tolerance had more extensive and deeper root systems (Hurd 1974).

The drought resistant cultivar had a much greater concentration of proline at peak drought stress and a rapid recovery to pre-stress levels on rewatering (Figure 3.9).

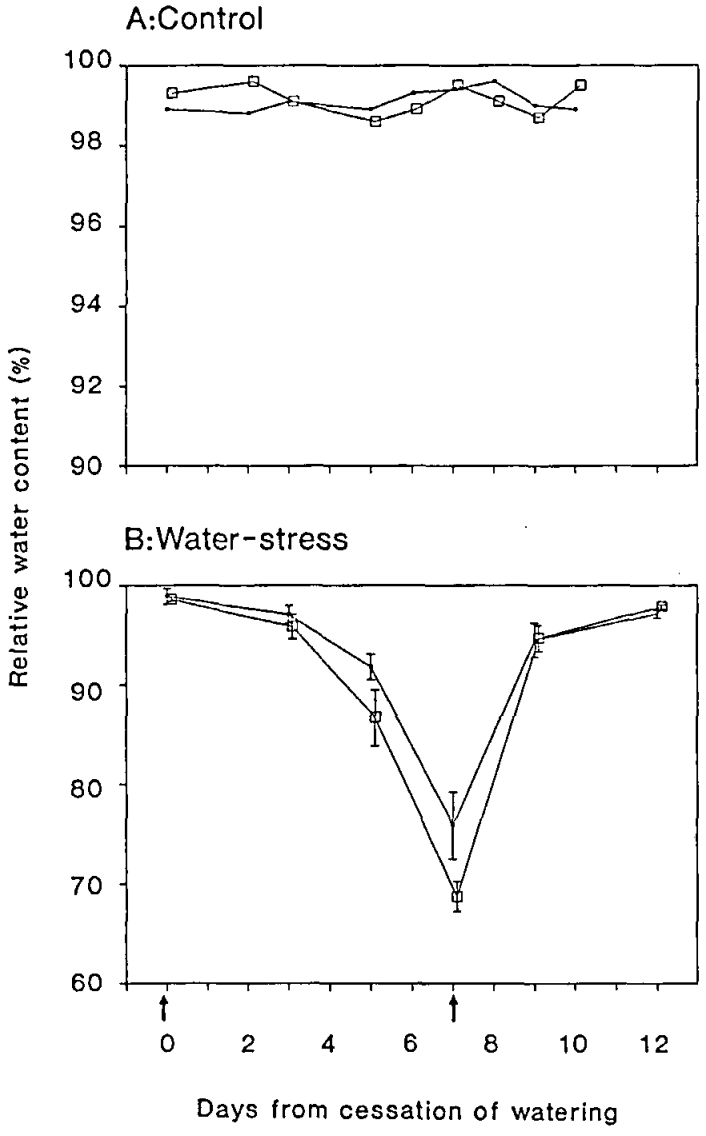


Figure 3.6: Relative water content of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52.

(-·-) = resistant cultivar; (-□-) = sensitive cultivar.

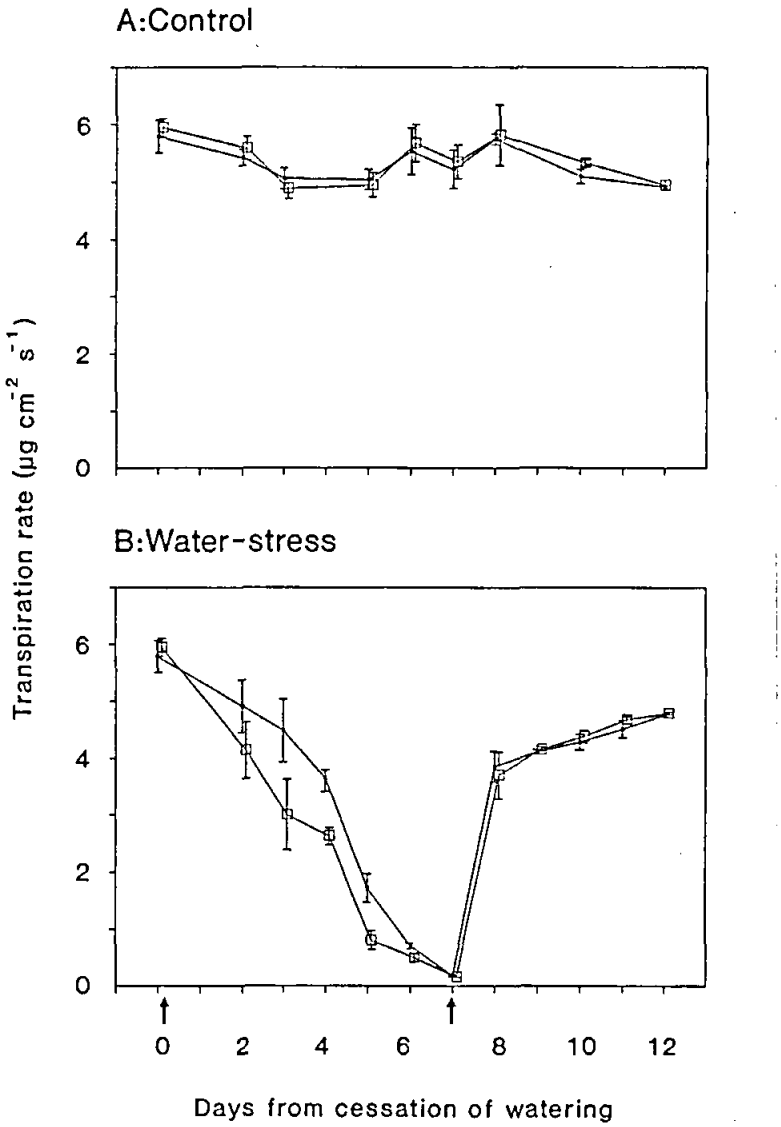


Figure 3.7: Transpiration rate of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52.

(-·-) = resistant cultivar; (-□-) = sensitive cultivar.

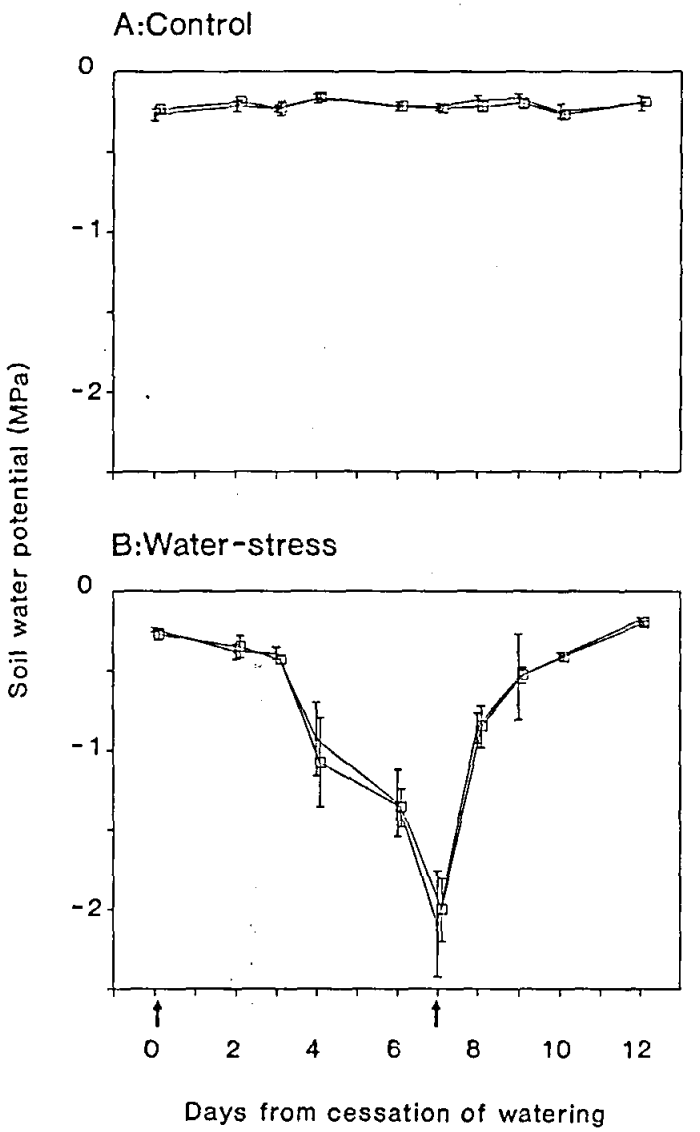


Figure 3.8: Soil water potential in the pots in which the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52 were growing. (-·-) = resistant cultivar ; (-□-) = sensitive cultivar.

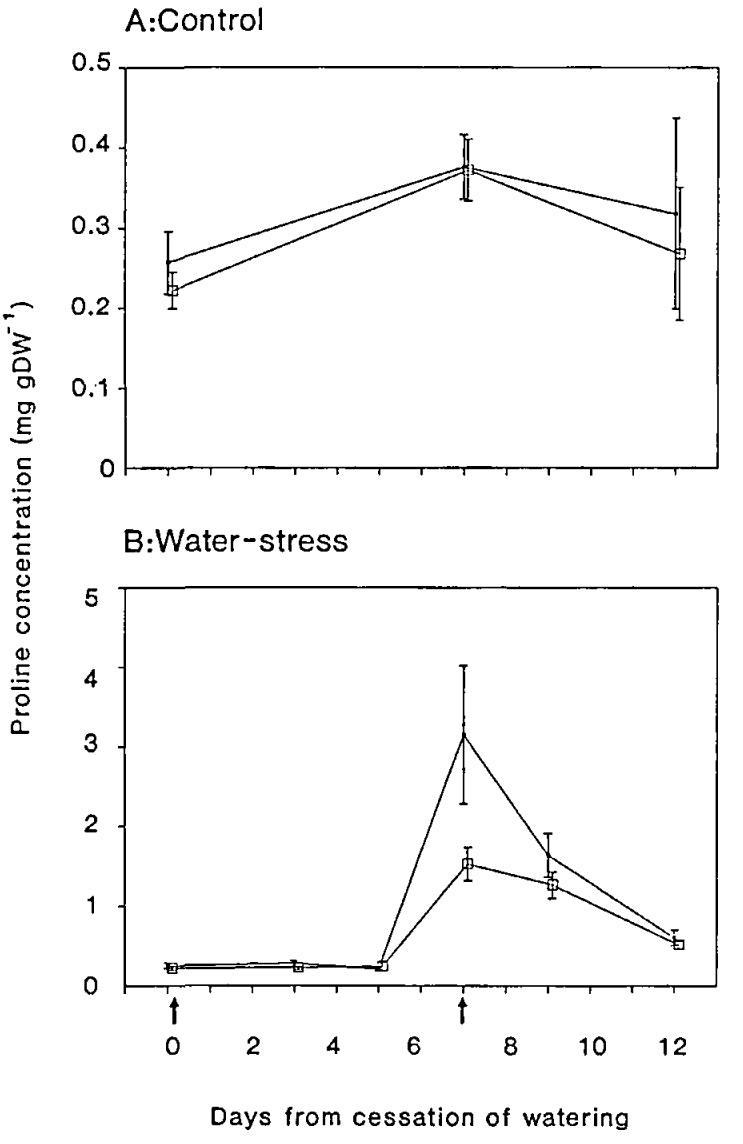


Figure 3.9: Proline concentration of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52.

(-·-) = resistant cultivar; (-□-) = sensitive cultivar.

In this study, the more drought resistant maize cultivar had increased root growth to deeper levels in drying soil, so it is possible that this increased root growth resulted from a greater osmotic adjustment brought about by a greater increase in proline concentration. However, proline is not the only contributing factor to increase root growth in drying soil. ABA, the levels of which increase in plants subjected to water stress, can also stimulate the growth of primary root axes (Yamaguchi and Street 1977; Saab, Sharp, Pritchard and Voetburg, 1990) thereby increasing the depth to which roots penetrate the soil profile (Watts, Rodriguez, Evans. and Davies, 1981). Drought resistance need not be attributed entirely to the physiology of the plant, and can be a result of phenotype. The resistant cultivar had higher levels of abscisic acid than the sensitive cultivar in the water-stress treatment (Figure 3.10), so it is possible that the same mechanisms of rooting response to abscisic acid are operating.

Drought resistance need not be attributed entirely to the physiology of the plant, and can be a result of phenotype. The two cultivars studied do differ in one important phenotypic characteristic that is related to yield under drought, and that is prolificacy. Under conditions of stress, if an ear is aborted then a prolific plant can rely on a second or subsequent ear to still produce a yield, whereas a non-prolific plant will lose its entire yield (Russel and Eberhart, 1968).

3.4.1.3. Conclusion

This study has established that the difference between the cultivars in their response to drought can indeed be attributed to a difference in their physiology and not just to a difference in phenotype. The difference in the physiological response is an increase in root growth to lower soil depths correlated with a larger increase in proline and ABA concentrations in the more drought resistant maize cultivar. It is also possible that a difference in gene expression underlies the difference in physiological responses.

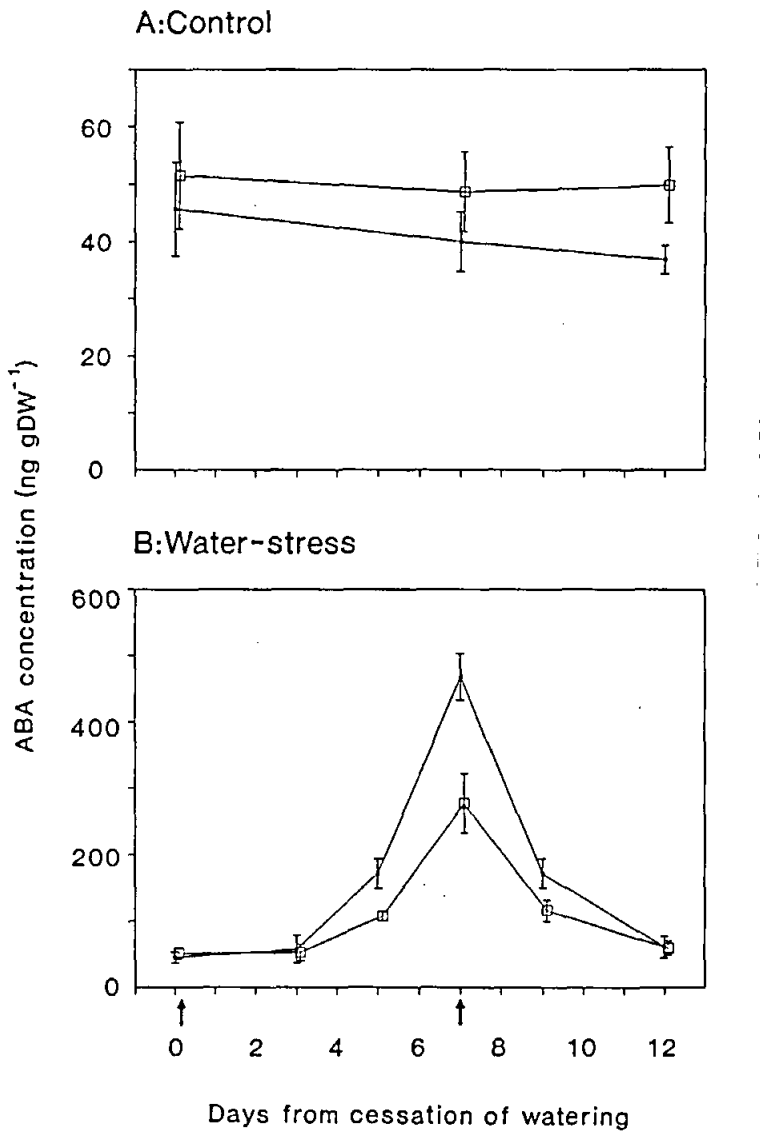


Figure 3.10: ABA concentration of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52

(-•-) = resistant cultivar; (-□-) = sensitive cultivar.

3.4.2. Differential Screening

3.4.2.1. RNA Isolation, cDNA Library Construction and Differential Plaque Cultivarization

Two maize cultivars with differing sensitivity to drought stress were studied. The cultivars are SR52, which is drought sensitive, and PAN473, which is drought tolerant. A differential screening of a water stress library was done on about 100 000 to 150 000 plaques per cultivar. The numbers of plaques cultivarizing more strongly with the water stress probe were 219 from the PAN473 library and 176 from the SR52 library. Those plaques cultivarizing more strongly with the water stress probe were isolated and reprobed to eliminate false positives. This second differential screening produced 24 positive plaques in PAN473 and 11 in SR52.

To determine the number of different clones represented in the isolated plaques, each plaque isolate was used to probe all the other positives. The 35 plaque isolates from the PAN473 and SR52 differential screening were found to represent ten different clones. Three clones are represented more than once, and six clones are represented only once. All the SR52 clones are present in the PAN473 isolates, and four are unique to the PAN473 isolates. Seven PAN473 isolates and three SR52 isolates did not cultivarize at all. The three clones that are represented more than once have been sequenced (Table 3.35, Figure 3.11).

3.4.2.2. Sequence Analysis of Two of the Drought - Related Genes in Maize

The paradigm of gene expression is: DNA makes messenger RNA and messenger RNA makes protein. If one wishes to know the function of a gene then one must know the polypeptide for which it codes, since this is the end product. Ten putative drought responsive cDNA clones were isolated from a maize drought stress cDNA library. Two of these clones were characterized (Figure 3.12 a, b). Clone A has a single open reading frame of 921 nucleotides with a derived amino acid sequence of 307 residues. There is a serine-rich region at the carboxyl-terminal end of the polypeptide. Only four amino acids make up 50% of the total sequence, and these are glycine, proline, arginine and serine. The predicted molecular weight is 33 775 Da. A hydropathy profile of the amino acid sequence shows clone A to be mainly hydrophilic.

Table 3.35: Results of the probing of the phage isolates showing the number of different cDNA species.

cDNA species	cDNA clone	
	PAN473	**SR52**
A. *rws*	3, 7, 17, 21	2, 9, 11
B. *rws*	11, 16, 24	3, 8
C. *rws*	5, 8, 19	7
D. *rws*	2	-
E. *rws*	6	-
F. *rws*	9	4
G. *rws*	10	-
H. *rws*	14	-
I. *rws*	15	1

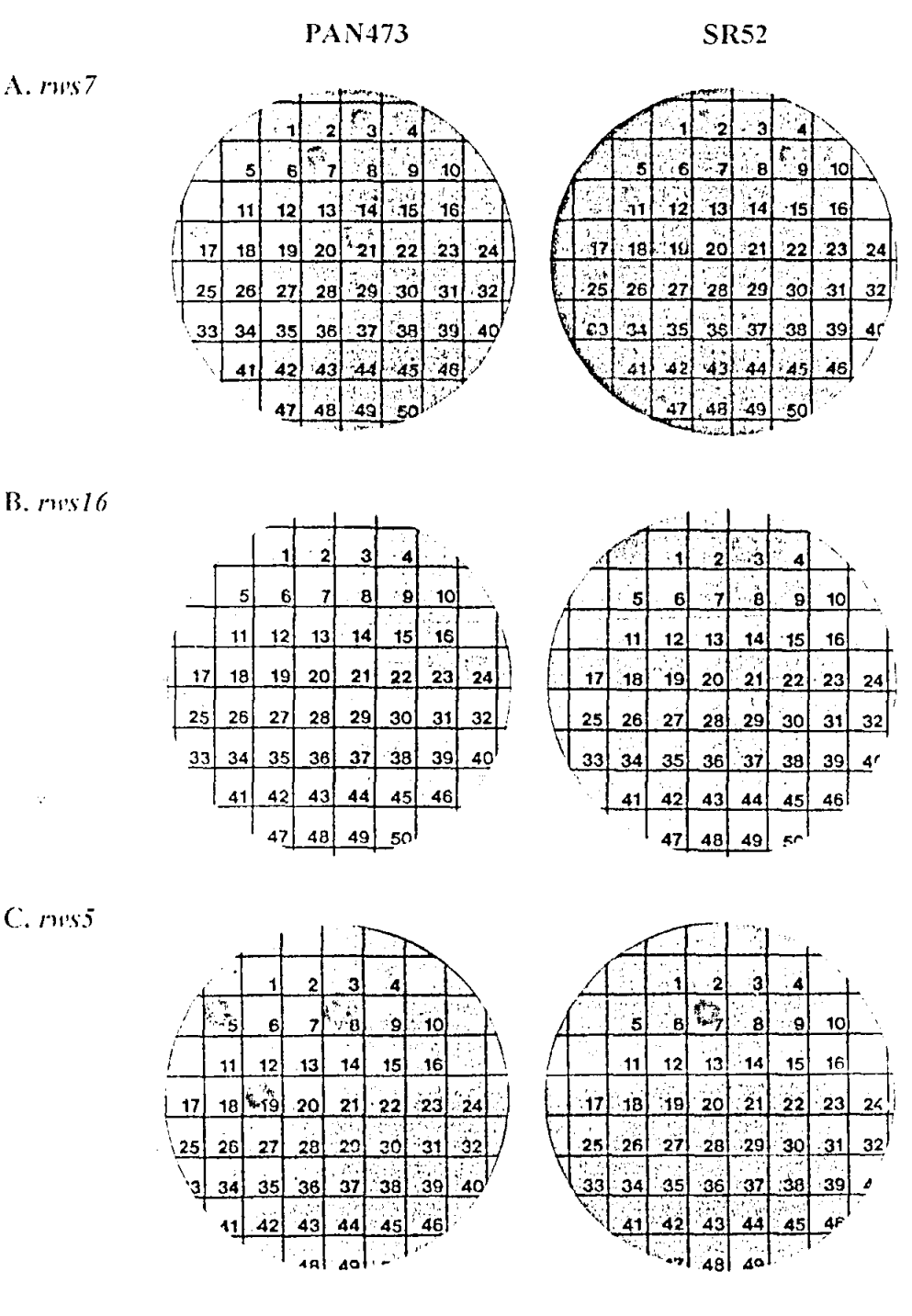


Figure 3.11: Autoradiographs of plaques cross-cultivarized with putative water stress cDNAs from the second round of differential screening to determine the number of different cDNA species represented. The putative water stress cDNA clones (24 PAN473 and 11 SR52) were plated out in a grid pattern. Replica membrane lifts were made of each plate and probed with [^{32}P]-labeled PCR product from each cDNA clone in turn. Plaque cultivarization with only three cDNA clones is shown namely *rws7*, *rws16* and *rws5*.

A BLAST search found no homologous amino acid sequences, but the characteristics of the polypeptide suggest that it is a dehydrin. Clone B has a coding sequence of 204 nucleotides. No methionine start codon was identified suggesting it is a partial sequence. A BLAST search found two homologous amino acid sequences, and both were chloride channel proteins. The one was an unpublished human chloride channel protein 210 amino acids in length to which clone B showed 33% identical amino acid homology and 56% positive amino acid homology. The other was a bovine chloride channel protein 437 amino acids in length, to which clone B showed 22% identical amino acid homology and 51% positive amino acid homology. Both genes showed increased transcription in response to drought stress in whole plants, and in response to desiccation and osmotic stress of detached leaves. No increase in transcription was found in response to exogenously applied abscisic acid or proline.

3.4.2.3. Characterization of Isolated Genes

The level of expression of a gene can change with the development of the plant, but in this experiment the controls did not show any observable change in mRNA levels of the three clones during the course of the drying cycle (Figure 3.13). The levels of mRNA increased with increasing water stress during the course of the drying cycle (Figure 3.13 a, b). Some other studies have shown gene expression to increase initially with the onset of drought, and to tail off with increasing water stress. The relative increases in mRNA levels are similar between this study and that of Stroeher, Boothe and Good, (1995). In this study the relative increases in the leaves over control levels were between eight- and seventeen fold compared with an eleven fold increase in a turgor-responsive gene from *B. napus* (Stroeher *et. al.* 1995).

Expecting a difference between the two cultivars in the pattern of expression of the three clones that can be correlated with their stress tolerance may be too simplistic. Some researchers have observed such correlations, but there does not seem to be consistency between studies that compare stress tolerant and sensitive varieties. Labhilili, Joudrier and Gautier, (1995) observed a difference between drought tolerant and drought sensitive *Triticum durum* varieties in the levels of dehydrin gene expression, with the drought tolerant varieties expressing the gene to higher levels in response to water stress than the drought sensitive varieties.

```
GAGTTCAAGTGATGATTCAGTAAGCTCAAAGACAGATAACAGCAGTTCTGACAGTGAAAA 60

GGGTGGCCATCGTACCAAGCGTTCCTTGCCGAAAGATAAAGAGAGTACCAAAATGACAAT 120
M T I   3

TTCAGAACAAGGAAGAACTTTTCAAGAAGCGGACAAAGGCAAACAAACAGTTACTACCAT 180
S E Q G R T F Q E A D K G K Q T V T T I   23

CAATCGATCACAATCACATGATGGAAGTAAACCCTCAAACAAAGATGGCAACGGGGCTGA 240
N R S Q S H D G S K P S N K D G N G A D   43

TGAAAGATCTGGAAATTACAATTCAGAAGATAGGCATGGTCCAGGTGGAAGCTCCAGGAA 300
E R S G N Y N S E D R H G P G G S S R N   63

CAATCCCATTCAGGTTGATGTAAATTTAACGAAACCAGTAAATGTAGATGGCAACACTGG 360
N P I Q V D V N L T K P V N V D G N T G   83

TGGTGATAATGCAGAGGCTGGCATGTCTAGAACTGGTGAAGGAAGGCACCTTCCAAGCAA 420
G D N A E A G M S R T G E G R H L P S N   103

TGAACCTGTGTCCACCAATGGTAAAGATTTAGGTGTGGGTTCTGCAGACAATGGGCAACC 480
E P V S T N G K D L G V G S A D N G Q P   123

TCAACGAATCAGGAAAGGACGTGGTTTTACTCAGAAGTATGGGTATCCACCCCGATACAG 540
Q R I R K G R G F T Q K Y G Y P P R Y R   143

GACGCCATCTCCCGAGCGTTCCCCTAGGTCAAACCCCCTACGATGGGGGGAGAGATATAT 600
T P S P E R S P R S N P L R W G E R Y I   163

AGATGGAATCATTTCAATAGGTATGAAAGAAATGGTCCTTACAGTAGACGCTCCCCCAGT 660
D G I I S I G M K E M V L T V D A P P V   183

AAGGAGATTCCATGGCTTGCCAAGAGCCAATAGCCCATCCAGGTACCCAAGAAGAGAACG 720
R R F H G L P R A N S P S R Y P R R E R   203

AATGAGGAGTAGGTCACGCAGCCCAGTGAGGCGCCATGACCGTGGAGGGTATCACCGCCC 780
M R S R S R S P V R R H D R G G Y H R P   223

CAGCCCAAGACGTAGCCGCAGCCCTGCAGAACCGAGACAGAATGTGAATGATAGCCCTCG 840
S P R R S R S P A E P R Q N V N D S P R   243

ATCAGGTCATGGCGGAGGCGATCCTGACCACAGCCCTCCTGCCAACAGGTCCAGATCGAA 900
S G H G G G D P D H S P P A N R S R S K   263

GAGCCGGGACCCCTTGGGACCCAGATCTCCTGATGCTGCCCCAGCCAAGAGGGAGAGCTC 960
S R D P L G P R S P D A A P A K R E S S   283

CAGATACAACCGCAGGCGCAGCAGCAGCTCAAGGTCCAGCAGCCCTGATGGAAACAAAGG 1020
R Y N R R R S S S S R S S S P D G N K G   303

CCTGGTTTCGTACTGAGAAGTAACGGCACAACAGTTTGCAGGCTCCATGGACCATGTTTC 1080
L V S Y *                   307

TTGCTAGTTTAGCATCATGTACTCTTTTGTAATGGCCTCTGAACTTGAGGTGTAGATCTA 1140

TATGTTCTGTT(An)                   1151
```

Figure 3.12a: Nucleotide and deduced amino acid sequence of *rws7*.

GAACGTGTCAGCGACTGATCTTAGCCTGGGGCCAAAGCTTTTCCACCTACAGATCGCACT 60
N V S A T D L S L G P K L F H L Q I A L 20

GGAGCATTTCAAAGGCTGGAAGATCCCAGAAAACCTAACCAATGTCCATGCCTACACCAA 120
E H F K G W K I P E N L T N V H A Y T K 40

GGCTCTTTTCAGCCGTGAATCTTTTGTCAAGACTAAGCCATCCGAGGAGCACGTGATTGC 180
A L F S R E S F V K T K P S E E H V I A 60

GGGATGGGCGCCCAAGGTGAATGCATAAGAGCCTTGTGCTTTGGTGCTACCTGGTGGACT 240
G W A P K V N A * 68

CCATCCATTTATCTTATCAGTCTTTGTCAGTGGTGTGGATAGGTGTCAAGTCTGTAGCCT 300

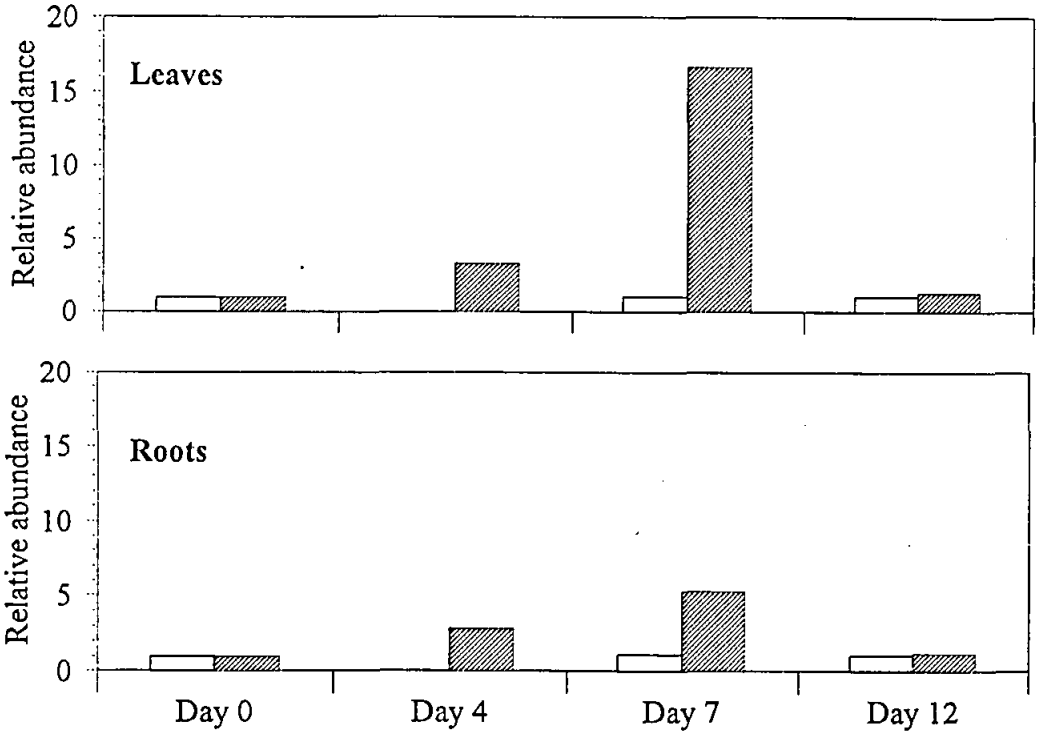
AGATGCATCATTCAGTTGGAATTATGCAGAGTCGTGTCTCGAGTACTCAGTTCTGGCTGT 360

TGCGGTTGTGCATCTTACCTTCTGTTGCTGGTTTTGTTTGAGGCCATGGGCATCTCTTGG 420

TCCTATGCTGTGAGAGCGCTGTAATGTGGGTACCAAATAATGGAGCTGCTTTTATGCT(A_n) 478

Figure 3.12b: Nucleotide and deduced amino acid sequence of *rws16*.

a



b

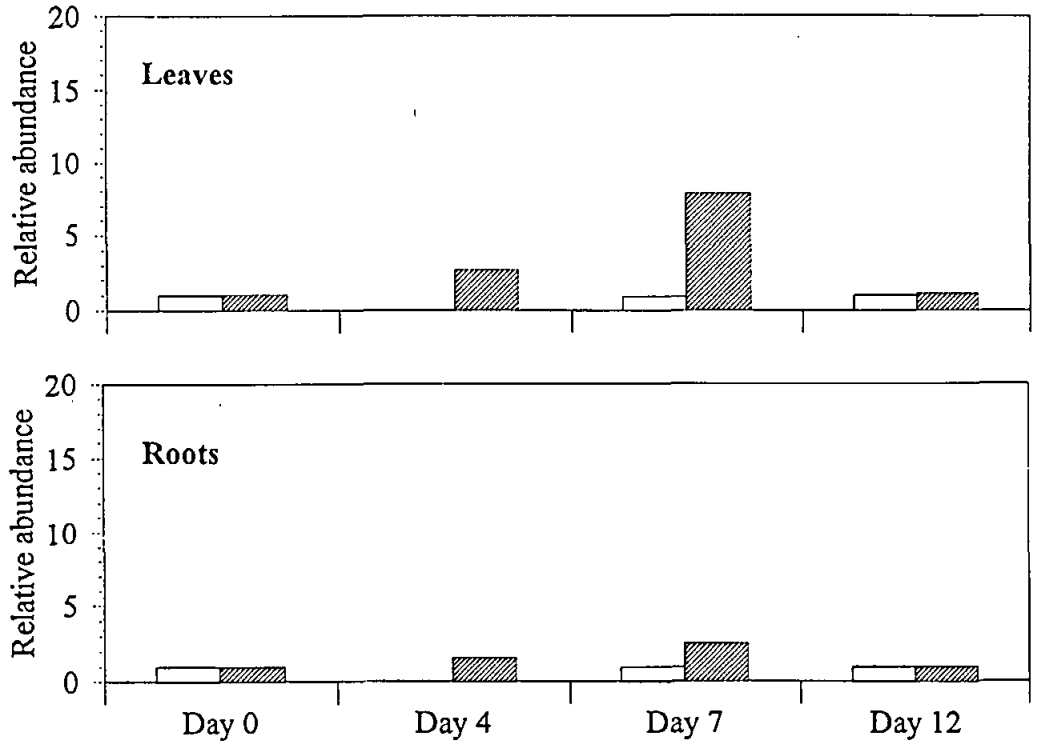


Figure 3.13: Expression of *rws7* transcripts in leaves and roots of the drought tolerant maize cultivar (a) and the maize sensitive cultivar (b) during a progressive soil drying cycle (day 0, 4 and 7) and recovery (day 12) compared with watered controls.

There was also a difference in the pattern of dehydrin gene expression. In the drought tolerant *T. durum* varieties the level of expression peaked at 60 hours after imposition of water stress, whereas in the drought sensitive varieties expression peaked at 30 hours and declined at 60 hours (Labhilili *et. al.* 1995). Houde, Danyluk, Laliberté, Rassart, Dhindsa and Sarhan, (1992) found similar results with *T. aestivum* in response to cold stress, with the tolerant varieties maintaining a high level of cold responsive mRNA transcripts throughout the duration of cold stress, and the sensitive varieties having a transient expression. On the other hand, similar work comparing the drought sensitive cultivated tomato *Lycopersicon esculentum* and the drought tolerant wild relative *L. pennellii* showed no difference in the levels of expression of four drought related genes that could explain the difference in drought tolerance (Kahn, Fender, Bray and O'Connell, 1993).

The expression of these four genes correlated with time to wilting, with *L. esculentum* taking a shorter time to reach wilting point under the same conditions of water stress as the wild relative *L. pennellii*. Again, the level of mRNA transcripts appears to reflect the physiological state of the plant tissue rather than any adaptive characteristic.

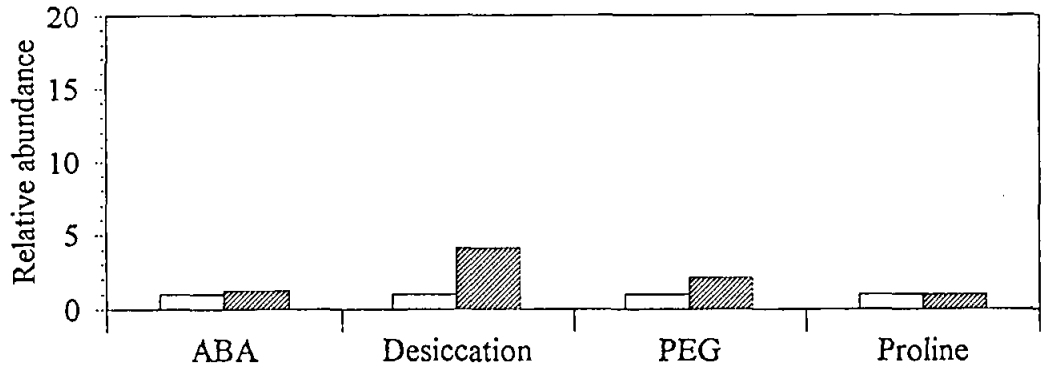
However, the interpretation of these results might be that the level of expression is merely reflecting the level of physiological stress in the plant, and that the sensitive varieties are stressed sooner after imposition of the stress treatment. In Labhilili *et. al.* (1995)'s study of *Triticum durum* mentioned above, the reduction in the level of dehydrin gene expression in sensitive varieties at 60 hours might be equivalent to the decrease in levels of expression of the turgor-responsive gene from *Brassica napus* upon wilting observed in Stroeher *et. al.* (1995)'s study, also mentioned above. The sensitive varieties may experience wilting sooner than the tolerant varieties. There was no increase in mRNA levels of any of the three clones in response to ABA treatment. Many drought-related genes do respond to ABA, but there are also many that do not. An ABA-independent response is also supported by the increase in levels of detached, desiccated leaves. The signal for many responses in the leaf is in the increased ABA flux in the transpiration stream initiated in the root. If these genes are responding in an ABA-independent manner then it would explain there increased mRNA levels in detached leaves.

An absence of a response to exogenous ABA does not of course mean that the gene does not respond to endogenous ABA. Endogenous ABA could be acting in conjunction with other cellular factors to induce gene expression. For instance it has been shown that the sensitivity of ABA can be mediated by concentration of ions in the cytosol, pH (Hartung and Slovik, 1991), tissue water potential (Saab *et. al.* 1990) and temperature (Allan, 1994). We cannot exclude the possibility therefore that ABA increase occurring in the maize leaves in response to gradual desiccation may be involved in the regulation of the expression of the three clones (Figure 3.14 a,b). Nevertheless there do appear to be at least two regulatory pathways involved in gene expression in response to water stress, since many genes have been shown to respond to exogenous application of ABA (for reviews see Skriver and Mundy, 1990; Chandler and Robertson, 1994).

3.4.2.4. Conclusion

One of the objectives of the stress treatment was to apply a mild stress to mimic as much as possible the conditions prevailing in the field, and to determine if any other genes are involved in the response to a mild stress. Many of the genes isolated already were isolated in response to severe stress occurring during embryogenesis or in extreme xerophytes such as the resurrection plant, so perhaps different genes are involved in the milder stress response. This part of the study showed some promise in that the genes that were completely sequenced were both novel. At present this novelty is limited since reports exist in the literature dehydrins and other researchers had previously isolated ion channel proteins. The identity and function of the remaining seven partially sequenced genes remains unknown at present, but the fact that they are novel hold promise that indeed different genes are involved the response to a milder stress.

a



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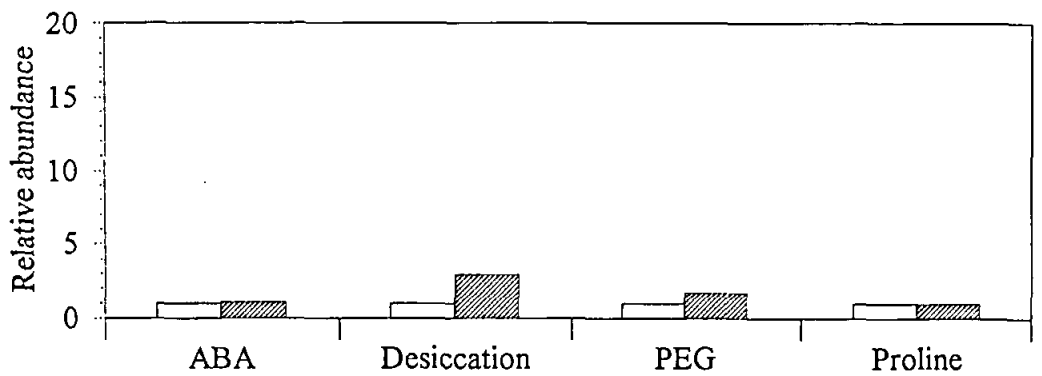
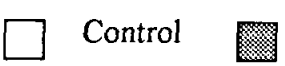


Figure 3.14: Expression of *rws7* transcripts in leaves of the drought tolerant maize cultivar (a) and the drought sensitive cultivar (b) during ABA, desiccation, osmoticum and proline treatments and controls



3.4.2.5. Two-cultivar Analysis of Water-stress Induced Genes from Maize

The interaction results between the two sequenced cDNA clones (rws7 pLexA and rws16 pB42AD) have been completed. There was no indication of an interaction between these two cDNA clones. The orientation of the clones and the reading frame has been confirmed. Figure 15 display typical results which were obtained in the study.

Figure 3.15(1PA) and (2PA) indicated positive controls. These are typical of positive results obtained with the lacZ reporter system. Figures 3.10(1PB) and (3PB) are false positives results. These are typical results obtained when the reporter system is activated but not by a protein - protein interaction.

The interaction study using rws16 pLexA and rws7 pB42AD had been completed with no positive interaction having been displayed.

This type of study has great potential for understanding the interaction of proteins that have been isolated as a result of an environmental stress. If two-cultivar studies are to continue then a much more complete study involving the construction of pLexA and pB42AD cDNA libraries will need to be contracted to allow a complete look at the interacting products of stress induced genes. A maize two - cultivar library has been obtained which uses a different reporter system and we plan to continue the work of studying the set of genes that have been isolated during this project.

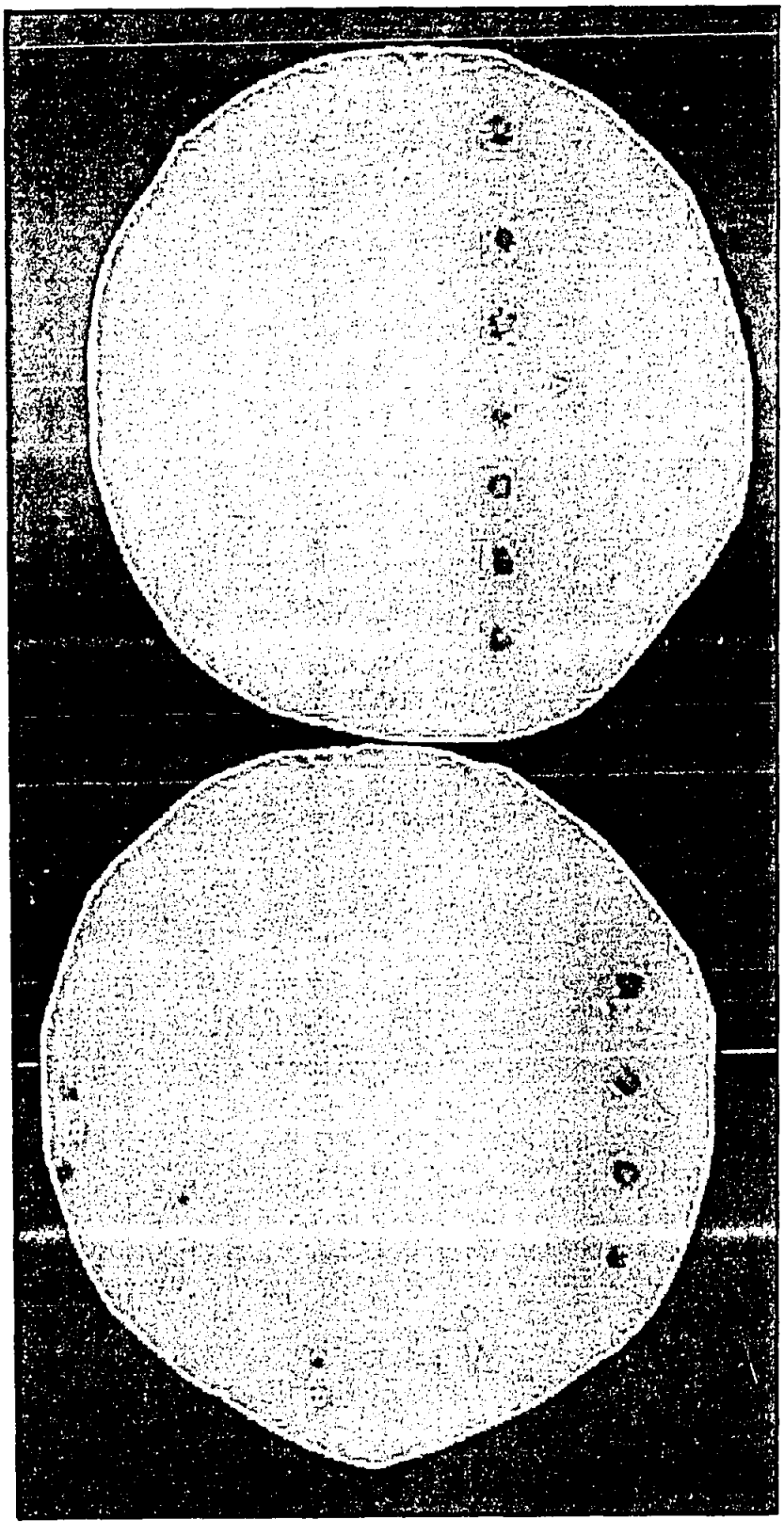


Figure 3.15 (1p): Interaction between the 2 sequenced cDNA clones (rws7 pLexA and rws16 pB42AD)

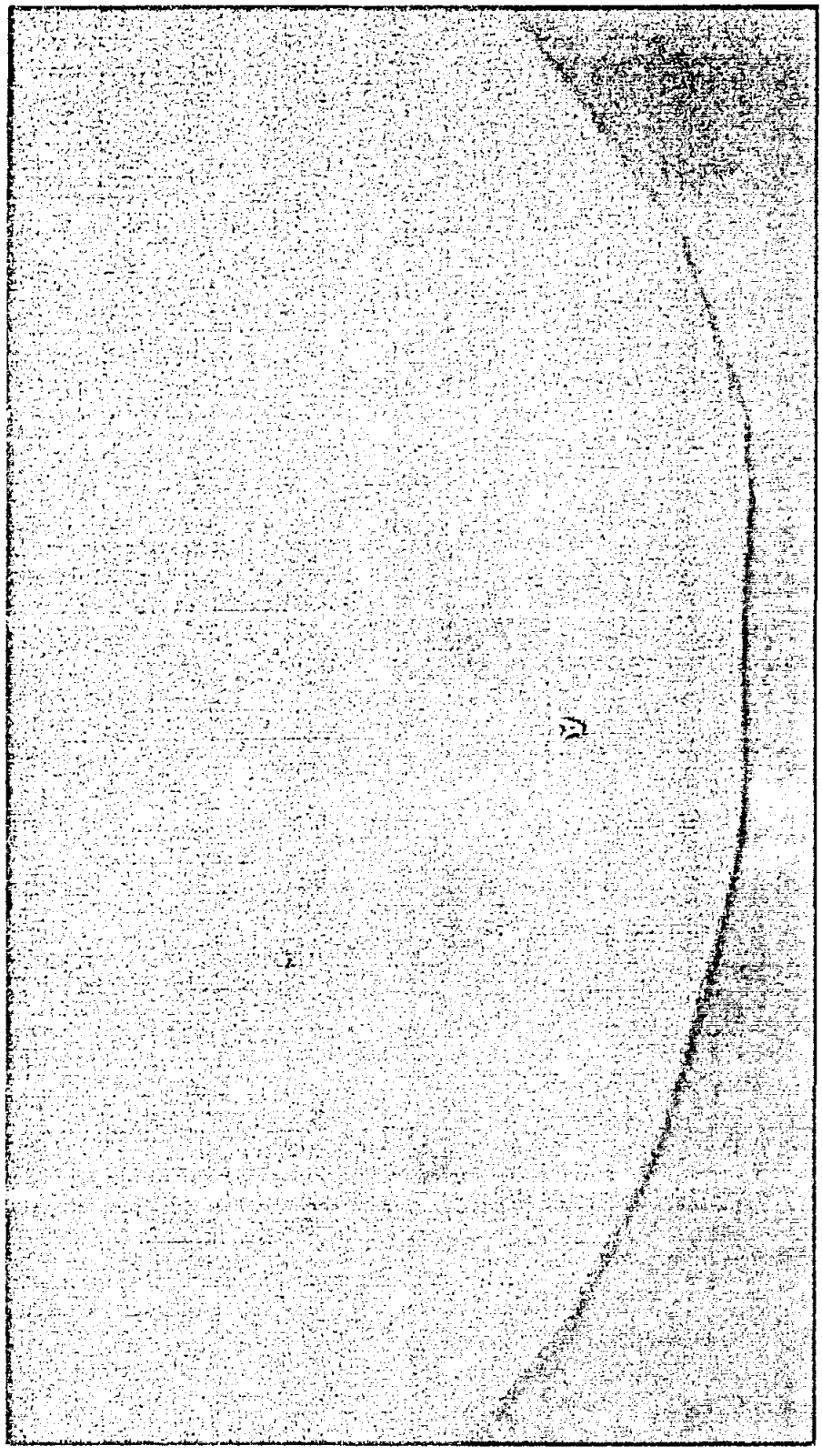


Figure 3.15 (2p): Interaction between the 2 sequenced cDNA clones (rws7 pLexA and rws16 pB42AD) (Continue)

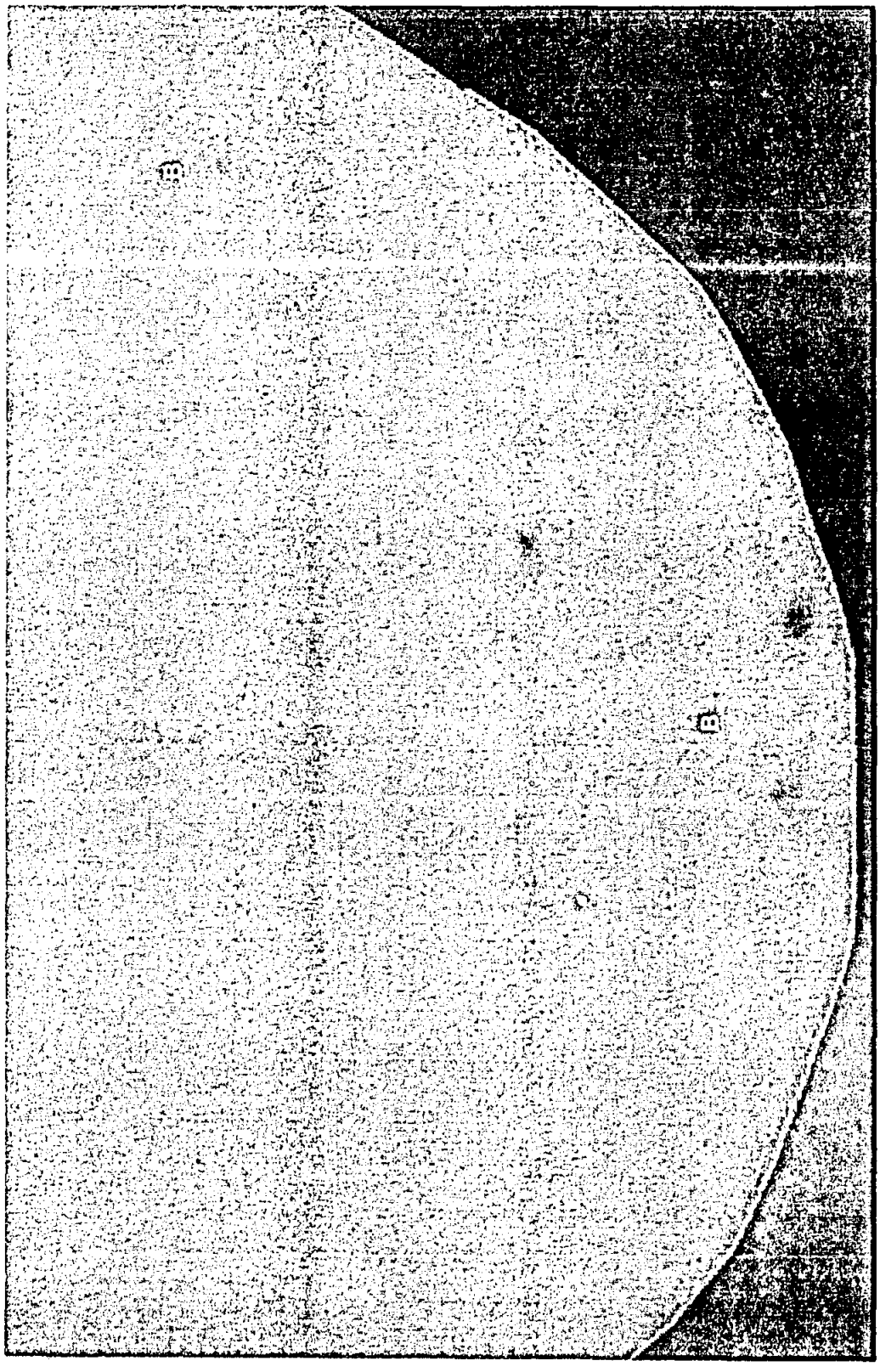


Figure 3.15 (3p): Interaction between the 2 sequenced cDNA clones (rws7 pLexA and rws16 pB42AD) (Continue)

3.4.3. Transformation of maize embryos with the Cu/Zn superoxide dismutase gene

3.4.3.1. Embryogenic Tissue

The best regeneration medium to obtain embryogenic tissue from maize line M162 W was MS-basal medium, supplemented with 1,3 g/l Proline (filter sterilized) and 3.3 mg/l Dicamba. Seventy eight percent embryogenic tissues were obtained with this medium. Dicamba was used as it limits the appearance of somaclonal variation. After two weeks of incubation on the regeneration medium, embryogenic tissues could be observed under a dissection microscope. These embryos were then transferred to MS-medium without any growth regulators, for further development of somatic embryo's. Within the first two weeks on MS-medium, the embryo's developed further and after three to four weeks, small plantlets could be obtained.

3.4.3.2. Manipulation of the superoxide dismutase gene for maize transformation

In this study, the ligation and transformation studies of pAHC25 with the *Arabidopsis thaliana* SOD gene, from the pcSODRH plasmid, is represented. The plasmid pcSODRH consists of a 788 base pair (bp) cDNA clone of a cytosolic Cu, Zn superoxide dismutase from *A. thaliana* in the *EcoRI* site of pBluescript (SK^+) (Figure 3.16).

The plasmid pAHC25 contains both a selectable marker (*bar*) and a scorable marker (GUS), each under the transcriptional control of a separate *Ubi-1* promoter (Figure 3.17). The maize *Ubi-1* promoter has a very high activity in monocotyledons (Christensen, Quail, 1992). The bar gene encodes for a phosphinotricin acyltransferase, which acetylates the NH_2 - terminal group of phosphinotricin, abolishing its herbicidal activity.

Plasmid DNA was isolated from *E.coli*, containing pcSODRH and pAHC25 separately, grown with the use of overnight cultures using Luria-Bertani broth. The pcSODRH was restricted with *EcoR1* to splice the SOD gene from the plasmid after which the SOD fragment was identified and isolated from a 1.5% LMP agarose gel.

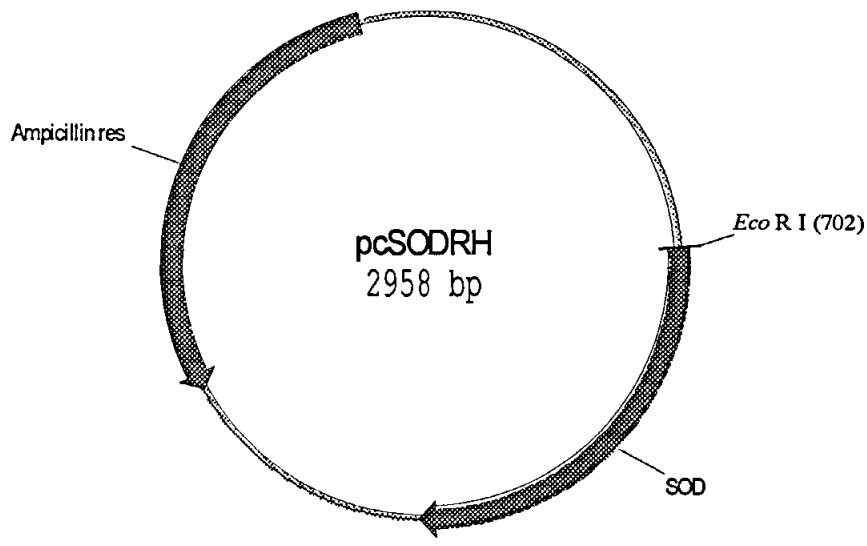


Figure 3.16: The plasmid pcSODRH. A 788 bp clone of a cytosolic Cu/Zn SOD from *A. thaliana* in the EcoR1 site of Blue script (SK+).

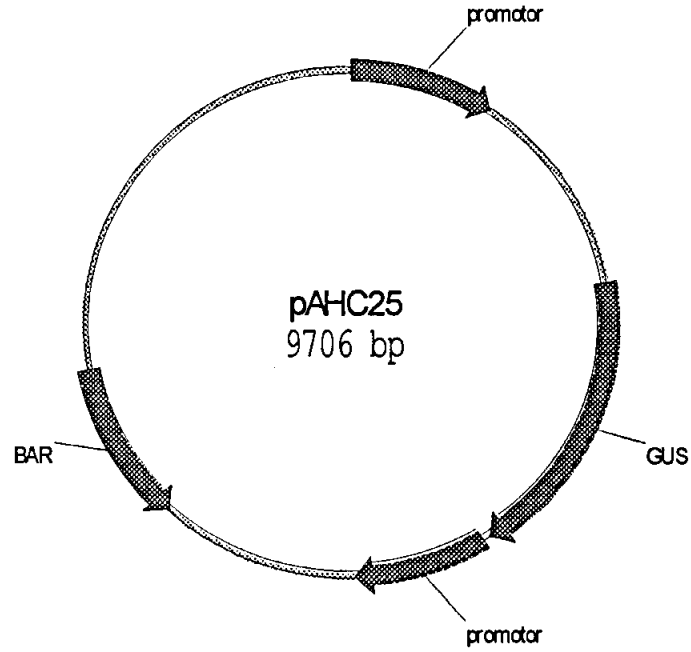


Figure 3.17: The plasmid pAHC25 containing both a selectable (bar) and a scorable (GUS) marker, each under the transcriptional control of a separate *Ubi-1* promoter.

The fragments were pured by means of Gelase treatment and consequently treated with Klenow enzyme to create blunt ends on both sides of the DNA fragment (Figure 3.18). Lanes 2-5 show the SOD gene as the lower band.

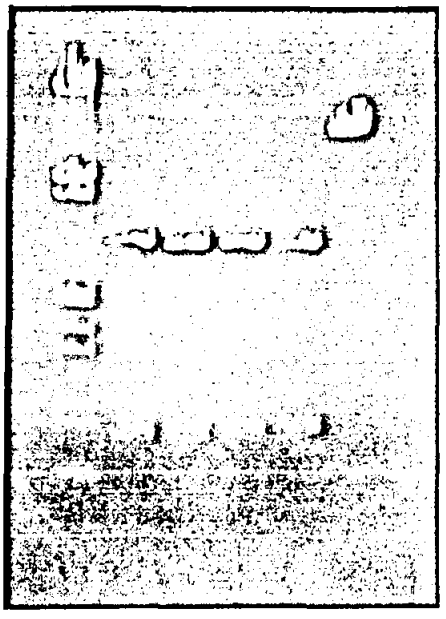
The plasmid pAHC25 was restricted with *Sma1* to create a linear fragment and isolated from a 1.5% LMP agarose gel and treated with alkaline phosphatase to eliminate the probability of circulation (Figure 3.18). Lane 6 shows the plasmid pAHC25 after restricted with *Sma I*. Ligation of the SOD insert and pAHC25 was performed and 8 colonies were identified as containing positive clones. Mini extractions were done on the 8 colonies to obtain DNA to be subjected to PCR analyses. DNA extracted various controls and DNA specific primers were used in the PCR reaction.

The PCR products were visualized on a 1% agarose gel stained with ethidium bromide. The negative control yielded no bands. The DNA of the eight colonies and the positive control yielded the expected band (Figure 3.19). Lanes 5-12 show the presence of the SOD gene in the extract. This implicated that the SOD gene had indeed been cloned into pAHC25 giving rise to a new plasmid pAHC25SOD.

To confirm furthermore the cloning of the SOD, DNA was extracted from the positive colonies. The DNA was subjected to enzyme restriction digestion of *Sal 1* and *Pst 1*. The digested samples were visualized on a 1% agarose gel (Figure 3.20). From this we could see that some of the samples had partially digested but 2 lanes, lane 6 and 7 clearly showed the correct bands.

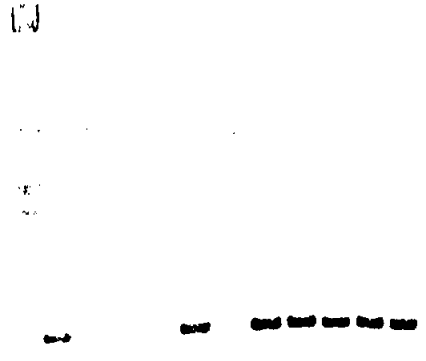
3.4.3.3. Particle Gun Bombardment

Tungsten particles were used as micro projectiles during bombardment and was sufficient surface sterilized with (70%) ethanol. The sequence of the chemicals is very important during the precipitation of the DNA to avoid clotting of the DNA. DNA precipitation was done by mixing 25µl tungsten with 5µl DNA plasmid (pACH25), 25µl $CaCl_2$ and 10µl spermidine. It is very important that the DNA is not used longer than 15 minutes after precipitation.



1 2 3 4 5 6

Figure 3.18: A 0.8% Agarose gel showing the EcoR1 and Sma1 digests on pcSODRH and pAHC25% respectively. Lane 1: Molecular marker III, Lane 2: pcSODRH EcoR1 digest, Lane 3: pcSODRH EcoR1 digest, Lane 4: pcSODRH EcoR1 digest, Lane 5: pcSODRH EcoR1 digest, Lane 6: pAHC25 Sma1 digest.



1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.19: PCR analysis displayed on a 1% agarose gel.
Lane 1: Molecular marker III, Lane 2: positive control, Lane 3: negative control, Lane 4: water control, Lane 5-12: positive bands reflecting the presence of the SOD gene.

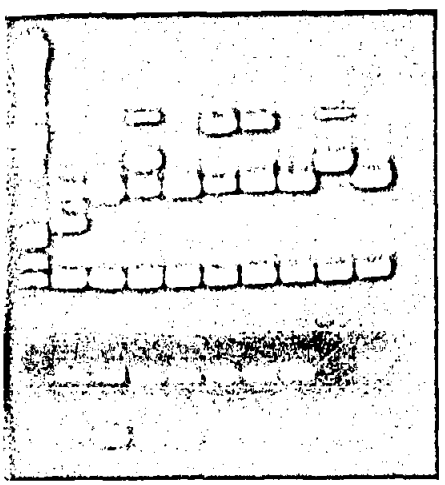


Figure 3.20: A 0.8% agarose gel showing DNA bands of different sizes after enzyme digestion of pAHC25SOD plasmid DNA with Pst1 and Sal1.
Lane 1: Molecular marker IV, Lane 2-10: Pst1 and Sal 1 restriction digests of extracted DNA.

An Ignite (PPT) range of (0.3,1.0, 2.0 and 3.0 mg/l) was used. The most efficient selective concentration level of Ignite was 3.0 mg/l. The result confirmed that the commercial formulation (Ignite) is suitable for maize transformation. The inert ingredients present in the commercial formulations do not have a detrimental effect on the growth and health of maize embryogenic calli.

3.4.3.4. Transformation of the Maize with the Plasmid pAHC25SOD

After bombardment and growth on high osmoticum medium the calli were put on MS medium supplemented with 0.3 mg/l PPT. The PPT acts as a selection medium to ensure that only the transformed calli will grow into mature plantlets. The calli developed into somatic embryos that gave rise to the development of the plantlets. Calli were submitted to GUS gene detection in confirming transformation. The blue dots observed are an indication of the amount of transformed calli (Figure 3.21). The plantlets were transferred to fresh MS medium every 2 weeks supplemented with a increasing range of PPT concentrations (Figure 3.22).

Unfortunately Bacillus sp. contamination took place just before hardening off of the first plantlets. After numerous discussions with the local pathologist we came to the conclusion that internal bacteria was the cause for the contamination. We could not get rid of the contamination since the bacteria are in the tissue of the plantlets. Various methods, like antibiotics (narrow and broad spectrum) and spore killing media were tried to terminate the contamination but to no avail. We were lucky to rescue some leave samples to be used for DNA extractions. We came to the conclusion that the contamination appeared only when the plant is put under stress should it be oxygen stress *(Bacillus* sp. thrive under anaerobic conditions) or the stress the PPT caused since the contamination did not show under normal growing conditions.

3.4.3.5. Confirmation of Transformation of pAHC25SOD Into Maize Calli

In order to be able to confirm the transformation of the pAHC25SOD into maize we collected leaf material from as much plantlets as possible. DNA extractions were executed on all the samples. The DNA was subjected to PCR analyses and the results are shown in Figure 3.23. Some of the

CHAPTER 4

CONCLUSION

Drought tolerance is a polygenetic trait as a combination of different traits contributes to this tolerance. These traits offer the opportunity for the development of screening methods. The first step in developing screening methods is understanding of the different physiological processes involved and the links between them. Secondly the screening method must be tested for reliable distinguishing between tolerant and sensitive cultivars. As pathways can play special roles in species, different species must be tested as well. Thirdly the screening method must be correlated to known tolerance obtained preferably from field data. The traits tested for the possible use as screening method included chlorophyll fluorescence, antioxidative enzymes, proline, polyamine and tetrazolium salt reduction. The species tested included potatoes, cotton, tobacco and maize.

The most promising physiological parameters to use as screening methods for drought tolerance in potato are the levels of Cu/Zn SOD activity, free proline accumulation and spermine levels. A chlorophyll fluorescence study as well as chlorophyll *a* and *b* level indicated a perfect correlation with known drought tolerance for the short growth period potato cultivars. This can be used as a screening method for short growth period potatoes. When drought stressed potato plants were compared to well watered control potato plants it was observed that the ability to maintain adequate or decreased concentrations of Cu/Zn superoxide dismutase correlated with known drought tolerance under field conditions. This may be due to the fact that drought tolerance is defined in terms of yield reduction and that selection for plants favours tuberization (avoidance of drought) rather than drought tolerance. The ability of potatoes to maintain adequate levels of superoxide dismutase activity seemed more important than an increase in enzyme activity. It was found that proline accumulation in potatoes is a function of growth period. Drought tolerant cultivars with a short growth period accumulated the highest levels of proline two weeks without water, cultivars with a medium growth period accumulated most proline three weeks without water while cultivars with a long growth period accumulated most proline after four weeks without water. The polyamine study revealed that the synthesis of spermine, agmatine and

spermidine were cultivar and age dependent. Spermine synthesis, after four weeks without water, showed a correlation with known drought tolerance in potato. The antioxidative enzyme pathway together with proline and polyamine pathway operates together in a biochemical combination in reduction of free radicals during stress conditions. It is suggested that these three parameters must be used when screening for drought tolerance in potato. With the use of these three parameters it can thus be concluded that the cultivars Aviva, Darius, Baraka, Late Harvest and Hoëvelder are tolerant and Raritan, Sebago and Kimberley Choice are sensitive to drought stress. With the dominant role which Cu/Zn superoxide dismutase plays in the drought mechanism, it was decided to use this gene in a transformation process in potato. It was observed that some of the putative transformants could withstand drought in the greenhouse for two weeks longer than the untransformed plants. Molecular techniques confirmed transformation of these plants. A TTC reduction assays also showed that the transformants were more drought tolerant than the untransformed plants. This finding is revolutionary, as it was believed that one gene can not contribute adequate to the tolerance of plants. This may be the start of a new dimension in breeding for drought tolerance hand in hand with gene manipulation.

The results obtained from an antioxidative enzyme study on six cotton cultivars indicated that Cu/Zn superoxide dismutase demonstrated an increase in the activity of the stress treated plants with regard to the control treated plants in two of the tolerant cultivars. Free proline accumulated in all the cultivars as a result of drought stress, but two tolerant cultivars accumulated more proline. Better correlation was however observed with chlorophyll fluorescence parameters Fm, qQ and Fv/Fm, which correlated to all drought tolerant cotton cultivars. Increased polyamine enzymes (ODC and ADC) level demonstrated an inverse correlation to drought tolerance. Cultivars with the highest increase corresponded to those having the greatest drought sensitivity, while cultivars with the lowest amount of enzyme increase corresponded to drought tolerant cultivars. The TTC viability assay proved to be a reliable method in screening for drought tolerance in the laboratory. With this method it was possible in ranking the cultivars from sensitive to tolerant. The enzymes involved in the reduction of the tetrazolium salt were evaluated. A higher succinic dehydrogenase (SDH) activity resulted in more tetrazolium salt reduction, which let to more viable plants. Tolerant cultivars have the ability to increase their SDH activity in order to increase viability. Malate dehydrogenase (MDH) activity increases in most of the cultivars.

However, the interaction between the enzymes is of great importance as they play a combined role in the electron transfer system. It is thus postulated that a cultivar needs increasing levels of both enzymes to be drought tolerant. From this information it is evident that the most promising traits to be used as screening methods for drought tolerance for cotton are chlorophyll fluorescence, polyamine enzymes and tetrazolium salt reduction.

The best screening traits for heat tolerance are the antioxidative enzymes (Cu/Zn SOD, peroxidase and glutathione reductase) and the TTC viability assay. When subjected to a heat stress, all the cultivars recorded lower Cu/Zn SOD activities for the stressed treatment than for the control treatment, indicated heat sensitivity. Increasing levels of SOD, peroxidase and glutathione reductase activities during heat stress corresponded with heat tolerance. The interaction between the enzymes is of great importance as they play a role in different parts of the antioxidase pathway during the elevation of stress. The tolerant cultivars have the ability to increase their enzyme levels after a heat treatment for two of the three enzymes tested. This is in contrast with the sensitive cultivars which do not have this ability and shown a decreasing tendency for two out of three enzymes. The free proline accumulation observed during a combined drought and heat stress indicated that heat sensitive cultivars accumulated less proline than heat tolerant cultivars. It confirms different mechanisms for heat and drought tolerance. A TTC study to determine the combined effect of heat and drought stress in the laboratory confirms the tolerance ranking observed with the proline study. Lower absorbance values were obtained during a heat treatment than during a drought treatment, correlated with lower Cu/Zn SOD levels during a heat treatment, indicated that the cultivars are more heat sensitive than drought sensitive.

As the TTC viability assay in cotton plants correlated with drought and heat tolerance, a heritability and combining ability study were conducted. The heritability observed for drought was $h^2 = 0.30$ and for the heat $h^2 = 0.33$. This indicated that the TTC viability assay can be used in a cotton-breeding programme as the differences between TTC values is heritable. Information from a TTC assay can be very valuable in a breeding programme in identify the correct parents, as well in identify the best climate for the cultivar.

The results obtained from a chlorophyll fluorescence study on seven tobacco cultivars insinuated that the cultivars demonstrated a significant decrease in photochemical quenching early in the drought stress period reacted as sensitive, in contrast with the cultivars demonstrated a significant decrease later in the stress period, which are more tolerant. The Fv/Fm ratio demonstrated a significant decrease over time in the sensitive cultivars and a significant increase in the tolerant cultivar Elsoma. The drought tolerant cultivars Elsoma and Domkrag accumulated the most free proline when subjected to a drought stress. The diamine/ polyamine ratio can be used as a screening method for drought in tobacco, as the tolerant cultivars demonstrated a significant decrease in this ratio. With the combined pathway of proline and polyamines working together in sustaining the tolerance mechanism in tobacco, it is postulated that the tobacco cultivars can be screened for tolerance using these two methods in combination.

It was established that maize inbred lines in the growing season can be evaluated for drought and heat tolerance using triphenyltetrazolium chloride reduction assays. It was established that there was a difference between the maize cultivars in certain physiological responses. The tolerant cultivar responded by growing its roots deeper into the wetter soil layer and thus being able to maintain a higher transpiration rate, a higher relative leaf tissue water content and a higher growth rate than the sensitive cultivar. Despite having a more favourable leaf water status, the tolerant cultivar had higher levels of abscisic acid and proline in the leaves under conditions of drought stress than the sensitive cultivar.

A differential screening isolated nine maize cDNA's that were more abundant in the drought stress treatment. Three of these cDNA's were isolated more than once and occurred in both cultivars, so the different sensitivities of these cultivars to drought could not be accounted for by the presence or absence of these genes. Two cDNA's were sequenced completely (rws7 and rws16), one was entirely novel but showed characteristics of dehydrins in its derived amino acid sequence, and the other showed homology to mammalian chloride channel proteins. The remaining seven showed no homology to existing nucleotide sequence in the GenBank databases. The fact that the isolated genes are novel, indicated that different genes are involved in the response to a milder stress. No indication of any interaction between rws7 and rws16 was found, indicated that they are indeed different.

A maize inbred line was transformed with the SOD gene using the particle gun method of direct gene transfer. Maize plants were regenerated and molecular tested for the presence of the gene in the maize genome. The transformants could however not be tested in the greenhouse for enhanced drought tolerance as a result of a *Baccilus sp.* contamination. These successfully transformed maize embryos open the way for any further transformation procedures. With the transformation procedure sorted out, any gene can now be transferred into maize embryos. Transgenic drought resistant maize plants in the field are now feasible.

Future research will include the field trials with the SOD transformed potatoes in evaluating drought tolerance. With the maize transformation procedure sorted out using maize embryos and particle gun transformation, it is recommended that maize plants be transformed with the maize genes identified with the differential screening method. The genes can also be used as selectable markers for drought screening. The SOD transformation of maize embryos will be repeated on a seed batch containing no *Bacillus sp.* contamination. All of these transgenic plants will afterwards be evaluated in the field for drought tolerance using the best screening technique(s) identified for each crop.

CHAPTER 5

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