PHYSIOLOGICAL AND GENE EXPRESSION RESPONSES TO WATER STRESS IN DROUGHT TOLERANT AND DROUGHT SENSITIVE MAIZE CULTIVARS

By

Brian Patrick O'Regan

Submitted in fulfilment
of the academic requirements for the degree of
Doctor of Philosophy
in the
Department of Botany
Faculty of Science
University of Natal
Pietermaritzburg
December 1996

ABSTRACT

Physiological characteristics of the response to water stress of two maize cultivars, the one drought resistant (PAN473) and the other drought sensitive (SR52), were compared. Mature plants were grown in one of two treatments, control and water stress. The drought resistant cultivar had a higher growth rate and a greater amount of roots in the lower soil levels than the drought sensitive cultivar in the water stress treatment. There was no difference between the cultivars in physiological characteristics in the control treatment, but in the water-stress treatment the drought resistant cultivar had a higher transpiration rate during the onset of water stress, and higher relative water content and levels of abscisic acid and proline throughout the period of water stress. A comparison between the cultivars in their gene expression response was done to determine if a correlation could be made with the difference in physiological response. A differential screening of water stress cDNA libraries identified nine different cDNA species which gave a signal with the water stress probe but not the control probe. Three of these cDNAs were represented by more than one cDNA clone. The cDNAs occurred in both libraries, therefore there was no difference between the cultivars in the presence or absence of the water stress responsive genes. The three genes represented by these cDNAs were named *rws7*, *rws16* and *rws5*. All three genes showed increased transcription in response to water stress in whole plants, and to desiccation and osmotic stress of detached leaves. No increase in transcription was found in response to exogenously applied abscisic acid or proline. No difference between the cultivars was observed in the pattern of transcription response. Two of the three cDNA species that are represented more than once were sequenced. *Rws7* had an open reading frame. A BLAST search found no homologous amino acid sequences, but the characteristics of the polypeptide suggest that it is a dehydrin. *Rws 16* had a partial coding sequence. A BLAST search found two homologous amino acid sequences, and both were chloride channel proteins. The remaining seven cDNA species were sequenced at their 5' ends, and no complete homologous nucleotide sequences were found.

PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, and at ARC-Roodeplaat Vegetable and Ornamental Plant Institute, Pretoria, under the supervision of Dr William Cress, and the cosupervision of Prof Johannes van Staden.

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

B. P. O'Regan

Brian Patrick O'Regan

December 1996

ACKNOWLEDGEMENTS

My sincere thanks and appreciation are extended to the following people and organizations who made this work possible:

The Water Research Commission for their financial support to the Drought Tolerance Project, of which this study formed a part.

Dr William Cress for giving me the opportunity to work in his laboratory, for his supervision and advice, and for financial support at the start of the project.

Prof Johannes van Staden for providing excellent research facilities in the Botany Department, University of Natal, Pietermaritzburg, and for proof reading this manuscript.

The members of the Molecular Biology Laboratory in the Botany Department, University of Natal, Pietermaritzburg, for their encouragement and companionship.

The Director of ARC-Roodeplaat and the Heads of the Biotechnology Division, Dr Dawie Ferreira and Dr Johan Brink, for giving their support to the project.

Anette van der Mescht and the other members of the "Drought Team" at ARC-Roodeplaat for their support and company during many eventful and enjoyable hours in the laboratory.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION 1

1.1. OVERVIEW OF DROUGHT RESISTANCE 1

1.2. MECHANISM OF WATER LOSS 4

1.2.1. Osmotic change 4

1.2.2. Turgor loss 5

1.2.3. Change in cellular pH and ABA 6

1.2.4. Abscisic acid as a signal 6

1.3. CELLULAR RESPONSE TO WATER STRESS 9

1.4. GENE EXPRESSION IN RESPONSE TO WATER STRESS AND ABA 11

1.5. RESPONSIVE ELEMENTS IN PROMOTER SEQUENCES 13

1.6. GENE PRODUCTS AND THEIR FUNCTIONS 16

1.7. ABSCISIC ACID 21

1.7.1. Synthesis and metabolism 21

1.8. PROLINE 22

1.8.1. Biosynthesis 22

1.8.2. Mode of action 23

1.8.3. Interaction between ABA and proline 24

1.9. OBJECTIVES AND NATURE OF THE STUDY 25

CHAPTER 2: PHYSIOLOGICAL DIFFERENCES BETWEEN DROUGHT RESISTANT AND DROUGHT SENSITIVE MAIZE CULTIVARS IN RESPONSE TO WATER STRESS 27

2.1. INTRODUCTION 27

2.2. METHODS 28

2.2.1. Study plants 28

2.2.2. Leaf and root growth 28

2.2.3. Water relations 29
2.2.4. Proline 30
2.2.5. Abscisic acid 31
2.3. RESULTS 31
2.3.1. Leaf and root growth 31
2.3.2. Water relations 34
2.3.3. Abscisic acid 39
2.3.4. Proline 39
2.4. DISCUSSION 42

CHAPTER 3: RNA ISOLATION, cDNA LIBRARY CONSTRUCTION AND DIFFERENTIAL PLAQUE HYBRIDIZATION 46
3.1. INTRODUCTION 46
3.2. METHODS 49
3.2.1. Plant growth 49
3.2.2. Total RNA extraction 49
3.2.3. Measurement of total RNA 51
3.2.4. Isolation of poly(A)$^+$ RNA 51
3.2.5. Measurement of poly(A)$^+$ RNA 52
3.2.6. cDNA synthesis 52
3.2.7. Measurement of cDNA 53
3.2.8. Cloning of cDNA into λgt10 53
3.2.9. Replica plating 55
3.2.10. Radiolabelled cDNA probe synthesis 55
3.2.11. Probe hybridization to replica membranes 55
3.2.12. Plaque isolation 56
3.2.13. Reprobing isolated plaques 56
3.2.14. Cross-hybridization of putative drought-related clones 57
3.2.15. Probe synthesis from the isolated λgt10 clones 57
3.3. RESULTS 59
3.3.1. Total RNA extraction 59

3.3.2. Isolation of poly(A)$^+$ RNA 60
3.3.3. cDNA synthesis 60
3.3.4. Cloning of cDNA into λgt10 61
3.3.5. Radiolabelled cDNA probe synthesis 63
3.3.6. Probe hybridization to replica membranes and plaque isolation . . 63
3.3.7. Cross-hybridization of cDNA clones 67
3.4. DISCUSSION 70

CHAPTER 4: SEQUENCE ANALYSIS OF TWO OF THE DROUGHT-RELATED CLONES 72
4.1. INTRODUCTION 72
4.1.1. Basic Local Alignment Search Tool 73
4.1.2. 5' region of mRNAs 73
4.1.3. 3' region of mRNAs 73
4.1.4. mRNA stability and regulation 75
4.1.5. Protein structure 76
4.1.6. The α-helix 76
4.1.7. The β-sheet 77
4.1.8. The random coil 78
4.1.9. Prediction of secondary structure 78
4.2. METHODS 81
4.2.1. Clone selection 81
4.2.2. Sequencing 81
4.2.3. Sequence analysis 83
4.3. RESULTS 83
4.3.1. Clone selection 83
4.3.2. Nucleotide sequence 83
4.3.3. Amino acid sequence 84
4.3.4. Hydropathy 88
4.3.5. Sequence homology 88
4.3.6. Sequence identity 91

4.3.7. Nucleotide and amino acid sequences of remaining seven clones . 91
4.4. DISCUSSION 96

CHAPTER 5: CHARACTERIZATION OF ISOLATED GENES 100
5.1. INTRODUCTION 100
5.2. METHODS 102
5.2.1. Plant growth 102
5.2.2. Drying cycle 102
5.2.3. Desiccation 103
5.2.4. Incubation conditions 103
5.2.5. Osmoticum 103
5.2.6. Proline 104
5.2.7. Abscisic acid 104
5.2.8. RNA extraction 104
5.2.9. RNA slot blots 104
5.2.10. Probe synthesis and hybridization 105
5.2.11. Poly(A)+ RNA quantification 105
5.2.12. *In situ* hybridization 106
5.3. RESULTS 107
5.3.1. Drying cycle 107
5.3.2. ABA, desiccation, PEG and proline treatments 108
5.3.3. *In situ* hybridization 121
5.4. DISCUSSION 126

CHAPTER 6: CONCLUSION 132
6.1. SUMMARY 132
6.2. FUTURE RESEARCH 133
6.3. EXAMPLES OF THE USE OF DROUGHT-RELATED GENES 134
6.3.1. Transformation with foreign genes 134
6.3.2. Overexpression of endogenous genes 135
6.3.3. Detrimental effects of plant transformation 136

6.3.4. Antisense strategies 136
6.3.5. Molecular markers 137
6.3.6. Determining the function of drought responsive genes 137

REFERENCES 139

APPENDIX A: REAGENTS AND SOLUTIONS 163

APPENDIX B: ABBREVIATIONS 166

LIST OF TABLES

Table 2.1. Soil water potential (MPa) of successive 20-cm soil layers in tubes 32

Table 3.1. Titre of control experiments of water stress treatment cDNA cloned into phage λgt10. 62

Table 3.2. Results of the probing of the phage isolates showing the number of different cDNA species. 68

Table 4.1. Amino acid composition of the derived polypeptide of *rws7*. 87

LIST OF FIGURES

Figure 2.1. Percentage of total root dry weight 33

Figure 2.2. Soil water potential in the pots 35

Figure 2.3. Relative water content 36

Figure 2.4. Transpiration rate 37

Figure 2.5. Diffusive resistance 38

Figure 2.6. ABA concentration 40

Figure 2.7. Proline concentration 41

Figure 3.1. Total RNA 59

Figure 3.2. Poly(A)$^+$ RNA 60

Figure 3.3. cDNA synthesised from poly(A)$^+$ RNA 61

Figure 3.4. PCR products of cDNA insert from randomly isolated plaques 62

Figure 3.5. Differential plaque hybridization 63

Figure 3.6. Second round of differential plaque hybridization 64

Figure 3.7. Autoradiographs of plaques cross-hybridised with putative water stress cDNAs 69

Figure 4.1. Nucleotide and deduced amino acid sequence of *rws7*. 85

Figure 4.2. Nucleotide and deduced amino acid sequence of *rws16*. 86

Figure 4.3. Hydropathy plot of the putative polypeptides of *rws7* and *rws16*. 89

Figure 4.4. Comparison of the deduced amino acid sequences of the putative *rws16* polypeptide, ClCP p64 from *Homo sapiens*, and p64 from *Bos primigenius* 90

Figure 4.5. Nucleotide and derived amino acid sequences of remaining clones 92

Figure 5.1. Expression of *rws7* transcripts in leaves and roots of the drought tolerant maize cultivar PAN473 during a progressive soil drying cycle 109

Figure 5.2. Expression of *rws16* transcripts in leaves and roots of the drought tolerant maize cultivar PAN473 during a progressive soil drying cycle 110

Figure 5.3. Expression of *rws5* transcripts in leaves and roots of the drought tolerant maize cultivar PAN473 during a progressive soil drying cycle 111

Figure 5.4. Expression of *rws7* transcripts in leaves and roots of the drought sensitive maize cultivar SR52 during a progressive soil drying cycle 112

Figure 5.5. Expression of *rws16* transcripts in leaves and roots of the drought sensitive maize cultivar SR52 during a progressive soil drying cycle 113

Figure 5.6. Expression of *rws5* transcripts in leaves and roots of the drought sensitive maize cultivar SR52 during a progressive soil drying cycle 114

Figure 5.7. Expression of *rws7* transcripts in leaves of the drought tolerant maize cultivar PAN473 during ABA, desiccation, osmoticum and proline treatments 115

Figure 5.8. Expression of *rws16* transcripts in leaves of the drought tolerant maize cultivar PAN473 during ABA, desiccation, osmoticum and proline treatments 116

Figure 5.9. Expression of *rws5* transcripts in leaves of the drought tolerant maize cultivar PAN473 during ABA, desiccation, osmoticum and proline treatments 117

Figure 5.10. Expression of *rws7* transcripts in leaves of the drought sensitive maize cultivar SR52 during ABA, desiccation, osmoticum and proline treatments 118

Figure 5.11. Expression of *rws16* transcripts in leaves of the drought sensitive maize cultivar SR52 during ABA, desiccation, osmoticum and proline treatments 119

Figure 5.12. Expression of *rws5* transcripts in leaves of the drought sensitive maize cultivar SR52 during ABA, desiccation, osmoticum and proline treatments 120

Figure 5.13. Hybridization of DIG-labelled probe to leaf cross sections of maize cultivar PAN473 122

Figure 5.14. Hybridization of DIG-labelled probe to root cross sections of maize cultivar PAN473. 125

CHAPTER 1:
INTRODUCTION

1.1. OVERVIEW OF DROUGHT RESISTANCE

Drought resistance is the term given to the range of mechanisms whereby plants withstand periods of dry weather. Three types of drought resistance are generally recognized (TURNER 1979; LEVITT 1980; JONES *et al.* 1981); drought escape, drought avoidance, and drought tolerance.

Drought escape is shown by ephemerals and is the ability of a plant to complete its life cycle before serious tissue water deficit occurs. In agricultural systems it is demonstrated by short growing season genotypes with a rapid development. Selection for rapid development has been particularly important in breeding for drought resistance in small grain cereals such as wheat and barley. However, drought escape does not entail any special physiological, biochemical or morphological mechanisms in the mature plant to cope with water deficit.

Drought avoidance does not escape drought, but does escape tissue desiccation during drought. High tissue water potentials can be maintained by absorbing more water from the soil, for instance by increasing hydraulic conductivity (ANDERSEN & PROEBSTING 1984; FISCHER *et al.* 1986) or increasing root volume and depth (FISCHER & TURNER 1978; TURNER 1979), or by conserving water through stomatal closure.

Drought tolerance allows the plant to maintain turgor during prolonged drought (HSIAO 1973; HSIAO *et al.* 1976). The most important mechanism for maintaining turgor is adjustment of the solute potential. Solute potential is determined by the concentration of inorganic ions and organic molecules in solution (MUNNS 1988). The organic solutes are mainly sugars, sugar alcohols, and the imino acid proline (MUNNS *et al.* 1979). So for a plant to maintain a low solute potential it can either absorb ions from the soil or synthesise organic

solutes.

Associated with drought tolerance at low water potentials is the induction of gene expression and gene products that limit damage to tissues and metabolic processes (BRAY 1993; CHANDLER & ROBERTSON 1994). To this end, the organic solutes that are synthesised, such as polyamines, sugar alcohols and proline, are those that do not appreciably interfere with metabolic processes. They are termed compatible solutes. Other solutes, such as trehalose and glycine betaine, act as osmoprotectants. Several gene products play a more active role. Chaperonins, to which class the heat shock proteins belong, act to renature proteins that have been denatured by the change in cellular water status, while proteases digest denatured proteins that are no longer functional. Some products bind to macromolecules within the cell, replacing water and thereby maintaining their structure and function. Many of these genes and their products were first isolated in the desiccation phase of embryo maturation and are thus termed late embryogenesis abundant (*lea*) genes. Although they were first isolated during embryogenesis, they have been shown to be induced also in mature tissue. A characteristic of many of these genes is that they are induced by several different stresses additional to desiccation, such as heat, cold and salinity.

How the plant detects desiccation and coordinates its response is largely speculative. It is thought that loss of turgor and/or change in solute potential are common signals which are detected by membrane proteins. These in turn trigger a signal transduction pathway leading to induction of physiological responses and gene expression. The same response being elicited by different stresses could therefore be explained if those stresses are mediated through a change in turgor or solute potential.

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 *et al.* 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 & 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 *et al.* 1989). Conserved domains of other protein products of *rab* genes suggest they can bind to nucleic acids (MUNDY & CHUA 1988) and thus may be capable of altering growth and development following stress (SKRIVER & 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.

Traits that are associated with maintaining yield during drought may be unrelated to the physiology of water relations in the plant. For instance, in maize, two traits that are linked to high yield in droughted environments are prolificacy (DU PLESSIS & DIJKHUIS 1967) and anthesis-silking interval (ASI) (HALL *et al.* 1980). Non-prolific maize plants produce one ear, and prolific maize plants produce more than one ear. 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 & EBERHART 1968). ASI is important because the longer the interval the greater is the chance that pollen shed will have ended before the silks mature, thus reducing yield by reducing pollination and seed set. Drought at this stage of growth lengthens ASI, so the effect on yield will be more critical in genotypes which already have a longer ASI (DU PLESSIS & DIJKHUIS 1967; HALL *et al.* 1980).

1.2. MECHANISM OF WATER LOSS

The perception of water loss resulting from water stress is in the main a poorly understood phenomenon. There is evidence that more than one stimulus is involved. Two of the stimuli resulting from water loss are a reduction in solute potential and a loss of turgor. Studies on *Escherichia coli* have shown that osmotic change is perceived by membrane proteins (FORST & INOUYE 1988), and it is possible that these bacterial processes could act as a model for gene regulation of osmotic stress in higher plants (HUGHES 1994). Proteins in the plasma membrane of higher plants have been implicated in the detection of turgor loss (DING & PICKARD 1993; GILROY *et al.* 1991; GILROY & JONES 1994).

1.2.1. Osmotic change

In bacteria, changes in the environment, whether external or internal, are sensed by a two-component system, a sensor component and a response regulator component. The sensor component is a histidine kinase in which a histidine residue autophosphorylates in response to a stimulus. The phosphorylation prevents the formation of hydrogen bonds and adds a negative charge thus altering a protein's shape and also its function. The histidine kinase can be an integral membrane protein or a cytoplasmic protein. The response regulator component is a protein containing an aspartate residue to which the phosphate is transferred, causing the response regulator to bind to an output protein which either activates or inactivates a signal transduction pathway (HUGHES 1994). The output protein is often a DNA-binding protein which can result in changes in gene expression (OTA & VARSHAVSKY 1993). This basic two-component system in bacteria is involved in a range of environmental responses, including osmoregulation (BOURRET *et al.* 1991).

Although examples of the two-component system are common in bacteria, only recently have such systems been observed in eukaryotes. A two-component system has been elucidated in yeast which senses extracellular osmolarity (MAEDA *et al.* 1994; OTA & VARSHAVSKY 1993). Low osmolarity activates the histidine kinase SLN1, which in turn inactivates the

response regulator protein Ssk1p by phosphorylating the aspartate residue. High osmolarity inactivates SLN1 allowing the now unphosphorylated Ssk1p to activate the MAP kinase cascade which results in an osmoregulatory response, such as accumulation of glycerol (MAEDA *et al.* 1994). A model proposed by OWEN & NAPIER (1988) has ABA activating a protein kinase which phosphorylates a set of specific enzymes.

1.2.2. Turgor loss

Stretch-activated ion channels have been detected in *Commulina communis* guard cells by patch clamp analysis (HEDRICH *et al.* 1990). Gradual application of suction to the plasma membrane opened successively more ion channels, as detected by a patch electrode. Stretch-activated channels which allow Ca^{2+} influx have also been detected in yeast (GUSTIN *et al.* 1988). The exact function of these channels remains to be elucidated, but it is widely accepted that they act as turgor sensors (SCHROEDER & HEDRICH 1989). Whether channels perceive a shrinking of the plasma membrane which would result from water loss has not been investigated, but in order to maintain some sort of osmotic or turgor equilibrium it would be expected.

Another exciting and recent line of research is into the mechanical sensing by integrins (TREWAVAS & KNIGHT 1994). Integrins are plasma membrane-spanning proteins which act as anchorage sites for the cytoskeleton. They can detect changes in cell conformation and respond by increasing nuclear Ca^{2+} concentration and activating a signal transduction pathway (SHANKAR *et al.* 1993). Although most of the work to date has been done on animal cells, proteins associated with the anchorage sites have been discovered in flowering plants (SANDERS *et al.* 1991), suggesting that integrins could also occur in plants. Their mechanism of action could be to respond to the conformational changes to the cytoskeleton brought about by loss of turgor thereby activating a signal transduction pathway.

1.2.3. Change in cellular pH and ABA

ABA is a weak acid, and the degree of dissociation of the ABA molecule depends on the pH of the medium, with low pH favouring the undissociated molecule, and high pH favouring the dissociated anion. Membranes are permeable to the undissociated molecule but impermeable to the dissociated anion. Therefore the relative pH of the different cellular compartments determines ABA distribution. As a result ABA tends to accumulate as the dissociated anion in the more alkali compartments such as the stroma of the chloroplast (COWAN *et al.* 1982). Upon desiccation there is an inhibition of plasma membrane-bound ATPases which results in changes in pH of the cellular compartments. The apoplast has the greatest change, to a higher pH, because of its small buffering capacity. The higher pH will favour the dissociated ABA anion, in which state it will not diffuse through the plasmalemma. The pH of the cytosol and the stroma decrease slightly, favouring the undissociated form which can diffuse through the membranes. The net effect is the diffusion of the undissociated molecule from the stroma and cytosol into the apoplast (HARTUNG *et al.* 1988; HARTUNG & SLOVIK 1991).

Although HARTUNG and SLOVIK (1991) reported a decrease in cytosolic pH in response to desiccation, other authors (IRVING *et al.* 1992; BLATT & ARMSTRONG 1993) reported an increase. This cytosolic alkalization was the result of activation of H^+-ATPase channels on the vacuolar membrane pumping H^+ from the cytosol into the vacuole (IRVING *et al.* 1992). However, even though there might be an increase in cytosolic pH, the cytosol is buffered more than the apoplast so the relative increase in pH will be greater in the apoplast (HARTUNG & SLOVIK 1991), so the net effect will still be for ABA to move into the apoplast. The opinion of most authors currently seems to support an alkalisation of the cytosol in response to desiccation.

1.2.4. Abscisic acid as a signal

The hormone ABA acts as a signal for desiccation, and many of the responses to desiccation can be elicited by artificial application of ABA. While a receptor site for ABA has not been unequivocally identified, there is evidence that one or more exist. Extracellular

application of ABA can cause stomatal closure (McAINSH *et al.* 1990) and inhibit stomatal opening (ANDERSON *et al.* 1994). ABA applied internally to the guard cells by microinjection caused no inhibition of stomata, which opened at the same rate as uninjected controls (ANDERSON *et al.* 1994). Taken together these data suggest an ABA receptor on the extracellular side of the plasma membrane, although they do not completely exclude the possibility of an intracellular receptor as well.

Evidence for an intracellular receptor is provided by ALLAN *et al.* (1994). They placed epidermal peels in ABA-free solution to remove any external source of ABA and microinjected guard cells with caged ABA. The release of active ABA from the caged compound was then effected by UV photolysis which resulted in stomatal closure. The apparent contradiction between ANDERSON *et al.* (1994) and ALLAN *et al.* (1994) in the response to microinjection of ABA could lie in the time scales of the two experiments. The response measured by ALLAN *et al.* (1994) was initiated within 5 min of the UV flash, whereas the response measured by ANDERSON *et al.* (1994) was more than 45 min after microinjection. It has been shown that within 30 min, 80% of intracellular ABA can be lost (WEYERS & HILLMAN 1979). In ANDERSON *et al.*'s (1994) experiment, therefore, the absence of continued stomatal closure after microinjection of ABA could be the result of loss of ABA and not an absence of an internal receptor. The evidence as it stands would therefore point to there being both extracellular and intracellular ABA receptors.

While the ABA receptors themselves have not been unequivocally identified, putative ABA binding proteins have been located on the plasmalemma of guard cells by affinity labelling (HORNBERG & WEILER 1984). Three proteins that have a high affinity for ABA were isolated, the one protein binding preferentially to the dissociated anion, the other two binding preferentially to the undissociated form of ABA. ZEEVAART & CREELMAN (1988) speculated that these proteins could be modulators of the H^+/ATPase which is inhibited by ABA. The existence of ABA-binding proteins, however, has not been confirmed by subsequent research.

Since guard cells do not have plasmodesmata (WEYERS & HILLMAN 1979), any

ABA from an external source must reach the guard cells via the apoplast. Upon desiccation, ABA does move from the existing pool in the mesophyll cells to the guard cells via the apoplast (HARTUNG *et al.* 1983) in large enough quantities to effect stomatal closure (RADIN & HENDRIX 1988), and this redistribution occurs before any change in bulk leaf ABA (CORNISH & ZEEVAART 1985; RADIN & HENDRIX 1988).

There is evidence also that ABA produced in the roots in drying soil is transported in the transpiration stream to the guard cells without there being any change in turgor or bulk ABA content in the leaf (BLACKMAN & DAVIES 1985; DAVIES & ZHANG 1991). GOWING *et al.* (1990) grew apple saplings with their roots divided between two containers, the one container watered and the other subjected to soil drying. Leaf growth was retarded even though there was no difference in shoot water relations compared with control plants. When the roots in the dry container were severed, normal leaf growth resumed to the same rate as control plants. The interpretation of these observations was that desiccation was detected by the roots in the dry soil container, and a signal was transmitted in the transpiration stream which inhibited leaf growth. When the roots in the dry soil were excised, the inhibitory signal could no longer reach the leaves, which resumed normal growth.

Although the identity of the signal was not investigated by GOWING *at al.* (1990), subsequent studies have demonstrated a correlation between soil drying, leaf stomatal conductance, and xylem ABA concentration (ZHANG & DAVIES 1990; TARDIEU *et al.* 1993; JANSSEN & MARKHART 1993), thus implicating ABA. However, the correlation with increased xylem ABA concentration could be due to stomatal closure reducing the transpiration flux. In order to determine that increased ABA concentration in the xylem is the causal agent and not just a result of stomatal closure, TARDIEU *et al.* (1993) injected ABA directly into the xylem of the stem of field-grown maize at a range of leaf water potentials. The relationship between xylem ABA concentration and stomatal conductance was similar to that in maize plants growing in a drying soil, thus suggesting that ABA was the causal agent.

In an opposite approach, ZHANG & DAVIES (1991) removed ABA from the xylem sap of laboratory-grown maize and found that the inhibition of stomatal conductance was also

removed. It would appear, therefore, that roots can be the primary sensors of water stress, and that signals initiated in the root can be transmitted by ABA in the transpiration stream to affect stomatal behaviour and leaf growth.

However, soil drying is not the only cause of desiccation. Increased vapour pressure deficit, for instance as the day temperature increases, can cause leaf desiccation in well-watered soil. Under conditions of constant soil water, TARDIEU and DAVIES (1992) showed that xylem ABA concentration remains relatively constant throughout the day. Increased transpirational flux in response to an increased vapour pressure deficit must therefore result in an increased ABA flux to the leaf, and stomata can close in response to an increase in ABA flux (TREJO *et al.* 1995). The response to leaf desiccation in well watered soil is therefore still mediated through ABA.

1.3. CELLULAR RESPONSE TO WATER STRESS

Most of the work on cellular responses to water stress has been done on guard cells. Although the details of the guard cell response to have yet to be elucidated, the general outline seems to be an initial response to either an increase in ABA concentration, or a redustribution of ABA within the guard cell. The change in ABA status effects a depolarisation of the plasma membrane resulting from an influx of cations, mainly Ca^{2+}, and an efflux of anions. This initial depolarisation activates two types of membrane channels, the R- and S-type, which lead to a long term depolarisation of the plasma membrane by anion efflux. The R- and S-type channels are sensitive to Ca^{2+} and are voltage-dependent. The depolarisation generates the driving force for the K^+ efflux through outward rectifying channels. At the same time, ABA inhibits K^+ influx through inward rectifying channels. The source of the Ca^{2+} is the apoplast and the vacuole (reviewed in GIRAUDAT *et al.* 1994).

Another effect of ABA on guard cells is the activation of H^+-ATPase channels which results in the alkalisation of the cytosol. This cytosolic alkalization activates a second Ca^{2+}-independent, pH-sensitive pathway of outward rectifying K^+ channels. The net effect is an

efflux of ions, a loss of turgor through osmotic adjustment, and closure of the stomata.

Evidence for the above outline comes from a multitude of sources. Calcium ion concentration in the cytosol is maintained at a low level by plasma membrane and vacuolar membrane ATPases which pump calcium into the apoplast and the vacuole (EVANS *et al.* 1991; WARD *et al.* 1995). Application of ABA leads to an increase in intracellular Ca^{2+} concentration due to opening of calcium channels (McAINSH *et al.* 1990; GILROY *et al.* 1991). Cytosolic Ca^{2+} combines with calmodulin or other Ca^{2+} binding proteins which in turn activate a signal transduction pathway via protein kinases (TREWAVAS & KNIGHT 1994). Direct application of Ca^{2+} inside guard cells from caged Ca^{2+} by UV photolysis leads to stomatal closure (GILROY *et al.* 1990). Elevated Ca^{2+} concentrations activates voltage dependent anion efflux channels which makes the membrane potential less negative. This membrane depolarization favours K^+ efflux down an electrochemical gradient through outward rectifying K^+ channels (SCHROEDER & HAGIWARA 1989; HEDRICH *et al.* 1990). ABA and Ca^{2+} act together to inhibit K^+ influx channels (SCHROEDER & HAGIWARA 1989; BLATT 1992) so that the net efflux of ions promotes stomatal closure through turgor loss (MacROBBIE 1989). About 90% of the guard cell's volume is taken up by the vacuole, and so most of the K^+ efflux from the guard cell will originate from the vacuole. WARD and SCHROEDER (1994) described two channels from the guard cell vacuole, both of which were activated by a rise in cytosolic Ca^{2+} concentration. The one, VK, was highly selective for K^+ ions, while the other, SV, was more selective for Ca^{2+} over K^+. So a rise in cytosolic Ca^{2+} can induce the release of both Ca^{2+} and K^+ from the vacuole.

There have been some investigations at the level of the individual guard cell, and they have found that although all guard cells close in response to applied ABA, the closure is not always associated with increased Ca^{2+} concentration (McAINSH *et al.* 1990; GILROY *et al.* 1991). These investigations suggest that there is also a Ca^{2+}-independent ABA signal transduction pathway, but some authors contend that this interpretation arises from limitations in the techniques, and that there is only one pathway which always involves Ca^{2+} (McAINSH *et al.* 1992; MacROBBIE 1992).

The sensitivity of guard cells to ABA can be modulated by other factors such as the concentration of inorganic ions in the xylem sap (GOLLAN *et al.* 1992; SCHURR *et al.* 1992), pH (HARTUNG & SLOVIK 1991), tissue water potential (SAAB *et al.* 1990) and temperature (ALLAN *et al.* 1994). DAVIES *et al.* (1994) suggested that the modulating effect of these factors allows the plant to integrate the input from different environmental cues, some of which might be contradictory, and adjust its response accordingly.

Most work on the cellular responses to ABA has been done on the guard cells, and their response to water stress is to lose turgor by K^+ and anion efflux. However, cells in the rest of the leaf require the opposite response, to increase their internal ionic concentration in order to absorb more water under dehydrating conditions. CURTI *et al.* (1993) found that cultured cells responded to osmotic stress by activating H^+-ATPase channels which in turn led to the uptake of K^+ and osmotic adjustment. ABA-induced changes have been observed in cell types other than guard cells. For instance, membrane depolarisation has been observed in seedlings and epidermal and mesophyll cells (MacROBBIE 1991), and an increase in pH and Ca^{2+} has been observed in roots, coleoptiles and hypocotyls (GEHRING *et al.* 1990).

ABA accumulation inhibits shoot elongation and promotes root elongation at low water potentials (SAAB *et al.* 1990), a response that would enable the plant to explore a greater soil volume for extraction of water. Responses of plants to exogenous ABA vary according to the state of the plant. For instance, well-watered and water stressed plants of the same species have different growth responses to applied ABA (WATTS *et al.* 1981), and is probably the result of differences between well watered and water stressed plants in such aspects as compartmentation of endogenous ABA and levels of other hormones.

1.4. GENE EXPRESSION IN RESPONSE TO WATER STRESS AND ABA

Under conditions of water stress, plants alter the expression of a variety of genes whose products have a wide range of functions (SKRIVER & MUNDY 1990; BRAY 1993;

CHANDLER & ROBERTSON 1994). The expression of some of these genes can also be modulated by application of ABA to unstressed tissue (GOMEZ *et al.* 1988; MUNDY & CHUA 1988). The nomenclature relating to these genes is quite confused and arises from the nature of the original investigation rather than any related function. For instance *lea* (late embryogenesis abundant), *rab* (responsive to ABA), *dhn* (dehydration induced) and *em* (early methionine-labelled) genes are all induced by ABA or desiccation. Some of the genes with similar names can have unrelated sequences (eg *rab17* and *rab28*), and other genes with an almost identical sequence can have different names (eg *rab17*, *d11* and *dhn*) (CHANDLER & ROBERTSON 1994).

Adding to the confusion is the lack of understanding of the function of many of the gene products. For instance, the protein osmotin was first isolated from tobacco subjected to salt stress, desiccation, and ABA (SINGH *et al.* 1985, 1989), hence its name. It was later found to belong to the group of pathogenesis related (PR) proteins which have antifungal activity (LIU *et al.* 1994). So although the osmotin gene is activated by osmotic stress, the function of its product is not related to the water relations of the plant.

ABA can also lead to the repression of gene expression and protein synthesis. LEONE *et al.* (1994) observed the repression of protein synthesis on application of ABA to potato cell suspension cultures. The majority of the proteins repressed by ABA application were found to be the same as those repressed by osmotic stress simulated by PEG solution (LEONE *et al.* 1994), thus implicating the role of ABA in the water stress response.

However, ABA does not account for all the responses to water stress observed. Some of the proteins induced by osmotic stress in potato cell suspension cultures were not induced by ABA (LEONE *et al.* 1994). Similar observations have been made on other plants such as pea (GUERRERO *et al.* 1990) and rice (BORKIRD *et al.* 1991). A gene from pea, *Cyp15a*, encoding a cysteine protease, had increased transcription in response to partial desiccation, osmoticum, and during germination, but not in response to externally applied ABA or gibberellins (JONES & MULLET 1995a). Cysteine proteases are thought to play a role in protein turnover, either in mobilising storage proteins on germination, or by degrading

denatured proteins in response to stress.

Furthermore, genes can respond to more than one signal transduction pathway. The *lti140* (low-temperature-induced) gene of *Arabidopsis thaliana* can respond by increased transcription to exogenous ABA, low temperature and desiccation (NORDIN *et al.* 1991). So although *lti140* is an ABA responsive gene, it is not ABA dependent.

Calcium is also implicated in the gene expression response to desiccation and ABA. Three cDNA clones have been isolated from chick-pea seeds that respond to desiccation and ABA application by increased transcription. When chick-pea seeds were incubated with water, $CaCl_2$, ABA, ABA + $CaCl_2$, and ABA + $CaCl_2$ chelating agents, the ABA + $CaCl_2$ treatment produced the highest level of transcription, whereas the ABA + $CaCl_2$ chelating agents treatment reduced transcription compared to ABA alone (COLORADO *et al.* 1994).

1.5. RESPONSIVE ELEMENTS IN PROMOTER SEQUENCES

The genes *rd29A* and *rd29B* were originally isolated from *Arabidopsis thaliana* in response to desiccation and were found to be tandemly arranged in the genome (YAMAGUCHI-SHINOZAKI & SHINOZAKI 1993). Analysis of their promoters revealed a nine bp drought responsive element (DRE), TACCGACAT, in *rd29A* that was rapidly induced by drought (YAMAGUCHI-SHINOZAKI & SHINOZAKI 1994). The same nine bp DRE was also identified in two other genes *kin1* and *cor6.6* from *Arabidopsis* which are also induced by desiccation as well as low temperature and osmoticum (WANG *et al.* 1995). These observations were made by transforming tobacco and *Arabidopsis* plants with the DRE promoter-GUS fusion gene and subjecting leaf discs to low temperature, ABA, osmoticum and desiccation. GUS activity was induced by ABA, osmoticum and desiccation, but not by low temperature. Northern analysis showed that low temperature did induce GUS mRNA. Regulation of these genes is therefore at the transcriptional level via the promoters in response to ABA, low temperature and desiccation, but low temperature reduces expression at the translational level (WANG *et al.* 1995). These results demonstrate that regulation is at both the

transcriptional and translational level, and that different stresses can induce gene expression via the same promoter.

The genes *kin1* and *cor6.6* contain also the palindromic core sequence, CACGTG, within an element designated the G-box, which is associated with ABA-induced expression and a DNA-binding protein (GUILTINAN *et al.* 1990). However, having the highly conserved G-box core sequence does not necessarily confer ABA responsiveness. Replacing all the base pairs in the G-box core sequence of ABA responsive element 1 (ABRE1) of the gene *HVA1* has no observable effect on promoter activity (STRAUB *et al.* 1994), suggesting that there are multiple regulatory elements working together to confer ABA/water stress responsiveness.

Multiple regulatory elements within a gene promoter were found by SHEN and HO (1995) in the barley ABA-responsive *HVA22* gene. The G-box, designated ABRE3, required an extra element, designated CE1 (coupling element 1), for ABA responsiveness. Elimination of either of these elements abolished ABA-responsiveness. Additionally they found a second response complex in the *HVA22* promoter region consisting of ABRE2 and an as yet unidentified second coupling element (CE2). SHEN & HO (1995) suggested that the interaction between ABRE and CE allowed for the specificity of gene response, so that the same gene could react to different environment and physiological cues. For instance, the presence of a G-box and another element is needed for gene responses to coumaric acid (LOAKE *et al.* 1992), UV (BLOCK *et al.* 1990) and white light (DONALD & CASHMORE 1990). Although the G-box elements are all similar between these different genes, the elements interacting with the G-box are different (SHEN & HO 1995).

SHEN & HO (1995) identified in the literature ten other ABA-responsive genes containing G-box/CE1-like element complexes in their promoter regions. One such gene, *rab16B*, contained three of these complexes (YAMAGUCHI-SHINOZAKI *et al.* 1989).

The existence of more than one response complex allows for an incremental expression level response, thus providing a mechanism to regulate the transcription level of the gene. This incremental expression is supported by the observation that mutation of ABRE1 results in a

reduction in the level of expression of the *HVA22* gene, and mutation of ABRE2 as well leads to a further reduction (SHEN & HO 1995).

The *Adh* (alcohol dehydrogenase) gene from *Arabidopsis* has been shown to have four regions of its promoter that are essential for expression in response to desiccation, cold and hypoxia (DOLFERUS *et al.* 1994). The activity of these regions was studied using *Adh* promoter::*gus* fusion expression with deletion mapping and site-specific mutagenesis of the promoter. Deletions and mutagenesis in region I increased GUS expression in all three stresses and in uninduced control conditions suggesting this region contains a repressor binding site, while mutations in region II reduced expression in all three treatments and the control suggesting this region contains a positive regulatory element. Region III contains two G-box elements, and mutation in G-box-1 reduced expression in response to cold and desiccation but not anoxia. Mutations in G-box-2 had little effect. Deletions and mutagenesis in region IV, the anaerobic responsive element (ARE), significantly reduced GUS expression in response to all three stresses and could be a general stress response element. (DOLFERUS *et al.* 1995). Again, this study demonstrates the interaction of a complex of elements in the promoter of a gene integrating the gene expression response to different environmental cues.

Proteins that interact with the G-box contain a basic region adjacent to a leucine zipper, which is characteristic of DNA binding proteins called bZIP proteins (LANDSCHULZ *et al.* 1988). The protein EmBP-1 binds to the ABRE sequence of the ABA-responsive *em* gene of wheat (GUILTINAN *et al.* 1990). Some bZIP proteins can form dimers (ARMSTRONG *et al.* 1992). VP1 protein is a transcription factor and is essential for the expression of the ABA-responsive *em* gene (McCARTY *et al.* 1991). VP1 does not have a DNA binding domain nor a dimerization motif so it is unlikely to bind directly to ABRE or CE. Possibly its function is to bind the bZIP proteins on ABRE and CE to the transcription factors and RNA polymerase II (COMAI *et al.* 1992).

WILLIAMS *et al.* (1992) studied the binding of seventeen G-box binding sequences with cauliflower nuclear extract using gel mobility shift assays and showed that there are two classes of G-box elements that interact with two types of G-box binding proteins. Within each

class of element, different binding sites vary in their affinity for binding proteins depending on the base sequence flanking the hexameric core. WILLIAMS *et al.* (1992) speculated on a hierarchical mechanism of gene expression based on the affinity of a particular binding site for a type of binding protein. If the availability of binding proteins are limited within the nucleus, then they will activate only those genes which possess sites to which the proteins have the greatest affinity. In cells expressing high levels of binding proteins, genes containing promoters with sites with a lower binding affinity will also be expressed.

Other *cis*-acting elements that are responsive to stimuli other than ABA have been found. The *rd29A* gene from *Arabidopsis* has both a slow response to stress that is ABA responsive, and a fast response that is ABA-independent. The promoter region of the *rd29A* gene has a *cis*-acting element that is involved in the rapid response to drought, high salt and low temperature but not to ABA (YAMAGUCHI-SHINOZAKI & SHINOZAKI 1994). This element was designated a DRE (desiccation-, high salt-, or low temperature-responsive element) and contains a nine bp core sequence (TACCGACAT), designated DR1, that is essential for stress inducible expression. Unlike the G box discussed above, DR1 does not need other elements in the promoter for expression. A DRE binding factor (DRBF1) was isolated which is always present in cells, even under unstressed conditions. The possible mode of function of DRBF1is that it interacts with a positive factor under stress conditions which enables it to bind with DR1 and initiate *rd28A* expression, or that it binds with a negative factor under non-stress conditions which inhibits binding to DR1.

Elucidation of the activity of the promoters of many of these genes has been done by transcriptionally fusing the promoter to a reporter gene such as β-glucuronidase (GUS) and testing by transient expression in transformed plants.

1.6. GENE PRODUCTS AND THEIR FUNCTIONS

Many of the gene products isolated in drought response studies do have an adaptive role since it has been shown that their presence enhances cellular function under conditions of

water stress. For instance, wheat seedlings show extreme desiccation tolerance and can resume growth after as much as 95% water loss. This ability is lost after the emergence of the first leaf, and this coincides with the presence of group 3 LEA proteins whose concentrations also decline after emergence of the first leaf (RIED & WALKER-SIMMONS 1993). Similarly, group 3 LEA proteins are found in the shoot but not the root of young seedlings, and the root does not show the same desiccation tolerance as the shoot (RIED & WALKER-SIMMONS 1993). However, the picture is not that clear since high levels of group 3 LEA proteins did not confer desiccation tolerance in rice (BRADFORD & CHANDLER 1992), so perhaps the group 3 LEA proteins form only part of a more complex response.

The only damage in response to stress which has so far been found *in vivo* is membrane rupture, which occurs in response to both freezing and excessive wilting (HINCHA *et al.* 1987). Proteins have been isolated that protect membranes from mechanical rupture (HINCHA et al 1990). It is possible that these same proteins may act in a similar way to protect against mechanical damage resulting from wilting. While not suggesting any specific mechanism, BLACKMAN *et al.* (1995) found that a group of ABA-responsive LEA proteins protected membrane integrity, as measured by electrolyte leakage, in soybean seedlings. When LEA proteins accumulated in response to ABA application and desiccation, membrane leakage declined, and cellular integrity was maintained when observed by light microscopy.

The proteins involved in these observations could be the heat shock proteins. After synthesis in response to heat stress, heat shock proteins are found distributed throughout the cell, mostly in the form of structurally bound aggregates which are converted to more soluble forms on recovery, suggesting their function has something to do with maintaining the integrity of cell structure (NOVER *et al.* 1989). Subsequent studies have shown that heat shock proteins are chaperonins and act to renature proteins that have been denatured by the change in cellular water status, a process that requires ATP (BRAY 1993).

Most of the *lea* gene products are hydrophilic suggesting that they reside in the cytoplasm and not cell membranes. They have been grouped according to their amino acid sequence similarities, and at least six groups have been categorised (BAKER *et al.* 1988;

DURE *et al.* 1989; DURE 1993). Group 1 LEA proteins contain a high proportion of charged amino acids and a random coil structure suggesting they bind water molecules. Group 2 LEA proteins have a 15-mer repeat and are thought to act as chaperones. Group 3 LEA proteins have an 11-mer amino acid motif that is repeated many times and are amphiphilic. Their predicted structure is a homodimer joined by the hydrophobic face, with the external charged surface sequestering ions, which are in a high concentration in desiccated cells, and preventing their crystallization. Group 4 LEA proteins have a conserved C-terminal random coil and are thought to replace water on the surfaces of macromolecules to maintain their structural integrity at low water potentials. Group 5 LEA proteins are thought to sequester ions in the same way as group 3.

Although the *lea* group of genes has been the most studied, many more drought-related genes have been isolated and their proposed functions span a wide range of cellular functions. The amino acid sequences of a variety of *rab* genes have also been derived (for a review, see SKRIVER & MUNDY 1990) and, similar to the *lea* genes, common characteristics of many of these gene products are their hydrophilic nature. There is a high preponderance of glycine and amino acids with hydroxyl groups, suggesting the possibility of forming amphiphilic helices containing highly charged amino acid residues (BAKER *et al* 1988; GOMEZ *et al* 1988; HARADA *et al* 1989). In addition, a set of desiccation-induced proteins, referred to as dehydrins, in barley and maize are also markedly hydrophilic and glycine-rich (CLOSE *et al.* 1989). A problem encountered by cells upon desiccation is the maintenance of the structural integrity of proteins and membranes with the removal of water. Since the bipolar nature of the water molecule is a fundamental component of the structure of proteins and phospholipids, the removal of water will modify their structure unless the bipolar charge is replaced by other molecules (CREIGHTON 1983). It is possible that the products of some of the *rab* genes take the place of water molecules associated with macromolecules and act to maintain the bipolar charge in the same manner as the group 4 LEA proteins. The hydroxyl groups could act to solvate molecular surfaces, and the freely rotating glycine residues could adopt a variety of shapes to fit the surfaces of macromolecules (BAKER *et al.* 1988). The presence of dehydrins alone is not enough to confer desiccation tolerance in soybean seeds (BLACKMAN *et al.* 1991). There is some evidence that to perform a desiccation tolerant role dehydrins may

interact with soluble sugars (BLACKMAN *et al.* 1992).

Many proteins induced in response to desiccation belong to the DHN/LEA/RAB family of proteins. The main unifying feature of this family are stretches of highly conserved amino acid sequences. At the N-terminal region many of the proteins contain a short repeat, DEYGNP. The central region contains a stretch of seven to nine serine residues (VILARDELL *et al.* 1990; GODAY *et al.* 1994), and it is possible that these proteins, such as RAB-17, could be involved in the signal transduction pathway since the serine residues can be phosphorylated (VILARDELL *et al.* 1990). WELIN *et al.* (1994) found the stretch of seven to nine serine residues to be followed by three acidic residues. In the C-terminal region a basic lysine-rich repeat is found in at least two copies. The proteins are hydrophilic (CLOSE *et al.* 1989). Although there are general similarities between LEAs, these similarities are never universal among all members of the LEA protein groups. This is most true for the dehydrins, or group 2 LEAs, which is a large family with great diversity of properties. Generally the dehydrins have several characteristics; hydrophilicity, the consensus sequence EKKGIMDKIKEKLPG at the carboxy terminus, a tract of six to nine serine residues (LABHILILI *et al.* 1995) and an absence of cysteine and tryptophan residues (CLOSE *et al.* 1989). Yet the cotton dehydrin D-11 does not have the consensus sequence (BAKER *et al.* 1988), and pea dehydrin does not have the serine tract (ROBERTSON & CHANDLER 1992).

Since channel proteins have been shown to be involved in responses to water stress, it is not surprising to find a drought-related gene product in peas which shows amino acid sequence homology to membrane-spanning proteins which act as K^+, Na^+ and Ca^{2+} channels (GUERRERO *et al.* 1990). The involvement of K^+ and Ca^{2+} in the response to ABA and desiccation has already been described above. The gene, *Trg31*, belongs to the MIP intrinsic membrane protein superfamily (JONES & MULLET 1995b). MIP proteins form membrane channels and have been found to be sensitive to turgor changes (PAO *et al.* 1991). By using *Trg31* promoter::*Gus* fusions and detecting GUS expression, the abundance of *Trg31* mRNA was found to be highest in non-elongating regions of pea roots, and in the root/shoot and cotyledon/hypocotyl junctions. The suggested function is a phloem unloading channel of solutes into the apoplast facilitating source to sink transport (JONES & MULLET 1995b).

A gene coding for a lysine-rich H1 histone was found to be drought- and ABA-inducible in *Lycopersicon pennellii* (WEI & O'CONNELL 1996). Assuming that this is an adaptive response, the proposed function of this gene could be modulating gene expression by altering the chromatin structure. It has been suggested that phosphorylation and dephosphorylation of lysine residues on the H1 histone controls its effect of condensation and decondensation of eukaryotic chromatin (ROTH & ALLIS 1992).

A thiol protease was isolated from pea shoots and was found to increase in pea shoots in response to desiccation but not ABA or heat stress (GUERRERO *et al.* 1990). Thiol proteases have been implicated in degrading vegetative storage proteins in the vacuole, thus making available amino acids for *de novo* protein synthesis in response to stress (GUERRERO *et al.* 1990).

Aquaporins are part of the membrane intrinsic protein (MIP) superfamily, of which there are more than twenty members so far described. The MIPs have six membrane-spanning domains which are a mixture of α-helix and β-strand, and cytoplasmic carboxyl- and amino-termini. None of the α-helices are amphiphilic. The channel protein has a tetrameric structure. Related proteins with the same conformation are the channel-forming integral proteins (CHIP) and the tonoplast intrinsic proteins (TIP). CHIPs are abundant in the plasma membrane of erythrocytes, and TIPs, as the name implies, are abundant in vacuolar membranes of plant cells (CHRISPEELS & AGRE 1994; CHRISPEELS & MAUREL 1994).The primary amino acid sequence of the amino terminal is 40% conserved between MIPs, CHIPs and TIPs. Aquaporins have a pore radius of between 1.5 Å (the radius of the H_20 molecule) and 2.0 Å (the radius of the smallest impermeable solutes such as urea), as determined by X-ray crystallography (CHRISPEELS & AGRE 1994). Induction of turgor-responsive aquaporins during water stress indicates that plants can adjust bulk water flow to restore turgor as the need arises. Two aquaporins that are induced by desiccation are the RD28 from *Arabidopsis thaliana* (YAMAGUCHI-SHINOZAKI *et al.* 1992) and Trg31 from pea shoots (JONES & MULLET 1995b). Other aquaporins such as γ-TIP are constitutively expressed (LUDEVID *et al.* 1992). This suggests that different aquaporins respond to different stimuli. γ-TIP is expressed most highly in elongating cells, so perhaps it is involved in supplying water to these rapidly growing

cells (LUDEVID *et al.* 1992).

1.7. ABSCISIC ACID

1.7.1. Synthesis and metabolism

Mevalonic acid (MVA) is the ultimate precursor of terpenoids and ABA. However, very little is known about the metabolic reactions involving ABA once they diverge from the established terpenoid pathway. The levels of ABA found in plants are so low that determining intermediates by the standard method of isolation of labelled compounds is very difficult. Current understanding is that ABA is synthesized in higher plants via a direct pathway from farnesyl pyrophosphate (FPP), and an indirect pathway from the breakdown of carotenoids. No intermediates between FPP and ABA unique to ABA synthesis have been identified with certainty, so understanding of the direct pathway remains limited (MILBORROW 1983).

Evidence suggests the indirect pathway involves the breakdown of carotenoids (HENSON 1984; MOORE & SMITH 1984; NEILL *et al.* 1986) by oxidative cleavage of xanthophylls, such as violaxanthin and neoxanthin, to xanthoxin and ABA (TAYLOR & BURDEN 1972; TAYLOR & BURDEN 1973; PARRY *et al.* 1988), although xanthoxin could be an intermediate in the direct pathway (ZEEVAART & CREELMAN 1988). Further evidence suggests that xanthophylls are the major precursors of ABA (PARRY *et al.* 1990), and that it is the indirect pathway that produces stress-induced ABA (NONHEBEL & MILBORROW 1986; CREELMAN *et al.* 1987). The site of ABA biosynthesis appears to be the cytosol (HARTUNG *et al.* 1981; HARTUNG *et al.* 1982).

Catabolic pathways from ABA are oxidation to phaseic (PA) and dihydrophaseic (DPA) acids (TINELLI *et al.* 1973; MURPHY 1984), which appears to occur in the cytosol (HARTUNG *et al.* 1980), and the formation of the conjugates abscisic acid glucose ester (ABAGE) (KOSHIMIZU *et al.* 1968) and abscisic acid glucoside (ABAGS) (LOVEYS & MILBORROW 1981). Catabolism to PA and DPA occurs under conditions of both full turgor

and water stress, whereas conjugation of ABA to ABAGE occurs only under conditions of prolonged water stress (ZEEVAART & BOYER 1982). ABAGE is irreversibly compartmentalised in the vacuole (LEHMANN & GLUND 1986). These conjugates are not converted back to ABA and so do not contribute to the pool of free ABA in the cell (MILBORROW 1978).

Although most of the free ABA in the cell occurs in the chloroplast (HARTUNG *et al.* 1982; HEILMAN *et al.* 1980), there is no evidence that the chloroplast is the site of ABA synthesis (COWAN & RAILTON 1986; HARTUNG *et al.* 1980; HARTUNG *et al.* 1981).

1.8. PROLINE

1.8.1. Biosynthesis

Proline biosynthesis in *Escherichia coli* (HAYZER & LEISINGER 1981) and *Saccharomyces cerevisiae* (TOMENCHOK & BRANDRISS 1987) involves a four step pathway mediated by three enzymes. The first step is the activation of L-glutamic acid by the enzyme γ-glutamyl kinase to form γ-glutamyl phosphate. This is converted to L-glutamate-γ-semialdehyde (GSA) by γ-glutamyl phosphate reductase. It is thought that these first two enzymes form a complex so that the unstable γ-glutamyl phosphate remains enzyme-bound. GSA spontaneously cyclizes to Δ^1-pyrroline-5-carboxylic acid (P5C), which is converted in the final step to proline by P5C reductase (P5CR). In *S. cerevisiae*, three genes, *PRO1*, *PRO2*, and *PRO3* code for the three enzymes γ-glutamyl kinase, γ-glutamyl phosphate reductase, and P5CR respectively (TOMENCHOK & BRANDRISS 1987). P5CR cDNA has also been isolated from soybean root nodules, and it showed 40% homology with *E. coli PRO3* (DELAUNEY & VERNA 1990).

In *S. cerevisiae* and plants, proline is also synthesized from arginine breakdown via ornithine and GSA (ADAMS & FRANK 1980). In both pathways P5CR is involved in the final step to proline.

1.8.2. Mode of action

Proline levels are elevated during and after stress (BLUM & EBERCON 1976; ITAI & PALEG 1982). Although it is not necessarily the amino acid with the highest concentration in plant tissue, its most striking characteristic is that it has by far the greatest increase in concentration in response to water stress (GIROUSSE *et al.* 1996). This suggests that it has a particular function in the water stress response, and this is supported by several lines of research.

A suggested role for proline is for osmotic adjustment. At low water potential, the increase in proline concentration accounts for most of the osmotic adjustment in the apical region of the growth zone of maize roots, and thus plays a major role in maintaining root elongation at low water potentials (VOETBURG & SHARP 1991). Common compatible solutes that are involved in osmotic adjustment are K^+ ions, proline, glycine-betaine, sucrose and sugar alcohols such as sorbitol. Compatible solutes for the most part do not carry a net electrical charge at physiological pH, near pH 7. K^+ is an exception to this generalization and may not offer as much protection against osmotic stress as the uncharged metabolites (CSONKA 1989). In certain bacteria, glycine-betaine is accumulated in preference to K^+ when internal concentrations of K^+ are potentially deleterious to enzyme function (SUTHERLAND *et al.* 1986).

There is a certain amount of debate as to whether proline acts only as a compatible solute to lower the solute potential of the cell, or also to protect proteins from denaturation in conditions of high electrolyte concentration such as would occur on desiccation. Proline appears to enhance a plant's growth during recovery from stress rather than its growth during stress (BLUM & EBERCON 1976; ITAI & PALEG 1982). For instance, application of proline during water stress had no effect on the growth of barley compared to controls, but growth during the recovery from water stress was enhanced (ITAI & PALEG 1982). SCHOBERT (1977) suggested that the proline molecule acts by the binding of its aliphatic portion to non-polar residues on proteins by hydrophobic interactions and exposing its charged imino and

carboxyl groups, thus coating the protein with a hydrophilic shell that enhances its solubility in an aqueous medium. However, the concentrations of proline that are required to increase significantly protein solubility are far in excess of those found in cells (SCHOBERT & TSCHESCHE 1978). Also those organic molecules that act as osmotic protectants are generally uncharged at physiological pH and so would have a negligible electrostatic interaction with proteins (ARAKAWA & TIMASHEFF 1985). Subsequent observations suggest that organic osmoprotectants are effective precisely because they do not interact with the surfaces of proteins, but instead stabilize protein structure by inducing preferential hydration of the macromolecules (ARAKAWA & TIMASHEFF 1983 and 1985). Although the weight of evidence supports the view that organic osmoprotectants act only as inert solutes that act to provide cell turgor, it does not explain why the number of preferred organic osmoprotectants are so few in number when there is a large variety of similar compounds available (CSONKA 1989).

1.8.3. Interaction between ABA and proline

ABA accumulation precedes proline accumulation in wilted barley leaves (STEWART & VOETBURG 1985) and in the growing region of the root apex in maize (OBER & SHARP 1994). The mechanism of ABA action on proline appears to be both by increasing proline synthesis from glutamate (STEWART 1980) and by inhibiting proline oxidation (DALLMIER & STEWART 1992). There is some debate in the literature about a causal link between ABA and proline accumulation. STEWART & VOETBURG (1987) showed ABA and proline accumulation are not causally linked, whereas OBER & SHARP (1994) showed the opposite, that an increase in ABA concentration is required for an increase in proline concentration. However, the levels of proline were severalfold lower in the study of STEWART & VOETBURG (1987), and OBER & SHARP (1994) suggested that ABA is required for high levels of proline to accumulate.

The effect of ABA on proline accumulation may not be direct, but could be mediated through osmotic adjustment since it is known that ABA has an effect on membrane ion channels and intracellular ion concentration. There is some evidence that ion influx involving

Na^+ and Cl^- or K^+ and Cl^- stimulates ABA-induced proline accumulation (PESCI 1988). It appears that it is Cl^- that elicits the increase in ABA-induced proline accumulation while requiring the simultaneous presence of K^+ or Na^+ (PESCI 1989).

The increase in proline levels during water stress appears to enhance a plant's growth on recovery from stress rather than its growth during stress (BLUM & EBERCON 1976; ITAI & PALEG 1982). Its action during water stress appears to be as a compatible solute to lower the osmotic potential of the cell, and to preferentially hydrate proteins to maintain their tertiary structure at low water potentials (ARAKAWA & TIMASHEFF 1985).

1.9. OBJECTIVES AND NATURE OF THE STUDY

Many of the drought related genes presently being studied were first isolated from the desiccation phase of embryogenesis or from the extremely drought tolerant resurrection plant. The extent of desiccation that is experienced by mature embryos and the desiccated resurrection plant is much more extreme than that experienced by a mature mesophyte such as maize during a growing season. Although many of these same genes are also induced in mature tissue in response to desiccation, it is possible that other genes may be important in the response to a milder stress. For this reason the aim of this study was to isolate drought related genes from mature maize plants subjected to the sort of water stress that could be expected in the field. Maize was chosen for the study because a lot of work has already been done on both the physiological and molecular responses of the crop.

It was decided that the nature of the study should be comparative between a drought resistant and a drought sensitive cultivar. If a difference in gene expression in response to water stress is observed, then the difference would suggest that the response is adaptive. The cultivars chosen were PAN473 which is drought resistant, and SR52 which is drought sensitive. The definition of drought resistance is based on yield; the yield of SR52 is reduced to a greater extent in dry environments than that of PAN473. In areas of relatively high rainfall SR52 has a higher yield than PAN473, but in lower rainfall areas PAN473 has a higher yield (KAISER

1990, pers. comm.[1]).

The possibility exists that the difference between the cultivars in drought resistance is phenotypic and not physiological. PAN473 is prolific and SR52 non-prolific (KAISER 1990, pers. comm.), so the difference could be attributable to prolificacy. The first part of the study, therefore, is an investigation of the response of certain drought related physiological characteristics to establish that there are physiological differences between the cultivars.

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 *et al.* 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.

[1]Dr H.W. KAISER, Pannar Seed Company (Pty.) Ltd., P.O.Box 19, Greytown 3500

CHAPTER 2:
PHYSIOLOGICAL DIFFERENCES BETWEEN DROUGHT RESISTANT AND DROUGHT SENSITIVE MAIZE CULTIVARS IN RESPONSE TO WATER STRESS

2.1. INTRODUCTION

Genotypic differences occur in the growth response of maize (*Zea mays* L.) to water stress (HALL *et al.* 1981; LORENS *et al.* 1987a & 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 *et al.* 1973; BEARDSELL & COHEN 1975; ACKERSON *et al.* 1979; QUARRIE 1980; ACKERSON 1983), osmotic adjustment (ACKERSON *et al.* 1979), ABA (BEARDSELL & COHEN 1975; QUARRIE 1980; ACKERSON 1983) and proline levels (BLUM & EBERCON 1976; HANSON *et al.* 1977; QUARRIE 1980; THAKUR & 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 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 plants were water stressed by soil drying in preference to other methods, since this would more accurately reflect what happens in the field.

2.2. METHODS

2.2.1. Study plants

The two *Zea mays* L. cultivars chosen for study were PAN473, a drought resistant cultivar, and SR52, a drought sensitive cultivar.

2.2.2. Leaf and root growth

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. Each tube was perforated at the base and at 10 cm intervals on opposite sides to allow drainage and aeration of the soil. After filling, the soil was watered to saturation and allowed to settle, whereupon more soil was added and rewatered. This was repeated until the soil remained level with the top of the tube. The soil surface was covered with vermiculite to keep it moist.

Seeds of both cultivars were germinated in seed trays containing vermiculite. On emergence of the hypocotyl, 25 seedlings per cultivar were selected for uniformity and planted, one in each tube. Fifteen plants per cultivar were allocated to one of two treatments, water-stress and control. The control plants were watered daily to run-off throughout the experiment. The water-stress plants were watered daily to run-off for the first fifteen days from planting, after which they were not watered. 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 glasshouse where maximum day temperatures varied between 23°C and 35°C and minimum night temperatures between 12°C and 20°C. The relative humidity varied between 29% and 53%, and PAR was generally between 600 and 950 μmol

m^{-2} s^{-1}.

On harvesting, the above-ground 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 20 cm depths and the bulk of the roots removed from each section by washing over a sieve. The roots and above-ground 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.

2.2.3. Water relations

A separate set of plants was used for the physiological measurements of water status and proline and ABA levels. The same soil mix was used, and seeds were germinated and planted in the same way. Plants were grown in seven-litre pots which were watered daily to a constant weight. They were grown in the glasshouse under the same conditions of temperature, humidity and light as described above.

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 porometer. 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 & WEATHERLEY (1962). Three leaf disks per plant were cut from the fifth leaf to emerge, their fresh weight measured, and floated on distilled water in a covered petri dish for 2 h after which the turgid weight was measured. The leaf disks were then dried in an oven and reweighed. 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.

2.2.4. Proline

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 homogenizing 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 *et al.*, 1973). Measurements were done on five plants per cultivar per treatment on six different days.

2.2.5. Abscisic acid

ABA concentration was determined by the method of HUBICK & REID (1980). A minimum of 1 g quantities of freeze-dried leaf tissue was homogenized with an extraction medium of methanol, ethyl acetate, and acetic acid, filtered through Whatman No. 1 filter paper, and the filtrates taken to dryness *in vacuo* at 35°C. Further freeze-drying of some of the samples was necessary. Two ml dichloromethane was added to the dry sample and 1 ml of the resulting extract loaded onto a silica Sep-pak cartridge. Contaminants were removed from the sample by a series of organic solvent mixtures before the ABA was eluted. The ABA fractions were bulked, taken to dryness, and the ABA redissolved in 10 ml 0.5 M phosphate buffer, pH 8.0 and transferred to a new flask. The pH was lowered to 2.5 and ABA partitioned into ethyl acetate.

The ethyl acetate fraction was taken to dryness and methylated with ethereal diazomethane. The ether was removed under N_2 gas. Five hundred microlitres ethyl acetate was added to the dry extract and gas chromatography performed using a Varian 3700 gas chromatograph equipped with a [^{63}Ni] electron capture detector and a 2 m x 3 mm glass column filled with 5% OV-17 on Chromosorb W-HP with N_2 as carrier gas.

2.3. RESULTS

2.3.1. Leaf and root growth

In the control treatment there was no significant difference between the sensitive and resistant cultivars in the mean whole plant dry weight (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.

There was a large difference between the treatments in the soil water potentials in the tubes, but no difference between the cultivars (Table 2.1.). A greater proportion of root dry weight occurred in the lowest 20 cm of the soil profile in the water-stress treatment than in the control treatment (Fig. 2.1.). The proportion of root dry weight in the lowest 20 cm of the soil profile in the stress treatment was greater in the resistant cultivar than in the sensitive cultivar.

Table 2.1. Soil water potential (MPa) of successive 20-cm soil layers in tubes in which the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52 were growing after a 15-day period during which tubes were either watered daily to run-off (control treatment) or not watered at all (water-stress treatment). Values are means ± s.e. of five determinations.

Soil depth	**Cultivar and treatment**			
	PAN473 (resistant)		**SR52 (sensitive)**	
	Control	**Water-stress**	**Control**	**Water-stress**
0-20 cm	-0.056±0.0057	-2.48±0.54	-0.069±0.010	-2.40±0.58
20-40 cm	-0.033±0.0031	-0.72±0.06	-0.030±0.0039	-0.84±0.08
40-60 cm	-0.025±0.0024	-0.38±0.05	-0.027±0.030	-0.37±0.06

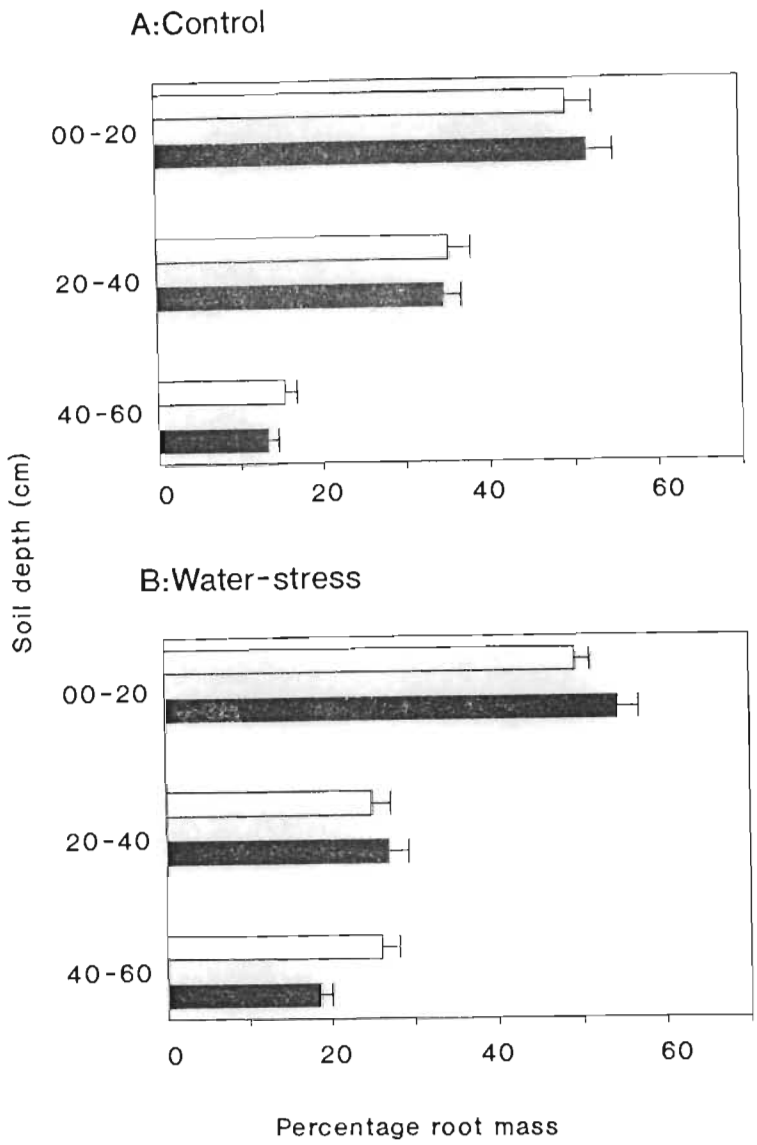


Figure 2.1. Percentage of total root dry weight of the drought resistant *Zea mays* L. cultivar PAN473 (open bars) and the drought sensitive cultivar SR52 (solid bars) in 20-cm soil layers after a 15-day period during which plants were either watered daily (A) or not watered after day 0 (B). Values are means ± standard error of ten determinations.

2.3.2. Water relations

In the pots the soil water potential in the control treatment varied between -0.16 and -0.27 MPa. In the stress treatment, the soil water potential reached -2.09 MPa at peak stress. There was no consistent difference between the cultivars in the soil water potentials (Fig. 2.2.).

No difference between the cultivars in the relative water content was observed in the control treatment. In the stress treatment the relative water content of both cultivars decreased, with that of the sensitive cultivar decreasing at a faster rate and to a lower value at peak stress than that of the resistant cultivar. Recovery to pre-stress levels in both cultivars upon rewatering was rapid (Fig. 2.3.).

There was little difference between the cultivars in transpiration rates in the control treatment. However, in the stress treatment the transpiration rate of the sensitive cultivar was lowered to a greater extent than that of the resistant cultivar during the onset of stress until the values were the same at peak stress. Recovery on rewatering was rapid, but the transpiration rates did not reach pre-stress levels. There was no difference between the cultivars in their recovery from water stress (Fig. 2.4.).

Diffusive resistances were similar between the two cultivars in the control treatment. In the stress treatment the diffusive resistance of the sensitive cultivar increased more rapidly at the onset of stress. Recovery was rapid on rewatering and there was little difference between the cultivars (Fig. 2.5.).

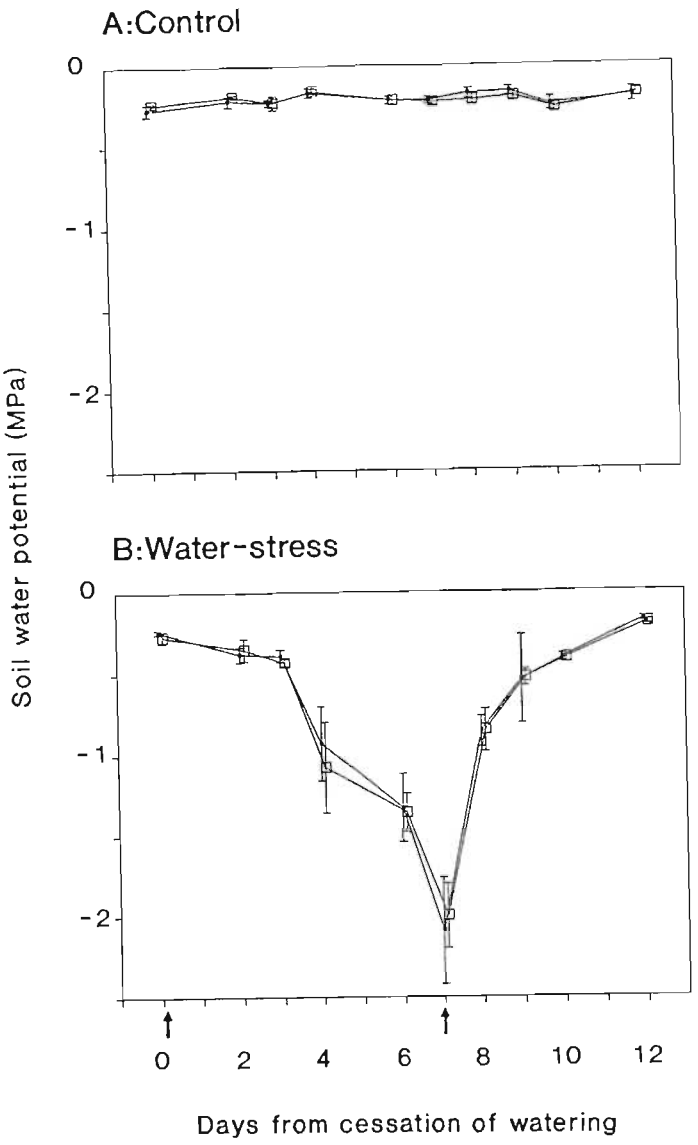


Figure 2.2. Soil water potential in the pots in which the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52 were growing. In the control treatment (A) pots were watered daily to a constant weight. In the water-stress treatment (B) watering was stopped when the plants were 40 days old (day 0, arrowed) and resumed when the plants were wilted (day 7, arrowed). Values are means ± standard error of five determinations. The points (-•-) represent the resistant cultivar and the open squares (-□-) represent the sensitive cultivar.

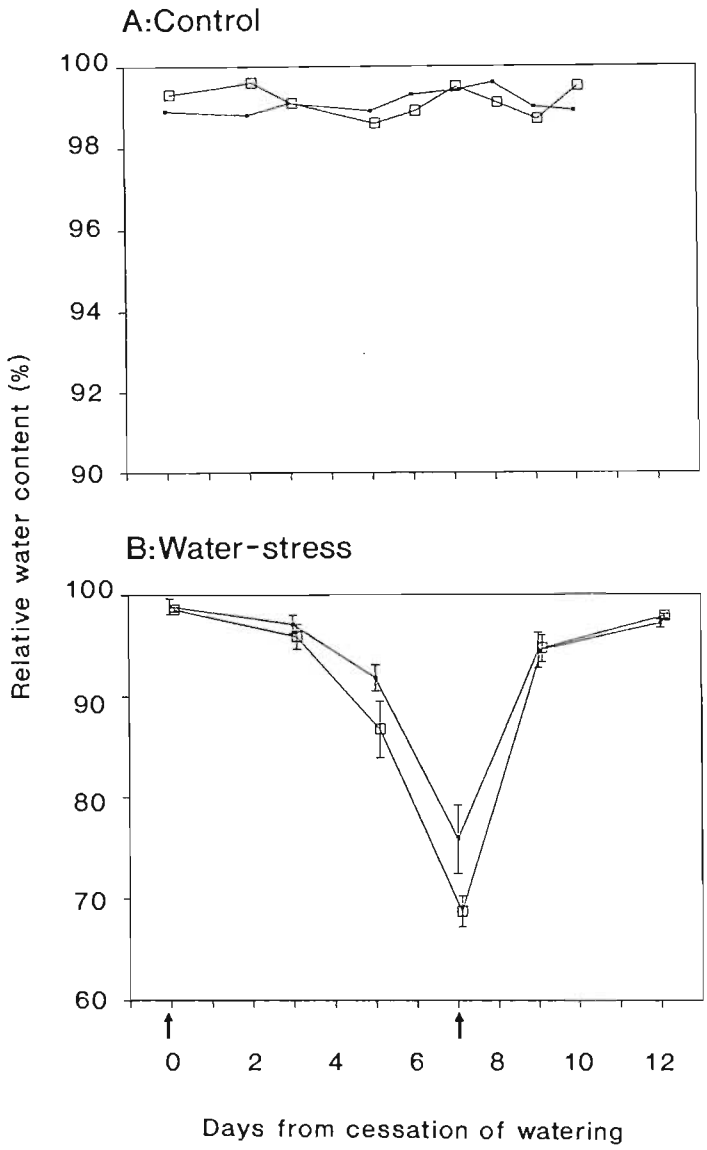


Figure 2.3. Relative water content of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52. In the control treatment (A) pots were watered daily to a constant weight. In the water-stress treatment (B) watering was stopped when the plants were 40 days old (day 0, arrowed) and resumed when the plants were wilted (day 7, arrowed). Values are means ± standard error of five determinations. The points (-•-) represent the resistant cultivar and the open squares (-□-) represent the sensitive cultivar.

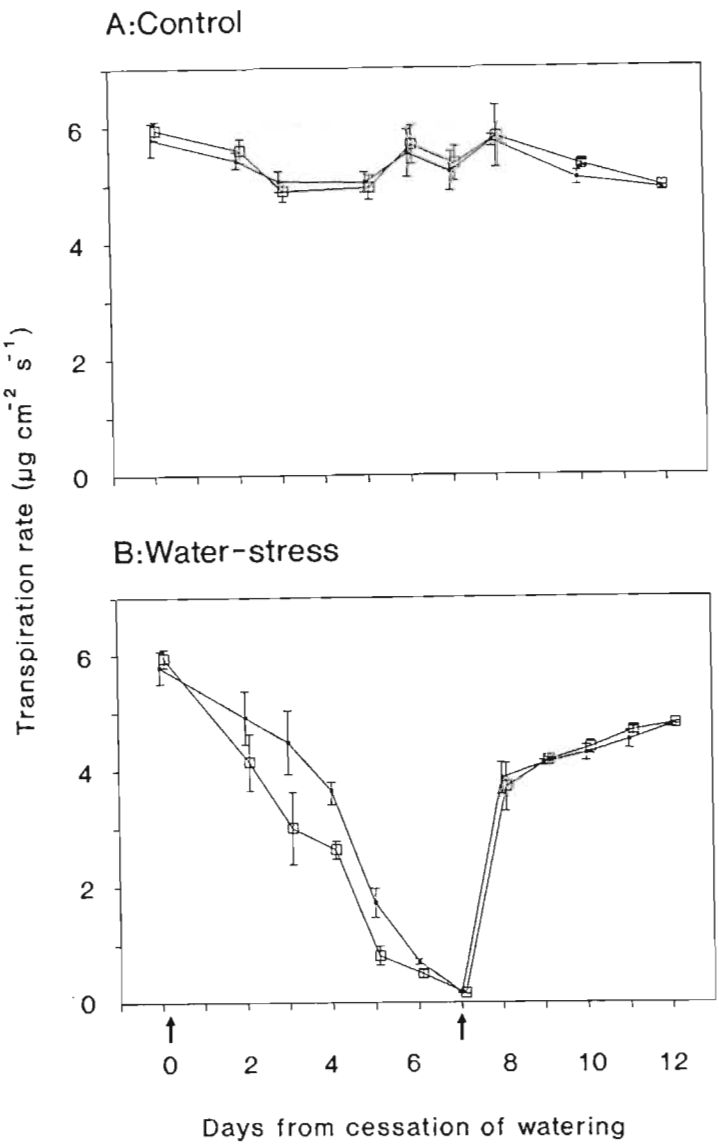


Figure 2.4. Transpiration rate of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52. In the control treatment (A) pots were watered daily to a constant weight. In the water-stress treatment (B) watering was stopped when the plants were 40 days old (day 0, arrowed) and resumed when the plants were wilted (day 7, arrowed). Values are means ± standard error of five determinations. The points (-•-) represent the resistant cultivar and the open squares (-□-) represent the sensitive cultivar.

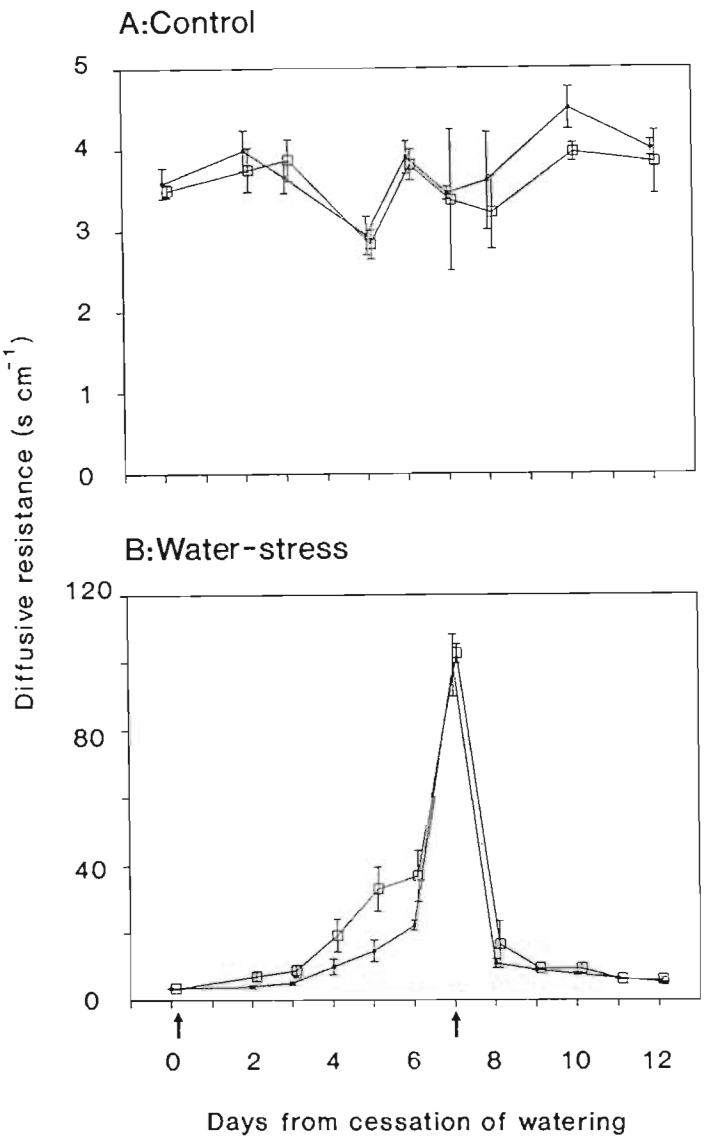


Figure 2.5. Diffusive resistance of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52. In the control treatment (A) pots were watered daily to a constant weight. In the water-stress treatment (B) watering was stopped when the plants were 40 days old (day 0, arrowed) and resumed when the plants were wilted (day 7, arrowed). Values are means ± standard error of five determinations. The points (-•-) represent the resistant cultivar and the open squares (-□-) represent the sensitive cultivar.

2.3.3. Abscisic acid

The concentration of ABA was slightly greater in the sensitive cultivar in the control treatment, but in the stress treatment the concentration of ABA increased to higher levels in the resistant cultivar (Fig. 2.6.). There was a significant difference between the cultivars in ABA content at peak stress (Student's *t*-test, t=3.35, p=0.010).

2.3.4. Proline

No difference between the cultivars in the concentration of proline was observed in the control treatment. In the stress treatment there was no change in proline concentration until after day six when there was a rapid increase, and a slow reduction in concentration following rewatering. The proline concentration at the end of the treatment on day 13 was higher than the pre-stress levels. The concentration of proline was significantly higher at peak stress in the resistant cultivar than the sensitive cultivar (Student's *t*-test, t=2.74, p=0.025) (Fig. 2.7.).

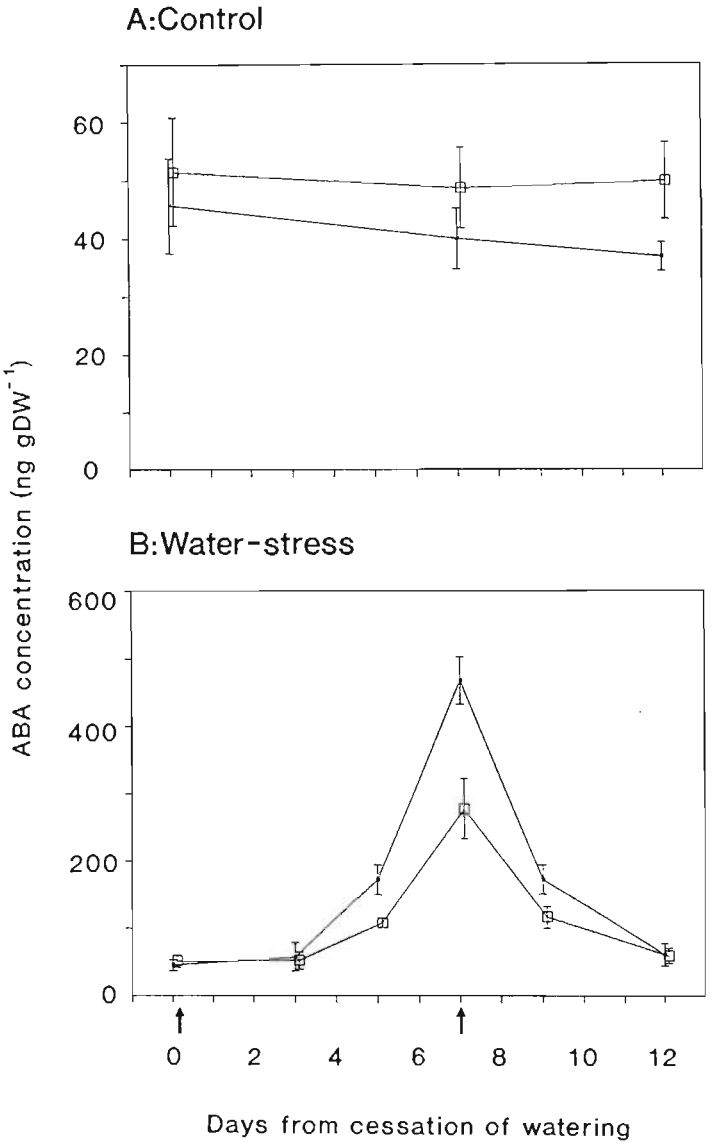


Figure 2.6. ABA concentration of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52. In the control treatment (A) pots were watered daily to a constant weight. In the water-stress treatment (B) watering was stopped when the plants were 40 days old (day 0, arrowed) and resumed when the plants were wilted (day 7, arrowed). Values are means ± standard error of five determinations. The points (-•-) represent the resistant cultivar and the open squares (-□-) represent the sensitive cultivar.

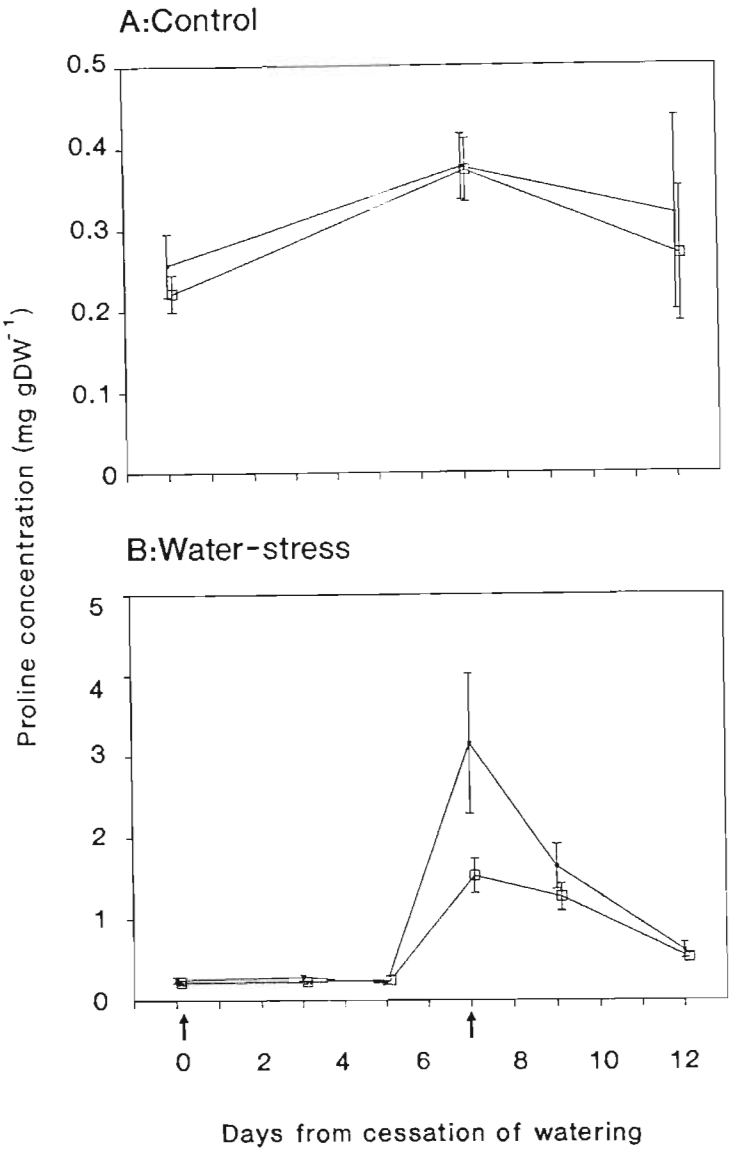


Figure 2.7. Proline concentration of the drought resistant *Zea mays* L. cultivar PAN473 and the drought sensitive cultivar SR52. In the control treatment (A) pots were watered daily to a constant weight. In the water-stress treatment (B) watering was stopped when the plants were 40 days old (day 0, arrowed) and resumed when the plants were wilted (day 7, arrowed). Values are means ± standard error of five determinations. The points (-•-) represent the resistant cultivar and the open squares (-□-) represent the sensitive cultivar.

2.4. DISCUSSION

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, at least in terms of whole plant growth in the stress treatment. Although the difference was small, it was significant, and resulted after only a seven-day soil drying cycle. Under optimal conditions in the control treatment the growth rate of the drought resistant cultivar PAN473 was lower than that of the sensitive cultivar SR52, but in the stress treatment its growth rate was reduced to a lesser extent than that of SR52. 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 *et al.* 1987). It appears that the adaptations required for tolerating stress conditions limit growth rate under optimal conditions.

These results compare well with the literature. LORENS *et al.* (1987b) investigated the growth of two maize hybrids that differed in their sensitivity to water stress. Under conditions of optimal water availability the growth rate and grain yield of the more sensitive hybrid was higher than that of the more resistant hybrid, but under conditions of severe water stress the positions were reversed and resistant hybrid had a significantly higher growth rate and grain yield. Under optimal watering conditions the differences between the hybrids were not great, but they were consistent.

The differences between the cultivars in the various parameters measured was 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. 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 water 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 water stress treatment up to the point of the drying cycle were therefore exposed to the same conditions of soil water potential.

The development of soil water deficits has been shown to induce deeper rooting (MALIK *et al.* 1979), and an increased supply of water from roots deeper in the soil profile when the upper layers have dried has been noted by other researchers (SHARP & DAVIES 1985; LORENS *et al.* 1987a). LORENS *et al.* (1987a) compared maize hybrids that differed in resistance to water stress and also found that the higher growth rate and greater final biomass under conditions of severe water stress of the more resistant hybrid resulted from a greater root density in the deeper zones of the soil profile. Similar genotypic differences have been found for wheat where cultivars with greater stress tolerance had more extensive and deeper root systems (HURD 1974).

The result of the changes in rooting characteristics was the maintenance of a higher relative water content and transpiration rate and a lower diffusive resistance in the stress resistant cultivar PAN473. The strategy of tapping soil water at greater soil depths can be regarded as an avoidance strategy, according to the definition of FISCHER & TURNER (1978) and TURNER (1979), since the plant is avoiding the physiological stress of low tissue water potentials by increasing its water uptake from an increased rooting depth. At the same soil water potential, the two cultivars were not necessarily suffering the same tissue water stress. Indeed, judging by its relative water content, it appears that the resistant cultivar was experiencing less tissue stress at the same soil water potential than the sensitive cultivar. These conditions are conducive for continued growth which was reflected in the greater growth of the resistant cultivar compared with the sensitive cultivar in the stress treatment. Although the drought resistant cultivar had a lower diffusive resistance during the early part of the water-stress treatment, both cultivars had complete stomatal closure at the same time. The important difference between the cultivars in this respect was in their ability to withstand the onset of water stress, with the resistant cultivar being able to postpone the onset of tissue desiccation through its deeper rooting response.

The drought resistant cultivar had a much greater concentration of proline at peak water stress and a rapid recovery to pre-stress levels on rewatering. Other studies have also reported a correlation between proline levels and drought resistance (SINGH *et al.* 1972; BLUM & EBERCON 1976; HANSON *et al.* 1977). An interesting feature of the proline response is that

there is a threshold during desiccation cycle beyond which there is a sudden increase in proline concentration. This threshold effect has also been observed by other researchers in *Brassica napus* (LARHER *et al.* 1993) and *Medicago sativa* (GIROUSSE *et al.* 1996), but no explanation has been offered for it. However, LARHER (1993) did observe that the induction of proline accumulation was a complex event involving the interaction between NaCl and sucrose. Proline accumulation was triggered first of all by an increase in NaCl concentration, and further enhanced by an increase in sucrose concentration. An increase in ionic and sucrose concentrations occur in response to osmotic stress. LARHER (1993) speculates that proline has a role as a protective agent for cytosolic enzymes and membrane structures, so perhaps the threshold for proline accumulation is the point at which the change in ionic strength of the cytosol begins to effect enzyme function.

It has been shown that an important site of proline action in maize in response to drying soil is in the region of the root apex (VOETBURG & SHARP 1991). The primary root of maize is able to elongate at low soil water potentials that completely inhibit shoot growth (SHARP *et al.* 1988), and proline can account for up to half of the osmotic adjustment required for continued elongation in the apical region of the root in maize (VOETBURG & SHARP 1991). 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 & STREET 1977; SAAB *et al.* 1990) thereby increasing the depth to which roots penetrate the soil profile (WATTS *et al.* 1981). The maintenance of primary root elongation in maize in drying soil is also aided by reversible loosening of the cellulose microfibrils in the cell wall, a response that requires increased ABA levels (WU *et al.* 1994). Increased levels of abscisic can also increase hydraulic conductivity of the root (LUDEWIG *et al.* 1988). In this study the resistant cultivar had higher levels of ABA than the sensitive cultivar in the water-stress treatment, so it is possible that the same mechanisms of rooting response to ABA is 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. Non-prolific maize plants such as SR52 produce one ear, and prolific maize plants such as PAN473 produce more than one ear. 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 & EBERHART 1968). 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 at 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, and this is what the remainder of the project sets out to investigate.

CHAPTER 3:
RNA ISOLATION, cDNA LIBRARY CONSTRUCTION AND DIFFERENTIAL PLAQUE HYBRIDIZATION

3.1. INTRODUCTION

There are three techniques commonly employed to investigate differential gene expression. These are differential plaque hybridization, subtraction hybridization, and polymerase chain reaction (PCR) differential display. All three techniques detect the increased abundance of mRNA transcripts in the treatment of interest, which in this study is the water stress treatment. Each technique has advantages and disadvantages.

Differential plaque hybridization involves the construction of a cDNA library of the treatment of interest in a suitable vector and screening the library with labelled cDNA probes to detect differential hybridization. A commonly used vector in this technique is phage λgt10 due to its high transformation efficiency and a 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 *et al.* 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 *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 visualising the clear plaques that result (MURRAY *et al.* 1977). The proportion of recombinant phage in the library is further increased on an *E. coli* strain such as NM514 that carries the *hfl*⁻ (high frequency lysogeny) 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 *et al.* 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 labelled 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 hybridizes with the homologous plaque on the replica membrane compared with the replica membrane hybridized to the control probe.

The disadvantages to the technique arise from the low abundance of the mRNAs of interest. Since the vast majority of genes are not water stress related and therefore will not be differentially expressed, many thousands of plaques have to be screened and this is time consuming. Also, due to the low abundance of any single mRNA species, the signal of the corresponding labelled cDNA will be weak. A large quantity of mRNA therefore needs to be isolated for probe synthesis to generate a strong enough signal. As a result, the technique is likely to isolate only those mRNAs that are in a high enough abundance to produce a strong enough signal, and these may not necessarily be the most important in the water stress response.

Construction of a subtracted cDNA library obviates some of these limitations by eliminating cDNAs that are common to both treatments, so that the subtracted library is enriched in cDNAs present in the treatment of interest (the water stress treatment). This will most easily isolate those genes whose expression is switched on *de novo* by the treatment (SAMBROOK *et al.* 1989). If the gene is expressed in both treatments, but the treatment response is an enhancement of expression, then a subtraction library might not isolate this clone. Also, the technique involves making first of all cDNA libraries of both treatments and then amplifying them. During amplification, the proportions of the different cDNA species will change, because some will amplify more readily than others, so that when the libraries are amplified, they are not truly representative of the original relative abundances.

Differential display by means of PCR was first described by LIANG & PARDEE (1992). Reverse transcription of mRNA from two treatments is performed using an oligo(dT)

3' primer with two additional 3' bases to synthesise a subset of first strand cDNAs. Theoretically, the two additional bases should provide enough specificity to synthesise cDNAs from one twelfth of the mRNAs present. The first strand cDNA is then used as the template for PCR amplification using the same 3' primer and a limited set of 5' primers, each ten random nucleotides in length. [^{35}S] dATP is used in the PCR to label the product which is separated on a 6% denaturing polyacrylamide gel and exposed to X-ray film. The bands that are brighter in one lane compared to the other correspond to treatment-enhanced mRNAs. This obviates the problem of faint signal since the PCR will amplify a mRNA that is present, irrespective of its concentration, as long as it has a sequence complementary to the primers. The time consuming screening of thousands of plaques that is involved in the differential plaque hybridization is also avoided.

There are limitations to the PCR differential display technique. The quality and quantity of mRNA have a significant effect on the results (LIANG *et al.* 1993). The number of mRNAs that are amplified will depend on the primers chosen. If there is no mRNA sequence complementary to a chosen primer, then that mRNA will not be amplified. To increase the chances of identifying a larger number of differentially expressed mRNAs, then a larger set of primers is required (BAUER *et al.* 1993). Difficulties can be encountered with "laddering" of some PCR products on the polyacrylamide gel (BAUER *et al.* 1993; LIANG *et al.* 1993) and in eluting and reamplifying bands from the gel (WILKINSON *et al.* 1995). Probes made from eluted bands can fail to detect a corresponding band in a Northern hybridization (LIANG *et al.* 1993; WILKINSON *et al.* 1995), which could be due to either low abundance of the original mRNA transcript, or an artefact of the technique. However, these problems seem to be due to the relative newness of the technique and undoubtedly they will by resolved with time and experience.

At the start of this study therefore it was decided to stay with the established techniques of subtractive hybridization and differential plaque hybridization. The subtractive hybridization was attempted first since this saves time in hybridization by first of all enriching for mRNAs expressed in one treatment compared to the other. However, no differential expression was detected by this method. The reason for this could be that the conditions of water stress

experienced in this study did not lead to *de novo* gene expression so no enrichment occurred during the subtraction. It was also felt that the extra number of steps involved in the subtraction could have had an influence on the results. It was therefore decided to pursue the simpler but more time-consuming technique of differential plaque hybridization. The requirement of large quantities of cDNA to generate a strong enough probe signal was not a limiting factor in this study since the plant material was abundant.

3.2. METHODS

3.2.1. Plant growth

Individual plants of the two maize cultivars were grown in PVC tubes in the glasshouse as previously described (Section 2.2.2.).

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.

3.2.2. Total RNA extraction

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 *et al.* 1979; AUSUBEL *et al.* 1989). All solutions and labware were treated with DEPC to inactivate ribonucleases. Total RNA was extracted by grinding 10 g leaf material in liquid nitrogen in a pestle and mortar to a fine powder. The powder was added to 100 ml grinding buffer (Appendix A.1.) and 33 ml TLE-equilibrated phenol in a 250 ml Oak Ridge centrifuge bottle. The mixture was homogenized for 2 min using a Janke & Kunkel Ultra-Turrax T25. Thirty 3 ml chloroform was added and the mixture briefly homogenized, then heated at 50°C for 20 min and centrifuged for 20 min at 18 000 x g in a Beckman JA-14 rotor. The aqueous layer was removed and extracted with phenol/chloroform by shaking at room temperature for 20 min. The mixture was centrifuged again for 15 min at 18 000 x g in a Beckman JA-14 rotor and the aqueous layer removed and kept. The aqueous layer was extracted with phenol/chloroform at least twice more (a total of minimum four times) until there was no white interface.

After the final phenol/chloroform extraction, ⅓ vol 8 M LiCl was added to the aqueous layer to a final concentration of 2 M and left overnight at 4°C to selectively precipitate the RNA.

The total RNA precipitate was collected by centrifuging for 20 min at 18 000 x g in a Beckman JA-14 rotor. The pellet was rinsed with 2 ml 2 M LiCl and resuspended in 3.6 ml water. The solution was removed to a 35 ml Oak Ridge tube and ⅓ vol 8 M LiCl was added to a final concentration of 2 M and the RNA precipitated again at 4°C for 2 h. The precipitate was collected by centrifuging for 20 min at 18 000 x g in a Beckman JA-20 rotor. The pellet was resuspended in 1.3 ml water. One tenth volume 3 M sodium acetate and 2.5 vol ethanol was added and precipitated overnight at -20°C. The precipitate was collected by centrifuging for 15 min at 18 000 x g in a Beckman JA-20 rotor. The pellet was resuspended in 666 µl water.

3.2.3. Measurement of total RNA

Ten µl total RNA was diluted in 1 ml water and the absorbence at 230 nm, 260 nm, 280 nm and 320 nm measured 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.

3.2.4. Isolation of poly(A)$^+$ RNA

Poly(A)$^+$ RNA was isolated from the total RNA following the method of AUSUBEL *et al.* (1989). A silanized plastic pipette tip plugged with silanized glass wool was washed with 10 ml 5 M NaOH and rinsed with water. For every 4 mg total RNA loaded, 1 ml dry oligo(dT) cellulose powder was added to 2 ml 0.1 M NaOH and poured into the pipette tip and equilibrated with loading buffer (Appendix A.2.) until the pH of the output was 7.5 (usually about 30 ml loading buffer). Four mg total RNA was denatured by heating at 70°C for 10 min and 10 M LiCl added to a final concentration of 0.5 M. The RNA solution was added to the column and washed with 2 ml loading buffer. The eluant was passed through the column twice more to ensure all of the poly(A)$^+$ RNA bound to the column. The column was rinsed with 4 ml middle wash buffer (Appendix A.3.) and the poly(A)$^+$ RNA eluted with 4 ml 2 mM EDTA/0.1% SDS. The column was reequilibrated with loading buffer and poly(A)$^+$ isolation repeated with the eluted RNA.

When the input total RNA was greater than 4 mg, all quantities and volumes were adjusted accordingly.

The RNA eluted after the second column purification was precipitated with 0.1 vol 3 M sodium acetate and 2.5 vol ethanol overnight at -20°C. The precipitate was collected by centrifuging for 30 min at 12 000 x g. The pellet was dried for 3 min in a vacuum desiccator

and resuspended in 100 µl TE buffer.

3.2.5. Measurement of poly(A)$^+$ RNA

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.

3.2.6. cDNA synthesis

cDNA was synthesised using the Amersham cDNA Synthesis System Plus (RPN 1256) following the protocol supplied with the kit. Twelve to eighteen-mer oligo(dT) was the first strand primer and the quantity of poly(A)$^+$ RNA substrate was 5 µg. The reaction was incubated at 42°C for 1 h with 100 U reverse transcriptase in a reaction volume of 50 µl. The first strand reaction mix was then incubated with 4 U *E. coli* ribonuclease H, to create nicks in the RNA strand of the double stranded hybrid, and 115 U *E. coli* DNA polymerase I, to synthesise the second strand using the nicked RNA as primers, in a reaction volume of 250 µl. The reaction was incubated successively at 12°C for 1 h and 22°C for 1 h. The enzymes were inactivated by heating at 70°C for 10 min. The reaction was then incubated with 10 U of T4 DNA polymerase for 10 min at 37°C to remove any 3' overhangs and create blunt ends. The reaction was stopped by adding EDTA (pH 8.0) to a final concentration of 10 mM. The reaction mix was extracted twice with an equal volume of phenol:chloroform 1:1 and once with an equal volume of chloroform, and precipitated with an equal volume of 4 M ammonium acetate and twice the combined volume of ethanol at -70°C for 15 min. The reaction mix was warmed to room temperature with shaking to redissolve the unreacted deoxynucleoside triphosphates, and centrifuged for 10 min in a microcentrifuge and the supernatant removed.

The pellet was washed with 2M ammonium acetate:ethanol 1:2, centrifuged for 2 min and aspirated, and washed finally with ethanol, centrifuged, and aspirated. The pellet was dried for 3 min in a vacuum desiccator and redissolved in 15 µl TE buffer.

3.2.7. Measurement of cDNA

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.

3.2.8. Cloning of cDNA into λgt10

cDNA from the water stress treatment only of both cultivars 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 adaptors containing one blunt end and one *Eco* RI end, converting the cDNA to *Eco* RI ends. The adaptors lacked 5' phosphates so that self-ligation of adaptors could not occur. Blunt-end ligation was done with 5 U T4 DNA ligase at 15°C for 16 h. The reaction was stopped by adding EDTA to a final concentration of 25 mM.

Separation of unreacted adaptors and size enrichment of cDNA greater than 500 bp was done by column purification. CE buffer (Appendix A.4.) was used as carrier and 120 µl fractions collected. Fractions 10-17, as recommended in the protocol, containing the size-enriched cDNA, were pooled and the water volume decreased by extracting with butan-1-ol.

The "adapted" cDNA ends were then phosphorylated by incubation with 40 U T4 polynucleotide kinase in a reaction volume of 400 µl at 37°C for 30 min. 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 precipitate was collected by centrifuging for 30 min. The supernatant was removed and the pellet washed in 70% ethanol, dried in a vacuum desiccator and resuspended in 20 µl TE buffer. 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 U T4 DNA ligase in a reaction volume of 10 µl and incubating at 15°C for 16 h. 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 (Appendix A.9.) using primers complementary to the *Eco*R1 site flanking regions of λgt10. The PCR reaction volume was 10 µl.

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.

3.2.9. Replica plating

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. The first membrane was placed on the agar surface of the plate for 1 min and the second membrane for 2 min. The membranes were marked to record the correct orientation. The membranes were then placed on filter paper soaked in denaturing solution (Appendix A.5.) for 7 min, neutralizing buffer (Appendix A.6.) for 3 min twice, washed in 2 x SSC and air-dried. DNA was fixed to the membrane by UV crosslinking. The original agar plates were stored at 4°C.

3.2.10. Radiolabelled cDNA probe synthesis

Radiolabelled 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 min with random hexanucleotides in reaction buffer (Boehringer Mannheim) and snap cooled on ice. DeoxyATP, dGTP and dTTP were added to a final concentration of 20 µM with 250 µCi [^{32}P]-labelled dCTP. Fifty U of Klenow enzyme (Boehringer Mannheim) were added and the reaction volume made up to 500 µl and incubated at 37°C for 1 h. 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 aliquots were collected from the end of the column in separate tubes and 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 hybridization. The percentage incorporation could be calculated from (cpm of the aliquots containing the incorporated radiolabel/total cpm) x 100.

3.2.11. Probe hybridization to replica membranes

The membranes were prehybridized for 2 h in hybridization buffer (Appendix A.7.) in

a Hybaid Hybridization Oven. Radiolabelled cDNA probe was denatured by boiling for 2 min and added to fresh hybridization buffer. The one replica membrane was probed with radiolabelled cDNA from the control treatment, and the other with radiolabelled cDNA from the water stress treatment. Hybridization was performed overnight at 68°C.

The membranes were washed twice with 2 x SSC/0.1% SDS at room temperature for 5 min, and twice with 0.1 x SSC/0.1% SDS at 68°C for 15 min. After the final wash the membranes were wrapped in clingfilm and exposed to X-ray film in a cassette with an intensifying screen at -70°C for between 14 and 28 days.

3.2.12. Plaque isolation

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 SM buffer at 4°C.

3.2.13. Reprobing isolated plaques

A second round of differential screening was done on these isolated plaques to confirm the first differential screening and to remove any false positives. The eluted phage in SM buffer from the first differential screening were toothpicked onto L-agar with NM514 in a grid pattern, fifty plaques per plate. Replica membrane lifts were made of each plate and probed with [^{32}P]-labelled cDNA from the control and water stress treatments as described above. 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.

3.2.14. Cross-hybridization of putative drought-related clones

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.

Each eluted bacteriophage clone from the second round of differential screening was toothpicked onto L-agar with NM514 in a grid pattern. Membrane lifts were made of each plate and probed with [^{32}P]-labelled cDNA insert from each clone in turn. Between hybridizations the membranes were stripped by incubating in 0.4 M NaOH at 45°C for 30 min, followed by washing buffer (Appendix A.8.) and incubating at 45°C for a further 15 min. Filters were autoradiographed to confirm the probe had been removed.

3.2.15. Probe synthesis from the isolated λgt10 clones

Probe was synthesised from the isolated λgt10 clones by PCR as previously described (Section 3.2.8.) using primers complementary to the flanking sequences of the *Eco*RI insertion site in λgt10. The reaction volume was 100 µl.

After PCR, the reaction mix was extracted twice with an equal volume of phenol:chloroform 1:1, and extracted twice more with an equal volume of chloroform. The DNA was ethanol precipitated with sodium acetate at -20°C for at least 1 h. The tube was centrifuged for 20 min in a microcentrifuge to pellet the DNA. The supernatant was removed and the pellet washed with cold 70% ethanol and centrifuged again for 10 min. The 70% ethanol was removed and the pellet dried in a vacuum desiccator and resuspended in 20 µl TE buffer, pH 7.5. The quantity of DNA was estimated by diluting 2 µl DNA solution 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.

The redissolved insert was then digested with *Eco*RI restriction enzyme to remove the primer sequences. This is necessary because the primer sequences, and any λgt10 sequences

between the primers, will hybridize with the λgt10 bound to the membrane and give a false signal. The 5' is 29 bp, and the 3' is 33 bp. Four μg DNA was added to 2 μl 10 x *Eco*RI restriction buffer (Boehringer Mannheim) and the volume made up to 20 μl with water. Four U of *Eco*RI restriction enzyme (Boehringer Mannheim) were added and the reaction incubated at 37°C for 3 h. The reaction was stopped by adding 2 μl 10 x DNA loading buffer and electrophoresing the reaction mix directly on a 1% SeaPlaque low-melting-temperature agarose gel. A DNA molecular weight marker and undigested PCR product were run in adjacent lanes to check the size of the digested PCR product. If the digestion was successful, then the band will be in a position 62 bp smaller than the undigested PCR product.

The gel was stained with ethidium bromide and visualised under UV illumination. The correct band was cut from the gel, placed in a 2 ml reaction tube, and the agarose digested with β-agarase (Boehringer Mannheim) according to the manufacturer's protocol. The weight of the excised agarose was determined and 0.04 vol 25 x agarase buffer (Boehringer Mannheim) added. The tube was incubated for 15 min at 65°C until the agarose was molten, then cooled to 45°C and 1 unit of β-agarase per 100 mg agarose added. Incubation took place at 45°C for 1 hour.

The digested oligosaccharides were pelleted by adding 0.1 vol 3 M sodium acetate (pH 5.5), chilling for 15 min on ice, and centrifuging for 15 min at 4°C. The supernatant was removed from the pellet and the DNA precipitated by adding 2.5 vol ethanol and placing at -20°C for at least 1 hour.

The DNA was then pelleted by centrifuging for 20 min followed by a 70% ethanol wash, drying in a vacuum desiccator and resuspending in 10 μl TE buffer pH 7.5.

A radiolabelled probe was synthesised from the redissolved DNA as previously described (Section 3.2.10.) using 1 μg DNA as template in a 100 μl reaction volume. Unincorporated [^{32}P]-labelled dCTP was separated on a Sephadex G50 column as previously described (Section 3.2.10.).

3.3. RESULTS

3.3.1. Total RNA extraction

From 10 g fresh weight starting material, recovery of total RNA was between 4.0 mg and 5.9 mg and the $A_{260/280}$ ratios were 2.1 or higher. The A_{230} values were all lower than the A_{280} values, and the A_{325} values were low, at 0.02 or lower.

When the total RNA was run on a gel, distinct bands, corresponding to ribosomal RNA, were clearly visible (Fig. 3.1.).

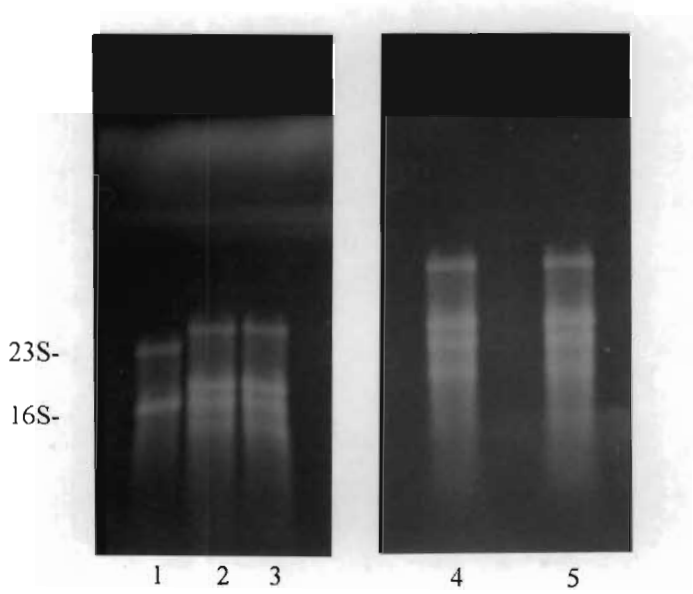


Figure 3.1. Total RNA extracted from control and droughted maize cultivars, run on a 1% agarose gel, and stained with ethidium bromide. Ribosomal RNA markers (lane 1), maize cultivar PAN473, drought treatment (lane 2), cultivar SR52, drought treatment (lane 3), cultivar PAN473, control treatment (lane 4) and cultivar SR52, control treatment (lane 5).

3.3.2. Isolation of poly(A)$^+$ RNA

Recovery of poly(A)$^+$ RNA was between 48 and 96 µg, which corresponds to between 1.2% and 1.9% of input total RNA. Again, $A_{260\,280}$ ratios were 2.1 or higher, and the A_{230} values were all lower than the A_{280} values, and the A_{325} values were low, at 0.02 or lower.

When run on a gel there is a smear indicating a broad size range of poly(A)$^+$ RNA. Faint banding is visible on the gel suggesting that some ribosomal RNA may have come through with the poly(A)$^+$ RNA (Fig. 3.2.).

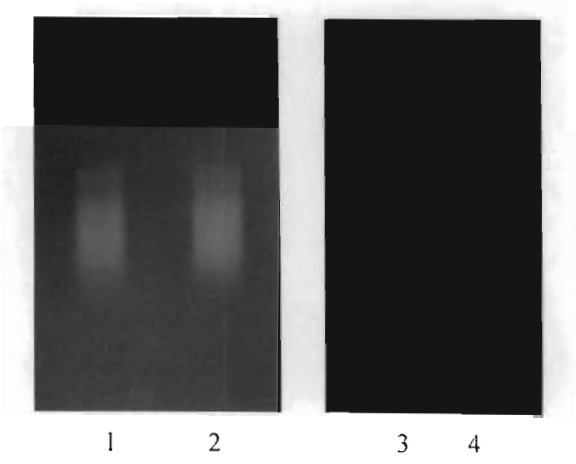


Figure 3.2. Poly(A)$^+$ RNA isolated from total RNA of control and droughted maize cultivars by oligo d(T) column selection, run on a 1% agarose gel, and stained with ethidium bromide. Maize cultivar PAN473, drought treatment (lane 1), cultivar SR52, drought treatment (lane 2), cultivar PAN473, control treatment (lane 3) and cultivar SR52, control treatment (lane 4).

3.3.3. cDNA synthesis

Estimated quantities of cDNA synthesized from 5 µg poly(A)$^+$ RNA ranged from 1.4 µg to 2.5 µg, which represents 28% to 50% transcription.

When the cDNA was run on a gel there was a visible size range from about 200 base pairs to about 6 000 base pairs (Fig. 3.3.). There is a bright band at about 2 kb on the cDNA gel.

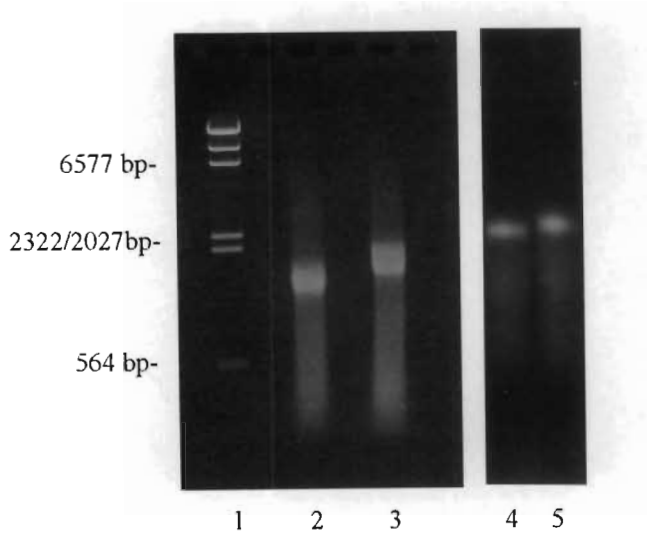


Figure 3.3. cDNA synthesised from poly(A)$^+$ RNA isolated from control and droughted maize cultivars, run on a 1% agarose gel, and stained with ethidium bromide. DNA molecular weight marker (lane 1), maize cultivar PAN473, drought treatment (lane 2), cultivar SR52, drought treatment (lane 3), cultivar PAN473, control treatment (lane 4) and cultivar SR52, control treatment (lane 5).

3.3.4. Cloning of cDNA into λgt10

The quantity of cDNA that gave the most number of transformants per unit weight when cloned into bacteriophage λgt10 proved to be 100 ng. The titre of the transformants was considerably higher than that of the background, so that the great majority of bacteriophage (96% or more) in the cDNA libraries of both maize cultivars contain inserts (Table 3.1.).

Table 3.1. Titre of control experiments of water stress treatment cDNA cloned into phage λgt10. Percentage recombinants was calculated using the formula ((titre of cDNA insert - titre of background)/titre of cDNA insert) x 100.

Reaction:	**Titre (pfu µg λgt10 arms^{-1}):**	**Percentage recombinants:**
λgt10 arms (background):	$7x10^4$	-
50 ng cDNA insert, PAN473:	$1.2x10^6$	94%
100 ng cDNA insert, PAN473:	$2.3x10^6$	97%
150 ng cDNA insert, PAN473:	$6.5x10^5$	89%
100 ng cDNA insert, SR52:	$1.8x10^6$	96%

When individual plaques were isolated and the cDNA insert amplified by PCR and run on a gel, bands were visible with a range in size (Fig. 3.4.).

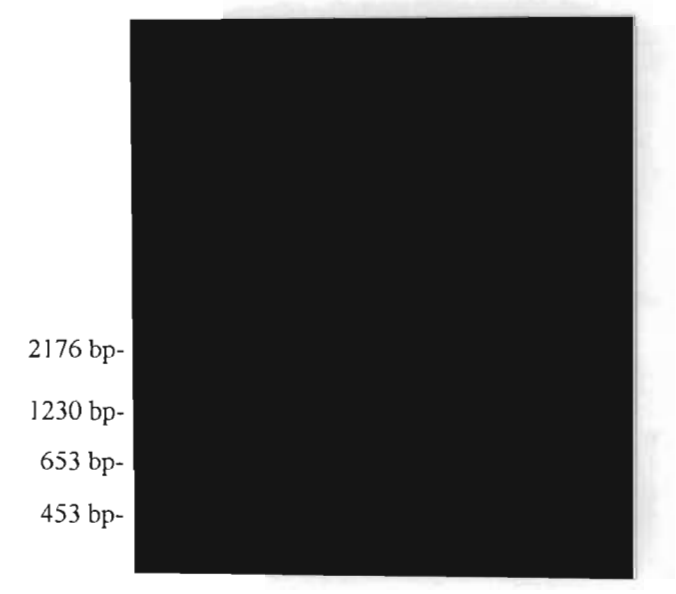


Figure 3.4. PCR products of cDNA insert from randomly isolated plaques, using primers complementary to the flanking sequences of the *Eco*RI insertion site of λgt10.

3.3.5. Radiolabelled cDNA probe synthesis

There was between 66% and 75% incorporation of [^{32}P]dCTP label into the cDNA probe.

3.3.6. Probe hybridization to replica membranes and plaque isolation

Approximately 150 000 plaques of each cultivar were screened. The number of plaques hybridizing more strongly with the water stress probe were 219 from the PAN473 library and 176 from the SR52 library (Fig. 3.5.).

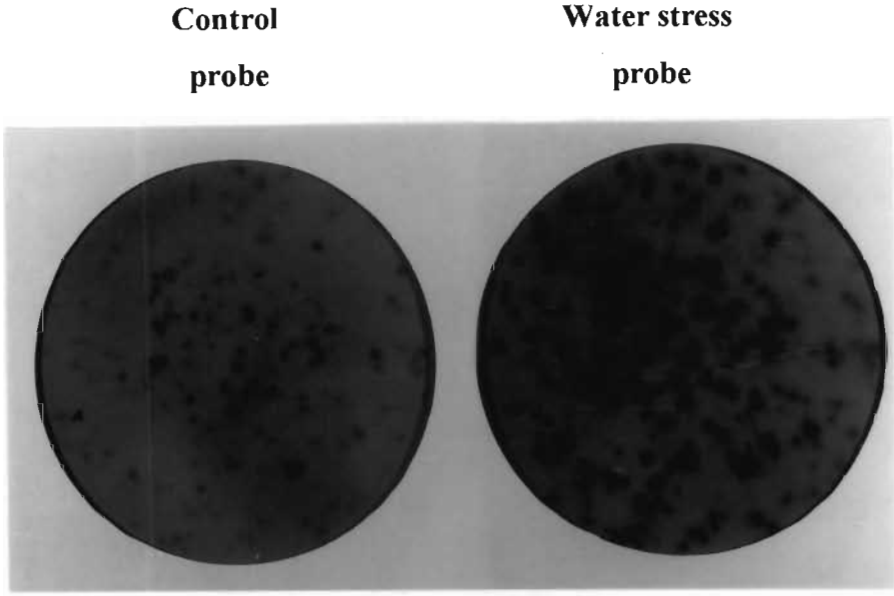


Figure 3.5. Differential plaque hybridization. Autoradiographs of a pair of replica membranes from plates containing plaques of a water stress cDNA library, the one membrane probed with [^{32}P]-labelled total cDNA from the control treatment (left), and the other with a [^{32}P]-labelled total cDNA from the water stress treatment (right). A plaque hybridizing more strongly with the water stress probe is arrowed.

When these isolates were reprobed for confirmation, most were found to be false positives. Twenty four positives of PAN473 and 11 positives of SR52 were reisolated by this second round of screening (Fig. 3.6.). For identification, the clones were numbered from 1 to 35 and given the designation *rws* for responsive to water stress.

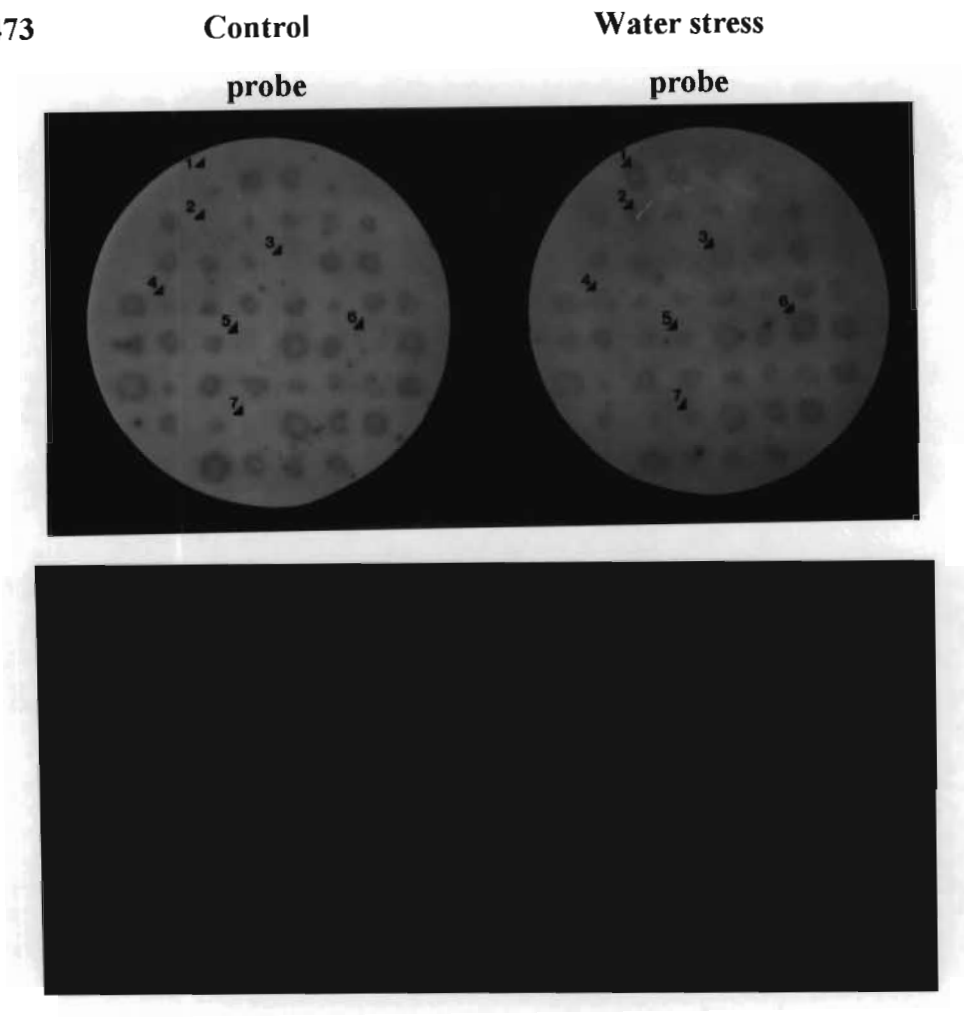


Figure 3.6. Second round of differential plaque hybridization. Autoradiographs of pairs of replica membranes from plates containing plaques isolated in the first round of differential screening of maize cultivars PAN473 (A) and SR52 (B). Eluted phage from the first differential screening were toothpicked onto L-agar plates in a grid pattern, fifty plaques per plate. Replica membrane lifts were made of each plate and probed with [^{32}P]-labelled cDNA from the control and water stress treatments. Plaques hybridizing more strongly with the water stress probe are arrowed.

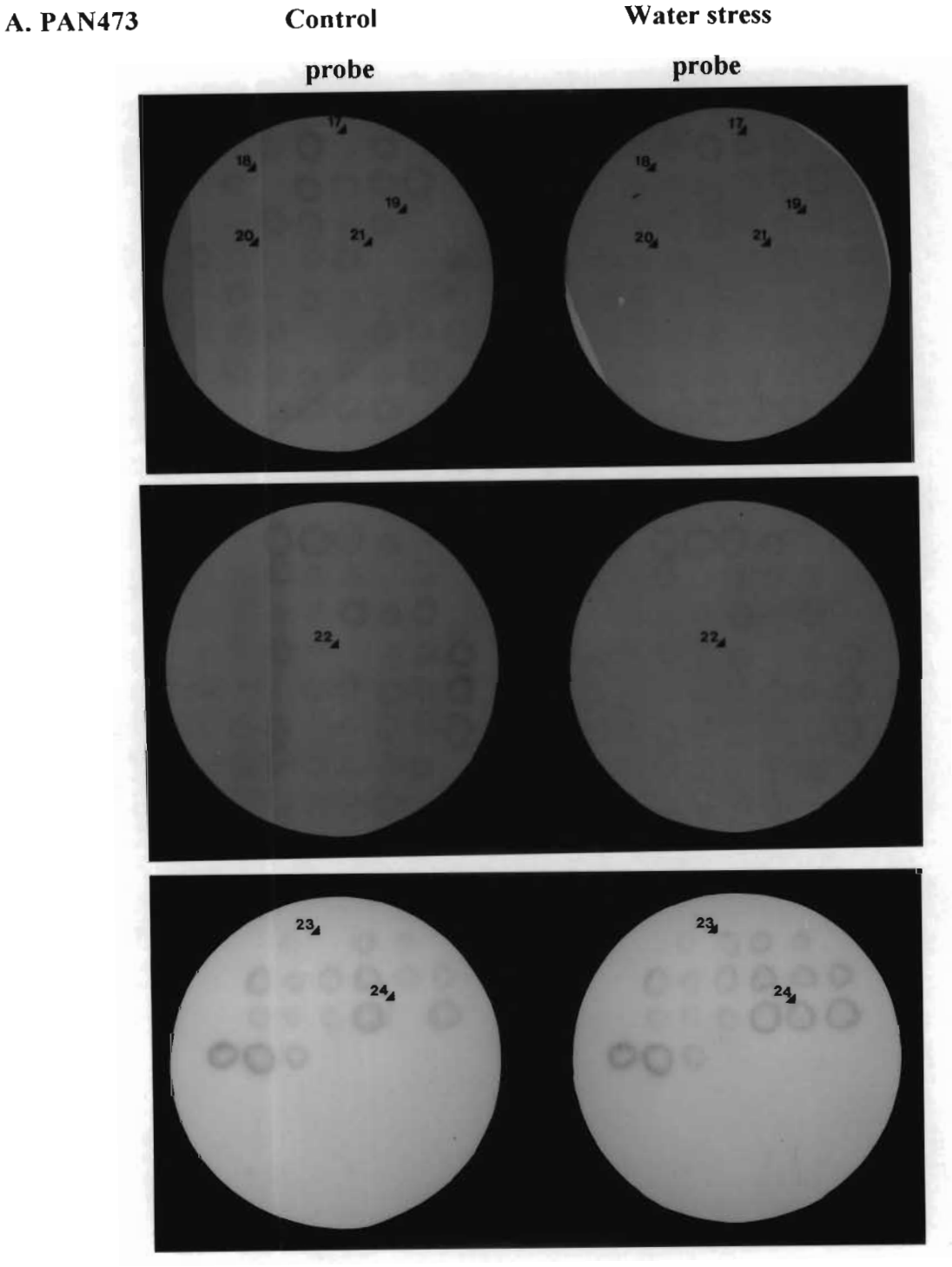


Figure 3.6. (cont'd). Autoradiographs of the second round of differential screening of maize cultivars PAN473 (A) and SR52 (B). Eluted phage from the first differential screening were toothpicked onto L-agar in a grid pattern, fifty plaques per plate. Replica membrane lifts were made of each plate and probed with [^{32}P]-labelled cDNA from the control and water stress treatments. Plaques hybridizing more strongly with the water stress probe are arrowed.

B. SR52 **Control probe** **Water stress probe**

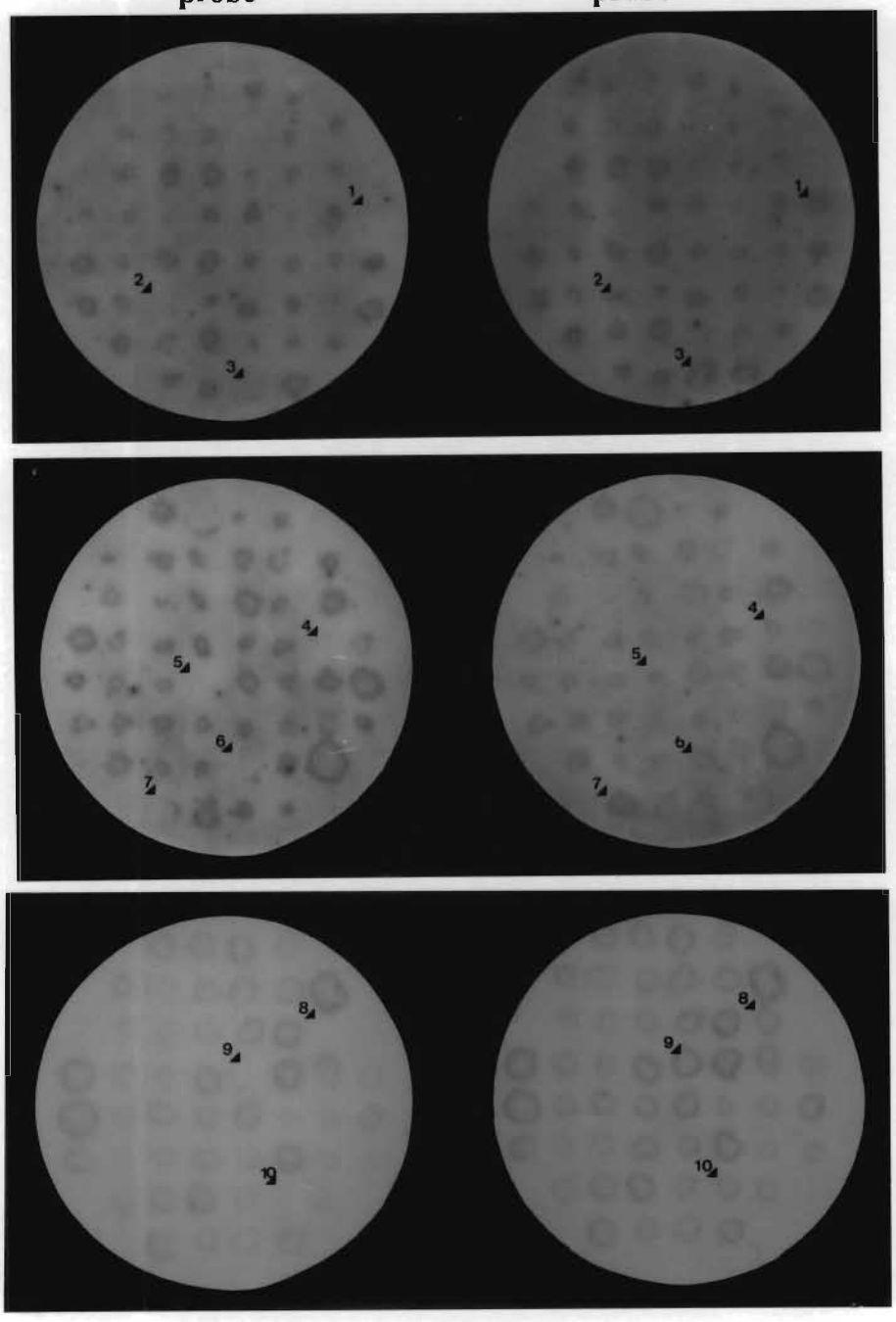


Figure 3.6. (cont'd). Autoradiographs of the second round of differential screening of maize cultivars PAN473 (A) and SR52 (B). Eluted phage from the first differential screening were toothpicked onto L-agar in a grid pattern, fifty plaques per plate. Replica membrane lifts were made of each plate and probed with [^{32}P]-labelled cDNA from the control and water stress treatments. Plaques hybridizing more strongly with the water stress probe are arrowed.

B. SR52

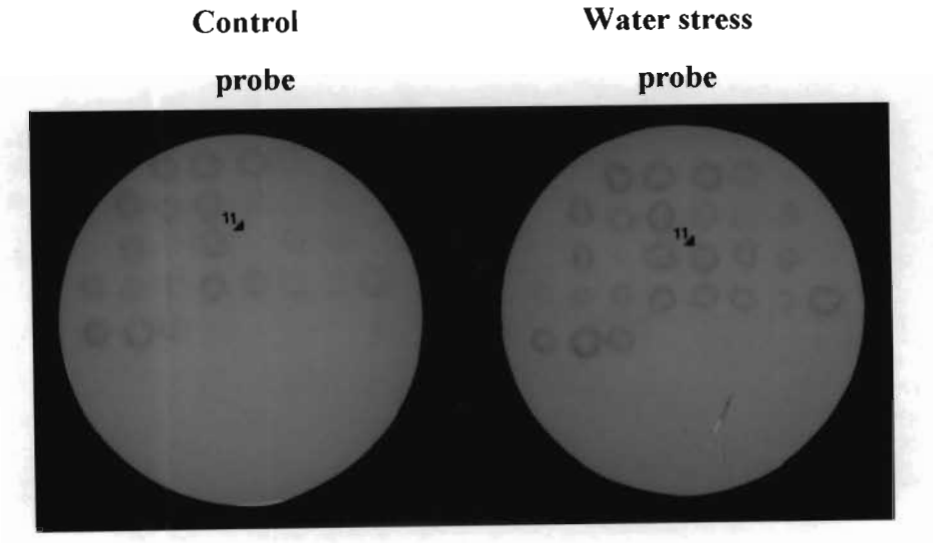


Figure 3.6. (cont'd). Autoradiographs of the second round of differential screening of maize cultivars PAN473 (A) and SR52 (B). Eluted phage from the first differential screening were toothpicked onto L-agar in a grid pattern, fifty plaques per plate. Replica membrane lifts were made of each plate and probed with [^{32}P]-labelled cDNA from the control and water stress treatments. Plaques hybridizing more strongly with the water stress probe are arrowed.

3.3.7. Cross-hybridization of cDNA clones

When the 35 cDNA clones (24 PAN473 and 11 SR52) isolated in the second round of differential screening were cross-hybridized with each cDNA clone in turn, they were found to represent nine different species of cDNA. Three cDNA species are represented by more than one clone per maize cultivar, and six cDNA species are represented only once per cultivar. All the SR52 cDNA species are represented in the PAN473 clones. Four cDNA species are unique to the PAN473 clones (Table 3.2.; Fig. 3.7.). Eight PAN473 isolates and three SR52 isolates did not hybridize at all.

Table 3.2. 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

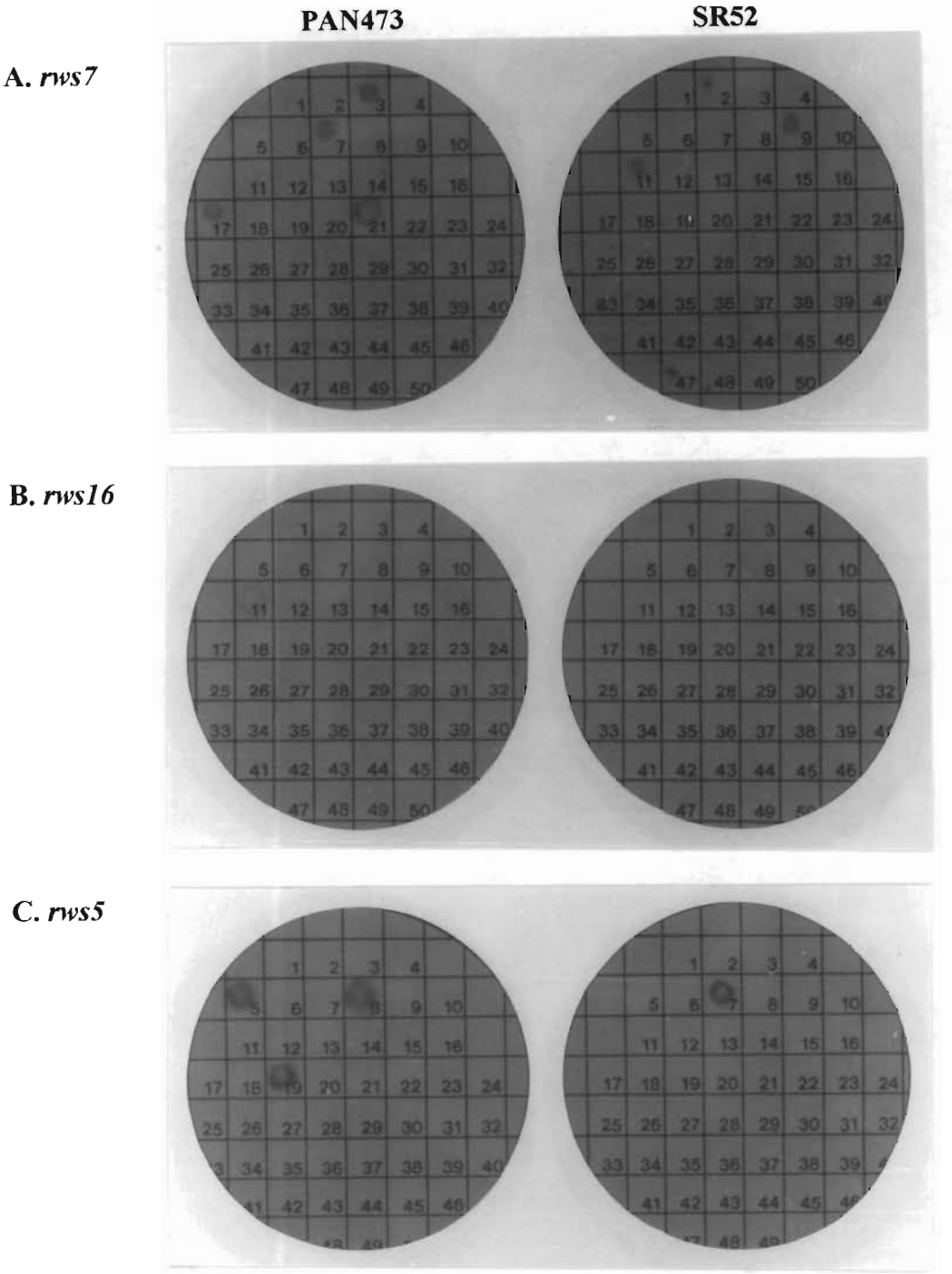


Figure 3.7. Autoradiographs of plaques cross-hybridised with putative water stress cDNAs from the second round of differential screening to determine the number of different cDNA species represented. The putative water stress cDNA clones (24 PAN473 and 11 SR52) were plated out in a grid pattern. Replica membrane lifts were made of each plate and probed with [^{32}P]-labelled PCR product from each cDNA clone in turn. Plaque hybridization with only three cDNA clones are shown, namely *rws7* (A), *rws16* (B) and *rws5* (C).

3.4. DISCUSSION

The success of cDNA synthesis and the construction of a cDNA library lies in having intact and pure poly(A)$^+$ RNA. An $A_{260/280}$ ratio of 2.1 or more obtained in this study corresponds to very pure RNA with negligible contamination with protein.The A_{230} readings were slightly lower than the A_{280} so there was also negligible contamination by phenol, urea, etc., which absorb at 230 nm. The low A_{325} readings also indicate there was negligible contamination by particulate matter which absorbs at this wavelength. All these values were within acceptable limits (AUSUBEL *et al.* 1989), so the extraction technique worked well. When the total RNA was run on a gel, distinct bands, corresponding to ribosomal RNA, were clearly visible, indicating the RNA was intact. Were the RNA degraded, the ribosomal RNA bands would appear diffuse and indistinct.

Recovery of poly(A)$^+$ RNA was quite high at between 1.2% and 1.9% of input RNA. Normal levels are around 1% of total RNA. The recovery of quantities of between 48 and 96 µg poly(A)$^+$ RNA in this study is enough for several cDNA synthesis reactions, since 5 µg is required for a single reaction. The recovered poly(A)$^+$ RNA was again very pure as estimated by the $A_{260/280}$ ratio.

When run on a gel there is a smear indicating a broad size range of poly(A)$^+$ RNA which is desired (Fig. 3.2.). Had there been degradation of the poly(A)$^+$ RNA, there would be a concentration at the small size range. Faint banding was visible on the gel suggesting that some ribosomal RNA may have come through with the poly(A)$^+$ RNA. This might account for the slightly higher percentage recovery of poly(A)$^+$ as mentioned above. Ribosomal RNA contamination might also account for the band at about 2 kb on the cDNA gel. An adenine rich sequence in the ribosomal RNA might have adhered to the oligo(dT) column and the oligo(dT) primer in the cDNA synthesis.

According to the cloning kit protocol, the titre of recombinant bacteriophage should be higher than 1 x 10^6 pfu µg λgt10 arms^{-1}, and the titre for both PAN473 and SR52 insert cDNA were higher than this figure indicating that the cloning reaction was successful. The level

of the background titre was also acceptably low so that the vast majority of plaques (96% or more) in the water stress libraries contain inserts.

A large quantity (10 µg) of input cDNA was used in the probe synthesis to produce a large enough signal with rare sequences (SAMBROOK *et al.* 1989). Even so, the X-ray film had to be exposed to the membranes for a long time, two weeks or more, to produce a spot that could be visualised. Because the original plates had to be kept for this length of time as well in order to isolate differentially hybridized plaques, the plating density was less than that recommended. During the exposure time the plaques continued growing while stored at 4°C, so if they were plated at the recommended density of about 3 000 per plate, at the end of this time period the plaques became confluent and individuals could not be distinguished. By plating out at less than 1 000 per plate, the plaques remained mainly separate. Even so, false positives were isolated after the first round of hybridization, possibly due to contamination from adjacent plaques.

However, care should be taken in the interpretation of these results since the use of cDNA for hybridization can introduce bias. The efficiency of reverse transcription varies between different poly(A)$^+$ RNA templates, so that a differential hybridization could result from the efficiency of reverse transcription rather than the relative abundance of the poly(A)$^+$ RNA. SCHERER *et al.* (1981) found this to be the case with a differential hybridization of stage-specific expression in *Drosophila* embryos, where clones which were strongly differential when hybridized with cDNA probe were only weakly or not differential when hybridized with poly(A)$^+$ RNA probe. To determine whether there is a true difference in transcription, a Northern hybridization would have to be done on the isolated differential clones.

SCHERER *et al.* (1981) also found that clones that cross-hybridized were not necessarily identical transcripts, but shared sequence homology along some of their length. Those clones that have been grouped together according to their cross-hybridization (Table 3.2.) are therefore not necessarily identical, and their identity would have to be confirmed by sequencing.

CHAPTER 4:
SEQUENCE ANALYSIS OF TWO OF THE DROUGHT-RELATED CLONES

4.1. INTRODUCTION

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 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 codons, the polyadenylation signals and the lack of introns, that enable the open reading frame to be recognised 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.

Although uracil (U) substitutes for thymine (T) in the RNA molecule, for convenience only T will be used in this discussion. Strictly speaking this is not incorrect in this study, as in many other studies, since it is the cDNA and not the original mRNA that is being analysed.

The information contained in the amino acid sequence can be extended by accessing a large library of sequences that have been derived and stored in computer databases. 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 *et al.* 1990).

4.1.1. Basic Local Alignment Search Tool

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 values +5 for identical nucleotide pairs, and -4 for mismatches (ALTSCHUL *et al.* 1990).

4.1.2. 5' region of mRNAs

The 5' leader sequences of plant mRNAs are AT-rich and vary in length from 9 to 193 nucleotides (JOSHI 1987b). The two most important functions of the 5' region are that it must be recognised by ribosomes, and it must have an initiation codon for translation. The mRNA initiation codon is the ATG triplet, and in 95% of mRNAs reviewed by KOZAK (1984) it is the first ATG that is the site of initiation. The sequence surrounding the initiation codon determines the efficiency of initiation of translation by the 40S ribosomal complex (KOZAK 1981). In a survey of 79 plant genes, JOSHI (1987b) found the consensus sequence for the start codon context to be TAAACA**ATGG**CT. Although many start codons occur in suboptimum contexts, there is a preponderance for A at the -3 from the first A of the ATG, and for GCT following the ATG (JOSHI 1987b). However, the situation may be more complex because there appears to be a variety of start codon contexts with each gene family having a slightly different consensus.

4.1.3. 3' region of mRNAs

The 3' region of mRNAs contains a translation stop codon and one or more

polyadenylation signals. There are three stop codons, TAA (ochre), TAG (amber) and TGA (opal). In both monocotyledonous and dicotyledonous plants, TGA and TAA are the preferred stop codons, with TAG occurring with the lowest frequency (ANGENON *et al.* 1990). The context of the stop codon is not random, with certain nucleotides being preferred in certain positions relative to the stop codon. This is particularly so for the TAG codon, and the reason for this is that the TAG codon is particularly leaky, and therefore the nucleotides flanking the TAG stop codon prevent misreading by a tRNA (ANGENON *et al.* 1990).

Unlike the highly conserved AATAAA consensus sequence in animals, polyadenylation signals are quite varied in plants. Several different base sequences are recognised, and more than one polyadenylation signal can exist on one mRNA molecule. Polyadenylation signals occur in the 3' end of the mRNA molecule, usually within a certain distance of the poly(A) tract. These signals have been grouped into classes, far upstream elements (FUEs), near upstream elements (NUEs) and the cleavage/polyadenylation sites (CSs) (HUNT 1994).

The FUEs are relatively distant from the CS, lying between 40 and 160 nucleotides from the CS (HUNT & MacDONALD 1989). There is no strict consensus sequence for FUEs, but there is a requirement for TG-richness (HUNT 1994).

The NUEs are usually located within 6 to 40 nucleotides upstream of the CS, but they can be as far as 130 nucleotides upstream (DEAN *et al.* 1986). The most common plant NUEs are AATAAA and AATAAT. Although there is no strictly conserved consensus in a NUE, the distinctive feature of most of them seems to be a YA (Y=pyrimidine) dinucleotide in a T-rich region (JOSHI 1987a). A sizeable proportion (about 15%) of plant poly(A)s have no AATAAA/T-like motif (HUNT 1994). There can be more than one NUE in a mRNA molecule.

Although there may be more than one NUE in the 3' untranslated region (UTR), only one of the sites is functional. This seems to be because there are constraints on the relative positions of the NUE and the CS which must lie within 40 nucleotides of each other. The NUE sequence itself cannot be the only recognition element because it can occur in the coding region and the introns of genes without acting as a polyadenylation signal. Therefore the context of

the NUE determines its function. For instance, the presence of the G/T cluster (the trinucleotide TGT repeated) about 30 nucleotides downstream appears to activate the NUE (McLAUCHLAN *et al.* 1985). By deletion analysis of the 3' end of a reporter gene, INGELBRECHT *et al.* (1989) found expression varied by as much as 60-fold depending on the context of the NUE. INGELBRECHT *et al.* (1989) also found a G/T-rich motif downstream from the polyadenylation signal to be important.

The poly(A) signals of plants involve a complex interaction between several FUEs, NUEs and the associated CS. There is a lack of sequence conservation in these 5' signals in plants. The possibility exists that different 3' *cis*-acting elements on the same gene can be recognised by different *trans*-acting factors leading to developmental or environmental control of 3' end formation.

4.1.4. mRNA stability and regulation

Regulation of gene expression can be at the transcriptional level, translational level, or at the level of mRNA stability.

INGELBRECHT *et al.* (1989) attached 3' ends from five different plant genes to a reporter gene and found a sixty-fold difference in the level of expression of the reporter gene. Interestingly enough the highest level of expression was with the 3' end from the ribulose-1,5-bisphosphate carboxylase small subunit gene, and the lowest level of expression was with the chalcone synthase gene 3' end. They speculated that the 3' end contributed to the regulation of the level of gene expression by modulating the stability of the mRNA transcript. The ribulose-1,5-bisphosphate carboxylase small subunit gene requires a high level of constitutive expression and it would therefore be advantageous if its mRNA was stable, whereas the chalcone synthase gene is transiently expressed in response to external environmental stimuli such as UV, and so an unstable mRNA would contribute to the rapid removal of the chalcone synthase enzyme on relief from environmental stress.

Translational regulation has been observed in the *rbcS* and *rbcL* gene of *Amaranthus* (BERRY *et al.* 1988; BERRY *et al.* 1990). The level of *rbcS* and *rbcL* mRNAs remained the same for 6 h after transfer of *Amaranthus* seedlings from light to dark. However, the incorporation of ^{35}S-methionine into the encoded proteins stops completely only 2 h after transfer. The mRNA was observed to remain bound to the polysomes suggesting regulation was at the level of translational elongation.

4.1.5. Protein structure

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 structure. 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 & CREIGHTON 1993).

4.1.6. The α-helix

The ordered structure of the α-helix is maintained by hydrogen bonding between the carbonyl group of one amino acid residue with the NH group of the fourth amino acid residue along the chain (DARBY & CREIGHTON 1993). With the exception of proline, all amino acid residues can occur in α-helices, although some occur with greater frequency than others. For instance, glycine occurs less frequently due to its rotational flexibility, whereas alanine has a strong propensity to form a helix (RICHARDSON & RICHARDSON 1989). Generally, amino acid residues with longer side chains occur less frequently because their side chains cannot easily be accommodated in the helix (DARBY & CREIGHTON 1993). Proline is the exception because, due to its shape, it incorporates a kink into the chain.

The structure of the α-helix can be stabilized by certain features. For instance, the

interspersion of polar and apolar amino acid residues in a repeating unit can stabilize the structure by causing alignment by mutual attraction/repulsion on different faces of the helix, the polar residues on one face, the apolar on the other (BAKER *et al.* 1988; DURE *et al.* 1989). Also, salt bridges can be formed between adjacent polar residues of opposite charge, such as glutamine and lysine, on different turns of the helix (MARQUSEE & BALDWIN 1987).

The average length of an α-helix is eleven amino acid residues, which corresponds to three turns of the helix, although variation from four to over forty amino acid residues has been found (RICHARDSON & RICHARDSON 1989).

4.1.7. The β-sheet

The other ordered component of protein secondary structure is the β-strand, in which the amino acid residues form an extended backbone with the side chains aligned on alternate sides in a single plane. Most β-strands are between five and ten residues long. The β-strand is not stable on its own, but occurs in β-sheets in which two or more β-strands align themselves parallel or antiparallel to each other. The structure is stabilized by hydrogen bonding between the carbonyl and NH groups that protrude at right angles with respect to the side chains. The β-sheets can be made up of β-strands that are located distantly on the polypeptide chain (DARBY & CREIGHTON 1993).

Parallel and antiparallel β-sheets have distinct hydrophilic and hydrophobic properties. Antiparallel β-sheets typically have alternating hydrophobic and hydrophilic amino acid residues, so that side chains on the one side of the sheet lie buried within the protein molecule, with the side chains on the other side exposed to the external medium. Parallel β-sheets typically have a central sequence of hydrophobic amino acid residues with hydrophilic residues at the ends, so that they lie buried within the structure of the proteins molecule (RICHARDSON & RICHARDSON 1989).

There is a greater preponderance of hydrogen bonding in β-sheets which makes them

more rigid than α-helices. Being more flexible, α-helices typically occur in transport proteins that need to change their shape to accommodate the molecule to be transported. For instance, the cytochromes and globins that transport oxygen, and the hemerythrins that transport iron are made up entirely of α-helices (CHOU 1989).

4.1.8. The random coil

Random coils are non-repetitive sequences that do not form a regular, recognisable secondary shape. Despite their name, they do form stable secondary structure. They tend to be rich in glycine, asparagine and proline residues which allow the polypeptide chain to bend so that the α-helices and β-strands can align to form the tertiary structure of the protein molecule. Whereas the α-helices and β-sheets usually form the hydrophobic core of the protein molecule, random coils usually occur on the surface. The sequence of amino acid residues in random coils is such that they do not interact with each other in an ordered way, as in the α-helices and β-strands, but they do interact with the aqueous solution by hydrogen bonding with water molecules. Random coils also interact with substrates and thereby contain the active site of enzymes within their structure (RICHARDSON & RICHARDSON 1989).

4.1.9. Prediction of secondary structure

The most widely used algorithm for predicting protein secondary structure is that of Chou & Fasman (PREVELIGE & 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

& FASMAN 1974a, 1974b, 1978a, 1978b).

There has been criticism of the Chou-Fasman algorithm. The algorithm fails to delimit helix, sheet and coil domains so that overlaps can occur in the predicted protein secondary structure. No rules were given to resolve these overlaps so that their predictions were only 45% accurate (NISHIKAWA 1983).

Also, the Chou-Fasman algorithm breaks down when predicting membrane protein secondary structure. The algorithm was derived from data for soluble proteins. Soluble proteins have β-sheets at their core because β-sheets are made up of hydrophobic amino acid residues. In a membrane protein the same situation will not prevail. The Chou-Fasman algorithm will predict hydrophobic β-sheets, but in the hydrophobic environment of the membrane, hydrophobic amino acid residues preferentially form α-helices since the necessity to saturate the hydrogen bonds intramolecularly is done more efficiently in an α-helix (JÄHNIG 1989).

To resolve this, JÄHNIG (1989, 1990) suggested an extension of the Chou-Fasman algorithm based on the hydropathy index of KYTE & DOOLITTLE (1982). If the hydropathy index is greater than or equal to 1.6 (hydrophobic) over a region of 20 contiguous residues (the number needed to span a membrane in an α-helix structure), then a membrane-spanning α-helix is predicted (JÄHNIG 1989, 1990). If the hydropathy index of twenty contiguous amino acid residues alternates between greater than 1.6 (hydrophobic) and less than 0.8 (hydrophilic) with a periodicity of 3.6 residues (the number of residues per turn of the helix), then a membrane-spanning amphiphilic α-helix is predicted (JÄHNIG 1990). If the hydropathy index alternates between greater than 1.6 (hydrophobic) and less than 0.4 (hydrophilic) with a periodicity of two residues over a region of ten contiguous amino acid residues (the number needed to span a membrane in a β-strand structure), then a membrane-spanning β-strand is predicted, but *only* if eight such strands are also predicted (JÄHNIG 1990). JÄHNIG's (1989, 1990) predictions proved much more reliable than those of CHOU & FASMAN (1974a, 1974b, 1978a, 1978b) for membrane proteins, but not for soluble proteins (JÄHNIG 1990).

Perhaps the strongest driving force in creating protein structure is the hydropathy of

amino acids. KYTE & DOOLITTLE (1982) calculated a hydropathic value for each amino acid based on its occurrence in the interior (hydrophobic) and exterior (hydrophilic) regions of soluble proteins of known structure, and on its transfer free energy between the water and vapour phases. The hydropathic index at each amino acid residue along the protein sequence is then calculated by averaging the hydropathic values of a moving window of several neighbouring amino acid residues (KYTE & DOOLITTLE 1982).

Protein folding is driven by the need to remove certain amino acid residues from the surface of the protein that is in contact with the solvent, whether the solvent is lipid (membrane) or aqueous (cytosol). In the lipid environment of membranes, hydrophobic residues will occur preferentially on the surface of the protein with hydrophilic residues removed to the internal space, whereas in the aqueous cytosol the position will be reversed (RICHARDSON & RICHARDSON 1989). In membrane-spanning proteins the α-helix is the most stable secondary structure. The lipid molecules of the membrane bilayer cannot participate in hydrogen bonding, and in the α-helix the hydrogen bonds can be contained within the turns of the helix and separated from the lipid bilayer (JÄHNIG 1989). An α-helix with a completely hydrophobic surface commonly acts as a membrane anchor domain (JÄHNIG 1990). One of the best characterized membrane proteins is bacteriorhodopsin, a light driven hydrogen ion pump involved in photosynthesis, which consists of seven membrane-spanning helices, two of which are amphiphilic (HENDERSON & UNWIN 1975).

Several amphiphilic α-helices can come together and form membrane channels, with their hydrophobic sides facing the hydrophobic membrane, and their hydrophilic sides facing inwards forming the channel. In such an arrangement, polar molecules can traverse the hydrophobic membrane by passing through the hydrophilic channel pore (JÄHNIG 1990). Aquaporins, proteins that form water-selective channels, have six putative membrane-spanning amphiphilic α-helices (reviewed in CHRISPEELS & AGRE 1994).

However, although the α-helix is the most stable secondary structure in the hydrophobic environment of the membrane, not all membrane proteins contain α-helices as membrane-spanning domains. The β-strand can also remove hydrogen bonds from the surface by forming

bonds between the strands, and several strands can come together in such an arrangement and form an amphiphilic β-barrel, with the outer face hydrophobic and the inner hydrophilic (PAULMICHL *et al.* 1992). Such a structure was found to exist in a mammalian chloride channel protein. The protein is a dimer containing eight membrane-spanning β-strands arranged in a β-barrel (PAULMICHL *et al.* 1992). Similarly, OmpA also exists as an eight-strand β-barrel (VOGEL & JÄHNIG 1986).

4.2. METHODS

4.2.1. Clone selection

Several of the isolated clones cross-hybridized. 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 (Table 3.2.). 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.hat is clones *rws7* and *rws16*, were sequenced along their entire length by primer walking.

4.2.2. 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.

A single plaque was cored out of an agar plate and eluted in 500 µl SM buffer. The insert was amplified by PCR (Appendix A.9.) in a 200 µl reaction volume, using primers

complementary to the *Eco*R1 site flanking regions of λgt10.

The PCR'd sample was purified by excising the band from the gel and digesting the agarose away with the enzyme agarase (Boehringer Mannheim), followed by ethanol precipitation and resuspension in 40 µl TE buffer. From a 200 µl PCR reaction the final yield after agarase digestion was about 2 µg DNA at a concentration of 50 ng μl^{-1}.

It was not necessary to perform the digestions with exonuclease and shrimp alkaline phosphatase since all single stranded primers and unincorporated dNTPs were removed by the gel digestion procedure.

Two 2 µl aliquots (about 200 ng dsDNA) of the treated PCR product DNA were removed for the annealing and labelling reactions. The one aliquot was annealed to the forward primer and the other aliquot to the reverse primer in separate reactions by denaturing at 100°C for 3 min then snap cooling on ice. A labelling reaction was then carried out using [^{35}S]-labelled dATP and unlabelled dC/G/TTPs and DNA polymerase. The reaction was carried out at room temperature for 5 min. The reaction was then terminated by adding aliquots to the termination mix of each of the four dideoxy nucleotides, and incubated at 37°C for 15 min. The reactions were stopped by adding 4 µl stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF).

To run on a polyacrylamide gel, the reaction mix was heated to 75°C for 2 min and snap cooled on ice. Samples of 2.5 µl were then loaded on the gel. Three separate loadings were done per sample and run for 1, 2 and 3 h. The gel was dried and exposed to film for between 24 and 72 h.

A complication arose because the sequence could not be read from the poly(A) tract of the clone. This meant that the sequence could only be read in one direction. The sequence from the poly(A) tract resulted in bands across all four lanes. This must have been due to incorrect reading of the poly(A) tract, either during PCR or phage replication, so that the resultant DNA molecules had tracts of varying lengths.

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

4.2.3. 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).

4.3. RESULTS

4.3.1. Clone selection

Of the two cDNA species that were chosen for sequencing, clone *rws7* was the longest fragment from species A, and *rws16* the longest fragment from species B (Table 3.2.).

4.3.2. Nucleotide sequence

Rws7 is 1 151 nucleotides long, up to and excluding the first A of the poly(A) tract. There is a single open reading frame of 921 nucleotides, starting at nucleotide 113 with the first methionine codon, and ending at nucleotide 1 033 before the first stop codon. There is a 3' untranslated region of 118 nucleotides. No typical polyadenylation signal was observed in the 3' untranslated region. The 5' untranslated region is A-rich (37%), and the 3' untranslated region is T-rich (36%). The AT proportion of the open reading frame is 49% (Fig. 4.1.A).

Rws16 is 478 nucleotides long, up to and excluding the first A of the poly(A) tract. There is a coding sequence of 204 nucleotides starting at nucleotide 2 and ending at nucleotide 205 before the first stop codon. No methionine start codon was identified. There is a 3' untranslated region of 273 nucleotides containing the AAATAA polyadenylation signal starting 24 nucleotides before the first A of the poly(A) tract. The 3' untranslated region is T-rich (36%). The AT proportion of the open reading frame is 48% (Fig. 4.1.B).

4.3.3. Amino acid sequence

The amino acid sequence of *rws7* derived from the coding region is 307 residues in length. There is a serine-rich region at the carboxyl-terminal end of the protein (Fig. 4.1.A). 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 (Table 4.1.).

The amino acid sequence derived from the coding region is 68 residues in length (Fig. 4.1.B).

```
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(A_n)                                                 1151
```

Figure 4.1. Nucleotide and deduced amino acid sequence of *rws7*. The nucleotide sequence of the noncoding strand, including the 5' and 3' untranslated regions up to the poly(A) tract (A_n), is shown. The amino acid sequence of the coding region is shown below the nucleotide sequence in the single letter code. An asterisk indicates the termination codon.

```
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(An)   478
```

Figure 4.2. Nucleotide and deduced amino acid sequence of *rws16*. The nucleotide sequence of the noncoding strand, including the 3' untranslated region up to the poly(A) tract (A_n), is shown. The amino acid sequence of the coding region is shown below the nucleotide sequence in the single letter code. An asterisk indicates the termination codon, and the polyadenylation signal is in bold.

Table 4.1. Amino acid composition of the derived polypeptide of *rws7*.

Amino acid:		**Percentage composition, *rws7*:**
A	Alanine	3.9
C	Cysteine	0.0
D	Aspartate	6.2
E	Glutamate	4.2
F	Phenylalanine	1.0
G	Glycine	11.4
H	Histidine	2.6
I	Isoleucine	2.6
K	Lysine	3.9
L	Leucine	2.6
M	Methionine	1.6
N	Asparagine	6.8
P	Proline	10.4
Q	Glutamine	2.9
R	Arginine	14.7
S	Serine	13.7
T	Threonine	3.9
V	Valine	4.2
W	Tryptophan	0.3
Y	Tyrosine	2.9
Acidic		10.4
Basic		18.6
Charged		29.0
Net charge		8.1
Hydrophobic		10.4
Residues		307
Molecular weight		33 775 Da

4.3.4. Hydropathy

A hydropathy profile of the amino acid sequence shows *rws7* to be mainly hydrophilic (Fig. 4.3.A).

A hydropathy profile of the amino acid sequence shows *rws16* to be slightly hydrophilic (Fig. 4.3.B).

4.3.5. Sequence homology

A BLAST search (ALTSCHUL *et al.* 1990) found one nucleotide sequence homologous to *rws7* which was a partial cDNA sequence from rice (SASAKI *et al.* 1994) 357 nucleotides in length (accession number D15868 in the GenBank™/EMBL Data Bank) to which *rws7* showed 78% homology over the whole length of the rice cDNA. There were no amino acid sequences that showed homology to *rws7*.

A BLAST search (ALTSCHUL *et al.* 1990) found two amino acid sequences homologous to *rws16*, and both were chloride channel proteins. The one was an unpublished human chloride channel protein 210 amino acids in length (accession number X87689 in the GenBank™/EMBL Data Bank) to which *rws16* showed 33% identical amino acid homology and 56% positive amino acid homology (Fig. 4.4.). The other was a bovine chloride channel protein 437 amino acids in length, termed p64 (accession number L16547 in the GenBank™/EMBL Data Bank) (LANDRY *et al.* 1993), to which *rws16* showed 22% identical amino acid homology and 51% positive amino acid homology (Fig. 4.4.).

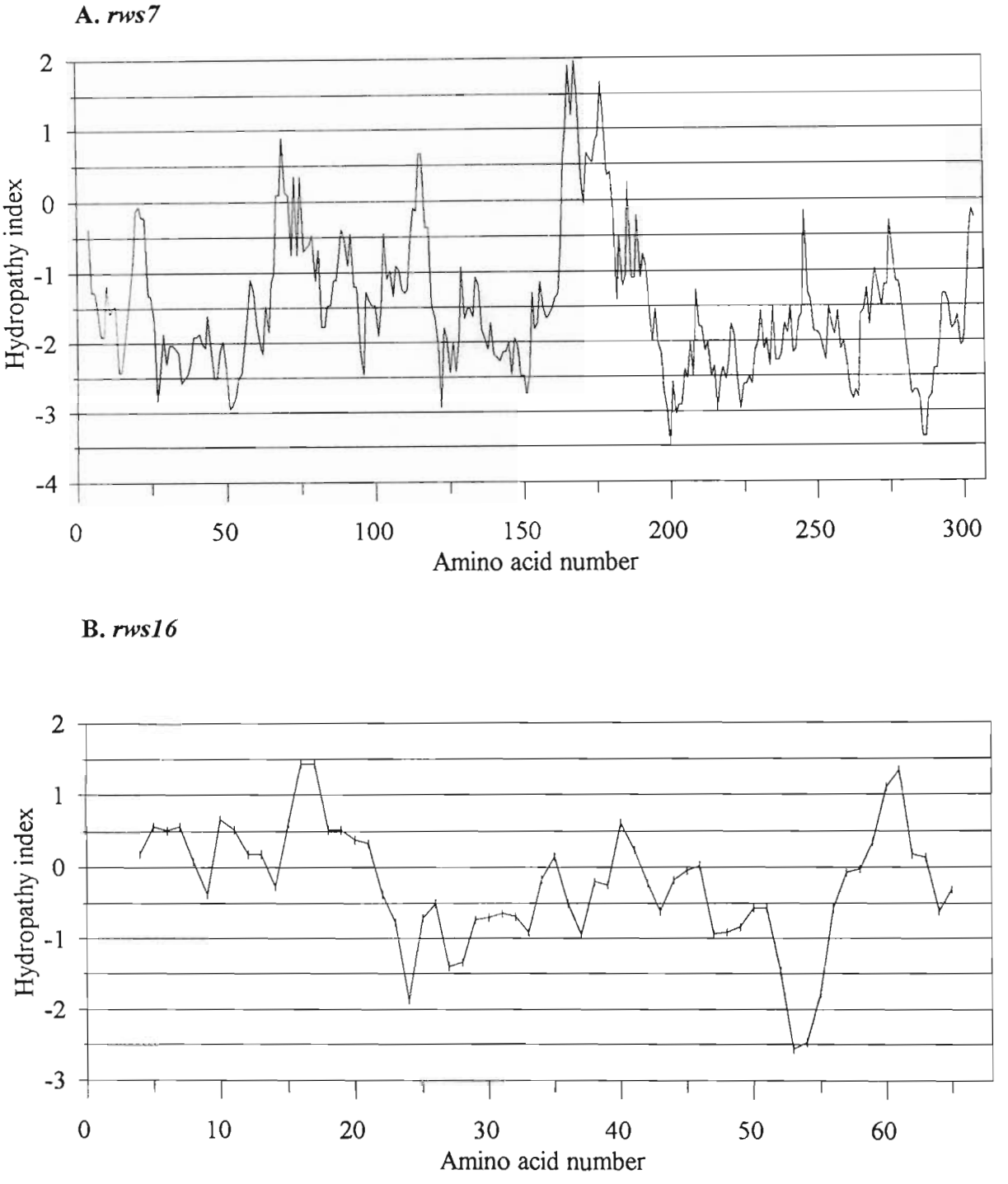


Figure 4.3. Hydropathy plot of the putative polypeptides of *rws7* (A) and *rws16* (B). The plot was made using a moving window of seven residues (KYTE & DOOLITTLE 1982).

```
BpP-64ClCP                                    MNDENYSTTIYNRVQTERVYEDSD   24

BpP-64ClCP    PAENGGPLYDEVHEDVRREDNLYVNELENQEYDSVAVYPVGRQGRTSASL   74

BpP-64ClCP    QPETGEYVLPDEPYSKAQDPHPGEPTEDEDISLEELLSPTKDQESDSEEP  124

BpP-64ClCP    QASDPEEPQASDPEEPQGPDPEEPQENGNEMEADLPSPSSFTIQNSRAFS  174

BpP-64ClCP    TREISPTSYSADDVSEGNESASASPEINLFVKAGIDGESIGNCPFSQRLF  224

BpP-64ClCP    MILWLKGVVFNVTTVDLKRKPADLHNLAPGTHPPFLTFNGDVKTDVNKIE  274

HsP-64ClCP    MVLWLKGVTFNVTTVDTKRRTETVQKLCPGGQLPFLLYGTEVHTDTNKIE   50

BpP-64ClCP    EFLEETLTPEKYPRLAAKHRESNTAGIDIFVKFSAYIKNTKQQSNAALER  324

HsP-64ClCP    EFLEAVLCPPRYPKLAALNPESNTAGLDIFAKFSAYIKNSNPALNDNLEK  100

BpP-64ClCP    GLTKALKKLDDYLNTPLPEEIDADTRGDDEKGSRRKFLDGDELTLA+CN+  374
rws16                                                 NVSATDLSL    9
HsP-64ClCP    GLLKALKVLDNYLTSPLPEEVDE TSAEDEGVSQRKFLDGNELTLA+CN+  149

BpP-64ClCP    L+++HVVK+VAKKYRNYDF+AEM+GLWR+L+NAYA+DE+TN+CAADSEIE  424
rws16         GPKLFHLQIALEHFKGWKIPENLTNVHAYTKALFSRESFVKTKPSEEHVI   59
HsP-64ClCP    L+++HIV+VVCKKYR+FT+++AFRG++R+LSNAYA++E+AS+C+DD+EIE  199

BpP-64ClCP    LAY+DVAKRLSRS     437
rws16         AGWAPKVNA          68
HsP-64ClCP    LAYEQVAK+LK       210
```

Figure 4.4. Comparison of the deduced amino acid sequences of the putative *rws16* polypeptide, ClCP p64 from *Homo sapiens*, and p64 from *Bos primigenius*. The plus signs show identical amino acid homology and the underlined letters show positive amino acid homology to *rws16* polypeptide.

4.3.6. Sequence identity

The remaining clones within each of the two cDNA species were sequenced at their 5' ends and they all showed identical sequence homologies with the fully sequenced clones.

4.3.7. Nucleotide and amino acid sequences of remaining seven clones

Of the seven clones which were sequenced at the 5' ends only of the sense strand, two (*rws2* and *rws9*) (Fig. 4.5.) have no stop codons in one of the three reading frames, indicating that this is a coding region and therefore represents only a partial sequence of the total RNA. All the other clones have stop codons in all three reading frames suggesting that they are 5' untranslated regions and that the entire open reading frame is present in the cDNA insert.

A BLAST search did not produce any homologous nucleotide sequences for the complete sequence query.

rws5

```
GGGGGAAAAAGATCGTCGTCGGGTTCTGAAGGATTTCTCGAAGATGCTTCATGATCAGAT      60
G  G  K  R  S  S  S  G  S  E  G  F  L  E  D  A  S  *  S  D
 G  E  K  D  R  R  R  V  L  K  D  F  S  K  M  L  H  D  Q  I
  G  K  K  I  V  V  G  F  *  R  I  S  R  R  C  F  M  I  R  *

AGAGAAGACTGTCCGTGTTTCTGTTGGAACAACAAGGACTATTGGCAAGCAGGATTGAGA     120
R  E  D  C  P  C  F  C  W  N  N  K  D  Y  W  Q  A  G  L  R
 E  K  T  V  R  V  S  V  G  T  T  R  T  I  G  K  Q  D  *  E
  R  R  L  S  V  F  L  L  E  Q  Q  G  L  L  A  S  R  I  E  K

AGTTAGGAAGCAAGGCATAGTACA                                          144
S  *  E  A  R  H  S  T
 V  R  K  Q  G  I  V
  L  G  S  K  A  *  Y
```

rws14

```
GATGAATTCACATTTTATTTCATTTTATACTCCTGAGTCCTGAAAATAACATGATAATAC      60
D  E  F  T  F  Y  F  I  L  Y  S  *  V  L  K  I  T  *  *  Y
 M  N  S  H  F  I  S  F  Y  T  P  E  S  *  K  *  H  D  N  T
  *  I  H  I  L  F  H  F  I  L  L  S  P  E  N  N  M  I  I  L

TTCCCATCGACAACGGCAATGACCTTATAAACACAACCGCTTAAGCCCTAAGCTACATAT     120
F  P  S  T  T  A  M  T  L  *  T  Q  P  L  K  P  *  A  T  Y
 S  H  R  Q  R  Q  *  P  Y  K  H  N  R  L  S  P  K  L  H  I
  P  I  D  N  G  N  D  L  I  N  T  T  A  *  A  L  S  Y  I  Y

ACTACTGAATATATTTACGACACTTTCGATGGTCATCAACACCCTACAGGTTTATATAAC     180
T  T  E  Y  I  Y  D  T  F  D  G  H  Q  H  P  T  G  L  Y  N
 L  L  N  I  F  T  T  L  S  M  V  I  N  T  L  Q  V  Y  I  T
  Y  *  I  Y  L  R  H  F  R  W  S  S  T  P  Y  R  F  I  *  H

ATATAGATTACACGACCAGAACATATACGT                                    210
I  *  I  T  R  P  E  H  I  R
 Y  R  L  H  D  Q  N  I  Y
  I  D  Y  T  T  R  T  Y  T
```

Figure 4.5. Nucleotide and derived amino acid sequences of remaining clones.

rws6

```
AATCTGCAAGCAGCATGGCGAGTTTATTATGATACAGGGAAGTGCGTGTCTGGCGTATTT      60
N  L  Q  A  A  W  R  V  Y  Y  D  T  G  K  C  V  S  G  V  F
 I  C  K  Q  H  G  E  F  I  M  I  Q  G  S  A  C  L  A  Y  F
  S  A  S  S  M  A  S  L  L  *  Y  R  E  V  R  V  W  R  I  S

CATTTAGCGTTCGAAGTACAACACTCGCACATATGTGTACATTATATTGTACATACTACA      120
H  L  A  F  E  V  Q  H  S  H  I  C  V  H  Y  I  V  H  T  T
 I  *  R  S  K  Y  N  T  R  T  Y  V  Y  I  I  L  Y  I  L  H
  F  S  V  R  S  T  T  L  A  H  M  C  T  L  Y  C  T  Y  Y  T

CAACATCTCGTTATGAATATTGTTACATCATCTACATCATGATGTGTACATACGACTCAA      180
Q  H  L  V  M  N  I  V  T  S  S  T  S  *  C  V  H  T  T  Q
 N  I  S  L  *  I  L  L  H  H  L  H  H  D  V  Y  I  R  L  N
  T  S  R  Y  E  Y  C  Y  I  I  Y  I  M  M  C  T  Y  D  S  T

CACTGTGACATATCTAGTATCAAGTATCACAGC                                  213
H  C  D  I  S  S  I  K  Y  H  S
 T  V  T  Y  L  V  S  S  I  T
  L  *  H  I  *  Y  Q  V  S  Q
```

rws9

```
ATTCGTCTGATTCATATAGCTCAGATACTTCAGACTCATTGTCCTATTCAGATAGTGGCT      60
I  R  L  I  H  I  A  Q  I  L  Q  T  H  C  P  I  Q  I  V  A
 F  V  *  F  I  *  L  R  Y  F  R  L  I  V  L  F  R  *  W  L
  S  S  D  S  Y  S  S  D  T  S  D  S  L  S  Y  S  D  S  G  S

CTGATTCAGAATCAGATTCATCAATGGATACTAGCTCGTCCAGTGATCATAGGCGTAAGA      120
L  I  Q  N  Q  I  H  Q  W  I  L  A  R  P  V  I  I  G  V  R
 *  F  R  I  R  F  I  N  G  Y  *  L  V  Q  *  S  *  A  *  E
  D  S  E  S  D  S  S  M  D  T  S  S  S  S  D  H  R  R  K  R

GAAGAAAGGATCTA                                                     134
E  E  R  I
 K  K  G  S
  R  K  D  L
```

Figure 4.5. (cont'd). Nucleotide and derived amino acid sequences of remaining clones.

rws10

```
ATTCGTCTGATTCATAAAGCTCAGATACTTCAGACTCATTGTCCTATTCTTCAGATAGTG      60
I  R  L  I  H  K  A  Q  I  L  Q  T  H  C  P  I  L  Q  I  V
 F  V  *  F  I  K  L  R  Y  F  R  L  I  V  L  F  F  R  *  W
  S  S  D  S  *  S  S  D  T  S  D  S  L  S  Y  S  S  D  S  G

GCTCTGATTCAGAATCAGATTCATCAATGGATACTAGCTCGTCCAGTGATCATAGGCGTA      120
A  L  I  Q  N  Q  I  H  Q  W  I  L  A  R  P  V  I  I  G  V
 L  *  F  R  I  R  F  I  N  G  Y  *  L  V  Q  *  S  *  A  *
  S  D  S  E  S  D  S  S  M  D  T  S  S  S  S  D  H  R  R  K

AGAGAAGAAAAGGATCTAAGAAGGATAAGCGCAAGCCACAAAGAGAAAGGGTAAACATAC      180
R  E  E  K  D  L  R  R  I  S  A  S  H  K  E  K  G  *  T  Y
 E  K  K  R  I  *  E  G  *  A  Q  A  T  K  R  K  G  K  H  T
  R  R  K  G  S  K  K  D  K  R  K  P  Q  R  E  R  V  N  I  Q

AAGGAGTAAGAG                                                      192
K  E  *  E
 R  S  K
  G  V  R
```

rws2

```
GGCAAGGAAGAATTGTTGTGTTCACTGTCTTGGGCAGTCTCTTGGTCCAATATGAATCAA      60
G  K  E  E  L  L  C  S  L  S  W  A  V  S  W  S  N  M  N  Q
 A  R  K  N  C  C  V  H  C  L  G  Q  S  L  G  P  I  *  I  N
  Q  G  R  I  V  V  F  T  V  L  G  S  L  L  V  Q  Y  E  S  T

CTTACACAGCATCTCCAAAAACTTCTAAAATTACTAGCTGAATGCCCGTGCGTTGCAACG      120
L  T  Q  H  L  Q  K  L  L  K  L  L  A  E  C  P  C  V  A  T
 L  H  S  I  S  K  N  F  *  N  Y  *  L  N  A  R  A  L  Q  R
  Y  T  A  S  P  K  T  S  K  I  T  S  *  M  P  V  R  C  N  G

GGAATATAT                                                         129
G  I  Y
 E  Y
  N  I
```

Figure 4.5. (cont'd). Nucleotide and derived amino acid sequences of remaining clones.

rws15

```
TCACCGAGAAGAGCATNATNTAATGACAACGCTAAAGCTGAAAATAGTTTGCTAGAACAT      60
S  P  R  R  A  X  X  N  D  N  A  K  A  E  N  S  L  L  E  H
 H  R  E  E  H  X  X  M  T  T  L  K  L  K  I  V  C  *  N  I
  T  E  K  S  X  X  *  *  Q  R  *  S  *  K  *  F  A  R  T  F

TCTGTGCCAACAAATAGATCACCCAAAGATCCAGATGAACATCAAGTTGACAAAGCAGCA     120
S  V  P  T  N  R  S  P  K  D  P  D  E  H  Q  V  D  K  A  A
 L  C  Q  Q  I  D  H  P  K  I  Q  M  N  I  K  L  T  K  Q  Q
  C  A  N  K  *  I  T  Q  R  S  R  *  T  S  S  *  Q  S  S  K

AAGTAGCTCGAAGGAACTAGGGAGAAAGGCGTGAGAAGAGAGCATGATTGTCATAAGATG     180
K  *  L  E  G  T  R  E  K  G  V  R  R  E  H  D  C  H  K  M
 S  S  S  K  E  L  G  R  K  A  *  E  E  S  M  I  V  I  R  *
  V  A  R  R  N  *  G  E  R  R  E  K  R  A  *  L  S  *  D  D

ATGAGCACTATAGCTGAATGCTGCA                                       205
M  S  T  I  A  E  C  C
 *  A  L  *  L  N  A  A
  E  H  Y  S  *  M  L
```

Figure 4.5. (cont'd). Nucleotide and derived amino acid sequences of remaining clones.

4.4. DISCUSSION

Since all the clones within each of the two cDNA species showed identical sequence homologies with the fully sequenced clones, it can be assumed that they are identical.

In *rws7*, there is an ATG codon close to the 5' end of the cDNA, and evidence for this being the start codon comes from its context relative to the 5' nucleotides. Typically, the 5' untranslated region is adenine-rich (JOSHI 1987b). This is the case in *rws7*, with adenine constituting 37% of the nucleotides in the 5' untranslated region up to the first ATG. PIATKOWSKI *et al.* (1990) isolated five desiccation responsive genes from the resurrection plant *Craterostigma plantagineum*, and four of these genes had A-rich sequences immediately upstream from the initiation codon. Similarly, GUERRERO *et al.* (1990) isolated three turgor-responsive genes from pea shoots, and two of these had A-rich sequences immediately upstream from the initiation codon, including the motif AAAGAAA, which is also repeated in *rws7*. The sequence around the initiation codon of *rws7* is in agreement with the consensus sequence of KOZAK (1981, 1984) where the -3 nucleotide is most commonly an A. The consensus sequence for the five nucleotides upstream of the start codon is CCACC which is in agreement with nucleotides -5 to -3 of *rws7*. The *rws7* sequence of CAAA in positions -4 to -1 of the start codon occurs in 2.34% of the 211 mRNAs reviewed by KOZAK (1984), whereas the percentage frequency expected by chance is 0.4%. However, in the same reading frame there are no stop codons 5' to the first ATG, whereas there are seven stop codons in the other two reading frames, so this ATG cannot be regarded with certainty as the start codon.

Rws16 must represent a partial sequence since no ATG codon was found at the 3' end of the sequence.

The sequences AAACAAA and AAAGAAA occur more than once in *rws7*. The significance of this is not known but it is observed in other gene sequences, for instance the *rab-17* gene from maize where AAACAAA occurs five bases from the 3' end (VILARDELL *et al.* 1990). Similarly, GUERRERO *et al.* (1990) isolated three turgor-responsive genes from pea shoots, and two of these had the motif AAAGAAA.

Although there is no AATAAA/T-like NUE motif within 40 nucleotides of the poly(A) tract in *rws7*, there is an AATGG motif at -41 nucleotides from the first A of the poly(A) tract which forms half of the AATGGAATGGA motif which is the NUE site in the *rbcS*-E9 gene (HUNT 1994), so this motif possibly acts as an NUE in *rws7*. It is possible that there are polyadenylation signals that are not yet recognised as such by researchers. For instance, GOSTI *et al.* (1995) isolated two desiccation-responsive genes from barley, neither of which have recognisable polyadenylation signals. A sizeable proportion (about 15%) of plant poly(A)s had no AATAAA/T-like motif (HUNT 1994).

There is no strict consensus sequence for FUEs, but there is a requirement for TG-richness (HUNT 1994), and the repetitive TGT motif has been shown to enhance the polyadenylation signal (McLAUCHLAN *et al.* 1985; INGELBRECHT *et al.* 1989). In *rws7*, the sequence TGT occurs more frequently in the 3'UTR compared with the coding region and the 5' UTR. The TGT motif does not occur in the 5'UTR, but it occurs six times in the coding region (0.33 times per 50 nucleotides) and six times in the 3'UTR (2.54 times per 50 nucleotides). Similarly to *rws7*, there is a repetitive TGT motif in the 3' UTR of *rws16*. The sequence TGT occurs more frequently in the 3'UTR of *rws16* compared with the coding region; TGT occurs four times in the open reading frame (0.98 times per 50 nucleotides) and thirteen times in the 3' UTR (2.38 times per 50 nucleotides).

The rice cDNA to which *rws7* showed homology was isolated randomly from a cDNA library (SASAKI *et al.* 1994) and so there is no indication of its function. However, the derived *rws7* polypeptide does show similarities to the LEA proteins. These similarities are the hydrophilic nature, the high proportion of glycine residues, the low abundance of cysteine (no residues) and tryptophan (one residue) and the serine-rich motif. However, this identification must remain tentative since *rws7* differs from other LEAs in the absence of the typical consensus sequences and the location of its serine tract. Common consensus sequences of LEA proteins are DEYGNP in the N-terminal region (VILARDELL *et al.* 1990; GODAY *et al.* 1994) and EKKGIMDKIKEKLPG at the carboxy terminus (LABHILILI *et al.* 1995), and *rws7* lacks both these sequences. In RAB-17 from maize (VILARDELL *et al.* 1990), D-11 from cotton (BAKER *et al.* 1988), RAB-21 from rice (MUNDY & CHUA 1988) the serine tract is

in the middle of the amino acid sequence, whereas in *rws7* it is at the carboxyl terminus.

The sequence of serine residues is susceptible to phosphorylation which might suggest an activating/deactivating role in a signal transduction pathway for *rws7* (VILARDELL *et al.* 1990; GODAY *et al.* 1994). On the other hand, the high preponderance of glycine and the hydrophilic nature, typical of many of the LEA proteins, might suggest a role in solvation of structural surfaces. Free rotation around the glycine peptide bond allows the protein to exist as an amorphous random coil, adjusting its shape to that of structural surfaces and thus solvating them with its hydrophilic residues (BAKER *et al.* 1988).

The p64 bovine chloride channel protein to which *rws16* shows homology comprises two and possibly four putative membrane-spanning domains. There are potential phosphorylation sites by protein kinase A and C, and casein kinase II (LANDRY *et al.* 1993). Expression cloning of p64 showed it to be expressed in internal membranes and not in the plasma membrane (LANDRY *et al.* 1993). If *rws16* is similarly expressed in internal membranes, then possibly it is expressed in the tonoplast to mobilise ions from the vacuole. Although vacuolar chloride channels have not been implicated in the cellular response to water stress, mobilisation of K^+ and Ca^{2+} from the vacuole through membrane channels has been shown to occur in osmotic adjustment to desiccation (WARD & SCHROEDER 1994).

It has been suggested that chloride channels found in the plasma membrane provide a mechanism for membrane depolarization which occurs during stomatal closure (SCHROEDER & HAGIWARA 1989). Patch clamp analysis has shown these channels allow Cl^- efflux which in turn activates K^+ efflux channels, and a role as a control mechanism for stomatal closure has been suggested for them (SCHROEDER & HAGIWARA 1989; SCHMIDT & SCHROEDER 1994).

Membrane channel proteins which increase in abundance in response to water stress have been isolated by other researchers. A gene which is responsive to desiccation in *Arabidopsis thaliana* encodes a protein, termed RD28, that has homology to a bovine major intrinsic protein, to soybean nodulin-26 and the glycerol facilitator from *Escherichia coli*

suggesting that it is a transmembrane channel protein containing six membrane spanning domains (YAMAGUCHI-SHINOZAKI *et al.* 1992). RD28 also shows homology to a turgor-inducible protein, Trg31, from pea shoots (GUERRERO *et al.* 1990; JONES & MULLET 1995). YAMAGUCHI-SHINOZAKI *et al.* (1992) speculated that RD28 transports small molecules across membranes, either across intercellular junctions between plant cells, or across intracellular membranes such as the tonoplast, thus controlling osmotic pressure in desiccation-stressed plant cells.

CHAPTER 5:
CHARACTERIZATION OF ISOLATED GENES

5.1. INTRODUCTION

Drought-related genes have been shown to respond to a range of stimuli, including ABA, turgor loss and osmoticum (for reviews, see SKRIVER & MUNDY 1990; BRAY 1993; CHANDLER & ROBERTSON 1994). It has been suggested, however, that the number of drought related genes that has been isolated might be biased towards ABA-responsive genes because response to ABA has often been the criterion for selection (GOSTI *et al.* 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 *et al.* 1990; YAMAGUCHI-SHINOZAKI *et al.* 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 & BRAY 1990; PLANT *et al.* 1991; ROBERTSON & 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 compartmentalisation of ABA within the cell is therefore at least as important as absolute levels of ABA (HARTUNG & 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 & HO 1995).

Since ABA plays such an important role in gene expression in response to stress, genes have been classed as either ABA-responsive or -independent depending on their response to exogenously applied ABA. ABA-responsive genes were first isolated because either their

expression correlated with high ABA levels in the plant, such as during embryogenesis and germination, or their expression was changed either on application of ABA or in ABA mutants (CHANDLER & ROBERTSON 1994). Although, as mentioned above, it is recognised that exogenously applied ABA does not reproduce accurately the state of the plant tissue under stress, it is still a useful criterion because it can isolate the stimulus that is leading to enhancement of expression. If ABA and water stress are applied at the same time there is no way of distinguishing the contribution of either stimulus. If a gene responds to exogenously applied ABA then it is straightforward to classify it as an ABA-responsive gene. On the other hand, a gene might be classified as ABA-independent because it does not respond to exogenously applied ABA, but this does not mean that ABA is not involved in that gene's response to stress under natural conditions.

Roots can be the primary sensors of water stress and the ABA signal can be initiated in the roots and transported in the transpiration stream to the leaves where a response is elicited before there is any change in bulk water status of the leaf (ZHANG & DAVIES 1990; TARDIEU *et al.* 1993). This is equivalent to applying ABA exogenously to leaf tissue, so under some natural conditions response to ABA can be elicited without the tissue itself being under stress.

The involvement of proline in the gene expression response to water stress has not been investigated. Since its accumulation in response to water stress is widespread in plants, the possibility exists that it could act as a signal molecule.

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 is adaptive, then the gene expression that induces this response would be different between drought sensitive and drought tolerant genotypes. The aim of this section of work, therefore, was to further characterize the response of the three isolated clones and compare that response between the two maize cultivars.

To this end the expression of the three clones in response to various treatments was

investigated. The treatments were a water stress cycle of pot-grown mature plants to determine the time course and levels of transcription during a drying cycle and recovery, desiccation of excised leaves to determine if the response is independent of signals from the root, ABA to determine if the three clones are ABA-responsive, osmoticum and proline.

In addition, *in situ* hybridization was done to investigate if the expression of the clones was tissue specific.

5.2. METHODS

5.2.1. Plant growth

Plants of the two maize cultivars were grown in the glasshouse as previously described (Section 2.2.2.). Plants were grown until the onset of pollen maturation.

5.2.2. Drying cycle

The plants were divided into two treatments, control and water stress. In the water stress treatment water was withheld and the plants subjected to a drying period lasting seven days. At the point in the stress period when the plants were judged to be at peak stress, that is when the leaves were still rolled the next morning, watering was resumed until the plants looked fully recovered on day twelve of the treatment. In the control treatment the plants were watered normally throughout.

Plants were harvested for RNA extraction at different times during the drying cycle. In the water stress treatment these were at day 0 (start of drying cycle), day 4, day 7 (peak stress) and day 12 (after recovery). In the control treatment the sampling times were day 0, day 7 and day 12. Three plants from each maize cultivar were used per sampling time. The first fully expanded leaf was removed, the midrib was excised, and only the lamina used for RNA

extraction. Roots were washed clean of soil and smaller roots removed and stored for RNA extraction. All three leaf and root samples from each sampling time were pooled and weighed, frozen in liquid nitrogen and stored at -70°C. All leaf and root samples were removed at the same time of day, that is late morning.

5.2.3. Desiccation

The first fully expanded leaves from five plants were removed and allowed to air-dry in the glasshouse at 28°C. The progress of desiccation was followed by weighing the leaves. After 24 h, when the leaves were wilted and had lost about 20% of their fresh weight, the laminae were removed, pooled, divided into ten-gram subsamples and stored at -70°C. The control was leaves removed at the same time, and the laminae separated from the midrib and immediately frozen in liquid nitrogen and stored at -70°C.

5.2.4. Incubation conditions

For the PEG, ABA and proline experiments, leaf discs 1.5 cm in diameter were punched out from the lamina. Ten leaf discs were placed in 25 ml of the appropriate medium in a petri dish, and ten petri dishes per treatment per cultivar (100 leaf discs) were used. Incubation was at 25°C for 24 h on a rotary shaker in a thermostatically controlled chamber. The leaf discs were then stored at -70°C.

5.2.5. Osmoticum

Polyethylene glycol 6000 (PEG 6000) was used as osmoticum. For the osmotic treatment, leaf discs were incubated in MS medium (MURASHIGE & SKOOG 1962) without sucrose and hormones (WANG *et al.* 1995). The control treatment contained no PEG, and the osmotic treatment contained 10% (w/v) PEG 6000 in the MS medium. A PEG concentration

of 10% has been shown to induce gene expression (NORDIN *et al.* 1991; LEONE *et al.* 1994).

5.2.6. Proline

For the proline treatment, leaf discs were incubated in medium containing 100 mM KCL and 1 mM (2-N-morpholino)ethanesulfonic acid (MES)-KOH, pH 6.1 (KLEIN & ITAI 1989). The control treatment contained no proline in the medium, and the proline treatment contained 100 µM dl-proline (Sigma). A concentration of 100 µM proline has been shown to affect physiological responses in plants (RAGHAVENDRA & REDDY 1987; KLEIN & ITAI 1989).

5.2.7. Abscisic acid

For the ABA treatment, leaf discs were incubated in medium containing 10mM MES buffer (pH 5.5 with Tris) with 500 µM $CaSO_4$. The control treatment contained no ABA, and the ABA treatment contained 100 µM (±)-cis,trans-ABA (Sigma).

5.2.8. RNA extraction

For RNA extraction, all 100 leaf discs per treatment/cultivar were pooled. As previously described, RNA was extracted by the phenol/SDS method and poly(A)+ RNA was isolated on an oligo d(T) cellulose column. The poly(A)+ RNA was resuspended at a concentration of 0.1 µg μl^{-1}.

5.2.9. RNA slot blots

Thirty µl (3 µg) poly(A)+ RNA was incubated at 65°C for 5 min in 3 vol denaturing solution (Appendix A.10.).

The solution was snap cooled on ice and 1 vol 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 2 h.

5.2.10. Probe synthesis and hybridization

Radiolabelled probe was synthesised from 100 ng of the PCR product of the λgt10 clone by random-primed labelling with Klenow enzyme as previously described. Radiolabelled probe was synthesised from *rws7*, *rws16* and *rws5* to a specific activity greater than 1 x 10^6 cpm μg^{-1}.

The membranes were hybridized 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.

5.2.11. 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.

5.2.12. *In situ* hybridization

In situ hybridization was done using a combination of the protocols described by McKHANN & 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. All solutions and plasticware was treated with DEPC, and all glassware baked as previously described to remove RNAses.

Samples were fixed in FAA for 24 h, after which they progressed through the following dehydration and embedding steps, 2 h 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 h. 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 1 h and dried overnight.

In preparation for DIG labelling, the slides were dewaxed and rehydrated by progression through the following rehydration series, 2 min 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 min, then rinsed twice in 2 x SSC for 5 min at 70°C, followed by water twice, 5 min per wash.

To reduce background by nonspecific binding, an acetic anhydride step was done. Slides were dipped in 100 mM triethanolamine in water for 5 min, after which acetic anhydride was added to a concentration of 0.25%, and incubated for 10 min.

The slides were rinsed twice in 2 x SSC for 5 min, then dehydrated by progression through an ethanol series, 30%, 70%, 95% and 100%, 2 min per step. Slides were air dried under vacuum for at least 1 h.

Labelling and detection with DIG was done using the Boehringer Mannheim protocol supplied with the labelling and detection kit. Template DNA was synthesized by PCR from the phage as previously described. One μg 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 hybridization 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. Hybridization was done with leaf and root sections from control and water stressed plants of both maize cultivars. Two control hybridizations were also done. A negative control was done by probing with labelled λgt10 DNA to determine the extent of nonspecific binding. A positive control was done by probing with total cDNA to determine that the reaction was working.

5.3. RESULTS

5.3.1. Drying cycle

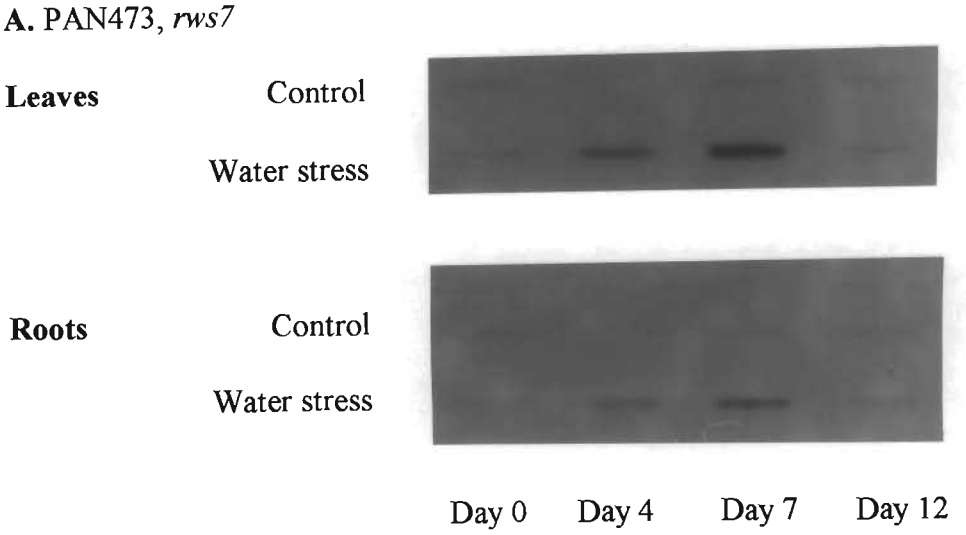
In the control treatment during the drying cycle there was no observable change in mRNA levels of all three clones in both leaf and root tissues of both cultivars. In the water stress treatment, mRNA levels rose severalfold to peak stress on day 7 and recovered to pre-stress levels by day 12 (Figures 5.1. to 5.6.).

In the drought sensitive cultivar (SR52), mRNA levels of all three clones in the leaves rose faster during the drying cycle (Figures 5.4. to 5.6.) so that they were higher on day 4 than those of the drought tolerant cultivar (PAN473) (Figures 5.1. to 5.3.). On day 4, in the leaves of SR52 there was an increase in mRNA levels of fivefold in *rws7* (Figure 5.4.), fivefold in *rws16* (Figure 5.5.) and elevenfold in *rws5* (Figure 5.6.), compared to an increase in the leaves of PAN473 of threefold for *rws7* (Figure 5.1.), threefold for *rws16* (Figure 5.2.) and eightfold for *rws5* (Figure 5.3.). There was no observable difference between the cultivars in the mRNA levels of each clone at peak stress on day 7 (Figures 5.1. to 5.6.).

There was a greater increase in mRNA levels in the leaves than in the roots in both cultivars. In the leaves of PAN473 at peak stress on day 7 the mRNA levels of *rws7* rose seventeenfold (Figure 5.1.), *rws16* eightfold (Figure 5.2.) and *rws5* fifteenfold (Figure 5.3.), and in the roots the increases were fivefold for *rws7* (Figure 5.1.), threefold for *rws16* (Figure 5.2.) and fourfold for *rws5* (Figure 5.3.). The levels of increase were very similar in SR52 (Figures 5.4. to 5.6.).

5.3.2. ABA, desiccation, PEG and proline treatments

Proline and ABA treatment of isolated leaf discs did not produce an increase in mRNA levels compared to controls in any of the three clones in both cultivars (Figures 5.7. to 5.12.). However, there was an increase in mRNA levels compared to controls in the desiccation treatment of detached leaves and osmotic treatment with PEG (Figures 5.7. to 5.12.). The increase in levels of mRNA was higher in the desiccation treatment of detached leaves than the osmotic treatment with PEG (Figures 5.7. to 5.12.).



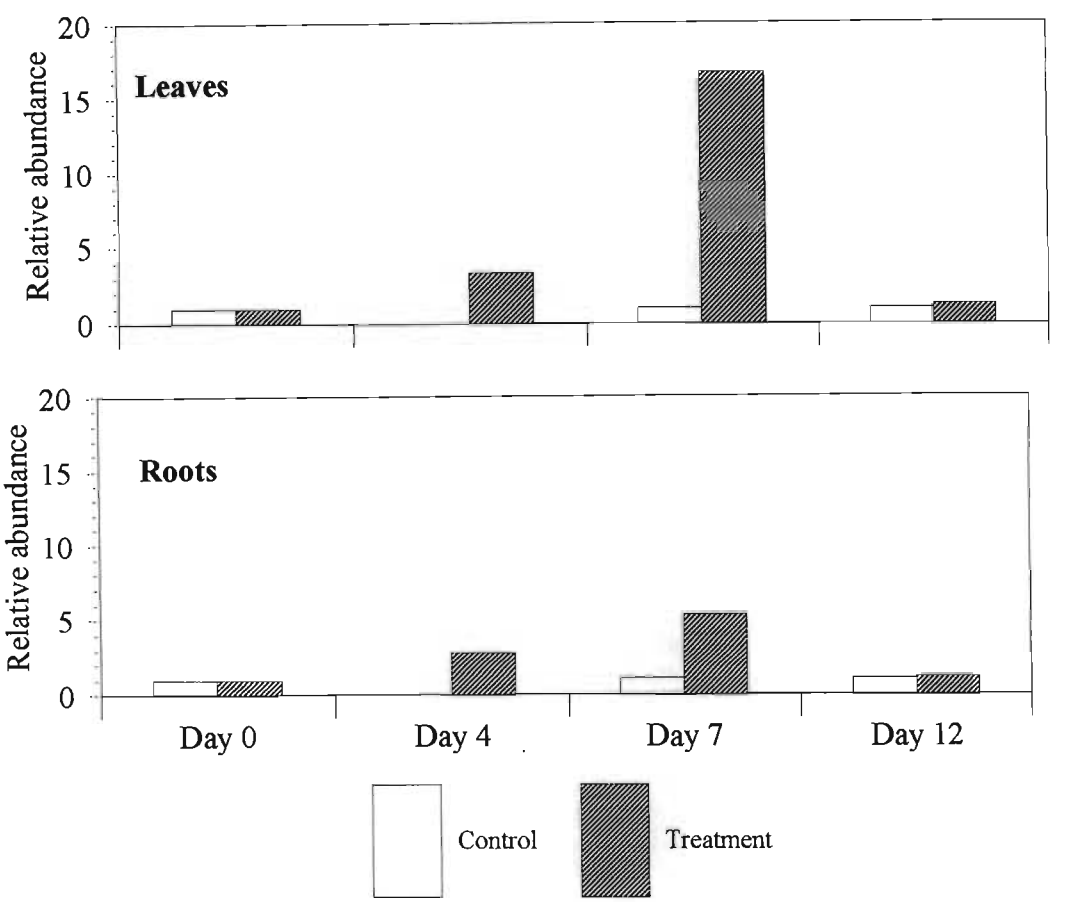
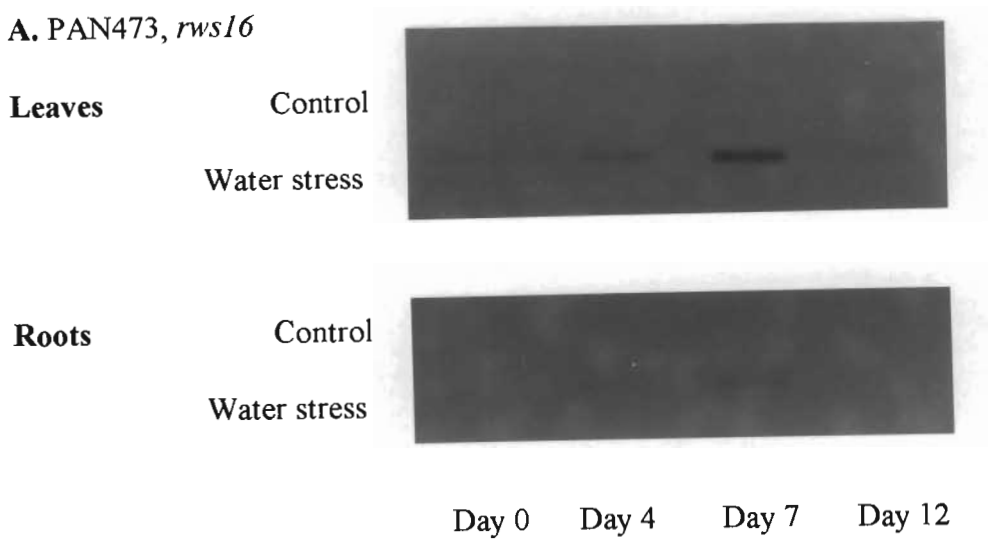


Figure 5.1. Expression of *rws7* transcripts in leaves and roots of the drought tolerant maize cultivar PAN473 during a progressive soil drying cycle (day 0, 4 and 7) and recovery (day 12) compared with watered controls. **A.** Equal amounts of poly(A)+ RNA (3 μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws7* cDNA and autoradiographed. **B.** Relative abundance of *rws7* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.



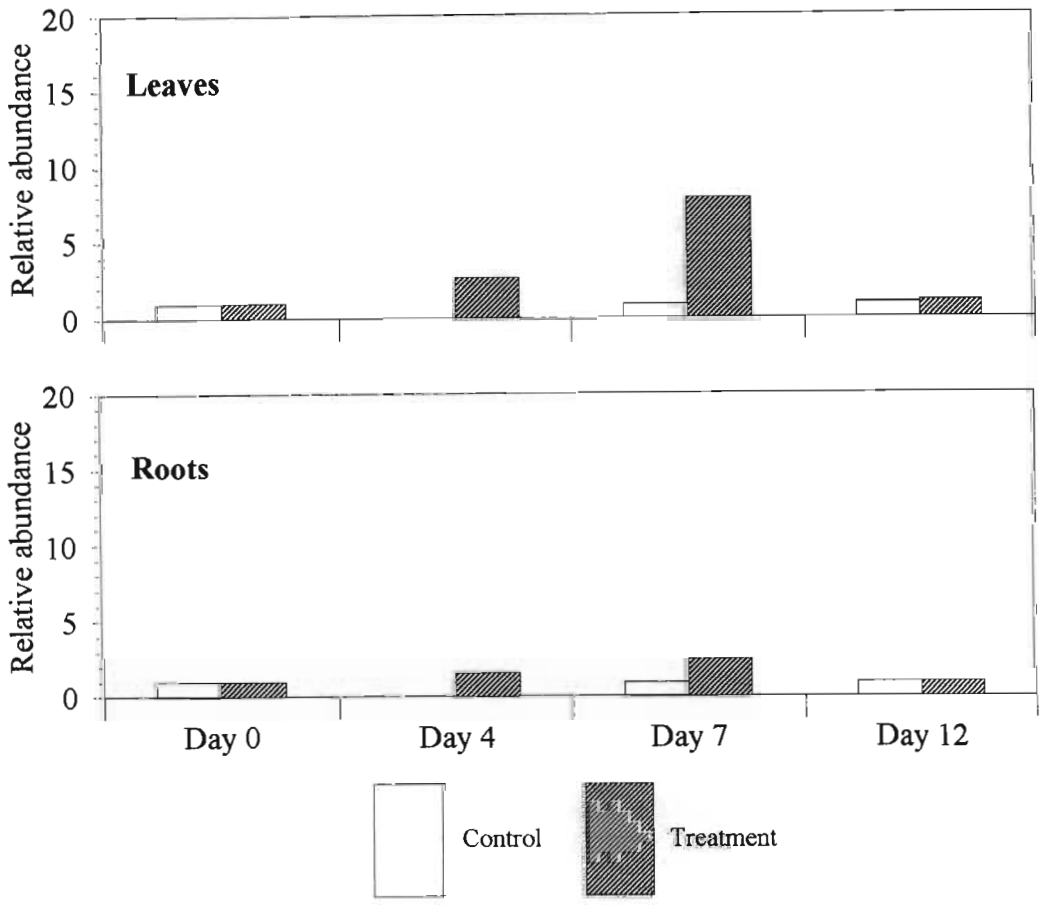
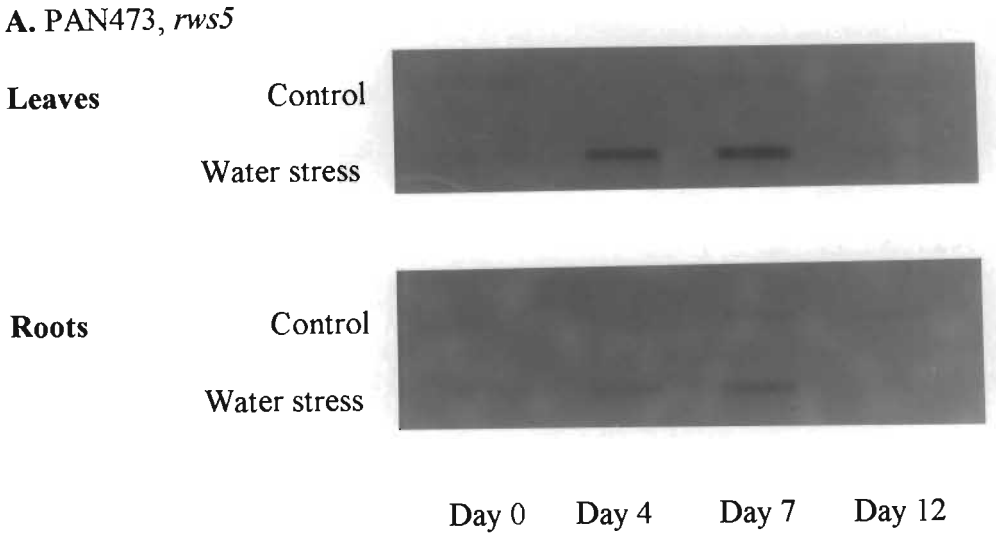


Figure 5.2. Expression of *rws16* transcripts in leaves and roots of the drought tolerant maize cultivar PAN473 during a progressive soil drying cycle (day 0, 4 and 7) and recovery (day 12) compared with watered controls. **A.** Equal amounts of poly(A)+ RNA (3 µg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws16* cDNA and autoradiographed. **B.** Relative abundance of *rws16* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.



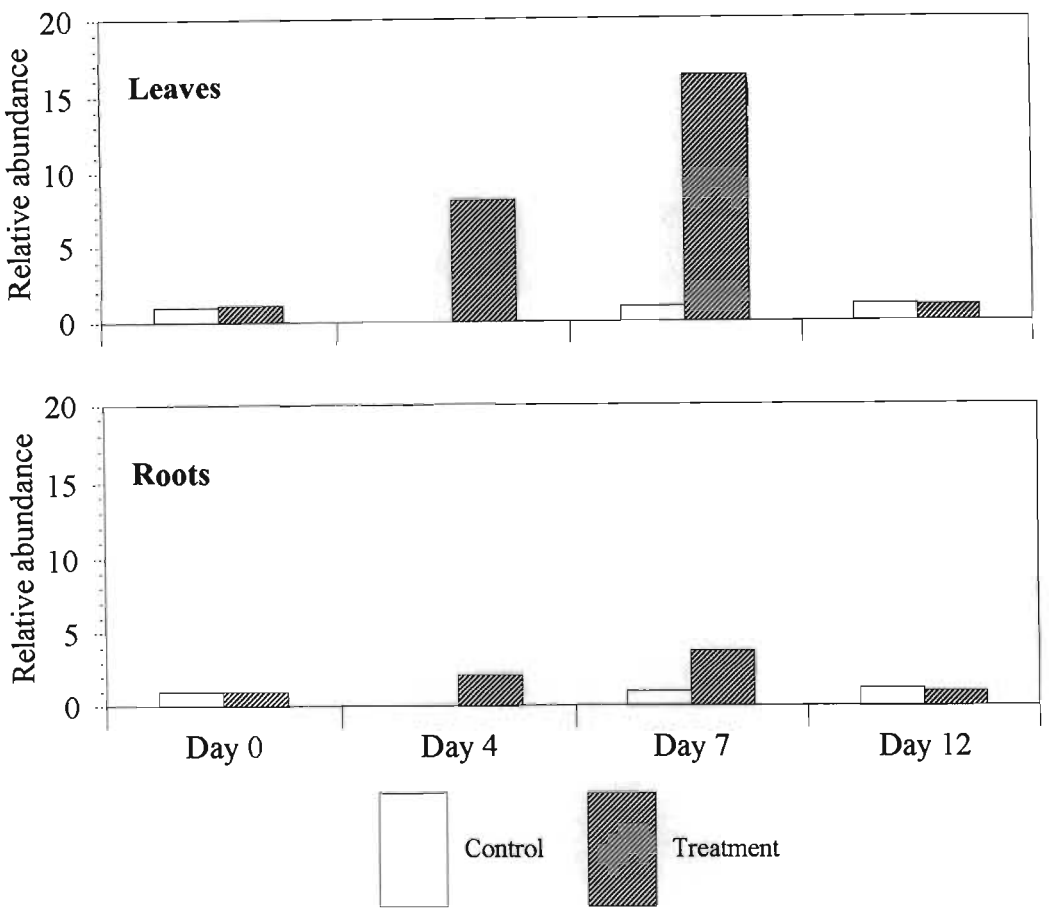
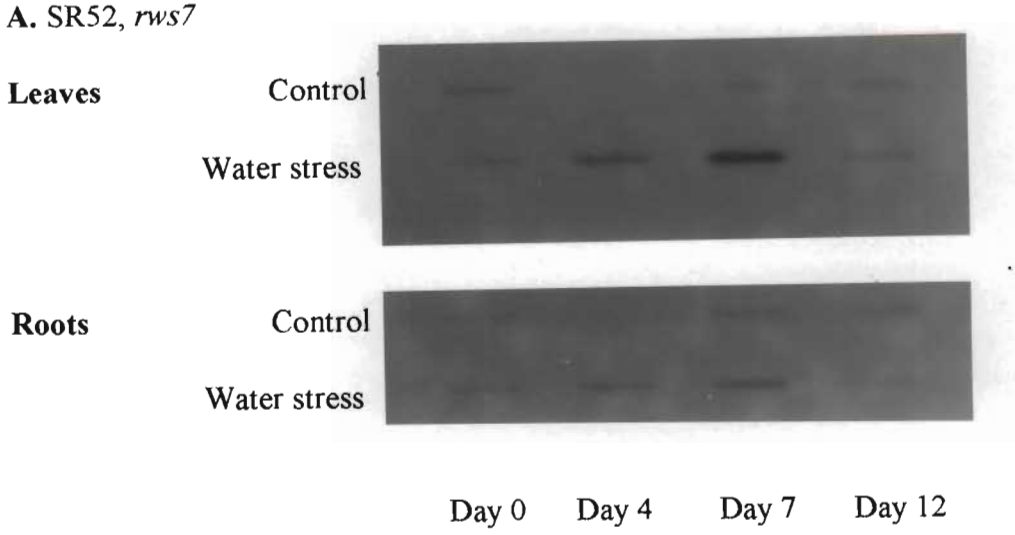


Figure 5.3. Expression of *rws5* transcripts in leaves and roots of the drought tolerant maize cultivar PAN473 during a progressive soil drying cycle (day 0, 4 and 7) and recovery (day 12) compared with watered controls. **A.** Equal amounts of poly(A)+ RNA (3 μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws5* cDNA and autoradiographed. **B.** Relative abundance of *rws5* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

A. SR52, *rws7*



B.

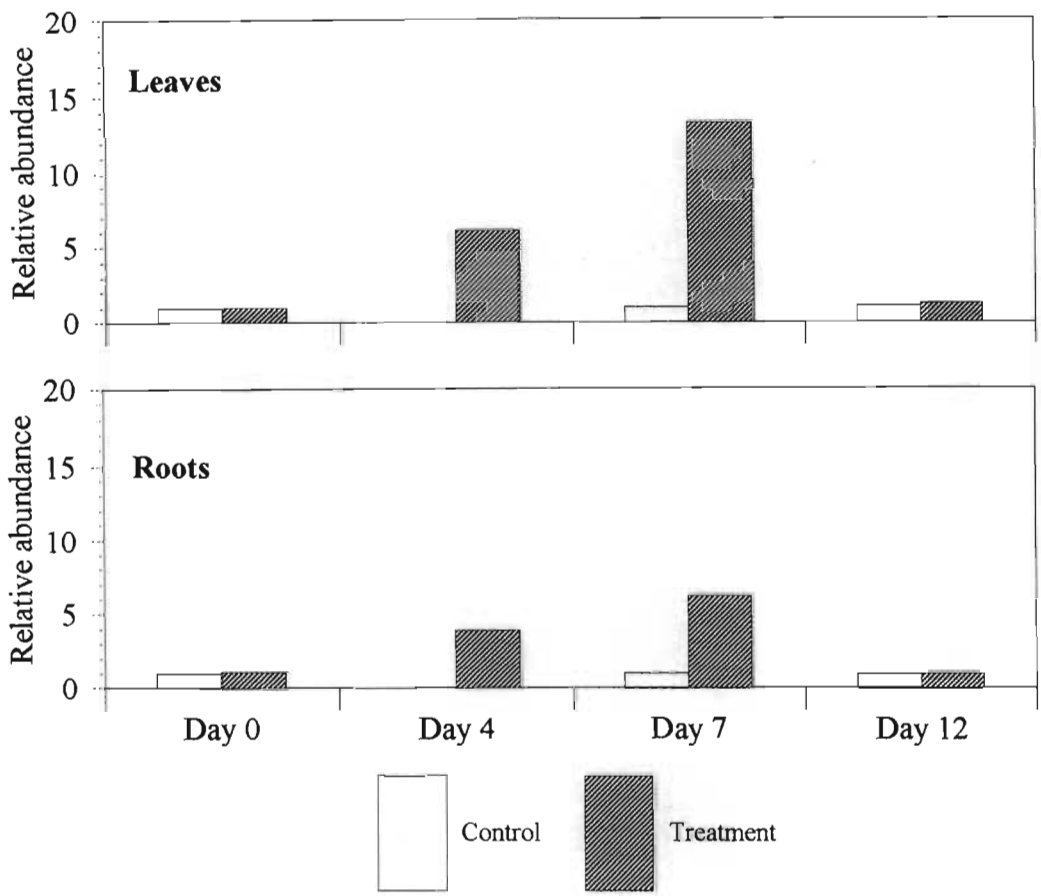
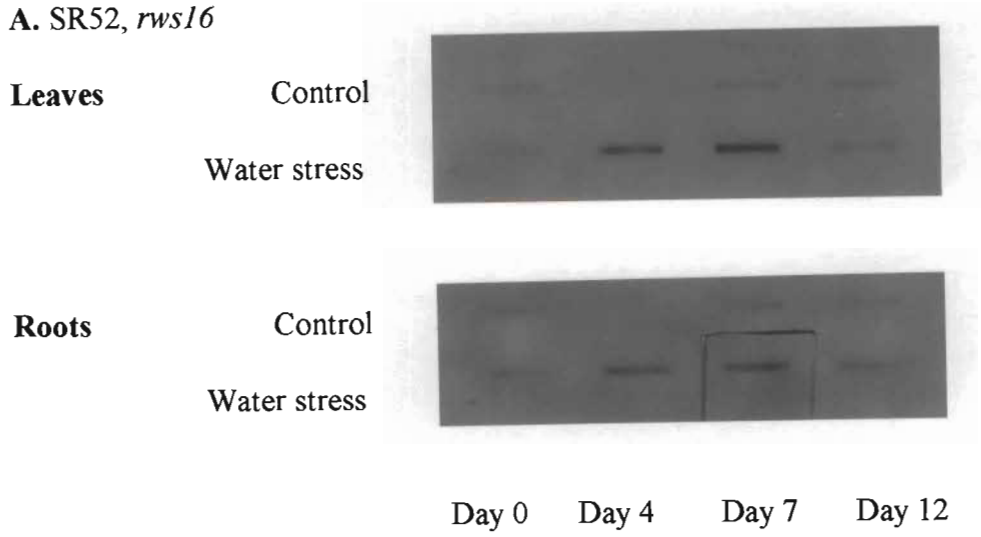


Figure 5.4. Expression of *rws7* transcripts in leaves and roots of the drought sensitive maize cultivar SR52 during a progressive soil drying cycle (day 0, 4 and 7) and recovery (day 12) compared with watered controls. **A.** Equal amounts of poly(A)+ RNA (3 μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws7* cDNA and autoradiographed. **B.** Relative abundance of *rws7* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.



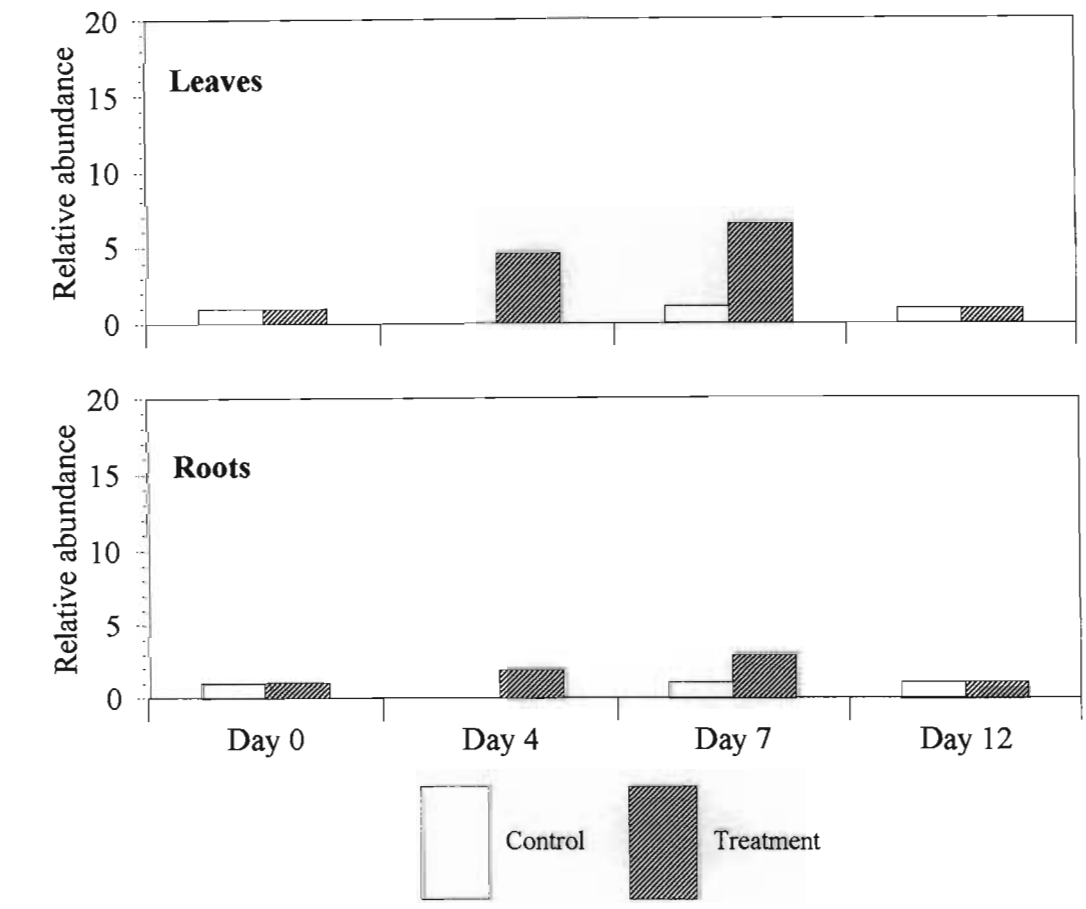


Figure 5.5. Expression of *rws16* transcripts in leaves and roots of the drought sensitive maize cultivar SR52 during a progressive soil drying cycle (day 0, 4 and 7) and recovery (day 12) compared with watered controls. **A.** Equal amounts of poly(A)+ RNA (3 μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws16* cDNA and autoradiographed. **B.** Relative abundance of *rws16* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

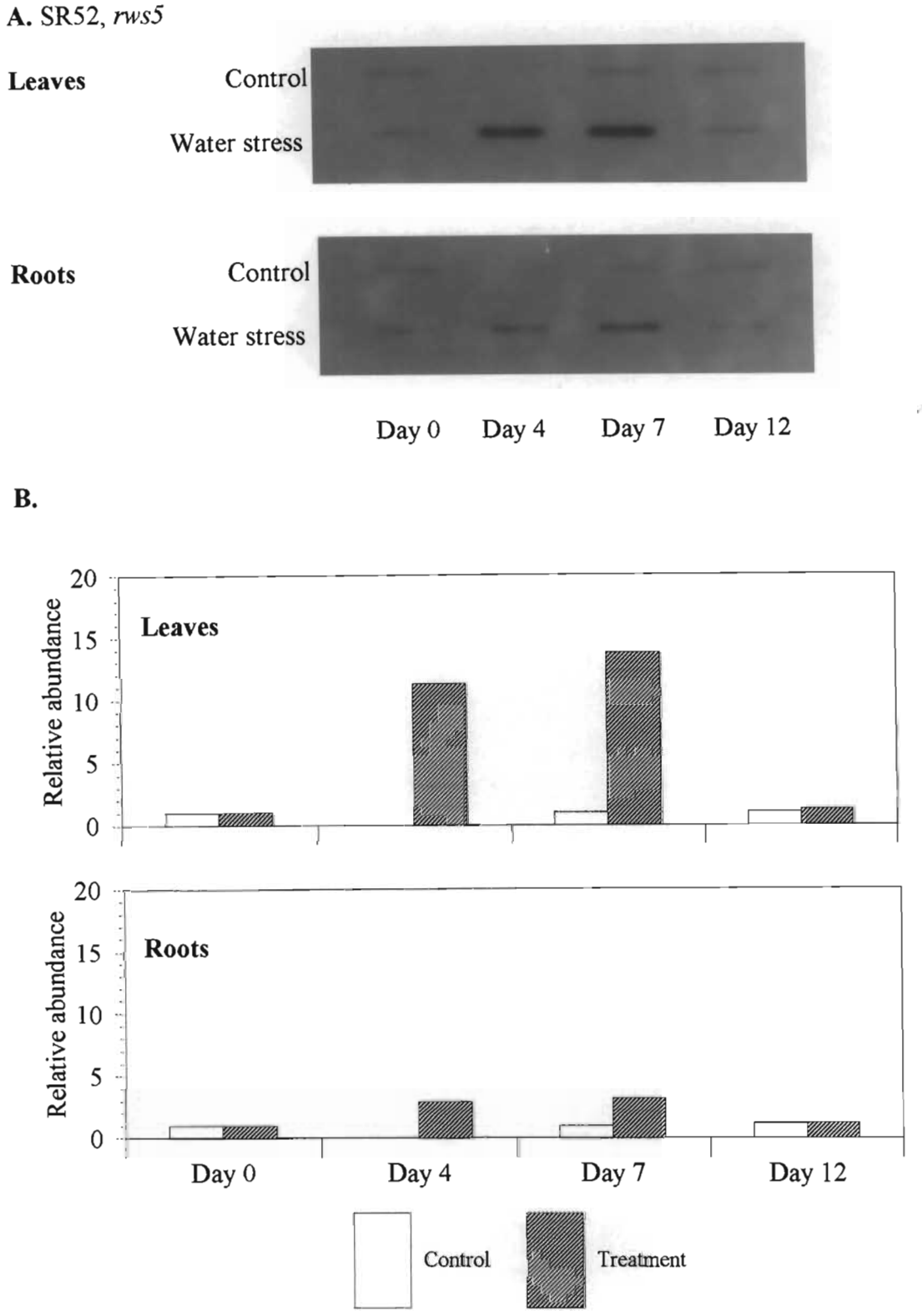
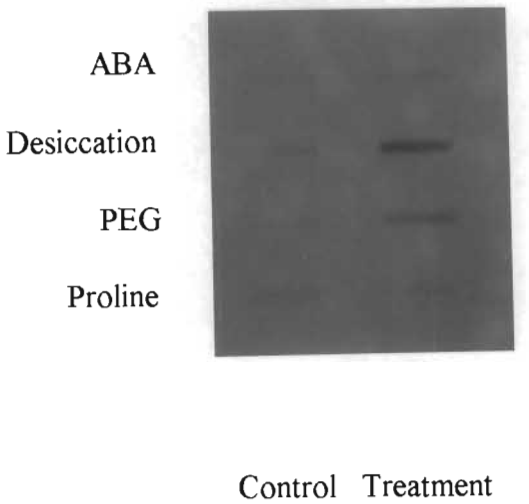


Figure 5.6. Expression of *rws5* transcripts in leaves and roots of the drought sensitive maize cultivar SR52 during a progressive soil drying cycle (day 0, 4 and 7) and recovery (day 12) compared with watered controls. **A.** Equal amounts of poly(A)+ RNA (3 μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws5* cDNA and autoradiographed. **B.** Relative abundance of *rws5* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

A. PAN473, *rws7*



B.

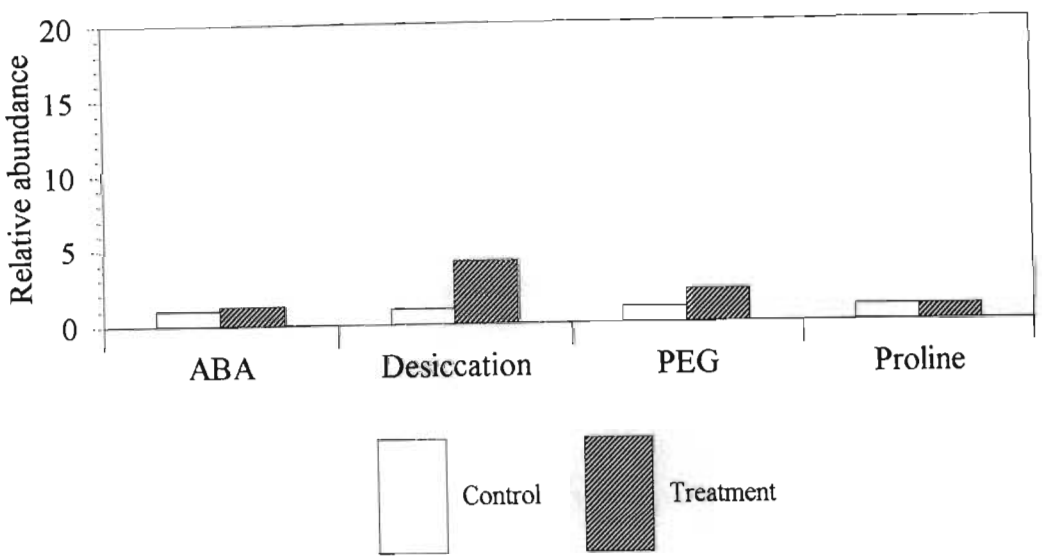
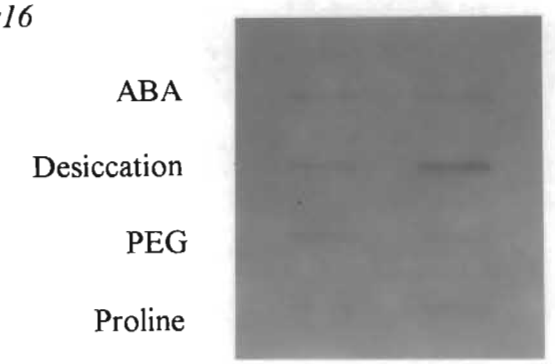


Figure 5.7. Expression of *rws7* transcripts in leaves of the drought tolerant maize cultivar PAN473 during ABA, desiccation, osmoticum and proline treatments and controls. **A.** Equal amounts of poly(A)+ RNA (3μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws7* cDNA and autoradiographed. **B.** Relative abundance of *rws7* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

A. PAN473, *rws16*



B.

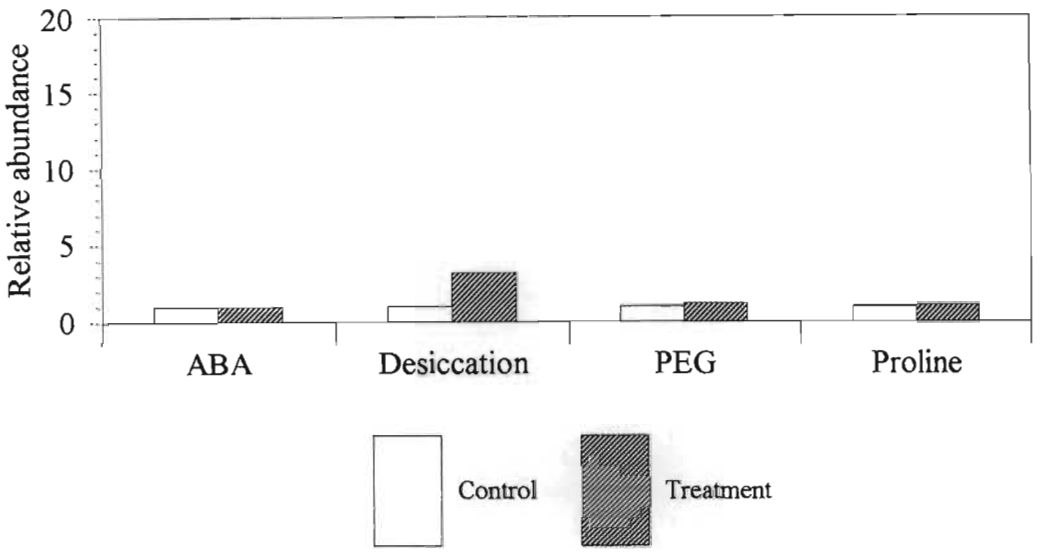
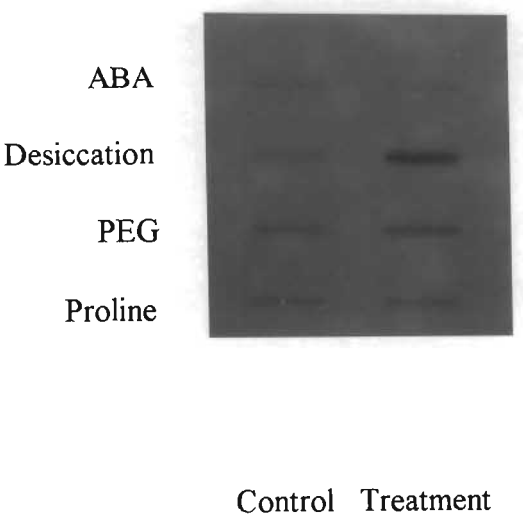


Figure 5.8. Expression of *rws16* transcripts in leaves of the drought tolerant maize cultivar PAN473 during ABA, desiccation, osmoticum and proline treatments and controls. **A.** Equal amounts of poly(A)+ RNA (3µg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws16* cDNA and autoradiographed. **B.** Relative abundance of *rws16* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

A. PAN473, *rws5*



B.

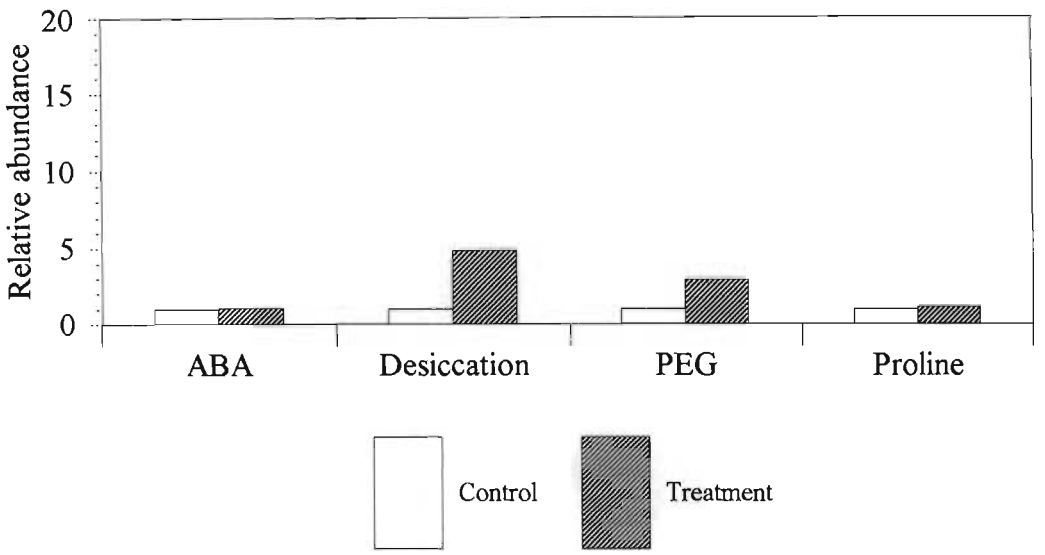
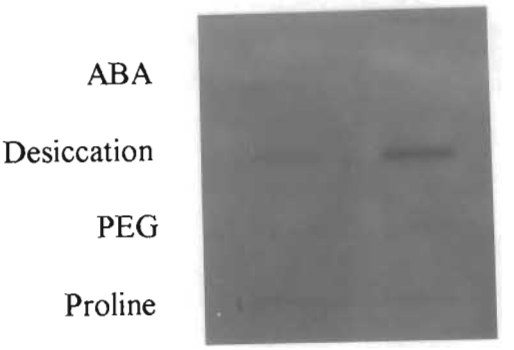


Figure 5.9. Expression of *rws5* transcripts in leaves of the drought tolerant maize cultivar PAN473 during ABA, desiccation, osmoticum and proline treatments and controls. **A.** Equal amounts of poly(A)+ RNA (3μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws5* cDNA and autoradiographed. **B.** Relative abundance of *rws5* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

A. SR52, *rws7*



B.

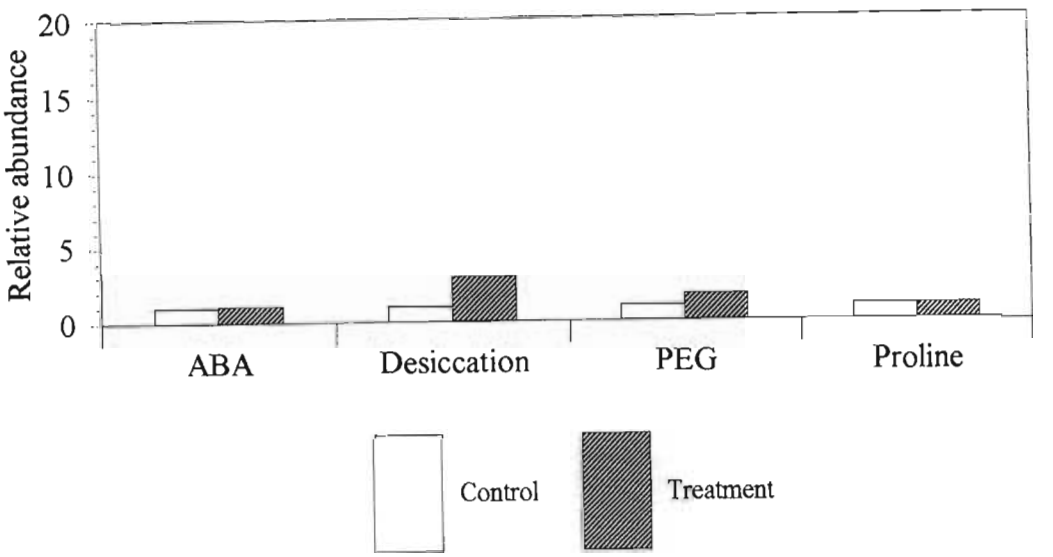
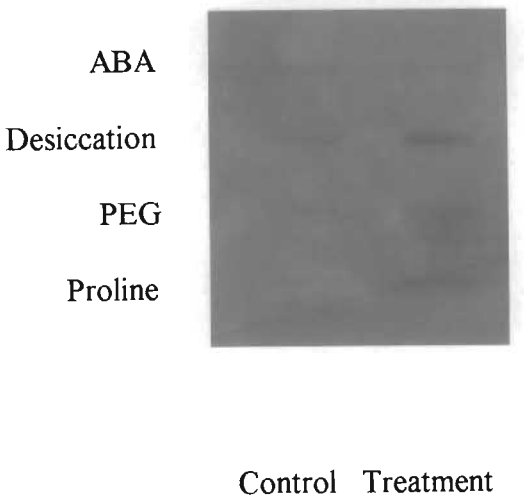


Figure 5.10. Expression of *rws7* transcripts in leaves of the drought sensitive maize cultivar SR52 during ABA, desiccation, osmoticum and proline treatments and controls. **A.** Equal amounts of poly(A)+ RNA (3μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws7* cDNA and autoradiographed. **B.** Relative abundance of *rws7* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

A. SR52, *rws16*



B.

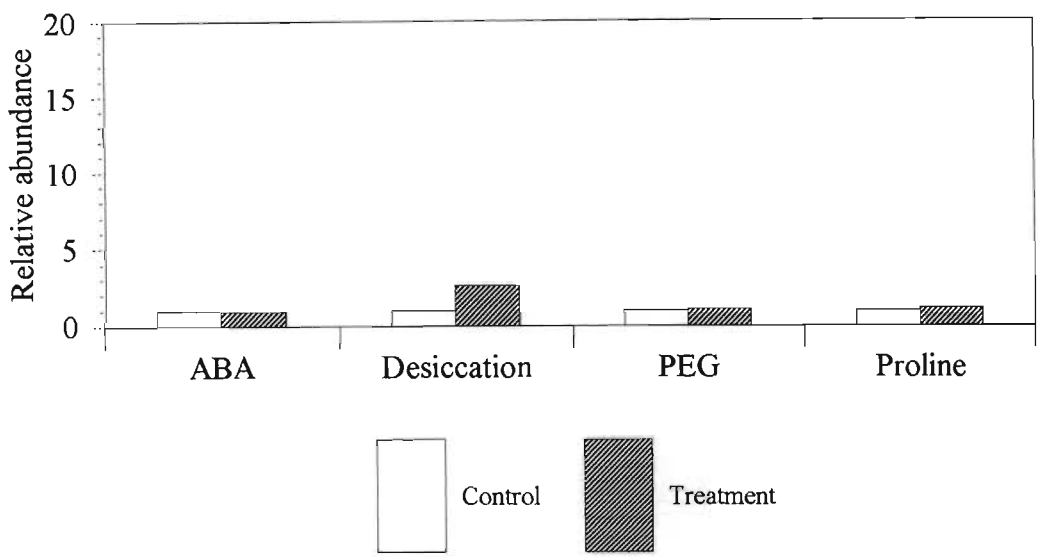
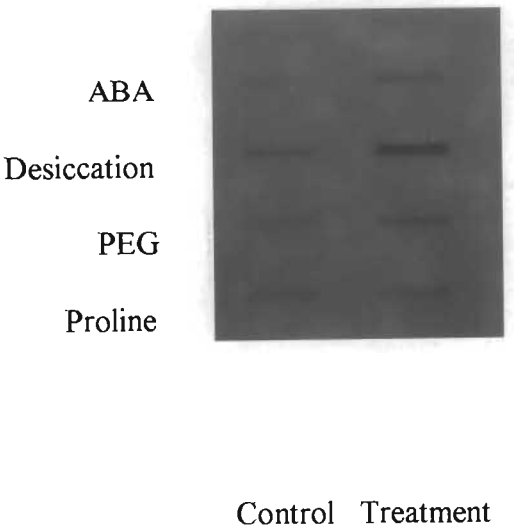


Figure 5.11. Expression of *rws16* transcripts in leaves of the drought sensitive maize cultivar SR52 during ABA, desiccation, osmoticum and proline treatments and controls. **A.** Equal amounts of poly(A)+ RNA (3μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws16* cDNA and autoradiographed. **B.** Relative abundance of *rws16* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

A. SR52, *rws5*



B.

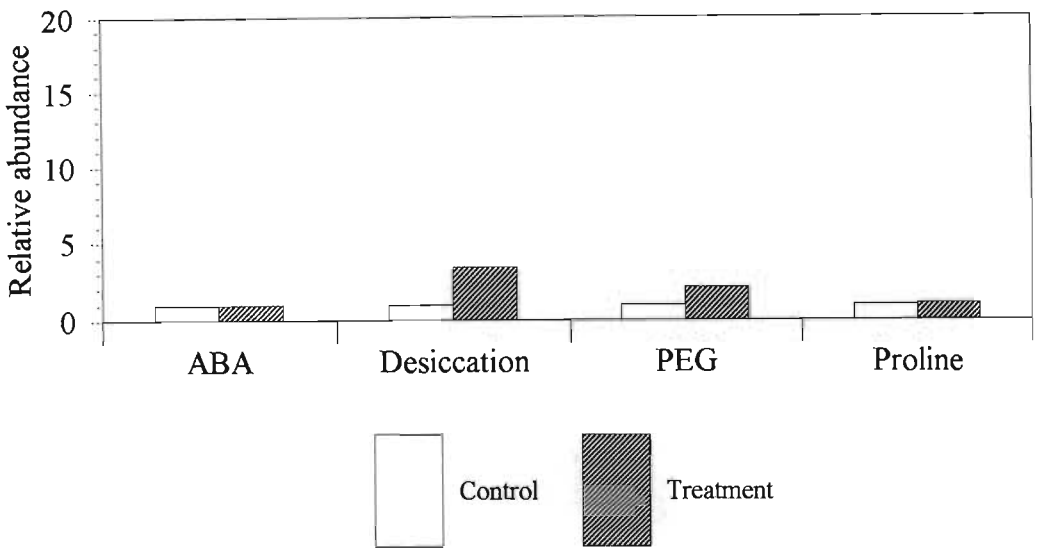
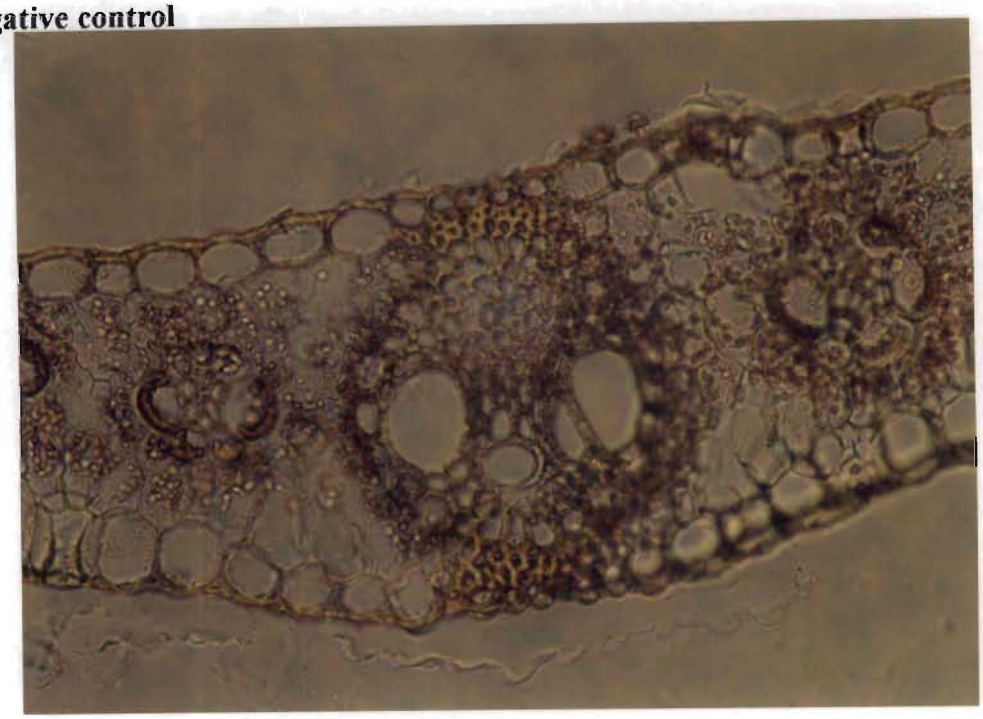


Figure 5.12. Expression of *rws5* transcripts in leaves of the drought sensitive maize cultivar SR52 during ABA, desiccation, osmoticum and proline treatments and controls. **A.** Equal amounts of poly(A)+ RNA (3μg) was slot blotted onto nylon membranes and hybridized with [^{32}P]-labelled *rws5* cDNA and autoradiographed. **B.** Relative abundance of *rws5* transcripts in the different treatments compared to controls as measured by spectrophotometry of the autoradiographs.

5.3.3. *In situ* hybridization

The positive control hybridization with the total cDNA produced a blue precipitate in the leaf sections (Fig. 5.13) but not the root sections (Fig. 14). Neither the negative control with λgt10 probe nor the probes from the three clones produced a visible blue precipitate when hybridized to the leaf cross sections (Figs. 5.13. and 5.14.). The reactions with the control treatments in the leaves are not shown, but no blue precipitate was visible. The reactions with maize cultivar SR52 are not shown, but they had the same results as those for cultivar PAN473.

A. Negative control



B. Positive control

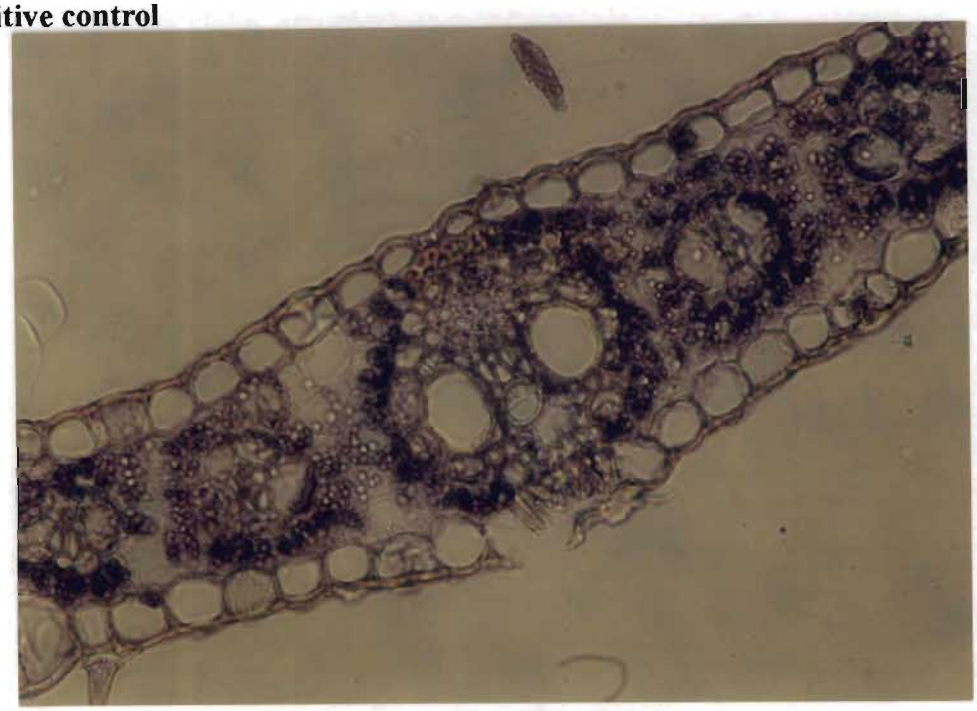
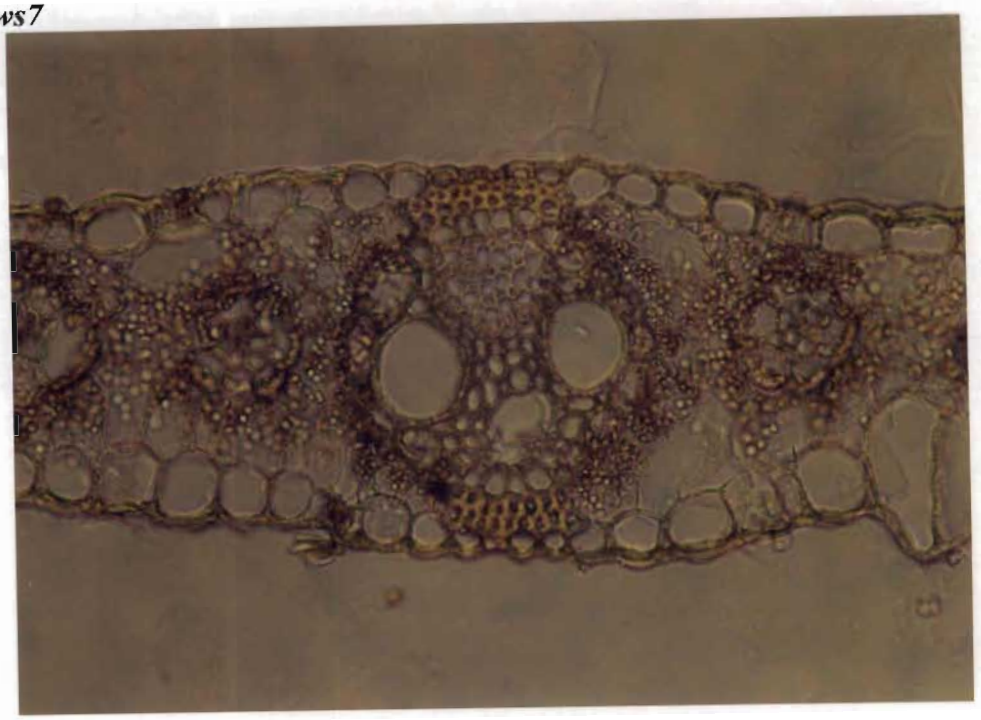


Figure 5.13. Hybridization of DIG-labelled probe to leaf cross sections of maize cultivar PAN473. Magnification x 250. **A.** Negative control with λgt10 probe. **B.** Positive control with total cDNA probe. **C.** *rws7* probe. **D.** *rws16* probe. **E.** *rws5* probe.

C. *rws7*



D. *rws16*

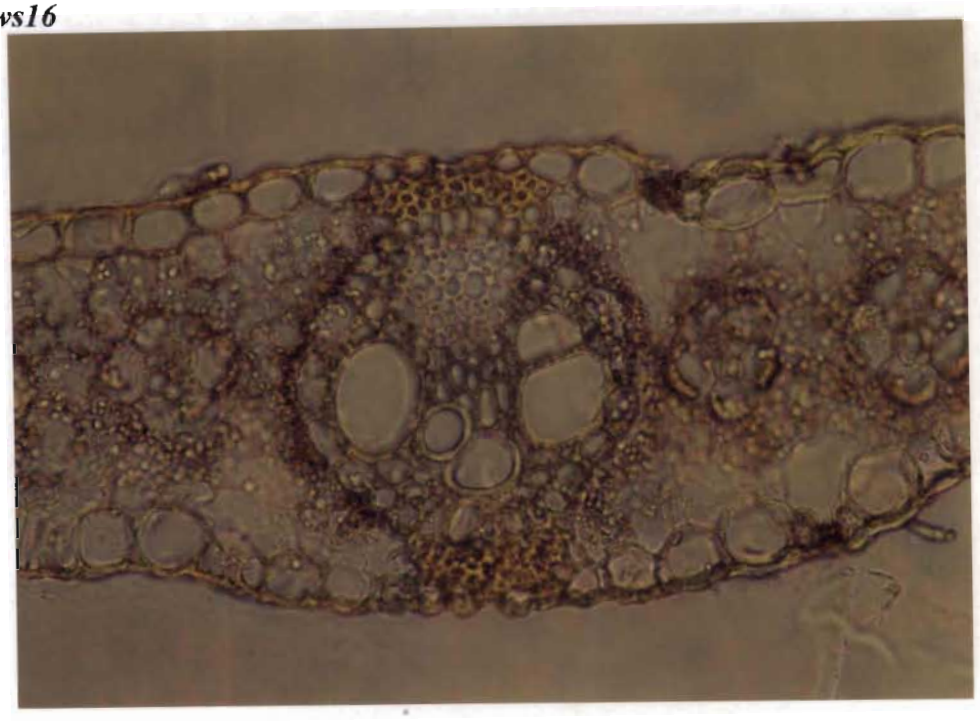


Figure 5.13. (cont'd). Hybridization of DIG-labelled probe to leaf cross sections of maize cultivar PAN473. Magnification x 250. **A.** Negative control with λgt10 probe. **B.** Positive control with total cDNA probe. **C.** *rws7* probe. **D.** *rws16* probe. **E.** *rws5* probe.

E. ***rws5***

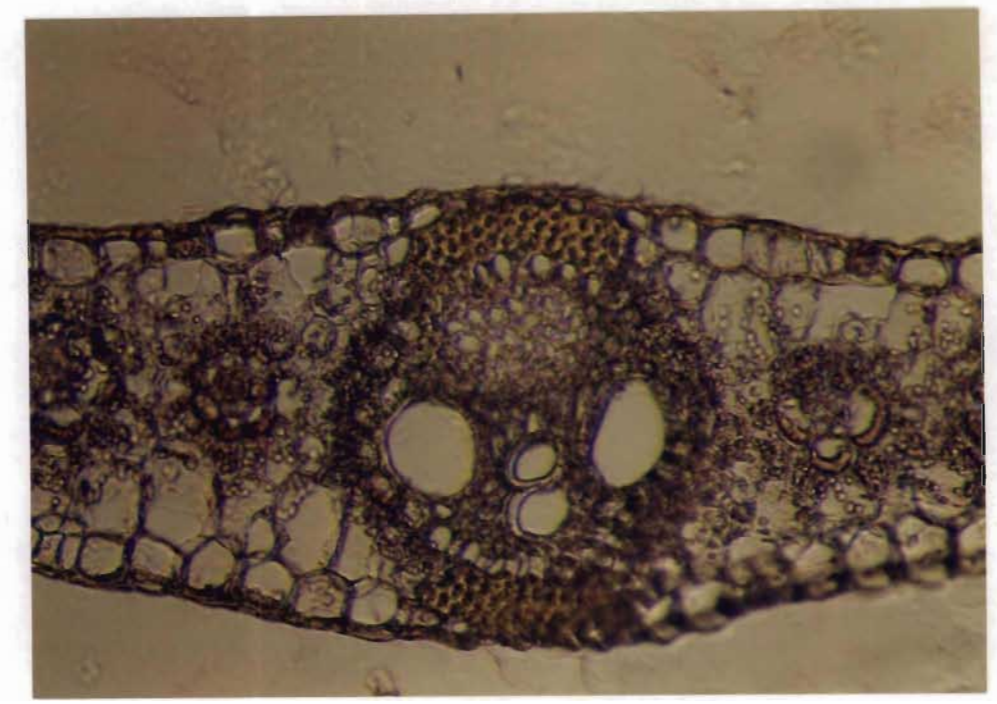
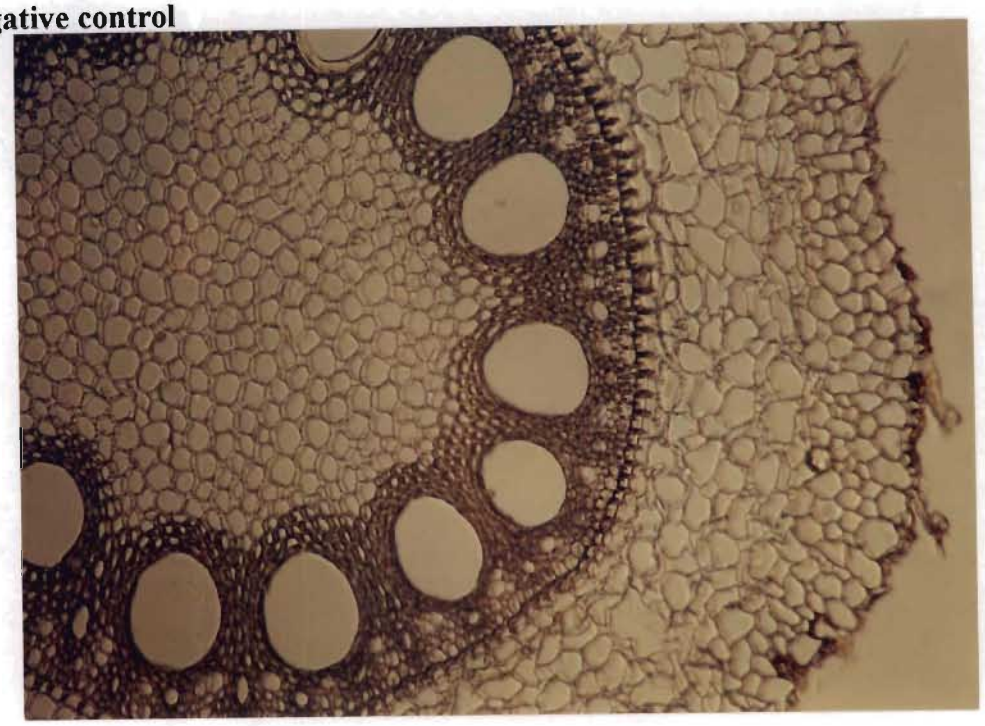


Figure 5.13. (cont'd). Hybridization of DIG-labelled probe to leaf cross sections of maize cultivar PAN473. Magnification x 250. **A.** Negative control with λgt10 probe. **B.** Positive control with total cDNA probe. **C.** *rws7* probe. **D.** *rws16* probe. **E.** *rws5* probe.

A. Negative control



B. Positive control

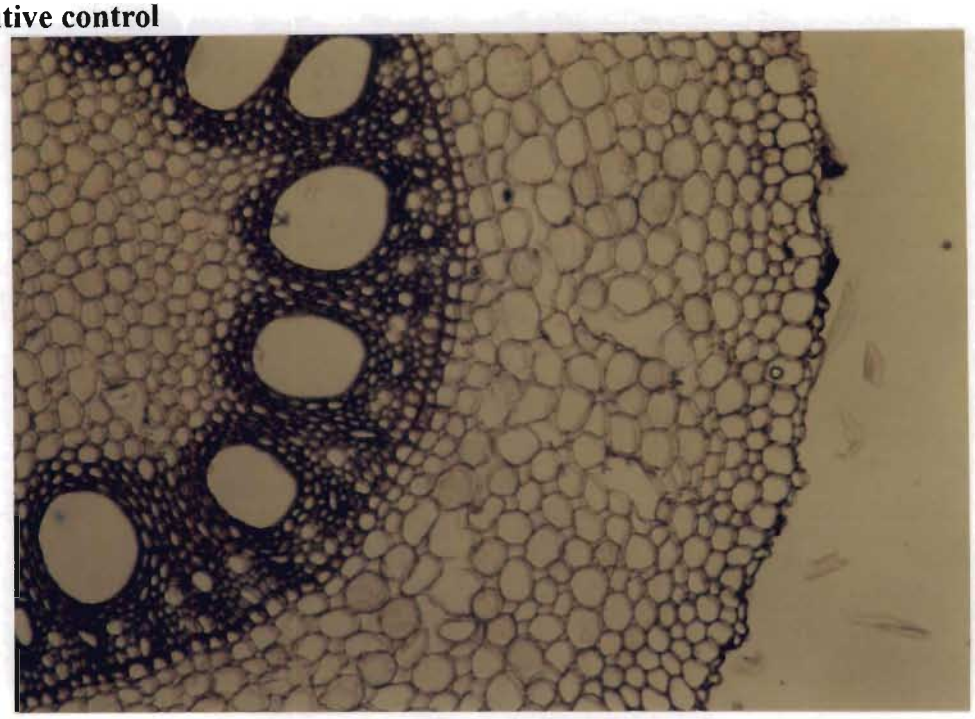


Figure 5.14. Hybridization of DIG-labelled probe to root cross sections of maize cultivar PAN473. Magnification x 125. **A.** Negative control λgt10 probe **B.** Positive control total cDNA probe.

5.4. DISCUSSION

The absolute levels of total mRNA was not measured, but it was assumed to stay the same between all treatments and controls. Where a change in the level of mRNA is detected, therefore, it more accurately represents a change in the proportion of that mRNA in the total population and not necessarily an absolute increase.

mRNA transcripts of all three clones were detected in the control treatments, so the response of these clones to water stress is not *de novo* transcription, but an enhancement of transcription.

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.

The levels of mRNA increased with increasing water stress during the course of the drying cycle. Some other studies have shown gene expression to increase initially with the onset of drought, and to tail off with increasing water stress. The level of expression of a turgor-responsive gene from *Brassica napus* gradually increased on droughting until the plants showed signs of wilting, after which expression levels decreased (STROEHER *et al.* 1995). In the present study the plants were rewatered at onset of wilting, so the level of transcription following wilting was not investigated.

The relative increases in mRNA levels are similar between this study and that of STROEHER *et al.* (1995). In this study the relative increases in the leaves over control levels were between eight- and seventeenfold compared with an elevenfold increase in a turgor-responsive gene from *B. napus* (STROEHER *et al.* 1995).

There was little observable difference between the cultivars in the levels of mRNA at peak stress on day 7, suggesting that the level of gene transcription is not the reason for the difference between the cultivars in their drought resistance. The drought sensitive cultivar

(SR52) did have higher levels of mRNA of all three clones at mid-stress on day 4 compared with the drought tolerant cultivar (PAN473), but this might be due to the fact that SR52 was experiencing greater physiological stress. As demonstrated in the physiological studies, SR52 did not extend its roots into deeper, wetter soil layers in response to soil drying as much as PAN473, and therefore experienced a lower tissue relative water content at the same soil water potential. If *rws7*, *rws16* and *rws5* respond to loss of turgor or lowered tissue water potential, then the increase in levels of mRNA indicated that SR52 has a lower turgor or water potential and has no adaptive significance.

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 which compare stress tolerant and sensitive varieties. LABHILILI *et al.* (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. 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 h after imposition of water stress, whereas in the drought sensitive varieties expression peaked at 30 h and declined at 60 h (LABHILILI *et al.* 1995). HOUDE *et al.* (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 *et al.* 1993). The expression of these four genes was found to be correlated with the 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.*'s (1995) study of *Triticum durum* mentioned above, the reduction in the level of dehydrin gene expression in sensitive varieties at 60 h 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.*'s (1995) study, also mentioned above. The sensitive varieties may experience wilting sooner than the tolerant varieties.

The three clones investigated had increased expression in both leaves and roots, and this has been shown to be the case in most other drought related genes (for review see BRAY 1991). Some genes, however, have been shown to be regulated exclusively in the roots, such as the *AtDr4* and *AtDr21* genes from *A. thaliana* (GOSTI *et al.* 1995) and the *Rab15* gene from wheat (JOSHI *et al.* 1992). Such a pattern of expression might be expected since the roots can be the primary source of the desiccation signal (BLACKMAN & DAVIES 1985) and their function as organs of water uptake is not shared by the rest of the plant, but the existence of unique drought-related proteins exclusive to the root have yet to be discovered. The protein products of the *AtDr* and *Rab* genes mentioned above show close similarities to proteins that occur in other parts of the plant. It is therefore unlikely, given existing evidence, that unique drought-related genes have been ignored by isolating genes from leaf tissue alone.

The levels of mRNA were higher in the leaves than in the roots. Again, this could be due to the fact that the roots were experiencing less physiological stress than the leaves since there is a gradient of water potential from the roots to the leaves and the atmosphere, so that the leaves would have a lower tissue water potential to drive the transpiration stream. Also the leaves are exposed to evaporative demand from the atmosphere.

It has been shown that the signal for physiological and gene expression responses can be initiated in the roots. But in this experiment detached leaves also showed an increase in mRNA levels of all three clones demonstrating that a root signal is not essential for their enhanced expression. It could be argued that the leaves responded to a wounding signal since

they were cut from the plant, but the levels of mRNA of all three clones was much the same between the control leaves that were cut from the plant and frozen straight away compared with the leaf discs that were used as controls in the PEG, ABA and proline treatments. The ratio between the four controls in any one experiment in the level of mRNA transcripts was never higher than 1:1.3 (Figures 5.7 to 5.12). If the clones were responding to a wound signal then the transcript levels would have increased in the leaf discs. A wound induced gene response has been shown to occur within 4 h of wounding (BREDERODE *et al.* 1991), so the incubation time of 24 h in this study would have been long enough to induce a wound response in the three clones. GROSSI *et al.* (1995) also found no wound induced response of drought induced barley genes.

LEONE *et al.* (1994) found that immersion of potato cell suspension cultures directly into 20% PEG led to induction of a different set of proteins compared to cultures gradually adapted to osmoticum by successive increases in PEG concentration. Direct immersion in 20% PEG can therefore be considered a shock which stimulates the plant to express a different set of shock responsive proteins. Immersion in 10% PEG can be considered a moderate water stress that can elicit gene expression (NORDIN *et al.* 1991) and for this reason a concentration of 10% was used in this study.

There was a slight increase over the control in mRNA levels in the osmotic treatment. PEG 6000 is used as an osmoticum because it does not enter the apoplastic space due to its large molecular weight (HOHL & SCHOPFER 1991). Osmotic treatment can simulate a loss of turgor, so the clones could be responding to a loss in turgor rather than to osmotic adjustment within the cells.

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.

In this study the response to drought is a slow response since the drying cycle lasted seven days. The same gene can be involved in both a rapid response and a slow response to stress which is mediated via different *cis*-acting elements in the promoter of the gene. YAMAGUCHI-SHINOZAKI & SHINOZAKI (1994) isolated the *rd28A* gene from *Arabidopsis* which had an ABA-independent rapid response and an ABA responsive slow response which were both regulated by different elements within the promoter region. The slow response was induced twenty min after the start of desiccation, and the slow response was induced after 3 h of desiccation. The present study differs in that the slow response is ABA independent.

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 (GOLLAN *et al.* 1992; SCHURR *et al.* 1992), pH (HARTUNG & SLOVIK 1991), tissue water potential (SAAB *et al.* 1990) and temperature (ALLAN *et al.* 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. 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 & MUNDY 1990; CHANDLER & ROBERTSON 1994).

The proline treatment did not produce an increase in mRNA levels. This perhaps is not surprising since proline has not been shown to produce responses, or at least only to a very limited extent. It has been shown to prevent the opening of stomata (RAJAGOPAL 1981; RAGHAVENDRA & REDDY 1987; KLEIN & ITAI 1989). KLEIN & ITAI (1989) found that L-proline inhibited stomatal opening in *Commelina communis* whereas D-proline had no such effect.

An increase in the level of an mRNA species does not necessarily mean that there will be a concomitant increase in the level of its protein. Regulation of the expression of a gene can

be at the posttranscriptional level by translational efficiency , mRNA stability or protein turnover (GALLIE *et al.* 1989; GALLIE 1993). The degradation of many transcripts may involve polyribosomes, so if translation and the formation of polyribosomes is inhibited during stress, the mRNA will not be degraded and will accumulate to higher levels. Nevertheless, in all the studies in the literature of gene responses to stress, where both mRNA and protein levels have been measured, the increase in mRNA transcript levels is associated with a corresponding increase in the level of the protein, so we can assume the relationship also holds in the present study.

The experiments in this section demonstrate that the three clones studied are regulated in a similar manner in the two maize cultivars, at least at the level of transcription. It is therefore unlikely that the level of transcription of the clones confers drought tolerance.

The absence of a visible blue precipitate with the negative control *in situ* hybridization indicates that there is no nonspecific binding, and the presence of a blue precipitate with the positive control total cDNA hybridization indicates that the reaction is working. However, the absence of a visible blue precipitate with the probes from the three clones indicates that the signal is too weak to be visualized.

CHAPTER 6:
CONCLUSION

6.1. SUMMARY

This study compared the response to water stress of two maize cultivars differing in their tolerance to drought. The study first established that there indeed was a difference between the cultivars in certain physiological responses to soil drying, in that the tolerant cultivar responded by growing a greater amount of roots in the deeper, wetter soil layer and was thus 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 ABA and proline in the leaves under conditions of water stress than the sensitive cultivar.

A differential screening isolated nine cDNAs that were more abundant in the water stress treatment, and three of these were isolated more than once. These three 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. The relative increase in transcription levels of the three genes under water stress also showed a similar pattern between the two cultivars, so again no difference between the cultivars in their sensitivity to water stress could be accounted for by transcription levels.

Of the nine isolated cDNAs, two were sequenced completely, and the other seven sequenced at their 5' ends. Of the two that were sequenced completely, 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.

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 ion channel proteins had previously been isolated by other researchers. 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.

6.2. FUTURE RESEARCH

There are several lines of research that can follow on from this study, all of which involve further characterizing the isolated genes. The first step is to sequence completely the remaining seven cDNAs, and perform a BLAST search to determine if there is homology to known sequences.

It would also be important to obtain full length sequences of the incomplete cDNAs. Full length clones of the cDNA can be obtained by hybridizing back either to the original poly(A)+RNA or to a genomic library. Poly(A)+RNA can be run on a gel and a Northern hybridization performed to determine the size of the complete transcript. The appropriate size fragment can be cut from a low melting point agarose gel and used to make a cDNA library. The library could then be screened with the appropriate cDNA clone to isolate those plaques containing the insert of interest. By isolating several of these clones and PCRing the insert a clone containing a full length insert can be isolated and sequenced. Alternatively a maize genomic library could be purchased and screened for fragments containing the gene which could then be sequenced, but this would contain the full gene with its introns.

Sequencing the gene from a genomic library would give useful information because any

promoter sequences upstream of the transcription start site could be determined. Homology to known promoter sequences might help further characterize the gene product. To investigate what conditions the promoter responds to, the promoter can be fused to a reporter gene such as GUS, and GUS expression investigated under a variety of conditions or in different tissues.

The location of the polypeptide within the cell can be determined by gold immunolabelling. The cDNA insert can be cloned into a high level expression system such as a baculovirus, the polypeptide extracted and monoclonal antibodies raised against it, assuming the polypeptide to be antigenic. The antibodies can be labelled with gold so that their location can be visualized under an electron microscope.

Maize plants can be transformed with the gene of interest under the control of a constitutive promoter to obtain overexpression. 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.

Finally, the use of the isolated genes as selectable markers for breeding for drought resistance can be investigated 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.

6.3. EXAMPLES OF THE USE OF DROUGHT-RELATED GENES

6.3.1. Transformation with foreign genes

Tobacco has been transformed with foreign genes involved in the biosynthesis of osmolytes such as mannitol, fructan and betaine, none of which is the endogenous osmoticum for tobacco, with mixed results. The *Escherichia coli mt1D* gene, which encodes for the

enzyme mannitol-1-phosphate dehydrogenase for mannitol synthesis, was introduced into tobacco under the control of a constitutive promoter. The transformed plants produced mannitol, which is not an endogenous osmolyte of tobacco, and had a greater fresh mass, height and new leaf and root accumulation when grown under high salt conditions compared with untransformed controls. There was no difference between transformed and untransformed tobacco plants in fresh mass accumulation when grown in the absence of NaCl (TARCZYNSKI *et al.* 1993).

Tobacco plants were transformed with the bacterial gene for fructan biosynthesis under the control of a constitutive promoter. When grown hydroponically in 10% PEG to induce osmotic stress, the transformed plants accumulated fructans and other non-structural carbohydrates to a higher level, and had a higher dry mass gain than untransformed controls (PILON-SMITS *et al.* 1995).

ISHITANI *et al.* (1995) transformed tobacco plants with the betaine aldehyde dehydrogenase gene, which synthesises betaine, from barley under the control of a constitutive promoter. In barley, betaine accumulates in response to osmotic stress. Immunoblots detected the presence of the enzyme and its activity in the transformed plants, but no increase in resistance to osmotic stress was observed. Either the biosynthetic pathway or the resistance mechanism is more complex than that demonstrated by the single gene.

6.3.2. Overexpression of endogenous genes

Many of the drought related genes that have been isolated occur in most if not all higher plants, so that many of the genes available for transformation may not be foreign to the plant of interest. It would therefore be more promising to introduce more copies of an endogenous gene to obtain overexpression

KISHOR *et al.* (1995) transformed tobacco with the gene encoding the Δ^1-pyrroline-5-carboxylate synthetase (P5CS) enzyme, which is involved in proline biosynthesis, to obtain

overexpression. The transformed plants produced a higher level of both the enzyme and proline compared with untransformed controls. Concomitant with these higher levels, transformed seedlings had a higher osmotic potential under conditions of water stress after ten days of withholding water, and a greater root biomass under conditions of salinity stress compared with untransformed controls. Under control conditions there were no significant differences between transformed and non-transformed plants in these parameters.

XU *et al.* (1996) transformed rice with the *lea* gene *HVA1* from barley. While the exact function of the protein is not known, it accumulated to high levels in the transformed plants which showed increased tolerance of water deficit and salinity by having higher growth rates, delayed development of damage such as wilting and leaf necrosis, and faster recovery on removal of the stress compared with untransformed controls. This correlated with higher levels of HVA1 protein in the transformed plants.

6.3.3. Detrimental effects of plant transformation

While the above examples show positive or neutral effects of transformation, some transformations may be detrimental to the overall functioning of a plant. This might be expected since the metabolic machinery is complex and interlinked and has evolved through time, and the engineering of a novel gene is likely to upset that balance. For instance, the engineering of increased oxygen radical scavenging would appear to be straightforward and beneficial, but it may reduce the levels of H_2O_2 which also acts as a signal in the pathogen response (MEHDY 1994; ALLEN 1995). The engineered plant may tolerate stress to a greater extent, but it may also be more susceptible to pathogen attack.

6.3.4. Antisense strategies

While it is difficult to imagine a situation where switching off a gene by antisense expression would confer drought tolerance, it is a technique which could be useful in

investigating the function of a gene. By switching off a gene and observing the resulting phenotype, an insight may be gained in the functioning of that gene. The antisense strategy is similar to the study of mutant plants which has elucidated the role of many stress responsive genes. OLIVER *et al.* (1993) transformed tobacco plants with the antisense gene for NADH-hydroxypyruvate reductase to investigate the role of photorespiration in stress protection.

6.3.5. Molecular markers

Anthesis-silking interval (ASI) is associated with drought tolerance in maize, and molecular markers have been used to map genomic segments associated with in ASI. Although the genes themselves have not been located, six quantitative trait loci (QTLs) associated with ASI have been detected which account for 47% of the phenotypic variance (RIBAUT *et al.*1996). It would be interesting to map the position of the genes isolated in this study to see if any of them coincide with these six QTLs.

6.3.6. Determining the function of drought responsive genes

Since the response of a plant to drought is so complex, interpretation of the results of any experiment must be done with caution. It is important to determine if the enhancement of the expression of a certain gene is adaptive or just a consequence of the stress. Certainly the accumulation of mRNA transcripts in itself is not conclusive proof that the protein product performs an adaptive role. Group 3 *lea* transcripts, for instance, accumulate in all tissues of desiccated wheat seedlings, but the protein product only accumulates in the shoots and scutellum (RIED & WALKER-SIMMONS 1993). It is after all the gene product itself that performs a function.

Plants have been transformed with drought related genes and in many instances this has not led to an alteration in drought tolerance (BRAY 1993). The absence of any observed response, however, does not mean the gene product has no adaptive role. The gene product may act in conjunction with other compounds, so that overexpression of the gene by itself

might not change drought tolerance. In water stressed cereal seedlings there is a large increase in the level of the group 2 LEA proteins, the dehydrins (CLOSE & CHANDLER 1990), so it is reasonable to expect them to be involved in adaptation to drought. However, the presence of dehydrins alone is not enough to confer drought tolerance, since they have been shown not to prevent desiccation injury when present in soybean seeds (BLACKMAN *et al.* 1991). ITURRIAGA *et al.* (1992) transformed tobacco plants separately with three genes encoding LEA proteins from the resurrection plant *Craterostigma plantaganeum*, but found no improvement in stress tolerance. It has been suggested that a number of LEA proteins have to be present simultaneously (BARTELS & NELSON 1994), or in conjunction with other substances such as sugars (BLACKMAN *et al.* 1992), to confer desiccation tolerance.

BARTELS & NELSON (1994) identified four basic steps in the investigation of tolerance mechanisms at the molecular level in tolerant plants; 1) to investigate differential gene action between stress and non-stress conditions, 2) to isolate genes correlated with stress tolerance, 3) to prove their role in stress tolerance by plant transformation, and 4) to use the isolated genes for crop improvement either by plant transformation or as a selective marker. At present, there are many examples of steps 1) and 2), including this study, and a few isolated examples of steps 3) and 4). The next phase of research should prove exciting as the role of these genes are elucidated and they are put to use in the breeding of plants for increased drought tolerance.

REFERENCES

ACKERSON, R.C. 1983. Comparative physiology and water relations of two corn hybrids during water stress. *Crop Science* 23:278-283.

ACKERSON, R.C., KRIEG, D.R. & SUNG, F.J.M. 1979. Osmoregulation and leaf conductance of field grown sorghum genotypes. *Crop Science* 20:10-14.

ADAMS, E. & FRANK. L. 1980. Metabolism of proline and the hydroxyprolines. *Annual Review of Biochemistry* 49:1005-1061.

ALLAN, C.A., FRICKER, M.D., WARD, J.L., BEALE, M.H. & TREWAVAS, A.J. 1994. Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. *The Plant Cell* 6:1319-1328.

ALLEN, R.D. 1995. Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiology* 107:1049-1054.

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. & LIPMAN, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410.

ANDERSEN, P.C. & PROEBSTING, W.M. 1984. Water and ion fluxes of abscisic acid-treated root systems of pear, *Pyrus communis*. *Physiologia Plantarum* 60:143-148.

ANDERSON, B.E., WARD, J.M. & SCHROEDER, J.I. 1994. Evidence for an extracellular reception site for abscisic acid in *Commelina* guard cells. *Plant Physiology* 104:1177-1183.

ANGENON, G., VAN MONTAGU, M & DEPICKER, A. 1990. Analysis of the stop codon context in plant nuclear genes. *Federation of European Biochemical Societies* 271:144-146.

ARAKAWA, T. & TIMASHEV, S.N. 1983. Preferential interactions of proteins with solvent components in aqueous amino acid solutions. *Archives of Biochemistry and Biophysics* 224:169-177.

ARAKAWA, T. & TIMASHEFF, S.N. 1985. The stabilization of proteins by osmolytes. *Biophysical Journal.* 47:411-414.

ARMSTRONG, G.A., WEISSHAAR, B. & HAHLBROCK, K. 1992. Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognise

diverse promoter elements with ACGT cores. *The Plant Cell* 4:525-537.

AUSUBEL F.M., BRENT R., KINGSTON R.E., MOORE D.D., SEIDMAN J.G., SMITH J.A. & STRUHL K. 1989. *Current Protocols in Molecular Biology.* John Wiley and Sons, N.Y.

BAKER, J., STEELE, C. & DURE, L. 1988. Sequence and characterization of 6 *Lea* proteins and their genes from cotton. *Plant Molecular Biology* 11:277-291.

BARRS, H.D. & WEATHERLEY, P.E. 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. *Australian Journal of Biological Sciences* 15:413-428.

BARTELS, D. & NELSON, D. 1994. Approaches to improve stress tolerance using molecular genetics. *Plant, Cell and Environment* 17:659-667.

BAUER, D., MULLER, H., REICH, J., RIEDEL, H.,AHRENKIEL, V., WARTHOE, P. & STRAUSS, M. 1993. Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Research* 21:4272-4280.

BEADLE, C.L., STEVENSON, K.R., NEUMANN, H.H., THURTELL, G.W. & KING, K.M. 1973. Diffusive resistance, transpiration, and photosynthesis in single leaves of corn and sorghum in relation to leaf water potential. *Canadian Journal of Plant Science* 53:537-544.

BEARDSELL, M.F. & COHEN, D. 1975. Relationships between leaf water status, abscisic acid levels, and stomatal resistance in maize and sorghum. *Plant Physiology* 56:207-212.

BERRY, J.O., CARR, J.P., & KLESSIG, D.F. 1988. mRNAs encoding ribulose-1,5-bisphosphate carboxylase remain bound to polysomes but are not translated in amaranth seedlings transferred to darkness. *Proceedings of the National Academy of Sciences USA* 85:4190-4194.

BERRY, J.O., BREIDING, D.E. & KLESSIG, D.F. 1990. Light-mediated control of translational initiation of ribulose-1,5-bisphosphate carboxylase in amaranth cotyledons. *The Plant Cell* 2:795-803.

BLACKMAN, P.G. & DAVIES, W.J. 1985. Root to shoot communication in maize plants of the effects of soil drying. *Journal of Experimental Botany* 36:39-48.

BLACKMAN, S.A., WETTLAUFER, S.H., OBENDORF, R.L. & LEOPOLD, A.C. 1991.

Maturation proteins associated with desiccation tolerance in soybean. *Plant Physiology* 96:868-874.

BLACKMAN, S.A., OBENDORF, R.L. & LEOPOLD, A.C. 1992. Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. *Plant Physiology* 100:225-230.

BLACKMAN, S.A., OBENDORF, R.L. & LEOPOLD, A.C. 1995. Desiccation tolerance in developing soybean seeds: The role of stress proteins. *Physiologia Plantarum* 93:630-638.

BLATT, M.R. 1992. K^+ channels of stomatal guard cells: Characteristics of the inward rectifier and its control by pH. *Journal of General Physiology* 99:615-644.

BLATT, M.R. & ARMSTRONG, F. 1993. K^+ channels of stomatal guard cells: Abscisic acid-evoked control of the outward rectifier mediated by cytoplasmic pH. *Planta* 191:330-341.

BLOCK, A., DANGL, J.L., HAHLBROCK, K. & SCHULZE, L.P. 1990. Functional borders, genetic fine structure, and distance requirements of *cis* elements mediating light responsiveness of the parsley chalcone synthase promoter. *Proceedings of the National Academy of Sciences USA* 87:5387-5391.

BLUM, A. & EBERCON, A. 1976. Genotypic responses of sorghum to drought stress III. Free proline accumulation and drought resistance. *Crop Science* 16:428-431.

BORKIRD, C., SIMOENS, C., VILLAROEL, R. AND VAN MONTAGU, M. 1991. Gene expression associated with water stress adaptation of rice cells and identification of two genes as hsp 70 and ubiquitin. *Physiologia Plantarum* 82:449-457.

BOURRET, R.B., BORKOVICH, K.A. & SIMON, M.I. 1991. *Annual Review of Biochemistry* 60:401-441.

BRADFORD, K.J. & CHANDLER, P.M. 1992. Expression of "dehydrin-like" proteins in embryos and seedlings of *Zizania palustris* and *Oryza sativa* during dehydration. *Plant Physiology* 99:488-494.

BRAY, E.A. 1993. Molecular responses to water deficit. *Plant Physiology* 103:1035-1040.

BREDERODE, F.T., LINTHORST, H.J.M. & BOL, J.F. 1991. Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. *Plant Molecular Biology* 17:1117-1125.

CHANDLER, P.M. & ROBERTSON, M. 1994. Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* 45:113-141.

CHAPIN, F.S.III, BLOOM, A.J. FIELD, C.B. & WARING, R.H. 1987. Plant responses to multiple environmental factors. *Bioscience* 37:49-57.

CHIRGWIN J.M., PRZBYLA A.E., MacDONALD R.J., RUTTER W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry* 18: 5294.

CHOU, P.Y. 1989. Prediction of protein structural classes from amino acid compositions. In: FASMAN, G.D. (ed), *Prediction of Protein Structure and the Principles of Protein Conformation*, pp. 549-586. Plenum Press, N.Y.

CHOU, P.Y. & FASMAN, G.D. 1974a. Conformational parameters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. *Biochemistry* 13:211-222.

CHOU, P.Y. & FASMAN, G.D. 1974b. Prediction of protein conformation. *Biochemistry* 13:222-245.

CHOU, P.Y. & FASMAN, G.D. 1978a. Prediction of secondary structure of proteins from their amino acid sequences. *Advances in Enzymology* 47:45-148.

CHOU, P.Y. & FASMAN, G.D. 1978b. Empirical predictions of protein conformation. *Annual Review of Biochemistry* 47:251-276.

CHRISPEELS, M.J. & AGRE, P. 1994. Aquaporins: Water channel proteins of plant and animal cells. *Trends in Biochemical Sciences* 19:421-425.

CHRISPEELS, M.J. & MAUREL, C. 1994. Aquaporins: The molecular basis of facilitated water movement through living plant cells? *Plant Physiology* 105:9-13.

CLOSE, T.J., KORTT, A.A. & CHANDLER, P.M. 1989. A cDNA-based comparison of dehydration-induced proteins dehydrins in barley and corn. *Plant Molecular Biology* 13:95-108.

CLOSE, T.J. & CHANDLER, P.M. 1990. Cereal dehydrins: Serology, gene mapping and potential functional roles. *Australian Journal of Plant Physiology* 17:333-344.

COHEN, A. & BRAY, E.A. 1990. Characterization of three mRNAs that accumulate in wilted tomato leaves in response to elevated levels of endogenous abscisic acid.

Planta 182:27-33.

COLORADO, P., RODRIGUEZ, A., NICOLAS, G & RODRIGUEZ, D. 1994. Abscisic acid and stress regulate gene expression during germination of chick-pea seeds. *Physiologia Plantarum* 91:461-467.

COMAI, L., TANESE, N. & TJIAN, R. 1992. The TATA-binding protein and associated factors are integral components of the RNA polymerase 1 transcription factor, SL1. *Cell* 68:965-976.

CORNISH, K. & ZEEVAART, J.A.D. 1985. Movement of abscisic acid into the apoplast in response to water stress in *Xanthium strumarium* L. *Plant Physiology* 78:623-626.

COWAN, I.R., RAVEN, J.A., HARTUNG, W. & FARQUHAR, G.D. 1982. A possible role for abscisic acid in coupling stomatal conductance and photosynthetic carbon metabolism in leaves. *Australian Journal of Plant Physiology* 9:489-498.

COWAN, A.K. & RAILTON, I.D. 1986. Chloroplasts and the biosynthesis and catabolism of abscisic acid. *Journal of Plant Growth Regulation* 4:211-224.

CREELMAN, R.A., GAGE, D.A., STULTS, J.T. & ZEEVAART, J.A.D. 1987. Abscisic acid biosynthesis in leaves and roots of *Xanthium strumarum*. *Plant Physiology* 85:726-732.

CREIGHTON, T.E. 1983. *Proteins: Structures and Molecular Properties*, Chapter 4. WH Freeman, New York.

CSONKA, L.N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews* 53:121-147.

CURTI, G., MASSARDI, F. & LADO, P. 1993. Synergistic activation of plasma membrane H^+-ATPase in *Arabidopsis thaliana* cells by turgor decrease and by fusicoccin. *Physiologia plantarum* 87:592-600.

DALLMIER, K.A. & STEWART, C.R. 1992. Effect of exogenous abscisic acid on proline dehydrogenase activity in maize (*Zea mays* L.). *Plant Physiology* 99:762-764.

DARBY, N.J. & CREIGHTON, T.E. 1993. *Protein Structure*, pp. 1-21, 23-41, 63-89. IRL Press, Oxford.

DAVIES, W.J. & ZHANG, J. 1991. Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Physiology and*

Molecular Biology 42:55-76.

DAVIES, W.J., TARDIEU, F. & TREJO, C.L. 1994. Update on long-distance signaling: How do chemical signals work in plants that grow in drying soil? *Plant Physiology* 104:309-314.

DEAN, C., TAMAKI, S., DUNSMUIR, P., FAVREAU, M., KATAYAMA, C., DOONER, H. & BEDBROOK, J. 1986. mRNA transcripts of several plant genes are polyadenylated at multiple sites *in vitro*. *Nucleic Acids Research* 14:2229-2240.

DELAUNEY, A.J. & VERNA, D.P.S. 1990. A soybean gene encoding Δ^1-pyrroline-5-carboxylate reductase was isolated by functional complementation in *Escherichia coli* and is found to be osmoregulated. *Molecular and General Genetics* 221:299-305.

DING, J.P. & PICKARD, B.G. 1993. Mechanosensory calcium-selective cation channels in epidermal cells. *Plant Journal* 3:83-110.

DOLFERUS, R., JACOBS, M., PEACOCK, W.J. & DENNIS, E. 1994. Differential interaction of promoter elements in stress responses of the *Arabidopsis Adh* gene. *Plant Physiology* 105:1075-1087.

DONALD, R.G.K. & CASHMORE, A.R. 1990. Mutation of either G-box or I-box sequences profoundly affects expression from the *Arabidopsis rbcS-1A* promoter. *EMBO Journal* 9:1717-1726.

DU PLESSIS, D.P. & DIJKHUIS, F.J. 1967. The influence of the time lag between pollen shedding and silking on the yield of maize. *South African Journal of Agricultural Science 10*: 667-674.

DURE-III, L., CROUCH, M., HARADA, J., HO, T.H.D., MUNDY, J., QUATRANO, R., THOMAS, T. & SUNG, Z.R. 1989. Common amino acid sequence domains among LEA proteins of higher plants. *Plant Molecular Biology* 12:475-486.

DURE-III, L. 1993. Structural motifs in Lea proteins. In: CLOSE, T.J. & BRAY, E.A. (eds), *Plant Responses to Cellular Dehydration during Environmental Stress. Current Topics in Plant Physiology, Vol 10*, pp. 91-103. American Society of Plant Physiologists, Rockville, MD.

EVANS, D.E., BRIARS, S.A. & WILLIAMS, L.E. 1991. Active calcium transport by plant cell membranes. *Journal of Experimental Botany* 42:285-303.

FISCHER, R.A. & TURNER, N.C. 1978. Plant productivity in the arid and semiarid zones. *Annual Review of Plant Physiology* 29:277-317.

FISCHER, E., RASCHKE, K. & STITT, M. 1986. Effects of abscisic acid on photosynthesis in whole leaves: changes in CO_2 assimilation, levels of carbon-reduction-cycle intermediates, and activity of ribulose-1,5-bisphosphate carboxylase. *Planta* 169:536-545.

FORST, S. & INOUYE, M. 1988. Environmentally regulated gene expression for membrane proteins in *Escherichia coli*. *Annual Review of Cell Biology* 4:21-42.

GALLIE, D.R. 1993. Posttranscriptional regulation of gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 44:77-105.

GALLIE, D.R, LUCAS, W.J. & WALBOT, V. 1989. Visualizing mRNA expression in plant protoplasts: factors influencing efficient mRNA uptake and translation. *The Plant Cell* 1:301-311.

GEHRING, C.A., IRVING, H.R. & PARISH, R.W. 1990. Effects of auxin and abscisic acid on cytosolic clacium and pH in plant cells. *Proceedings of the National Academy of Sciences USA* 87:9645-9649.

GILROY, S., READ, N.D. & TREWAVAS, A.J. 1990. Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. *Nature* 346:769-771.

GILROY, S., FRICKER, M.D., READ, N.D. & TREWAVAS, A.J. 1991. Role of calcium in signal transduction of *Commelina* guard cells. *The Plant Cell* 3:333-334.

GILROY, S. & JONES, R.L. 1994. Perception of gibberellin and abscisic acid at the external face of the plasma membrane of barley (*Hordeum vulgare* L.) aleurone protoplasts. *Plant Physiology* 104:1185-1192.

GIRAUDAT, J., PARCY, F., BERTAUCHE, N., GOSTI, F. & LEUNG, J. 1994. Current advances in abscisic acid action and signalling. *Plant Molecular Biology* 26:1557-1577.

GIROUSSE, C., BOURNOVILLE, R. & BONNEMAIN, J.-L. 1996. Water deficit-induced changes in proline and some other amino acids in the phloem sap of alfalfa. *Plant Physiology* 111:109-113.

GODAY, A., JENSEN, A.B., CULIANEZ-MACIA, F.A., ALBA, M.M., FIGUERAS, M.,

SERRATOSA, J., TORREMT, M. & PAGES, M. 1994. The maize abscisic-acid-responsive protein Rab17 is located in the nucleus and interacts with nuclear localization signals. *The Plant Cell* 6:351-360.

GOLLAN, T., SCHURR, U. & SCHULZE, E.-D. 1992. Stomatal responses to drying soil in relation to changes in the xylem sap composition of *Helianthus annuus*. I. The concentration of cations, anions and amino acids in, and pH of the sylem sap. *Plant Cell and Environment* 15:551-559.

GOMEZ, J., SANCHEA-MARTINEZ, D., STIEFEL, V., RIGAU, J., PUIGDOMENECH, P. & PAGES, M. 1988. A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. *Nature* 334:262-264.

GOSTI, F., BERTAUCHE, N., VARTANIAN, N. & GIRAUDAT, J. 1995. Abscisic-acid-dependent and -independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*. *Molecular and General Genetics* 246:10-18.

GOWING, D.J.G., DAVIES, W.J. & JONES, H.G. 1990. A positive root-sourced signal as an indicator of soil drying in apple, *Malus* X *domestica* Borkh. *Journal of Experimental Botany* 41:1535-1540.

GROSSI, M., GULLI, M., STANCA, M. & CATTIVELLI, l. 1995. Characterization of two barley genes that respond rapidly yo dehydration stress. *Plant Science* 105:71-80.

GUERRERO, F.D., JONES, J.T. & MULLET, J.E. 1990. Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. *Plant Molecular Biology* 15:11-26.

GUILTINAN, M.J., MARCOTTE, W.R. & QUATRANO, R.S. 1990. A plant leucine zipper protein that recognises an abscisic acid response element. *Science* 250:267-271.

GUSTIN, M.C., ZHOU, X-L., MARTINEC, B. & KUNG, C. 1988. A mechanosensitive ion channel in the yeast plasma membrane. *Science* 242:762-765.

HALL, A.J., GINZO, H.D., LEMCOFF, J.H. & SORIANO, A. 1980. Influence of drought during pollen-shedding on flowering, growth and yield of maize. *Zeitschrift für Acker- und Pflanzenbau* 149:287-298.

HALL, A.J., LEMCOFF, J.H. & TRAPANI, N. 1981. Water stress before and during

flowering in maize and its effects on yield, its components, and their determinations. *Maydica* 26:19-38.

HANSON, A.D., NELSEN, C.E. & EVERSON, E.H. 1977. Evaluation of free proline accumulation as an index of drought resistance using two contrasting barley cultivars. *Crop Science* 17:720-726.

HARADA, J.J., DELISLE, A.J., BADEN, C.S. & CROUCH, M.L. 1989. Unusual sequence of an abscisic acid-inducible mRNA which accumulates late in *Brassica napus* seed development. *Plant Molecular Biology* 12:395-401.

HARTUNG, W., GIMMLER, H., HEILMAN, B. & KAISER, G. 1980. The site of abscisic acid metabolism in mesophyll cells of *Spinacia oleracea*. *Plant Science Letters* 18:359-364.

HARTUNG, W., HEILMAN, B. & GIMMLER, H. 1981. Do chloroplast play a role in abscisic acid synthesis? *Plant Science Letters* 22:235-242.

HARTUNG, W., GIMMLER, H. & HEILMAN, B. 1982. The compartmentation of-ABA-biosynthesis, ABA-metabolism, and ABA-conjugation. In: WAREING, P.F. (ed), *Plant Growth Substances*, pp. 325-333. Academic, London.

HARTUNG, W., KAISER, W.M. & BURSCHKA, C. 1983. Release of abscisic acid from strips under osmotic stress. *Zeitschrift für Pflanzenphysiologie* 97:131-138.

HARTUNG, W., RADIN, J.W. & HENDRIX, J.L. 1988. Abscisic acid movement into the apoplastic solution of water-stressed cotton leaves: role of apoplastic pH. *Plant Physiology* 86:908-913.

HARTUNG, W. & SLOVIK, S. 1991. Physicochemical properties of plant growth regulators and plant tissues determine their distribution and redistribution: stomatal regulation by abscisic acid in leaves. *New Phytologist* 119:361-382.

HAYZER, D.J. & LEISINGER, T. 1981. Proline biosynthesis in *Escherichia coli*. *The Biochemistry Journal* 197:269-274.

HEDRICH, R., BUSCH, H. & RASCHKE, K. 1990. Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. *EMBO Journal* 9:3889-3892.

HEILMAN, B., HARTUNG, W. & GIMMLER, H. 1980. The distribution of abscisic acid between chloroplasts and cytoplasm of leaf cells and the permeability of the

chloroplast envelope for abscisic acid. *Zeitschrift für Pflanzenphysiologie* 97:67-78.

HENDERSON, R. & UNWIN, P.N.T. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* 257:28-32.

HENSON, I.E. 1984. Inhibition of abscisic acid accumulation in seedling shoots of pearl millet (*Pennisetum americanum* [L.] Leeke) following induction of chlorosis by norflurazon. *Zeitschrift für Pflanzenphysiologie* 1145:35-43.

HINCHA, D.K., HOFNER, R., SCHWAB, K.B., HEBER, U. & SCHMITT, J.M. 1987. Membrane rupture is the common cause of damage to chloroplast membranes in leaves injured by freeaing or excess wilting. *Plant Physiology* 83:251-253.

HINCHA, D.K., HEBER, U. & SCHMITT, J.M. 1990. Proteins from frost-hardy leaves protect thylakoids against mechanical damage in vitro. *Planta* 180:416-419.

HOHL, M. & SCHOPFER, P. 1991. Water relations of growing maize coleoptiles. *Plant Physiology* 95:716-722.

HORNBERG, C. & WEILER, E.W. 1984. High-affinity binding sites for abscisic acid on the plasmalemma od *Vicia faba* guard cells. *Nature* 310:321-324.

HOUDE, M., DANYLUK, J., LALIBERTÉ, J.P., RASSART, E., DHINDSA, R. & SARHAN, P. 1992. Cloning, characterization and expression of a cDNA encoding a 50 kilodalton protein specifically induced by cold acclimation in wheat. *Plant Physiology* 99:1381-1387.

HOYT, M.A., KNIGHT, D.M., DAS, A., MILLER, H.I. & ECHOLS, H. 1982. Control of phage λ development by stability and synthesis of cII protein: Role of the viral *cIII* and host *hflA*, *himA*, and *himD* genes. *Cell* 31:565-573.

HSIAO, T.C. 1973. Plant responses to water stress. *Annual Review of Plant Physiology* 24: 519-570.

HSIAO, T.C., ACEVEDO, E., FEREERES, E. & HENDERSON, D.W. 1976. Stress metabolism: water stress, growth, and osmotic adjustment. *Philosophical Transactions of the Royal Society of London, Series B* 273: 479-500.

HUBICK, K.T. & REID, D.M. 1980. A rapid method for the extraction and analysis of abscisic acid from plant tissue. *Plant Physiology* 65: 523-525.

HUGHES, D.A. 1994. Histidine kinases hog the limelight. *Nature* 369:187-188.

HUNT, A.G. 1994. Messenger RNA 3' end formation in plants. *Annual Review of Plant*

Physiology and Plant Molecular Biology 45:47-60.

HUNT, A.G. & MACDONALD, M. 1989. Deletion analysis of the polyadenylation signal of a pea ribulose-1,5-bisphosphate carboxylase small subunit gene. *Plant Molecular Biology* 13:125-138.

HURD, E.A. 1974. Phenotype and drought tolerance in wheat. *Agricultural Meteorology* 14:39-55.

INGELBRECHT, I.L.W., HERMAN, L.M.F., DEKEYSER, R.A., VAN MONTAGU, M.C. & DEPICKER, A.G. 1989. Different 3' end regions strongly influence the level of gene expression in plant cells. *The Plant Cell* 1:671-680.

IRVING, H.R., GEHRING, C.A. & PARISH, R.W. 1992. Changes in cytoplasmic pH and calcium of guard cells precede stomatal closure. *Proceedings of the National Academy of Sciences USA* 89:1790-1794.

ISHITANI, , M., NAKAMURA, T., HAN, S.Y. & TAKABE, T. 1995. Expression of the betaine aldehyde dehydrogenase gene in tobacco in response to osmotic stress and abscisic acid. *Plant Molecular Biology* 27:307-315.

ITAI, C. & PALEG, L.G. 1982. Response of water stressed *Hordeum distichum* L. and *Cucumis sativus* to proline and betaine. *Plant Science Letters* 25:329-335.

ITURRIAGA, G., SCHNEIDER, K., SALAMINI, F. & BARTELS, D. 1992. Expression of desiccation-related proteins from the resurrection plant *Craterostigma plantaganeum* in transgenic tobacco. *Plant Molecular Biology* 20:555-558.

JÄHNIG, F. 1989. Structure prediction for membrane proteins. In: FASMAN, G.D. (ed), *Prediction of Protein Structure and the Principles of Protein Conformation*, pp. 707-717. Plenum Press, N.Y.

JÄHNIG, F. 1990. Structure predictions of membrane proteins are not that bad. *Trends in Biochemical Sciences* 15:93-95.

JANSSEN, M.G. & MARKHART, A.H. III. 1993. Gradual soil drying promotes stomatal closure and xylem ABA increase in common tepary bean (abstract No. 878). *Supplement to Plant Physiology* 102:153.

JONES, J.T. & MULLET, J.E. 1995a. A salt- and dehydration-inducible pea gene, *Cyp15a*, encodes a cell-wall protein with sequence similariey to cysteine proteases. *Plant Molecular Biology* 28:1055-1065.

JONES, J.T. & MULLET, J.E. 1995b. Developmental expression of a turgor-responsive gene that encodes an intrinsic membrane protein. *Plant Molecular Biology* 28:983-996.

JONES, M.M., TURNER, N.C. & OSMOND, C.B. 1981. Mechanisms of drought resistance. In: PALEG, L.G. & ASPINALL, D. (eds), *The Physiology and Biochemistry of Drought Resistance in Plants*, pp 15-37. Academic Press, Australia.

JOSHI, C.P. 1987a. Putative polyadenylation signals in nuclear genes of higher plants: a compilation and analysis. *Nucleic Acids Research* 15:9627-9640.

JOSHI, C.P. 1987b. An inspection of the domain between putative TATA box and translation start site in 79 plant genes. *Nucleic Acids Research* 16:6643-6653.

JOSHI, C.P., KING, S.W. & NGUYEN, H.T. 1992. Molecular cloning and characterization of a cDNA encoding a water stress protein (WSP23) from wheat roots. *Plant Science* 86:71-82.

KAHN, T.L., FENDER, S.E., BRAY, E.A. & O'CONNELL, M.A. 1993. Characterization of expression of drought- and abscisic acid-regulated tomato genes in the drought resistant species *Lycopersicon pennellii*. *Plant Physiology* 103:597-605.

KISHOR, P.B.K., HONG, Z., MIAO, G.-H., HU, C.-A.A. & VERMA, D.P.S. 1995. Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* 108:1387-1394.

KLEIN, A. & ITAI, C. 1989. Is proline involved in stomata regulation of *Commelina communis* plants recovering from salinity stress? *Physiologia plantarum* 75:399-404.

KOSHIMIZU, K., INUI, M., FUKUI, H. & MITSUI, T. 1968. Isolation of (+)-abscisic-β-d-glucopyranoside from immature fruit of *Lupinus luteus*. *Agricultural and Biological Chemistry* 32:789-791.

KOZAK, M. 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. *Nucleic Acids Research* 9:5233-5252.

KOZAK, M. 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Research* 12:857-872.

KYTE, J. & DOOLITTLE, R.F. 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157:105-132.

LABHILILI, M., JOUDRIER, P. & GAUTIER, M.-F. 1995. Characterization of cDNAs encoding *Triticum durum* dehydrins and their expression patterns in cultivars that differ in drought tolerance. *Plant Science* 112:219-230.

LANDRY, D., SULLIVAN, S., NICOLAIDES, M., REDHEAD, C., EDELMAN, A., FIELD, M., AL-AWQATI, Q. & EDWARDS, J. 1993. Molecular cloning and characterization of p64, a chloride channel protein from kidney microsomes. *The Journal of Biological Chemistry* 268:14948-14955.

LANDSCHULZ, W.H., JOHNSON, P.F. & McKNIGHT, S.L. 1988. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* 240:1759-1764.

LARHER, F., LEPORT, L., PETRIVALSKY, M. & CHAPPART, M. 1993. Effectors for the osmoinduced proline response in higher plants. *Plant Physiology and Biochemistry* 31:911-922.

LEHMANN, H. & GLUND, K. 1986. Abscisic acid metabolism-vacuolar/extravacuolar distribution of metabolites. *Planta* 168:559-562.

LEONE, A., COSTA, A., TUCCI, M. & GRILLO, S. 1994. Comparative analysis of short- and long-term changes in gene expression caused by low water potential in potato (*Solanum tuberosum*) cell-suspension cultures. *Plant Physiology* 106:703-712.

LEVITT, J. 1980. Measurement of drought avoidance. In: LEVITT, J. (ed), *Responses of Plants to Environmental Stresses. Volume 2. Water, Radiation, Salt and Other Stresses*, Chapter 6. Academic Press, New York.

LIANG, P. & PARDEE, A.B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967-971.

LIANG, P., AVERBOUKH, L & PARDEE, A.B. 1993. Distribution and cloning of eukaryotic mRNAs by means if differential display: refinements and optimization. *Nucleic Acids Research* 21:3269-3275.

LIU, D., RAGHOTHAMA, K.G., HASEGAWA, P.M. & BRESSAN R.A. 1994. Osmotin overexpression in potato delays development of disease symptoms. *Proceedings of the National Academy of Sciences USA* 91:1888-1892.

LOAKE, G.J., FAKTOR, O., LAMB, C.J. & DIXON, R.A. 1992. Combination of H-box (CCTACCN 7CT) and G-box (CACGTG) cis elements is necessary for feed-forward stimulation of chalcone synthase promoter by the phenylpropanoid-pathway intermediate ρ-coumaric acid. *Proceedings of the National Academy of Sciences USA* 89:9230-9234.

LORENS, G.F., BENNET, J.M. & LOGGALE, L.B. 1987a. Differences in drought resistance between two corn hybrids. I. Water relations and root length density. *Agronomy Journal* 79:802-807.

LORENS, G.F., BENNET, J.M. & LOGGALE, L.B. 1987b. Differences in drought resistance between two corn hybrids. II. Component analysis of growth rates. *Agronomy Journal* 79:808-813.

LOVEYS, B.R. & MILBORROW, B.V. 1981. Isolation of and characterization of 1'-*O*-abscisic acid-β-*D*-glucopyranoside from vegetative tomato tissue. *Physiologia Plantarum* 46:79-84.

LUDEVID, D., HÖFTE, H., HIMELBLAU, E. & CHRISPEELS, M.J. 1992. The expression pattern of the tonoplast intrinsic protein γ-TIP in *Arabidopsis thaliana* is correlated with cell enlargement. *Plant Physiology* 100:1633-1639.

LUDEWIG, M.L., DÖRFFLING, K. & SEIFERT, H. 1988. Abscisic acid and water transport in sunflowers. *Planta* 175:325-333.

MacROBBIE, E.A.C. 1989. Calcium influx at the plasmalemma of isolated guard cells of *Commelina communis*. *Planta* 178:231-241.

MacROBBIE, E.A.C. 1991. Effect of ABA on ion transport and stomatal regulation. In: DAVIES, W.J. & JONES, H.G. (eds), *Abscisic Acid Physiology and Biochemistry*, pp. 153-168. BIOS Scientific Publishers, Oxford.

MacROBBIE, E.A.C. 1992. Calcium and ABA-induced stomatal closure. *Philosophical Transactions of the Royal Society of London, Series B* 338:5-18.

MAEDA, T., WURGLER-MURPHY, S.M. & SIATO, H. 1994. A two-component system that regulates an osmosensing MAP kinase in yeast. *Nature* 369:242-245.

MALIK, R.S., DHANKAR, J.S. & TURNER, N.C. 1979. Influence of soil water deficits on root growth in cotton seedlings. *Plant and Soil* 53:109-115.

MARQUSEE, S. & BALDWIN, R.L. 1987. Helix stabilization by Glu-...Lys+ salt bridges

in short peptides of *de novo* design. *Proceedings of the National Academy of Sciences USA* 84:8898-8902.

McAINSH, M.R., BROWNLEE, C. & HETHERINGTON, A.M. 1990. Abscisic acid-induced elevation of guard cell C^{2+} precedes stomatal closure. *Nature* 343:186-188.

McAINSH, M.R., BROWNLEE, C. & HETHERINGTON, A.M. 1992. Visulaizing changes in cytosolic-free Ca^{2+} during the response of stomatal guard cells to abscisic acid. *The Plant Cell* 4:1113-1122.

McCARTY, D.R., HATTORI, T., CARSON, C.B., VASIL, V., LAZAR, M. & VASIL, I.K. 1991. The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* 66:895-905.

McKHANN, H.I. & HIRSCH, A.M. 1993. *In situ* localization of specific mRNAs in plant tissues. In: GLICK, B.R. & THOMPSON, J.E. (eds), *Methods in Plant Molecular Biology and Biotechnology*, pp. 179-205. CRC Press, Boca Raton.

McLAUCHLAN, J., GAFFNEY, D., WHITTON, J.L. & CLEMENTS, J.B. 1985. The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNAs 3' termini. *Nucleic Acids Research* 13:1347-1368.

MEHDY, M.C. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiology* 105:467-472.

MILBORROW, B.V. 1978. The stability of conjugated abscisic acid during wilting. *Journal of Experimental Botany* 29:1059-1066.

MILBORROW, B.V. 1983. Pathways to and from abscisic acid. In: ADDICOTT, F.T. (ed), *Abscisic Acid*, pp. 79-111. Praeger, New York.

MOORE, R. & SMITH, J.D. 1984. Growth, graviresponsiveness and abscisic acid content of *Zea mays* seedlings treated with fluoridone. *Planta* 162:342-344.

MUNDY, J. & CHUA, N-H. 1988. Abscisic acid and water-stress induce the expression of a novel rice gene. *EMBO Journal* 7:2279-2286.

MUNNS, R. 1988. Why measure osmotic adjustment? *Australian Journal of Plant Physiology* 15:717-726.

MUNNS, R., BRADY, C.J. & BARLOW, E.W.R. 1979. Solute accumulation in the apex and leaves of wheat during water stress. *Australian Journal of Plant Physiology*

6:379-389.

MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15:473-497.

MURPHY, G.J.P. 1984. Metabolism of R,S-[2-^{14}C] abscisic acid by non-stressed and water-stressed detached leaves of wheat (*Triticum aestivum* L.). *Planta* 160:250-255.

MURRAY, N.E., BRAMMAR, W.J. & MURRAY, K. 1977. Lambdoid phages that simplify the recovery of *in vitro* recombinants. *Molecular and General Genetics* 150:53-61.

NEILL, S.J., HORGAN, R. & PARRY, A.D. 1986. The carotenoid and abscisic acid content of viviparous kernels and seedlings of *Zea mays* L. *Planta* 169:87-96.

NISHIKAWA, K. 1983. Assessment of secondary structure prediction of proteins: comparison of computerized Chou-Fasman method with others. *Biochimica et Biophysica Acta* 748:285-299.

NONHEBEL, H.M. & MILBORROW, B.V. 1986. Incorporation of ^{2}H from $^{2}H_2O$ into ABA in tomato shoots: evidence for a large pool of precursors. *Journal of Experimental Botany* 37:1533-1541.

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

NOVER,L., SCHARF, K.D. & NEUMANN, D. 1989. Cytoplasmic heat shock granulesare formed from precursor particles and are associated with a specific set of mRNAs. *Molecular and Cellular Biology* 9:1298-1308.

OBER, E.S. & SHARP, R.E. 1994. Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials. I. Requirement for increased levels of abscisic acid. *Plant Physiology* 105:981-987.

OLIVER, M.J., FERGUSON, D.L., BURKE, J.J. & VELTEN, J. 1993. Inhibition of tobacco NAPh-hydroxypyruvate reductase by expression of a heterologous antisense RNA derived from a cucumber cDNA: Implications for the machanism of action of antisense RNAs. *Molecular and General Genetics* 239:425-434.

OTA, I.M. & VARSHAVSKY, A. 1993. A yeast protein similar to bacterial two-

component regulators. *Science* 262:566-569.

OWEN, J.H. & NAPIER, J.A. 1988. Abscisic acid: New ideas on its role and mode of action. *Plants Today* 2:55-59.

PAO, G.M., WU, L.-F., JOHNSON, K.D., HOFTE, H., CHRISPEELS, M.J., SWEET, G., SANDAL, N.N. & SAIER, M.H. 1991. Evolution of the MIP family of integral membrane transport protiens. *Molecular Microbiology* 5:33-37.

PARRY, A.D., BABIANO, M.J. & HORGAN, R. 1990. The role of *cis*-carotenoids in abscisic acid biosynthesis. *Planta* 182:118-128.

PARRY, A.D., NEILL, S.J. & HORGAN, R. 1988. Xanthoxin levels and metabolism in the wild-type and wilty mutants of tomato. *Planta* 173:397-404.

PAULMICHL, M., LI, Y., WICKMAN, K., ACKERMAN, M., PERALTA, E. & CLAPHAM, D. 1992. New mammalian chloride channel identified by expression cloning. *Nature* 356:238-241.

PESCI, P. 1988. Ion fluxes and abscisic acid-induced proline accumulation in barley leaf segments. *Plant Physiology* 86:927-930.

PESCI, P. 1989. Involvement of Cl^- in the increase in proline induced by ABA and stimulated by potassium chloride in barley leaf segments. *Plant Physiology* 89:1226-1230.

PIATKOWSKI, D., SCHNEIDER, K., SALAMINI, F. & BARTELS, D. 1990. Characterisation of five abscisic acid-responsive clones isolted from the desiccation-tolerant plant *Craterostigma plantagineum* and their relationship to other water-stress genes. *Plant Physiology* 94:1682-1688.

PILON-SMITS, E.A.H., EBSKAMP, M.J.M., PAUL, M.J., JEUKEN, M.J.W., WEISBEEK, P.J. & SMEEKENS, S.C.M. 1995. Improved performance of transgenic fructan-accumulating tobacco under water stress. *Plant Physiology* 107:125-130.

PLANT, A.L., COHEN, A., MOSES, M.S. & BRAY, E.A. 1991. Nucleotide sequence and spatial expression pattern of a drought- and abscisic acid-induced gene of tomato. *Plant Physiology* 97:900-906.

PREVELIGE, P. & FASMAN, G.D. 1989. Chou-Fasman prediction of the secondary structure of proteins. In: FASMAN, G.D. (ed), *Prediction of Protein Structure and*

the Principles of Protein Conformation, pp. 391-416. Plenum Press, N.Y.

QUARRIE, S.A. 1980. Genotypic differences in leaf water potential, abscisic acid and proline concentration in spring wheat during water stress. *Annals of Botany (London)* 46:383-394.

RADIN, J.W. & HENDRIX, J.L. 1988. The apoplastic pool of abscisic acid in cotton leaves in relation to stomatal closure. *Planta* 174:180-186.

RAGHAVENDRA, A.S. & REDDY, K.B. 1987. Action of proline on stomata differs from that of abscisic acid, G-substances, or methyl jasmonate. *Plant Physiology* 83:732-734.

RAJAGOPAL, V. 1981. The influence of exogenous proline on the stomatal resistance in *Vicia faba*. *Physiologia Plantarum* 52:292-296.

RIBAUT, J.-M., HOISINGTON, D.A. & DEUTSCH, J.A. 1996. Identification of quantitative trait loci under drought conditions in tropical maize. 1. Flowering parameters and the anthesis-silking interval. *Theoretical and Applied Genetics* 92:905-914.

RICHARDSON, J.S. & RICHARDSON, D.C. 1989. Principles and patterns of protein conformation. In: FASMAN, G.D. (ed), *Prediction of Protein Structure and the Principles of Protein Conformation*, pp. 1-98. Plenum Press, N.Y.

RIED, J.L. & WALKER-SIMMONS, M.K. 1993. Group 3 late embryogenesis abundant proteins in desiccation-tolerant seedlings of wheat (*Triticum aestivum* L.). *Plant Physiology* 102:125-131.

ROBERTSON, M. & CHANDLER, P.M. 1992. Pea dehydrins: identification, characterisation and expression. *Plant Molecular Biology* 19:1031-1044.

ROTH, S.Y. & ALLIS, C.D. 1992. Chromatin condensation: does histone H1 dephosphorylation play a role? *Trends in Biochemical Sciences* 17:93-98.

RUSSEL, W.A. & EBERHART, S.A. 1968. Test crosses of one- and two-ear types of cornbelt maize inbreds. II. Stability of performance in different environments. *Crop Science* 8:248-251.

SAAB, I.N., SHARP, R.E., PRITCHARD, J. & VOETBURG, G.S. 1990. Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. *Plant Physiology* 93:1329-1336.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

SANDERS, L.C., WANG, C.S., WALLING, LL. & LORD,E.M. 1991. A homolog of the substrate adhesion factor vitronectin occurs in four species of flowering plants. *The Plant Cell* 3:629-635.

SASAKI, T., SONG, J., KOGA-BAN, Y., MATSUI, E., FANG, F., HIGO, H., NAGASAKI, H., HORI, M., MIYA, M. & MURAYAMA-KAYANO. 1994. Toward cataloguing all rice genes: large-scale sequencing of randomly chosen rice cDNAs from a callus cDNA library. *Plant Journal* 6:615-624.

SCHERER, G., TELFORD, J., BALDARI, C. & PIRROTTA, V. 1981. Isolation of cloned genes differentially expressed at early and late stages of *Drosophila* embryonic development. *Developmental Biology* 86:438-447.

SCHMIDT, C. & SCHROEDER, J.I. 1994. Anion selectivity of slow anion channels in *Vicia faba* guard cells: Large nitrate permeability. *Plant Physiology* 106:383-441.

SCHOBERT, B. 1977. Is there an osmotic regulatory mechanism in algae and higher plants. *Journal of Theoretical Biology* 68:17-26.

SCHOBERT, B. & TSCHESCHE, H. 1978. Unusual solution properties of proline and its interaction with proteins. *Biochimica et Biophysica Acta* 541:270-277.

SCHROEDER, J.I. & HAGIWARA, S. 1989. Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* 338:427-430.

SCHROEDER, J.I. & HEDRICH, R. 1989. Involvement of ion channels and active transport in osmoregulation and signaling of higher plant cells. *Trends in Biochemical Sciences* 14:187-192.

SCHURR, U., GOLLAN, T. & SCHULZE, E.-D. 1992. Stomatal responses to drying soil in relation to changes in the xylem sap composition of *Helianthus annuus*. II. Stomatal sensitivity to abscisic acid imported from the sylem sap. *Plant Cell and Environment* 15:561-567.

SHANKAR, G., DAVISON, I., HELFRICH, M.P., MASON, W.T. & HORTON, M.A. 1993. Integrin receptor mediated mobilisation of intranuclear clacium in rat osteoclasts. *Journal of Cell Science* 105:61-68.

SHARP, R.E. & DAVIES, W.J. 1985. Root growth and water uptake by maize plants in drying soil. *Journal of Experimental Botany* 170:1441-1456.

SHARP, R.E., SILK, W.K. & HSIAO, T.C. 1988. Growth of the maize primary root at low water potentials. I. Spatial distribution of expansive growth. *Plant Physiology* 87:50-57.

SHEN, Q.X. & HO, T.H.D. 1995. Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel *cis*-acting element. *The Plant Cell* 7:295-307.

SINGH, T.N., ASPINALL, D. & PALEG, L.G. 1972. Proline accumulation and varietal adaptability to drought in barley: a potential metabolic measure of drought resistance. *Nature* 236:188-190

SINGH, T.N., PALEG, L.G. & ASPINALL D. 1973. Stress metabolism. I. Nitrogen metabolism and growth in the barley plant during water stress. *Australian Journal of Biological Science* 26: 45-56.

SINGH, N.K., HANDA, A.K., HASEGAWA, P.M. & BRESSAN, R.A. 1985. Proteins associated with adaptation of cultured tobacco cells to NaCl. *Plant Physiology* 79:126-137.

SINGH, N.K., NELSON, D.E., KUHN, D., HASEGAWA, P.M. & BRESSAN, R.A. 1989. Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. *Plant Physiology* 90:1096-1101.

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

SKRIVER, K., OLSEN, F.L., ROGERS, J.C. & MUNDY, J. 1991. *Cis*-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. *Proceedings of the National Academy of Sciences USA* 88:7266-7270.

SOBRADO, M.A. 1990. Drought responses of tropical corn 1. Leaf area and yield components in the field. *Maydica* 35:221-226.

STEWART, C.R. 1980. The mechanism of abscisic acid-induced proline accumulation in barley leaves. *Plant Physiology* 66:230-233.

STEWART, C.R. & VOETBERG, G. 1985. Relationship between stress-induced ABA and proline accumulations and ABA-induced proline accumulation in excised barley

leaves. *Plant Physiology* 79:24-27.

STEWART, C.R. & VOETBERG, G. 1987. Abscisic acid accumulation is not required for proline accumulation in wilted leaves. *Plant Physiology* 83:747-749.

STRAUB, P.F., SHEN, Q. & HO, T.-H.D. 1994. Structure and promoter analysis of an ABA- and stress-regulated barley gene, *HVA1*. *Plant Molecular Biology* 26:617-630.

STROEHER, V.L., BOOTHE, J.G. & GOOD, A.G. 1995. Molecular cloning and expression of a turgor-responsive gene in *Brassica napus*. *Plant Molecular Biology* 27:541-551.

SUTHERLAND, L., CAIRNEY, J., ELMORE, M.J., BOOTH, I.R. & HIGGINS, C.F. 1986. Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on the intracellular potassium. *Journal of Bacteriology* 168:805-814.

TARCZYNSKI, M.C., JENSEN, R.G. & BOHNERT, H.J. 1993. Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science* 259:508-510.

TARDIEU, F. & DAVIES, W.J. 1992. Stomatal response to abscisic acid is a function of current plant water status. *Plant Physiology* 98:540-545.

TARDIEU, F., ZHANG, J. & GOWING, D.J.G. 1993. A model of stomatal control by both ABA concentration in the xylem sap and leaf water status: test of the model and of alternative mechanisms for droughted and ABA-fed field-grown maize. *Plant, Cell and Environment* 16:413-420.

TAYLOR, H.F. & BURDEN, R.S. 1972. Xanthoxin, a recently discovered plant growth inhibitor. *Proceedings of the Royal Society of London, Series B* 180:317-346.

TAYLOR, H.F. & BURDEN, R.S. 1973. Preparation and metabolism of 2-[^{14}C]-*cis,trans*-xanthoxin. *Journal of Experimental Botany* 24:873-880.

THAKUR, P.S. & RAI, V.K. 1981. Growth characteristics and proline content in relation to water status in two *Zea mays* L. cultivars during rehydration. *Biologia Plantarum* 23:98-103.

TINELLI, E.T., SONDHEIMER, E., WALTON, D.C., GASKIN, P. & MacMILLAN, J. 1973. Metabolites of 2-^{14}C-abscisic acid. *Tetrahedron Letters* 1973:139-140.

TOMENCHOK, D.M. & BRANDRISS, M.C. 1987. Gene-enzyme relationships in the

proline biosynthetic pathway of *Saccharomyces cerevisiae*. *Journal of Bacteriology* 169:5364-5372.

TREJO, C.L., CLEPHAM, A.L. & DAVIES, W.J. 1995. How do stomata read abscisic acid signals? *Plant Physiology* 109:803-811.

TREWAVAS, A. & KNIGHT, M. 1994. Mechanical signaling, calcium and plant form. *Plant Molecular Biology* 26:1329-1341.

TURNER, N.C. 1979. Drought resistance and adaptation to water deficits in crop plants. In: MUSSEL, H. & STAPLES, R.C. (eds), *Stress Physiology in Crop Plants*, pp. 343-372. Wiley-Interscience, New York.

VILARDELL, J., GODAY, A., FREIRE, M.A., TORRENT, M., MARTINEZ, C., TORNE, J.M. & PAGES, M. 1990. Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. *Plant Molecular Biology* 14:423-432.

VOETBURG, G.S. & SHARP, R.E. 1991. Growth of the maize primary root at low water potentials III. Role of increased proline deposition in osmotic adjustment. *Plant Physiology* 96: 1125-1130.

VOGEL, H. & JÄHNIG, F. 1986. Models for the structure of outer-membrane proteins of *Escherichia coli* derived from Raman spectroscopy and prediction methods. *Journal of Molecular Biology* 190:191-199.

WANG, H., DATLA, R., GEORGES, F., LOEWEN, M. & CUTLER, A.J. 1995. Promoters from *kin1* and *cor6.6*, two homologous *Arabidopsis thaliana* genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum and dehydration. *Plant Molecular Biology* 28:605-617.

WARD, J.M. & SCHROEDER, J.I. 1994. Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *The Plant Cell* 6:669-683.

WARD, J.M., PEI, Z.-M. & SHROEDER, J.I. 1995. Roles of ion channels in initiation of signal transduction in higher plants. *The Plant Cell* 7:833-844.

WATTS, S., RODRIGUEZ, J.L., EVANS, S.E. & DAVIES, W.J. 1981. Root and shoot growth of plants treated with abscisic acid. *Annals of Botany (London)* 47:595-602.

WEI, T. & O'CONNELL, M.A. 1996. Structure and characterization of a putative drought-inducible H1 histone gene. *Plant Molecular Biology* 30:255-268.

WELIN, B.V., OLSON, Å., NYLANDER, M. & PALVA, E.T. 1994. Characterization and differential expression of *dhn/lea/rab*-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*. *Plant Molecular Biology* 26:131-144.

WEYERS, J.D.B., & HILMAN, J.R. 1979. Uptake and distribution of abscisic acid in *Commelina* leaf epidermis. *Planta* 144:167-172.

WILKINSON, J.Q., LANAHAN, M.B., CONNER, T.W. & KLEE, H.J. 1995. Identification of mRNAs with enhanced expression in ripening strawberry friut using polymerase chain reaction differential display. *Plant Molecular Biology* 27:1097-1108.

WILLIAMS, M.E., FOSTER, R. & CHUA, N.-H. 1992. Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. *The Plant Cell* 4:485-496.

WU, Y., SPOLLEN, W.G., SHARP, R.E., HETHERINGTON, P.R. & FRY, S.C. 1994. Root growth maintenance at low water potentials. *Plant Physiology* 106:607-615.

XU, D., DUAN, X., WANG, B., HONG, B., HO, T.-H.D. & WU, R. 1996. Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiology* 110:249-257.

YAMAGUCHI, T. & STREET, H.E. 1977. Stimulation of excised cultured roots of soya bean by abscisic acid. *Annals of Botany (London)* 41:1129-1133.

YAMAGUCHI-SHONIZAKI, K., MUNDY, J. & CHUA, N-H. 1989. Four tightly linked *rab* genes are differentially expressed in rice. *Plant Molecular Biology* 14:29-39.

YAMAGUCHI-SHINOZAKI, K., KOIZUMI, M., URAO, S. & SHINOZAKI, K. 1992. Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. *Plant Cell Physiology* 33:217-224.

YAMAGUCHI-SHINOZAKI, K. & SHINOZAKI, K. 1993. Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. *Molecular and General Genetics* 236:331-340.

YAMAGUCHI-SHINOZAKI, K. & SHINOZAKI, K. 1994. A novel *cis*-acting element in

an *Arabidopsis* gene is involved in responsiveness to drought, low temperature or salinity. *The Plant Cell* 6:251-264.

ZEEVAART, J.A.D. & BOYER, G.L. 1982. Metabolism of abscisic acid in *Xanthium strumarium* and *Ricinus communis*. In: WAREING, P.F. (ed), *Plant Growth Substances*, pp. 335-342. Academic, London.

ZEEVAART, J.A.D. & CREELMAN, R.A. 1988. Metabolism and physiology of abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology* 39:439-473.

ZHANG, J. & DAVIES, W.J. 1990. Changes in the concentration of ABA in xylem sap as a function of changing soil water status can account for changes in leaf conductance and growth. *Plant Cell and Environment* 13:277-285.

ZHANG, J., SCHURR, U. & DAVIES, W.J. 1987. Control of stomatal behaviour by abscisic acid which apparently originates in the roots. *Journal of Experimental Botany* 38:1174-1181.

APPENDIX A:
REAGENTS AND SOLUTIONS

1. Grinding buffer

0.18 M Tris.Cl
0.09 M LiCl
4.5 mM EDTA
1% sodium dodecyl sulphate
pH 8.2

2. Loading buffer

0.5 M LiCl
10 mM Tris.Cl
1 mM EDTA
0.1% sodium dodecyl sulphate
pH 7.5

3. Middle wash buffer

0.15 M LiCl
10 mM Tris.Cl
1 mM EDTA
0.1% sodium dodecyl sulphate
pH 7.5

4. CE buffer

2.5 mM Tris.Cl

0.25 mM EDTA

pH 8

5. Denaturing solution

1.5 M NaCl

0.5 M NaOH

6. Neutralizing buffer

1.5 M NaCl

0.5 M Tris.Cl

1 mM EDTA

pH 7.2

7. Pre/hybridization buffer

5 x SSC

1.0% Blocking Reagent (w/v)

10 mM maleic acid

15 mM NaCl

0.1% N-lauroylsarcosine

0.02% SDS

pH 7.5

8. Washing buffer

0.1 x SSC
0.1% SDS
0.2 M Tris.Cl
pH 7.5

9. PCR conditions per 50 µl reaction volume

1 x PCR buffer (Boehringer Mannheim)
0.2 M dNTP's
5 µl eluted phage in SM buffer
0.5 µM forward primer
0.5 µM reverse primer
2.5 U Taq DNA polymerase

Temperature cycling was as follows:

94.5°C	30 s	)
64°C	30 s	} 35 cycles
72°C	45 s	)
72°C	10 min	

10. RNA denaturing solution

formamide	500 µl
37% formaldehyde	162 µl
10 x MOPS	100 µl

APPENDIX B:
ABBREVIATIONS

A	absorbance
ABA	abscisic acid
bp	base pair
cm	centimetre
cpm	counts per minute
DEPC	diethylpyrocarbonate
EDTA	ethylenediaminetetraacetic acid
g	gram
h	hour
µg	microgram
µl	microlitre
µM	micromolar
µmol	micromole
m	metre
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
Mpa	megapascal
N_2	nitrogen
ng	nanogram
nm	nanometre
OD	optical density
PAR	photosynthetically active radiation
PCR	polymerase chain reaction

rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
s.e.	standard error
vol	volume
U	unit
UV	ultraviolet