

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

**Resources for the Investigation of the Desiccation
Tolerance Mechanisms of the Roots of *Xerophyta
humilis* (Bak.) Dur and Schinz.**

Michael Jack Gardner

**Submitted in partial fulfilment of the academic requirements for a M.Sc. degree in the
Department of Molecular and Cell Biology, University of Cape Town.**

**Cape Town
2002**

PREFACE

The experimental work described in this report was carried out in the Department of Molecular and Cell Biology, University of Cape Town, from February 2001 to September 2002, under the supervision of Professors N. Illing and J. Farrant.

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

Michael Jack Gardner
September 2002

ශ්‍රී ලංකා විපක්ෂ

University of Cape Town

ACKNOWLEDGEMENTS

My sincere thanks are due to the individuals who have contributed towards this work and who have assisted me during the course of its preparation.

My supervisors, Professors Nicci Illing and Jill Farrant, for their insight, encouragement and guidance.

Helen Collett, for her assistance with the technical aspects of this thesis - for her quiet cynicism, sense of humour, supply of cappuccinos and her endless bravery in proofreading this document.

'Professor' Mohammed Jaffer for expert assistance with the microscopy work.

Keren Cooper, for her wizardry with graphics, and her willingness to assist in times of technical distress and/ or sense of humour failure.

My friends both inside and outside the labs – for tips, solutions, laughs, headaches and copious amounts of illicit chocolate. Also Duncan, who knows why – and Hannah and Kelvin who probably wish they did too.

Mrs. B. Thomas of Concord-Harmony Trust and the National Research Foundation, (Pretoria) for their generous financial assistance.

My parents Betty and Rob, for their love, support and understanding over difficult years.

Abstract

Although roots play an integral role in the sensing and amelioration of environmental stresses, there are no reports that specifically detail their involvement in the desiccation tolerance mechanisms of resurrection plants. Very little is known about even the general anatomy and physiology of the roots of plants such as *X. humilis*, and almost nothing about the molecular responses that confer their ability to survive desiccation. This report details foundational studies of the functional anatomy and large-scale molecular responses of the roots during dehydration and rehydration.

Anatomical studies revealed that the roots undergo transitions similar to those observed and characterized in the leaves - namely division of vacuoles, glass formation and loss of internal detail. Electrolytic leakage data confirmed that little membrane damage was incurred during desiccation, but analysis of proline and sucrose levels suggested that these osmocompatible solutes are only minor contributors to the preservation of structural integrity. Roots rehydrated several times faster than the aerial portions of the plant. The idea that the conspicuous exodermis and the unusual *velamen radicum* are involved in this rapid absorption of water is discussed.

The documented involvement of two-component systems in osmosensing led to an attempt to clone homologues present in *X. humilis* roots. Primers were designed to conserved regions of plant histidine kinase and response regulator genes and utilized in degenerate RT-PCR reactions. The root-specific *Arabidopsis thaliana* histidine kinase 1 (*ATHK1*) clone was employed concurrently to study the copy number and regulation of related histidine kinases in *X. humilis*.

To address the lack of molecular resources available for resurrection plant research, high-quality root- and stress-specific cDNA libraries were constructed from plants at various stages of desiccation and rehydration. These will be used to complement leaf libraries constructed from the same plants. The libraries were evaluated and screened to assess their quality. The usefulness of β -tubulin and other housekeeping genes as probes in this screening is discussed with reference to northern blot analyses. An attempt was made to reduce the redundancy levels of individual sequences in these libraries through the use of a normalization procedure adapted from that employed by the IMAGE consortium. Normalized libraries were screened to determine the extent of the reduction in tubulin frequencies. The success of this method relative to other subtractive hybridizations is discussed, alongside the prospects for large-scale sequencing and microarray analysis of the transcriptome of the roots of *X. humilis*.

This study describes novel findings relating to the physiological, anatomical and molecular features of the roots of *X. humilis*. In addition, it has generated several resources that may form the basis of the extensive characterization of the genetic elements utilized by these plants in stress amelioration.

Table of Contents

Preface	i
Acknowledgements	iii
Abstract	iv
Figures	ix
Tables	xiv
Abbreviations	xv
1 Introduction: Mechanisms of desiccation tolerance	1
1.1 Resurrection Plants	1
1.1.1 <i>Xerophyta humilis</i> and the genus <i>Xerophyta</i>	3
1.2 Mechanisms of Desiccation Tolerance	4
1.2.1 The sensing of environmental stresses	6
1.2.2 Gene regulation in water stress responses	8
1.2.3 Gene expression and cellular responses to desiccation and rehydration	10
1.3 Targeted Approaches to Dissection of Stress-Response Pathways	16
1.4 Summary and Aims	19
2 Anatomy And Physiology: Root responses to water flux	20
2.1 Introduction	20
2.1.1 Metabolic adjustment and the preservation of structural integrity	21
2.2 Materials and Methods	24
2.2.1 Plant material	24
2.2.2 Water content and water potential determinations	24
2.2.3 Microscopy	25
2.2.4 Electrolytic leakage	26
2.2.5 Sugar analysis	27
2.2.6 Proline analysis	27

2.3	Results	27
2.3.1	Gross morphology	27
2.3.2	Anatomy	28
2.3.3	Water contents and water potentials	35
2.3.4	Electrolytic leakage	35
2.3.5	Proline and sugars	37
2.4	Discussion	39
3	Targeting Candidate Genes: Stress sensing and signalling systems	44
3.1	Introduction	44
3.2	Materials and methods	47
3.2.1	Probe preparation	47
3.2.2	Southern and northern blot analysis	47
3.2.3	Primer design	48
3.2.4	Degenerate primer PCR	48
3.2.5	Cloning	50
3.2.6	Screening and sequencing	50
3.3	Results	51
3.3.1	Northern and southern analysis using <i>ATHK1</i>	51
3.3.2	Primer design	52
3.3.3	Optimization of degenerate RT-PCR and cloning	54
3.3.4	Sequence analysis	56
3.4	Discussion	57
4	cDNA Library Construction: A molecular entry into desiccation tolerance	61
4.1	Introduction	61
4.1.1	The production of high quality libraries	62
4.1.2	cDNA libraries in water stress research	65
4.2	Materials and methods	66
4.2.1	Plant material	66
4.2.2	RNA extraction and analysis	67
4.2.3	mRNA Isolation	68
4.2.4	Library construction	68
4.2.5	Evaluation of libraries	71
4.2.6	Screening with β -tubulin	72
4.2.7	Northern blot analysis	73
4.2.8	Data analysis and management	74

4.3	Results	74
4.3.1	RNA and mRNA isolation and evaluation	74
4.3.2	cDNA synthesis and cloning	76
4.3.3	Library evaluation	77
4.3.4	β -tubulin screens	80
4.4	Discussion	83
5	Normalization of cDNA Libraries: A rational approach to gene discovery	89
5.1	Introduction	89
5.1.1	Re-association kinetics in normalization	90
5.1.2	Kinetic methods of normalization	92
5.1.3	The Soares method	93
5.1.4	HAP column chromatography	95
5.1.5	The effectiveness of normalization	96
5.1.6	normalization and plant desiccation tolerance	97
5.2	Methods	99
5.2.1	Mass excision of pBluescript phagemid	99
5.2.2	Isolation of single stranded library DNA	100
5.2.3	Purification of single stranded tracer	101
5.2.4	Generation of the driver DNA	102
5.2.5	Hybridization	102
5.2.6	Purification of the normalized DNA population	103
5.2.7	Electroporation	104
5.2.8	Preparation of libraries for storage and arraying	104
5.2.9	Evaluation of the normalized libraries	104
5.3	Results	105
5.3.1	Preparation of Tracer and driver DNA	105
5.3.2	Hybridization and purification	109
5.3.3	Electroporation	110
5.3.4	Screening	111
5.4	Discussion	113
6	Conclusions	117
7	References	119

Figures

Chapter 1

- Figure 1.1: Phylogenies representing the evolution of land plants. Asterisks indicate taxa that display vegetative desiccation tolerance (Figure from Oliver *et al.*, 2000). 3
- Figure 1.2: Photographs of (A) hydrated and (B) dehydrated *X. humilis* plants. 4
- Figure 1.3: A basic model depicting the possible processes involved in the onset, detection and amelioration of desiccation stress. 5
- Figure 1.4: An overview of phases of gene expression in response to desiccation. 10
- Figure 1.5: An example of a microarray slide. Spots indicate the position of independent cDNAs that have been competitively targeted by populations of Cy3/ Cy5 labeled mRNA. The relative intensity of the red: green channels for an individual spot is indicative of the different levels of a particular mRNA in the sample (Image obtained from the *Arabidopsis* Functional Genomics Consortium). 17

Chapter 2

- Figure 2.1: Photographs showing the gross morphology of *X. humilis*. (A) The intact plant, and (B) separation of the living and older (dead) roots. 29
- Figure 2.2: Scanning electron micrographs of freeze fractured *X. humilis* roots showing (A) the outer root (B) the schlerenchymatous exodermis (C-D) parenchyma cells forming the living root cortex. cw = cell wall, cx = cortex, en = endodermis, ex = endodermis, sc = schlerenchyma, v = *velamen radicum*. Magnifications and scale bars are indicated on the images. 30

- Figure 2.3:** Transmission electron micrographs showing cross sections through cells of the cortex of a hydrated *X. humilis* root. (A-C) Cortical parenchyma cells [7 000 x] and (D) the cortical cells bordering the lignified stele [8 000 x]. cw = cell wall, ls = lignified stelar cell, n = nucleus, v = vacuole. 31
- Figure 2.4:** Scanning electron micrographs of freeze-fractured (A-B) older (dead) and (C-D) desiccated *X. humilis* roots. Shattering of the dry roots is evident from the presence of the glass-like shards (s). cx = cortex, ex = exodermis, x = xylem. Magnifications and scale bars are indicated on the images. 33
- Figure 2.5:** Transmission electron micrographs of cross sections through dehydrated *X. humilis* roots. (A) The intact stele [1 000 x] (B) metaxylem vessels [2 000 x] (C) cortical cells filled with vesicles [10 000 x] (D) young vacuolated cortical cells (*image inverted for clarity) [60 000 x]. cw = cell wall, dcw = damaged cell wall, s = stele, v = vessel, vs = vesicle containing electron dense substance. 34
- Figure 2.6:** Changes in the water status of the roots and leaves of *X. humilis*. Relative water contents during (A) dehydration and (B) rehydration. (C) Water potentials during rehydration. Vertical bars represent standard deviations of the means. 36
- Figure 2.7:** Electrolytic leakage of the roots and leaves of *X. humilis*. Values are expressed as percentages of the hydrated controls. Standard deviations of the means were set at 5 %. 36
- Figure 2.8:** Glucose, fructose and sucrose levels in roots of *X. humilis*. Changes in glucose levels are non-significant ($P = 0.95$). Standard deviations are represented by vertical bars. The leaf data is presented as an internal control. 38
- Figure 2.9:** Proline levels in the leaves of *X. humilis* as determined by reference to a standard proline curve constructed under the same experimental conditions. Dehydrated inner leaves of *E. nindensis* were used as a control. 39

Chapter 3

- Figure 3.1:** The functioning of two component systems (A) A simple two-component system in which a phosphate group is transferred directly from the histidine kinase to the response regulator. (B) A multiple phosphorelay system involving a hybrid type histidine kinase (images from Schaller *et al.*, 2001). 46

Figure 3.2:	Southern blot analysis showing the representation of ATHk1-related sequences in genomic DNA of <i>X. humilis</i> .	51
Figure 3.3:	Multiple sequence alignments of selected histidine kinases *AHK1-3: <i>Arabidopsis</i> histidine kinase; ATHK1: <i>Arabidopsis thaliana</i> histidine kinase 1; PEHK: <i>Populus</i> ATHK1 homologue; CKR1: Cytokinin receptor 1.	52
Figure 3. 4:	Multiple sequence alignments of response regulators. *PARR = <i>Arabidopsis</i> response regulators 1-5; PZMRR = <i>Zea mays</i> response regulators 1-6, PDCRR = <i>Daucus carota</i> response regulator.	53
Figure 3.5:	Effect of annealing temperatures and DMSO on product yield from RT-PCR. (A) 45°C, (B) 50°C and (C) 55°C. 1 = histidine kinase primers, no DMSO, 2 = histidine kinase primers, 5 % DMSO, 3 = response regulator primers, no DMSO, 4 = response regulator primers, 5% DMSO. Optimized PCR conditions were used.	55
Figure 3.6:	Amplification of cloned inserts by colony PCR. Lanes 1-9 = products from selected colonies, lane 10 = negative control, M = marker (<i>Pst</i> II digested lambda DNA).	55
Figure 3.7:	Characterization of the <i>RstA</i> homologue. (A) Conserved domain detection. (B) Partial Amino acid sequence alignment of <i>RstA</i> homologue and <i>AthK1</i> .	56
Figure 3. 8:	PCR from cDNA prepared by RT from poly(A) RNA. Lane 1 = histidine kinase primers + DMSO, 2 = response regulator primers + DMSO, M= marker.	57

Chapter 4

Figure 4.1:	The Lambda-Zap vector (Image from the Uni-Zap Manual Stratagene).	63
Figure 4.2:	RNA isolated at varying water contents from roots of (A) dehydrating and (B) rehydrating <i>X. humilis</i> plants. 1-2 µg of RNA was loaded per lane. The sizes and positions of the 18S and 28S ribosomal subunits are indicated. RWC = relative water content, HAW = hours after watering.	74

Figure 4.3:	(A) Poly(A) ⁺ RNA derived from pooled dehydration (lanes 1-2) and rehydration samples (lanes 3-5). Approximately 0.5 µg RNA from several separate isolations was loaded per lane. (2) Autoradiograms of [³² P] dATP labelled cDNA derived from root RNA at different water contents. PD = partially dehydrated, D = dehydrated, PR = partially rehydrated, un = unincorporated nucleotides.	75
Figure 4.4:	Photographs of (A) first-strand and (B) second-strand synthesis reactions. 1-2 µl of DNA was loaded per lane and electrophoresed on alkaline agarose gels in the presence of ethidium bromide. D = pooled dehydration samples, R = pooled rehydration samples.	76
Figure 4.5:	Evaluation of library inserts. Agarose gels of colony PCR reactions indicating the presence of inserts in 12 randomly selected clones from the (A) dehydration and (B) rehydration libraries.	78
Figure 4.6:	Pie charts indicating the shift in the percentage of blue and white clones in (A) dehydration and (B) rehydration libraries during the excision procedure.	79
Figure 4.7:	Graphs indicating (A) the percentage of colonies containing inserts and (B) the average sizes of inserts in colour selected colonies.	80
Figure 4.8:	Representative autoradiographs indicating the representation of β-tubulin isoforms in the (A) dehydration and (B) rehydration libraries. Approximately 30 000 pfu were screened per membrane.	81
Figure 4.9:	Northern blot analysis of (A) β-tubulin II (B) β-actin and (C) 18S RNA expression in the leaves and roots of <i>X. humilis</i> . 10 µg of total RNA was loaded per lane. Controls indicated sensitivity to the pg level. * Actin blot carried out once only.	82

Chapter 5

Figure 5.1:	A typical C ₀ t curve of a complex DNA solution.	91
Figure 5.2:	An outline of the available methods of normalization. The driver DNA is denatured and annealed to the tracer in the presence of blocking oligonucleotides to achieve the required C ₀ t time (adapted from Bonaldo <i>et al.</i> , 1996).	95

Figure 5.3:	Effect of the helper phage multiplicity of infection (MOI) level on the recovery of single stranded DNA from the dehydration and rehydration cDNA libraries. Lane numbers 1-4 indicate a MOI of 1×10^x , M = marker (<i>Pst</i> II digested lambda DNA).	105
Figure 5.4:	Single stranded DNA recovered from the dehydration and rehydration cDNA libraries. Lane numbers indicate the amount (in μ l) of DNA loaded. M = marker (<i>Pst</i> II digested lambda DNA).	106
Figure 5.5:	S1 nuclease digests of the single stranded dehydration and rehydration library DNA. Lanes: 1 = S1 nuclease digested sample, 2 = original sample, M = marker (<i>Pst</i> II digested lambda DNA).	107
Figure 5.6:	Recovery of purified single stranded DNA from HAP columns. Input = 10 % of original amount of DNA loaded. Lane numbers indicate fraction numbers. M = marker (<i>Pst</i> II digested lambda DNA).	107
Figure 5.7:	Representative images of the desalting and gel purification of HAP purified single stranded DNA. (A) Sigma spin column desalting of dehydration library DNA (B) gel and Qiagen column purification of rehydration library DNA. Lanes: 1 = DNA before purification, 2 = tracer DNA after purification, M = marker (<i>Pst</i> II digested lambda DNA).	108
Figure 5.8:	Driver DNA produced by PCR from the dehydration and rehydration tracer DNA. Lanes: 1 = dehydration driver DNA, 2 and 3 = rehydration driver DNA, 4 = negative (no primer) control, 5 = template DNA, M = marker.	109
Figure 5.9:	PCR of inserts from normalized single stranded DNA samples. Lanes: 1 = negative (no primer) control, 2 = 10 % of PCR reaction using R _{15A} library DNA as a template, M = marker (Promega 100 bp ladder).	110
Figure 5.10:	Amplification of cloned inserts by colony PCR. Lanes 1- 11 represent inserts from selected colonies. Marker and controls not shown.	112
Figure 5.11:	Autoradiograph of a cloneset of the R ₂ library probed with β -tubulin I-III by colony hybridization.	112

Tables

Chapter 1

Table 1.1:	Some important genes differentially expressed during water stress in resurrection plants.	15
------------	---	----

Chapter 3

Table 3.1:	Degenerate primers for two component system genes.	49
Table 3.2:	Parameters tested for degenerate PCR optimisation.	54

Chapter 4

Table 4.1:	Summary of key library data.	77
Table 4.2:	Average insert sizes and insert size ranges for dehydration and rehydration libraries.	78
Table 4.3:	Summary of the statistics relating to the percentage of positive plaques detected in the β -tubulin screen.	81

Chapter 5

Table 5.1:	Summary of the normalized libraries generated by electroporation into DH10B™ cells.	111
Table 5.2:	Factors affecting the reaction rate (data from Soares and Bonaldo, 1998).	115

Abbreviations

A	adenosine		
ABA	abscisic acid	dNTP	deoxynucleotide
ABRE	ABA responsive element		triphosphate
ABREB	ABRE binding protein	EtOH	ethanol
AMP	ampicillin	EST	expressed sequence tag
AThk1	<i>Arabidopsis thaliana</i> histidine kinase 1	fdt	fully desiccation tolerant
ATP	adenosine triphosphate	HAP	hydroxyapatite
AWC	absolute water content	HOG	high osmolarity growth
bp	base pairs	Kb	kilobase
BLAST	basic local alignment search tool	LB	luria broth
BSA	bovine serum albumin	LEA	late embryogenesis abundant
cDNA	complementary DNA	mdt	modified desiccation tolerant
cfu	colony forming units	mRNA	messenger ribonucleic acid
CRE	C repeat element	PCR	polymerase chain reaction
DAG	diacylglycerol	Pfu	plaque forming units
DMSO	dimethyl sulfoxide	RWC	relative water content
DNA	deoxyribonucleic acid	SEM	scanning electron microscopy
DRE	drought response element	TEM	transmission electron microscopy
DREB	DRE binding protein	Tm	melting temperature
dT	deoxythymidine	U	units
		UTR	untranslated region

Introduction

The importance and mechanisms of desiccation tolerance

1.1 Resurrection Plants

Water, as the ubiquitous biological solvent, has largely governed the pattern of the evolution of biochemical systems. Very few organisms are capable of enduring the absence of water for prolonged periods of time while retaining structural, organizational and functional integrity (Somero, 1992; Bewley and Oliver, 1992; Vertucci and Farrant, 1995; Gaff, 1997). Consequently, it is difficult to understand the ability of certain vegetative plant tissues to survive the loss of the bulk of their cellular water.

Desiccation tolerance (or poikilohydry) in vegetative tissues is defined as the ability of an organism to equilibrate its internal water potential with that of moderately dry air and then resume normal functioning after rehydration (Alpert, 2000). It is one of the three classical strategies of dealing with osmotic stress (the other two being avoidance and escape; Ludlow, 1984) and is predominantly a rare peculiarity of lower order plants, and plants that inhabit environments that are subject to rapid, unpredictable changes in the availability of water (Gaff, 1977). The frequency of the occurrence of such hyper-variable habitats in South Africa, Australia and South America means that desiccation tolerant, or 'resurrection plants' are well represented in the floras of these regions (Gaff, 1989; Alpert, 2000).

Although the distinction is by no means clear-cut, desiccation tolerant plants fall into two broad categories, namely fully desiccation tolerant plants (fdt) and modified desiccation tolerant (mdt) plants (Oliver *et al.*, 1998; Farrant *et al.*, 1999). All fdt plants studied to date are from the lower order groups such as the Bryophyta (true mosses). They can

withstand (and indeed have no means of preventing) the extremely rapid loss of protoplasmic water, but are dependent on large-scale structural and functional repair after rehydration (Oliver *et al.*, 1998). Of more immediate importance to any research with the ultimate goal of crop improvement are the mdt plants. These plants are largely representatives of the pteridophyte and angiosperm families. They are equipped with a protective approach to desiccation tolerance - having morphological and biochemical mechanisms that retard initial water loss, and institute protective features during dehydration and rehydration (Dace *et al.*, 1998; Oliver *et al.*, 1998; Farrant *et al.*, 1999). This category is further represented by both homoichlorophyllous and poikilochlorophyllous plants based on the fact that the former retain their chlorophyll during desiccation while the latter actively break it down (Gaff, 1977; Bewley, 1979; Tuba *et al.*, 1998). Poikilochlorophylly is the most derived desiccation tolerance mechanisms and is a peculiarity of certain monocotyledon families (Oliver *et al.*, 2000).

Based on the existence of these distinct forms of desiccation tolerance and on a phylogeny from the consensus of several synthetic studies (Figure 1.1), Oliver *et al.* (2000) proposed that vegetative desiccation tolerance evolved on a number of occasions.

The prevalence of the fdt phenotype amongst the bryophytes and cryptograms suggests that the initial evolution of desiccation tolerance was a critical factor governing the colonization of land by early fresh water flora (Oliver *et al.*, 2000). This primitive phenotype was lost from vegetative tissue because of the slow growth rates associated with the energy deficits attached to constitutive protection. It is likely that many of the molecular features of this phenotype were recruited for water stress responses and for the production of desiccation tolerant propagules (Oliver *et al.*, 2000). The evolutionary advantages of the increased dispersal and survival of such tolerant seeds led to the retention of this feature in angiosperms. This presumably allowed the re-acquisition of vegetative tolerance as angiosperms migrated into marginal and variable habitats. Oliver *et al.* (2000) identified at least 8 independent cases of the re-evolution of

desiccation tolerance, with the last being the introduction of poikilochlorophyll within certain monocotyledon families.

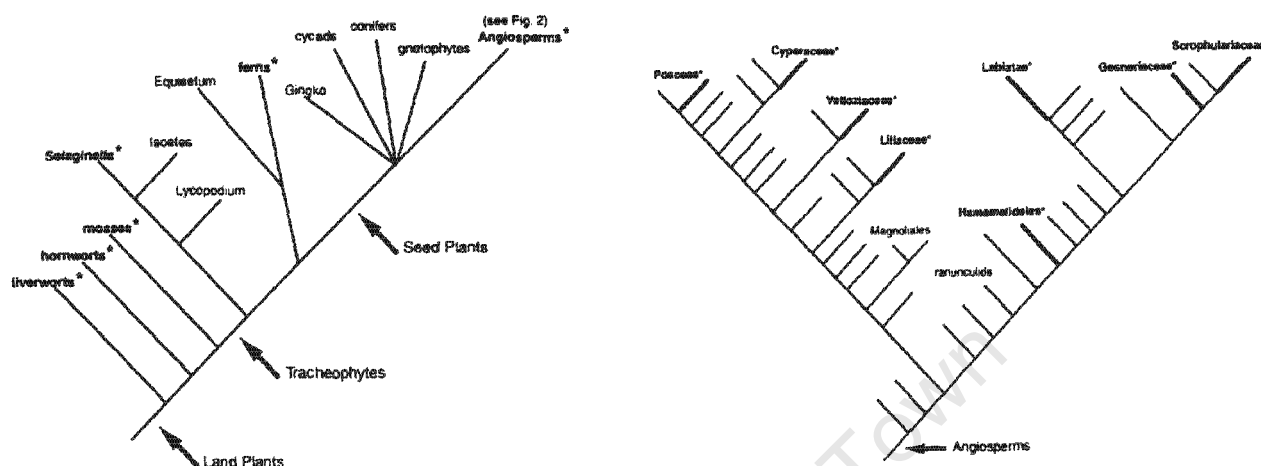


Figure 1.1: Phylogenies representing the evolution of land plants. Asterisks indicate taxa that display vegetative desiccation tolerance (figure from Oliver *et al.*, 2000).

1.1.1 *Xerophyta humilis* and the genus *Xerophyta*

Xerophyta humilis (Bak.) Dur. and Schinz (Figure 1.2) is a member of the Velloziaceae, a family which contains more desiccation tolerant members than any other plant taxon, having no less than 200 desiccation tolerant species in 8 genera (Gaff, 1977; Kubitzki, 1998; Porembski and Barthlott, 2000).

The genus *Xerophyta* is characterized by leaves which fold inward to assume a tight v-shape during desiccation (Gaff, 1989). The stomata are confined to longitudinal furrows that permit inrolling or outrolling of the leaves, depending on the water status (Porembski and Barthlott, 2000). Unlike those of related plants, the leaves of *X. humilis* do not fold over the apical bud during drying (Gaff, 1989; Dace *et al.*, 1998; Farrant *et al.*, 1999; Farrant, 2000). As a poikilochlorophyllous representative, *X. humilis* actively

breaks down its thylakoids and chlorophyll (Dace *et al.*, 1999; Farrant, 2000; Figure 1.2). This is thought to be a mechanism of limiting photo-oxidative damage (Smirnoff, 1993) and may enable poikilochlorophyllous plants to survive lower relative humidity than homoichlorophyllous relatives (Gaff, 1989).



Figure 1.2: Photographs of (A) hydrated and (B) dehydrated *X. humilis* plants.

Although several important processes occur at the anatomical level during desiccation and rehydration, there are no known studies of the changes undergone by the roots of *X. humilis* during the same progression. In Chapter 2 of this dissertation, some of the anatomical responses of the roots to desiccation and rehydration have been explored.

1.2 Mechanisms of Desiccation Tolerance

An overview of the processes that may be involved in the sensing and response to water loss in the roots and leaves of a desiccation tolerant plant are illustrated in Figure 1.3.

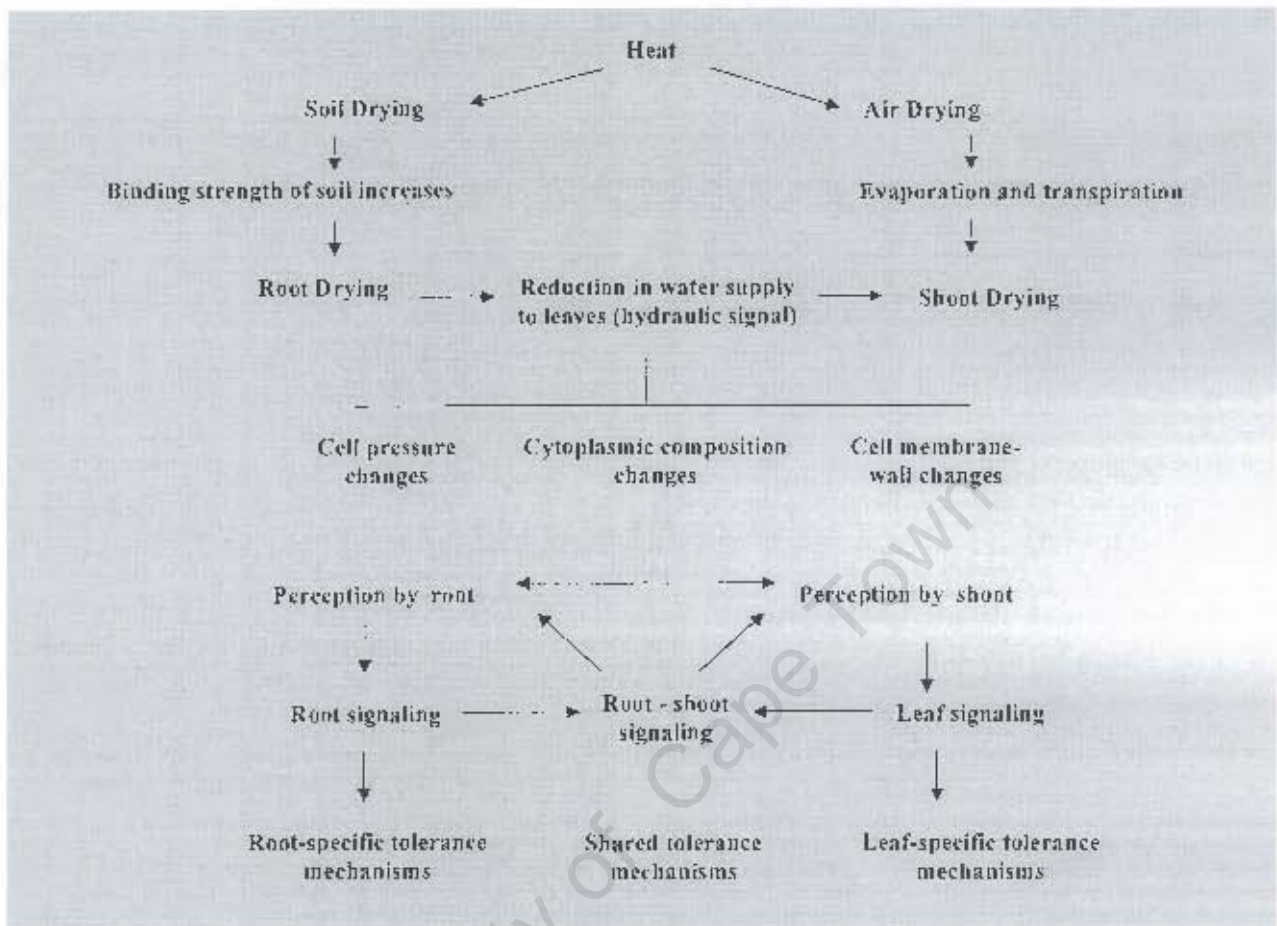


Figure 1.3: A basic model depicting the possible processes involved in the onset, detection and amelioration of desiccation stress.

It is evident that in order for the aerial parts of the plant to survive desiccation, the roots must themselves be desiccation tolerant. However, it cannot be assumed that roots and shoots respond in the same manner during osmotic stress (Scott, 2000). There is reasonable evidence to suggest a convergence on some common features (Somero, 1992; Alpert, 2000; Lopez-Bucio *et al.*, 2000), but it is likely that the roots exhibit alternate means of responding to, and surviving, water flux. The most critical of these differences – in terms of the plant's survival – is the facility with which roots detect subtle shifts in their osmotic environment.

1.2.1 The sensing of environmental stresses

An intrinsic part of the ability of all plants to survive osmotic stress is the rapidity with which they are able to detect the onset of dry periods. This predictive, or early warning feature is necessary in *X. humilis*, which if dried down rapidly, is incapable of implementing the features required to recover from the desiccated state (Farrant *et al.*, 1999). The importance of the roots in inducing desiccation tolerance was recognized by Gaff, who noted that leaves, when detached from most hydrated mdm plants, display tolerance levels equivalent to normal crop plants, while leaves removed from slightly stressed plants survive complete drying (Gaff, 1997).

Features of the cellular environment that may serve as triggers for stress recognition include decreases in turgor, changes in cell volume, membrane area (Bray, 1997), changes in the membrane fluidity (Bowse and Xin, 2001), cell wall-plasma membrane connections and changes in the solute concentrations (Felix *et al.*, 2000). In yeast both the *Sho1p* trans-membrane protein and the *Sln1* (Synthetic lethal N-end of rule 1) protein, a member of the yeast two-component system, respond to high osmolarity and both regulate the high osmolarity growth (HOG) phosphorylation cascade that is involved in the osmotic response (Bray, 1997). The *Sho1p* receptor is thought to respond to alterations in the tension between the cell wall and the cell membrane while the mechanisms of *Sln1* stress perception is unclear (Bray, 1997; Urao *et al.*, 1999). It has been reported that plants also make use of two-component systems, though these are less abundant in plants than in yeast (Urao *et al.*, 1999; Urao *et al.*, 2000). In *Arabidopsis*, a root-specific trans-membrane hybrid-type histidine kinase, with structural similarity to *Sln1* functions as an osmosensor and presumably initiates downstream phosphorylation cascades (Urao *et al.*, 1999). Similar or divergent histidine kinases may be involved in desiccation tolerance mechanisms in *X. humilis*. This possibility and alternate mechanisms of stress sensing and signaling are explored in greater detail in Chapter 3.

Non-hydraulic root-derived signals such as abscisic acid (ABA) and calcium are also central to conferring stress responsiveness to the aerial portions (Davies and Zhang, 1991; Griffiths and Bray, 1996; Bray, 1997; Croker *et al.*, 1998; Starck *et al.*, 1998).

Calcium fluxes bring about diverse downstream effects by altering the activity of calmodulin and other calcium-binding proteins, and by affecting membrane permeability and gene expression (Bowler and Fluhr, 2000; Reddy, 2001). It is thought that calcium fluxes may arise in response to the ionic changes during water loss that precede the direct perception of water deficit (Rengel, 1992; Reddy, 2001), but the rapidity and organelle specificity of such fluxes make this a problematic area of research in non-model plants. Consequently, the role of calcium has not been studied in detail in resurrection plants.

ABA accumulates in the leaves of desiccation tolerant plants such as *Myrothamnus flabelifolius*, *Craterostigma plantagineum* and *Sporobolus stapfianus* early on in the desiccation cycle (Ingram and Bartels, 1996; Sherwin and Farrant, 1998) and exogenous ABA induces tolerance in a number of these plants (Ingram and Bartels, 1996; Gaff, 1997). This ABA may be sourced from the roots, which produce increased amounts of ABA when under water stress and which store conjugated ABA for more rapid responses (Gowing *et al.*, 1993; Jackson, 1997; Sauter and Hartung, 2000). Sauter *et al.* (2001) have shown that small perturbations in the conditions around the roots cause rapid modification of ABA fluxes in the plant. This ABA induces responsiveness in the shoots through a complex system of gene regulation and secondary signaling (Ingram and Bartels, 1996; Seki *et al.*, 2001). The downstream effects of this include stomatal closure in order to conserve water loss through transpiration (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990; Ingram and Bartels, 1996; Jackson, 1997; Mariaux *et al.*, 1998b), ABA synthesis in the leaves (Sauter *et al.*, 2001).

ABA provokes several changes in the root systems themselves. These include (1) rapid release of solutes into the xylem, (2) an increase in the volume of water flowing through the roots, (3) increased ion transport and (4) a decrease in root hydraulic conductivity

(Hose *et al.*, 2000). ABA increases the rate of cell-to-cell transport across the root cylinder, presumably by altering the functioning of plasma-membrane water channels (Hose *et al.*, 2000). Effects are likely to include the regulation of genes vital in the control of desiccation tolerance mechanisms in the root (Ingram and Bartels, 1996). However, since few studies have examined the nature of ABA accumulation in the roots and the dependence of shoot survival on root-derived ABA in desiccation tolerant systems, many details of this signalling system remain unclear.

In general, few details of the perceptual mechanisms of desiccation tolerant plants are understood. Where they are understood in non-tolerant systems, it is uncertain whether they can be applied to tolerant systems.

1.2.2 Gene regulation in water stress responses

One of the primary molecular effects of stress signaling is the activation of transcription factors that bind to *cis*-acting elements in the promoter regions of required genes. In water stress responses, there are two classical, inter-dependant systems – the drought response element (DRE) and the ABA responsive element (ABRE; Smirnov and Bryant, 1999)

The DRE, also known as the C repeat element (CRE) has a 5 bp core CCGAC and is present in multiple copies in the promoters of many stress-responsive genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al.*, 1997). It is bound by the DRE binding proteins (DREBs) which drive the expression of target genes involved in the dehydration, cold and salinity responses of *Arabidopsis* (Bray, 1997; Stockinger *et al.*, 1997; Smirnov and Bryant, 1999; Kizis *et al.*, 2001). Overexpression of DREB1 proteins in *Arabidopsis* generates a drought and cold resistant phenotype, although this also associated with severe reductions in growth rate (Gilmour *et al.*, 2000; Kizis *et al.*, 2001).

ABA-dependent expression is largely governed by the ABRE that is bound by the bZIP ABRE binding proteins (ABREBs; Ingram and Bartels, 1996; Bray, 1997). The consensus sequence of the element is RYACGTGGYR*, with the ACGT forming the core. The ABRE does not act autonomously and is usually associated with coupling elements that regulate the ABA-dependent gene expression. Trans-acting factors such as the seed-associated *viviparous* (VP1) protein may also be required in conjunction with ABREBs, as indicated by studies that have shown that expression in *Arabidopsis* of a gene from *Craterostigma plantagineum* requires the presence of the VP1 homologue (Furini *et al.*, 1996; Bray, 1997; Chandler and Bartels, 1997).

Seki *et al.* (2001; 2002) have speculated on the relationship between ABA-dependent and independent gene expression, but their distinction between the two should not be taken as absolute. Recent work on the DREB genes has shown that the expression of some of these transcription factors is dependent on the accumulation of ABA (Kizis *et al.*, 2001) in contrast to the classical contention that they drive ABA-independent responses (Shinozaki and Yamaguchi-Shinozaki, 1997; Stockinger *et al.*, 1997). It is likely that the utilization of genes in stress responses is more complex than a linear model will convey. This is borne out by the fact that several other co-operative and independent promoter elements have been identified (Bray, 1997; Smirnov and Bryant, 1999; Bartels and Salamini, 2001; Fowler and Thomashow, 2002). In desiccation tolerant plants there is likely to be an additional interaction with the classical light responsive promoters in leaf tissues that accumulate anthocyanins (eg: *Craterostigma* and *Myrothamnus*) and in tissues that break down chlorophyll (eg: *Xerophyta*).

The identification of *cis*-acting elements in promoters constitutes a vital component of research into the molecular mechanisms of desiccation tolerance. Yet, this characterization rests on a prior knowledge of the genes that are co-ordinately regulated under stress conditions. The limited information available for such genes in desiccation tolerant systems is outlined below.

* Y = pyrimidine base, R = purine base

1.2.3 Gene expression and cellular responses to desiccation and rehydration

The onset of desiccating conditions results in the rapid implementation of features that 'chaperone' the plant into the appropriate physiological condition. In *Xerophyta* species, there are discernable changes in protein composition and synthesis rates (Tymms *et al.*, 1982; Tymms and Gaff, 1984) and in the activity of pre-existing enzyme systems upon drying and rehydration (Dace *et al.*, 1998; Farrant, 2000). Oztur *et al.* (2002) have estimated that 15 % of the transcriptome may be involved in responses to osmotic stress. For an average plant this means that between 3000 and 5 000 genes must be targeted for up- or down regulation during periods of substantial water flux.

Molecular studies have identified three main areas of gene control during desiccation and rehydration, namely (1) reduction of water loss, (2) implementation of protection, stabilization and quiescence mechanisms during dehydration and (3) repair, recovery and prevention of damage during rehydration (Bewley, 1979; Ingram and Bartels, 1996; Oliver *et al.*, 2000).

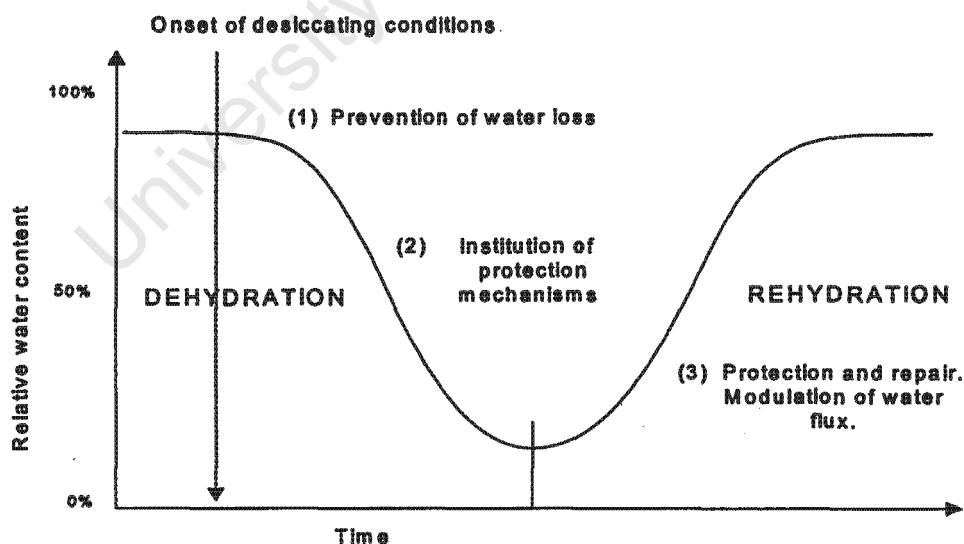


Figure 1.4: An overview of phases of gene expression in response to desiccation.

I) Regulation of water loss

Apart from transcription factors and proteins that govern the regulation of gene expression (eg: histones and related proteins), there are a number of products that are required to stabilize the plant system during the early phases of water deficit. Amongst these, aquaporin and H⁺-ATPase genes are some of the most rapidly expressed and activated (Mariaux *et al.*, 1998; Mundree and Farrant, 2000; Seki *et al.*, 2001). They help to increase the flow rate of water through stressed roots and to correctly partition the remaining water in order to avoid irreparable damage (Kirch *et al.*, 2000). They may also assist in the deliberate release of water from the plant below critical water contents. Aquaporin homologues in microbes have a well-established role in the transport of osmocompatible solutes such as glycerol (Maurel and Chrispeels, 2001) and may also contribute to the gradual replacement of water during later phases of desiccation.

Such changes are not restricted to the accumulation of proteins alone. Alterations in the nature of structural membrane proteins and membrane lipids may contribute to the modulation of water loss and of the damage incurred by such loss (Vertucci and Farrant, 1995; Mariaux *et al.*, 1998). These changes often include the switch from hydrophobic to hydrophilic proteins – an alteration that may be important in the binding of remaining water. The role of lipid composition and membrane structure is receiving renewed attention in plant and seed biology because the loss of membrane integrity is a classical feature of desiccation sensitivity (Smith and Berjak, 1995; Vertucci and Farrant, 1995; Hilhorst, 1998).

II). Protection, stabilization and quiescence during dehydration

The accumulation of several types of compounds occurs during the intermediate phase of drying. These compounds assist in the reduction of metabolic activity and contribute to the protection and stabilization of the cellular constituents prior to onset of completely anhydrous conditions, often through the formation of cytoplasmic glasses. Compounds accumulated in both drought resistant and desiccation tolerant plants include non-

reducing sugars (Vertucci and Farrant, 1995; Ghasempour *et al.*, 1998), complex carbohydrates (Ingram *et al.*, 1997), osmocompatible solutes (Franco and Melo, 2000) and proline (Iyer and Caplan, 1998; Gibon *et al.*, 2000). Aspects of proline and carbohydrate metabolism will be addressed in greater detail in Chapter 2.

During desiccation free radicals accumulate due to the higher failure rate of chemical reactions in an increasingly water-poor matrix (Vertucci and Farrant, 1995; Suna and Leopold, 1997). However, this damage is limited in part by the down-regulation of chloroplast and photosystem II genes, and the dismantling of the chloroplast constituents (Tuba *et al.*, 1996; Dace *et al.*, 1998; Farrant, 2000; Oztur *et al.*, 2002). This characteristic feature of poikilochlorophyllous plants serves to reduce light-chlorophyll interactions, and thus, photo-oxidation in conditions in which photosynthesis is not possible or conducive to survival (Sherwin and Farrant, 1998). There is also an extensive reliance on the upregulation of anti-oxidant mechanisms to overcome free-radical induced damage. Such systems include gamma-glutamylcysteine synthetase (ECS) responsible for the synthesis of glutathione (May *et al.*, 1998), glutathione reductase, catalase, superoxide dismutase and ascorbate peroxidase (Sherwin and Farrant, 1998; Farrant, 2000). Lee *et al.* (2000) have also demonstrated that the cys-peroxiredoxin family of enzymes act as anti-oxidants when coupled to sulfhydryl reducing systems in dehydrated seeds. Consequently, it is expected that the genes related to anti-oxidant systems will form a large component of any stress-related responses.

Additional factors that constitute a large component of the molecular responses to desiccation are the late-embryogenesis accumulated (LEA) and dehydrin (or LEA-D11) proteins (Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Egerton-Warburton *et al.*, 1997; Bartels and Salamini, 2001). Although these proteins have no known catalytic activity, they are highly conserved, suggesting that they are important in protecting the integrity of cellular constituents, perhaps by water binding or replacement during the dry state (Dure, 1993; Vertucci and Farrant, 1995; Colmenero-Flores *et al.*, 1999). This role is facilitated by their extensive hydrophilicity, due mainly to the glycine-rich motifs that

retain water (Franco and Melo, 2000; and references therein). Other demonstrated roles for LEA proteins include the sequestration of ions (Dure, 1993), molecular chaperone activity and the transport of nuclear-targeted proteins during stress (Goday *et al.*, 1994; Close, 1996; Colmenero-Flores *et al.*, 1999).

Heat-shock proteins, which are thought to protect enzyme (and notably photosystem II) functioning in stressed plants (Schroda *et al.*, 1999), appear to be acquired in response to stress in a non-organ-specific manner and may also be a feature of root responses to desiccation in *X. humilis*.

III) Repair, recovery and prevention of damage during rehydration

Comparatively little work has been completed on the molecular processes which prevent cell death upon rehydration. There must be extensive mechanisms in place to limit and repair the damage done to cellular machinery incurred during the initial phases of the process. In *T. ruralis* the synthesis of at least 25 proteins is terminated, and that of 74 initiated during rehydration (Velten and Oliver, 2001). The latter proteins – termed rehydrins – may play an important role in attenuation of cellular damage and in stabilizing membranes which are extremely susceptible to shearing (Vertucci and Farrant, 1995; Hilhorst, 1998; Hoekstra *et al.*, 2001; Velten and Oliver, 2001). In *Xerophyta* species metabolic recovery is comparatively slow due to the requirement for repair of the photosynthetic apparatus (Tuba *et al.*, 1998; Oliver and Bewley, 2000). The initial independence of recovery from nuclear control may also allow time for the genomic irregularities resulting from desiccation to be identified and repaired (Dace *et al.*, 1998). Such genome repair is said to occur in seeds (Smith and Berjak, 1995), and thus will presumably occur in roots. The use of replacement histones may also be a critical feature of the stabilization and regulation of nuclear activities during this period (Velten and Oliver, 2002).

Protection mechanisms must also include the enzymes responsible for mitochondrial recovery and carbohydrate metabolism that allow resumption of respiration and rapid

removal of accumulated osmoprotectants and toxins (Ingram and Bartels, 1996). In the roots, a large role may be played by water and ion channels, which remove embolisms that are a consequence of xylem emptying (Netting, 2000). Rehydration thus poses a unique challenge to the plant, and calls for the expression or activation of a number of specific genes. The vast majority of these have not been identified.

There are two important features of the above discussion. The first is that the majority of the genes considered crucial are active in leaf tissues, largely because there are no published reports of gene expression studies in the roots of desiccation tolerant plants. The second is that very few of these 'stress genes' are actually directly associated with desiccation tolerant plants. Despite the vast literature on the molecular responses to desiccation, relatively few genes have been cloned from resurrection plants. A number of these are listed in the table below (Table 1.1).

Nevertheless, what emerges most clearly from the literature on the molecular mechanisms of desiccation tolerance and stress resistance is that these are complex phenomena, and that elucidation of the key features will rely initially on broad-scale analyses. The value of high quality cDNA libraries as the basic currency in the cataloging of the vast array of molecular responses will be outlined in chapter 4.

Table 1.1: Some differentially expressed genes cloned from resurrection plants

Plant	Gene(s)	Identity	Reference
<i>C. plantagineum</i>	<i>pcC -</i>	Dehydrins	Piatkowski <i>et al.</i> (1990)
	<i>pcC -</i>	LEAs	"
	<i>pcC 37-31</i>	Early-light-inducible protein	Bartels <i>et al.</i> (1992)
	<i>GapC-Crat</i>	Cytosolic GPD	Velasco <i>et al.</i> (1994)
	<i>Tkt3, 7, 10</i>	transketolases	Bernacchia <i>et al.</i> (1995)
	<i>pSPS1</i>	Sucrose-phosphate synthase	Ingrams & Bartels (1996)
	<i>pSSI; pSS2</i>	Sucrose synthases	"
	<i>Cpm5, 10</i>	Myb transcription factor	Iturraga <i>et al.</i> (1996)
	<i>CpABI-3</i>	<i>ABI-3</i> homologue	Chandler and Bartels (1997)
	<i>CpSPS1, 2</i>	Sucrose phosphate synthase	Ingram <i>et al.</i> (1997)
	<i>CpPIPa, h, c</i>	Plamamembrane intrinsic proteins	Mariaux <i>et al.</i> (1998)
	<i>CpIIP</i>	Tonoplast intrinsic protein	"
	<i>CpPLD1, 2</i>	Phospholipase D	Frank <i>et al.</i> (2000)
	<i>CpALDH3, 4</i>	Aldehyde dehydrogenase	Kirch <i>et al.</i> (2001)
	<i>CpHB3-7</i>	Homeodomain leucine zipper genes	Deng <i>et al.</i> (2002)
	<i>CpR18</i>	SAP transcription factor	Hilbricht <i>et al.</i> (2002)
	<i>CpPTPs</i>	Plastid targeted protein family	Philips <i>et al.</i> (2002)
<i>T. ruralis</i>	<i>Trs</i>	Rehydrins (18 genes)	Scott and Oliver (1994)
	<i>Tr288, 155</i>	Rehydrins	Oliver <i>et al.</i> (1995)
	-	Ubiquitin	O'Mahony and Oliver (1999)
	<i>Rps14, 16</i>	Ribosomal proteins	Wood <i>et al.</i> (2000)
	<i>S3a</i>	Ribosomal protein	Duff <i>et al.</i> (1999)
	<i>rh153</i>	Replacement histone	Velten and Oliver (2002)
<i>S. stapfianus</i>	<i>Rab2</i>	GTP-binding protein	O'Mahony and Oliver (1999)
	-	Polyubiquitin	O'Mahony and Oliver (1999b)
	<i>SDG137c</i>	Small glycine rich protein	Neale <i>et al.</i> (2000)
	<i>SDG43c</i>	Glycine and proline rich protein	"
	<i>SDG134c</i>	Protein initiation factor	"
	<i>SDG69c</i>	Early light inducible protein	"
	<i>SDG37c</i>	Serine/threonine phosphatase	"
	<i>SDG50c</i>	Gamma tonoplast intrinsic protein	"
<i>X. viscosa</i>	<i>AldrXV4</i>	Aldose reductase	Mundree <i>et al.</i> (2000)
	<i>XVT6</i>	Dehydrin	Mundree and Farrant (2000)
	<i>XVT8</i>	LEA	Ndima <i>et al.</i> (2001)
	<i>XVT15</i>	Lectin	Mundree and Farrant (2000)
	<i>PAPXV</i>	Acid Phosphatase	"
	<i>RHXV</i>	Rehydrin	"
	<i>CBPXV</i>	Calcium binding protein	"
	<i>GSXV</i>	Galactinol synthase	"

1.3 Targeted Approaches to Dissection of Stress-Response Pathways

In a complex background of gene flux such as that described above, it is extremely difficult to target vital components of the desiccation tolerance mechanisms using traditional molecular techniques. In addition, reverse genetic approaches are limited by the lack of tissue-cultured material and by the slow germination and growth rates of desiccation tolerant species (Oliver and Bewley, 2001). The recent development of microarrays (Schena *et al.*, 1995) has, however, made the comprehensive analysis of the molecular responses to desiccation possible.

Microarrays are basically extensions of the Southern and reverse northern blot procedures (Eisen and Brown, 1999; Lander, 1999). In their most common form, they involve the immobilization of defined cDNAs (the 'probes'; Phimister, 1999) on glass or perspex slides (Eisen and Brown, 1999). Slides consisting of up to 1000 genes per cm² and 30 000 independent probes in total are now commonly available (Lander, 1999). For transcript profiling, two populations of RNA or mRNA (the 'targets', Phimister, 1999) are labeled with different fluorescent dyes and hybridized to the chip. Comparison of the signal intensities for the two dyes allows differential levels of target to be identified (Figure 1. 5; Epstein and Butow, 2000). The range of applications for microarrays is expanding extremely rapidly to include the use of oligonucleotide probes and genomic DNA in the identification of drug targets, evolutionary relationships, disease causing mutations and organ or tissue specific expression patterns (Epstein and Butow, 2000). Essentially the technique may be modified and adjusted to suit the research in question.

The power of the microarray analysis is considerable and is being demonstrated in plant research on an increasing number of occasions. The regulation of the *Arabidopsis* transcriptome has been studied during the cell cycle (Vandepoele *et al.*, 2002), seed development (Girke *et al.*, 2000), circadian rhythms (Harmer *et al.*, 2000), plant development (Zhu *et al.*, 2000) and in response to oxidative stress (Desikan *et al.*, 2001), nitrate flux (Wang *et al.*, 2001) and a variety of abiotic stresses (Seki *et al.*, 2001; Cheong *et al.*, 2002; Fowler and Thomashow, 2002; Seki *et al.*, 2002).

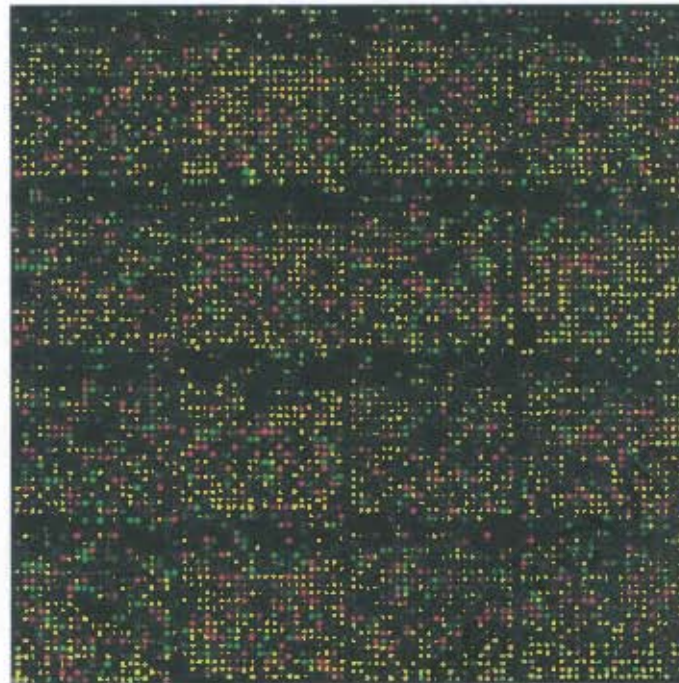


Figure 1.5: An example of a microarray slide. Spots indicate the position of independent cDNAs that have been competitively targeted by populations of Cy3/ Cy5 labeled mRNA. The relative intensity of the red: green channels for an individual spot is indicative of the different levels of a particular mRNA in the sample (Image obtained from the *Arabidopsis* Functional Genomics Consortium).

In studying plant responses to water deficit, Seki *et al.* (2001) arrayed 1 300 *Arabidopsis* cDNAs and identified 44 drought- and 19 cold-inducible genes, of which 30 and 10 respectively were novel. They have recently expanded the analysis to include 7 000 full length cDNAs, and have consequently identified an additional 53, 277 and 194 genes that are dramatically up-regulated in response to drought, cold and salinity stress, respectively (Seki *et al.*, 2002). Based on the results, the authors have proposed that there is a large overlap in the utilization of genes for the amelioration of divergent stresses - a contention that has been supported by the analysis of the differential expression 8 200 *Arabidopsis* genes in response to pathogen attack, hormone treatment, heat shock and osmotic stress (Cheong *et al.*, 2002). However, because the

conditions for water deficit used by Seki *et al.* (2001; 2002) involved direct air-drying of plants removed from agar plates, generalized stress responses would be expected, suggesting that these results may be misleading. Nevertheless, Bray has recently compiled a list of 130 genes that are upregulated in response to water deficit in *Arabidopsis* (Bray 2002). The bulk of these appear to be related to signaling and removal of toxins. A similar pattern of expression has been shown for drought and salt stressed barley (Oztur *et al.*, 2002).

The use of microarrays has undoubtedly enhanced the ability of researchers to target important genes more effectively than previously possible. But, while such microarrays are useful in the categorization of molecular features that govern the responses of plants to stress, they only provide limited insight into the features that might be involved in desiccation tolerance. The lack of low-redundancy genesets or large-scale sequence information prohibits the production of cDNA or oligonucleotide arrays for the analysis of the responses of non-model organisms such as *X. humilis*. Consequently the initial step in any systematic approach to transcriptome analysis is the production of the molecular resources that underlie such research (Clark *et al.*, 1999). The generation of normalized (or low-redundancy) EST libraries as a means of addressing this is outlined in chapter 5.

It is important to bear in mind that RNA expression profiles do not always correlate with protein expression or activation patterns (Kuhlemeier, 1992; Wood and Oliver, 1999; Bray, 2002) and that verification functionality of identified genes is imperative. Nevertheless, it is envisioned that molecular resources for the analysis of the transcriptomes of desiccation tolerant plants will become increasingly available in the near future, and facilitate the genome-wide comparison of different strategies to the survival of water deficit stress.

1.4 Summary and Aims

It is clear that there is a critical lack of information about the role of the roots in desiccation tolerance. Studies of the changes that occur in non-tolerant plants, and in the leaves of desiccation tolerant plants have provided a basic framework of information which may pinpoint features that are relevant to desiccation tolerance in *X. humilis*. Based on such information, this study aims to examine the role of the roots in the tolerance of desiccation and rehydration in *X. humilis* by:

1. determining the changes in the water contents and water potentials of roots in relation to those of the leaves;
2. observing anatomical changes in the roots and comparing these to known changes in the leaves;
3. creating cDNA libraries of transcripts involved in desiccation and rehydration in the roots;
4. normalizing the cDNA libraries in order to allow for efficient sequencing and arraying of a low-redundancy gene-set;
5. analyzing the representation and expression of selected genes during desiccation and rehydration; and
6. cloning and characterizing two component systems in *X. humilis* roots.

Physiology and Anatomy

Root responses to water flux

2.1 Introduction

Plants experience both chemical and physical stresses during desiccation and rehydration (Vertucci and Farrant, 1995). If unchecked, these stresses result in loss of the structural and functional integrity of the cellular environment. The preservation of anatomical order thus requires significant adjustments at the metabolic, ultrastructural and whole plant levels. The ability to mobilize the vast array of resources required is often a distinguishing factor between tolerant and non-tolerant plants.

Several important processes occur at the anatomical level in the leaves of *Xerophyta* during desiccation and rehydration. As water is lost, the large vacuoles subdivide and are filled with stable metabolites as a means of retaining outward pressure on the cell membrane (Farrant, 2000). The chloroplasts are dismantled and the mitochondria lose their cristae (Hallam and Luff, 1980; Sherwin and Farrant, 1998; Farrant, 2000). The leaves become increasingly rich in anthocyanins as the chlorophyll is removed and eventually fold along the midrib as complete desiccation is approached. Unlike in other desiccation tolerant plants, the folding of the leaves of *X. humilis* is not related to folding of cell walls (Tuba *et al.*, 1993; Sherwin and Farrant, 1998; Farrant, 2000). The process of rehydration appears to involve the direct reversal of these changes (Dace *et al.*, 1998).

Unfortunately, there are no known studies of the changes undergone by the roots of *X. humilis* during this progression. Indeed details of the root anatomy of the genus *Xerophyta* are generally lacking. The members of this genus, like several other arborescent monocotyledons, have persistent pseudostems, consisting of fibrous leaf bases surrounded by adventitious roots. Root production shows an annual pattern,

and the roots are unusual in that they possess a *velamen radicum* (an outer sheath of dead cells) of one to several cell layers thick; Porembski and Barthlott, 2000). The *velamen* is a common trait of the Orchidaceae where it assists in rapid water uptake, and hence it may be an important part of the functional morphology of desiccation tolerant members of the genus *Xerophyta* (Porembski and Barthlott, 2000). It is possible that the changes during drying in these organs mirror those observed in the leaves, but this is not certain.

2.1.1 Metabolic adjustment and the preservation of structural integrity

Cell membranes are regarded as the primary site of desiccation injury in plants largely because elevated electrolyte leakage during rehydration is one of the most diagnostic features of damage (Senaratna and McKersie, 1983; McKersie, 1996). Numerous ultrastructural studies have confirmed that membrane disorganization and rupture is a common phenomenon in sensitive plants. However, cell wall shearing, large-scale agglutination of cellular components and metabolic disintegration due to free radical damage are also common consequences of desiccation-induced damage (Vertucci and Farrant, 1995; Walters *et al.*, 2002).

Several altered metabolic processes make the anatomical adjustment to desiccation possible. Of these, carbohydrate metabolism is thought to have a particularly important role. Oligosaccharide accumulation has been convincingly shown to protect the integrity of membranes and membrane functioning in anhydrous conditions, possibly by preventing Maillard-type reactions (Ingram and Bartels, 1996; Hoekstra *et al.*, 1997; Suna and Leopold, 1997). Sucrose in particular is thought to interact with membrane lipids at low water content. Through the formation of hydrogen bonds between the hydroxyl groups of the sugar and the phosphate group of the phospholipid, sucrose acts as a replacement for water and maintains the hydrophobic-hydrophilic orientation of the membrane phospholipids (Ingram *et al.*, 1997; Oliver *et al.*, 2001). Sucrose deposition may also maintain cytoplasmic pressure against the cell wall, thereby preventing cell collapse in the desiccated state (Ingram and Bartels, 1996; Ghasempour *et al.*, 1998; Farrant, 2000).

Carbohydrates play an integral role in the formation of cytoplasmic glasses that stabilize the cellular environment. Oligosaccharides, especially raffinose and stachyose prevent crystallization of sucrose, phase separation and membrane deterioration (Suna and Leopold, 1997). Vitrification also helps reduce the metabolic rate and thus, the damage incurred by free radicals. This is aided by the upregulation of enzymes involved in the synthesis of non-reducing sugars and disaccharides, which removes monosaccharides that act as the substrates for energy conversion and free-radical production (Vertucci and Farrant, 1995; Ingram *et al.*, 1997; Ghasempour *et al.*, 1998). Increased quantities of malate and citrate are excreted into the soil from the roots of several crop plants. Their removal serves to reduce the availability of glycolytic precursors and intermediates, thereby reducing respiratory rates (Serraj *et al.*, 1999; Lopez-Bucio *et al.*, 2000). In general, the resultant reduction in metabolic activity may well be a vital component of desiccation tolerance as studies have shown that metabolic dysfunction and unabated respiration are a feature of desiccation sensitive radicles (Farrant *et al.*, 1997; Leprince *et al.*, 2000).

Proline accretion is a well-documented plant response to stress that may well have a role in physical preservation of the cellular environment. Because of its zwitterionic, highly hydrophilic characteristics, it is able to accumulate to high levels in the cells without interfering with metabolic processes (Verslues and Sharp, 1999). It is correlated to chlorophyll loss and decreases in mitochondrial activity in canola (Gibon *et al.*, 2000), and the products of its metabolism affect gene expression and selectively inhibit the translation of certain mRNAs (Iyer and Caplan, 1998). Yet, the primary roles of proline appear to be as a protectant, pH regulator and energy source (Aziz *et al.*, 1999; Franco and Melo, 2000). It has been shown to reduce enzyme denaturation and membrane damage by shielding these against free radicals and physical stresses and is osmoprotective and cryoprotective to higher plant cells when applied exogenously (Iyer and Caplan, 1998). Unlike perturbing solutes which readily enter the hydration sphere of proteins and cause unfolding, compatible osmolytes tend to be excluded from the hydration sphere of proteins and stabilize folded protein structures. On the other hand, proline catabolism may maintain $\text{NADP}^+/\text{NADPH}$ ratios at values compatible with metabolism and may provide reducing equivalents that support

mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damage (Hare and Cress, 1997). Despite this, however, the relationship between proline and stress tolerance remains uncertain. Some authors maintain that it is merely a response to stress, rather than a means of ameliorating it (Hare and Cress, 1997).

A number of protein products actively maintain the structural and functional integrity of plant cells during water stress and desiccation. Among these osmotic responsive genes are LEA proteins, dehydrins, heat shock proteins, oleosins, lipoxygenases, osmotin, and ion and water channels (Franco and Melo, 2000). These are often glycine-rich, hydrophilic proteins that retain water, exclude ions, chaperone molecules, assist in the formation of glasses and generally stabilize the structure of the hypo-osmotic cell (Franco and Melo, 2000).

Obviously, alterations in protein composition and carbohydrate and proline levels are not the only shifts involved in the preservation of metabolic and anatomical order. Polyphenols, quaternary ammonium compounds (such as betaines), polyamines and alcohols all have similar functions (Franco and Melo, 2000). However, their roles are less well understood and it is not clear to what extent the function is unique to each. What is clear is that failure to accumulate a certain set of such metabolites leads to rupture of membranes and cell walls, which in turn results in measurable electrolyte leakage and observable cellular damage.

Since little is known about the roots of *X. humilis* in relation to the features discussed above, this chapter outlines an investigation of the functional anatomy of these organs. The study aimed to determine what changes occur in these organs during desiccation and rehydration, whether cellular integrity is maintained, and whether proline and simple carbohydrates are involved in the preservation of the cellular environment.

2.2 Materials and Methods

2.2.1 Plant material

Xerophyta humilis (Bak.) Dur and Schinz specimens were collected in the Buffelskloof Nature Reserve, potted in a mixture of peat, river sand and potting soil and maintained in a greenhouse as described by Sherwin and Farrant (1996) and Farrant *et al.* (1999). For the experiments described below, plants were transferred to a Phytotron® unit set to 16 h light and 8 h dark cycles for dehydration cycles. Light intensity during the day was $1500 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ as measured by a Li-Cor light meter (Li-Cor, USA), while the temperature and humidity were maintained at $25 \pm 2^\circ\text{C}$ and 50-65 % respectively. During the dark phases the temperature was kept at $18 \pm 2^\circ\text{C}$. Dehydration of the plants was commenced by withholding water, and rehydration by extensive re-watering of the soil.

Leaves removed from the plants were briefly wiped with dry paper towel, flash frozen in liquid nitrogen. The roots were excised and the soil and debris removed by vigorous rubbing (in the case of the fully dehydrated roots) or by brief washing (in the case of hydrated or partially hydrated samples). These bulk samples were then carefully separated into living and dead roots by visual inspection based on colour, size and fragility. Except for microscopical studies, in which the living and dead roots were examined, only the living roots were used. These were flash frozen in liquid nitrogen and stored with the leaves at -70°C until required. In all cases unfrozen samples were set aside for water content and water potential determination as described below.

2.2.2 Water content and water potential determinations

Water contents were measured gravimetrically as described by Tymms *et al.* (1982) and Sherwin and Farrant (1998). Three samples consisting of a minimum of 5 leaves or roots each were weighed on a Mettler (Mettler, USA) five-place balance and desiccated for 24 h at 105°C . Dry samples were transferred to a desiccator containing silica gel and left to cool to room temperature. The dry masses were determined and

the absolute water content (AWC) and relative water content (RWC) calculated. Water content at full turgor was predetermined by hydrating several plants for 24 h in a closed environment. The average water contents were determined as described above, but 7 replicates of four leaves or roots each were used.

Water potentials were obtained indirectly through the measurement of water activity by a series 100 Aqualab (Aqualab, U.S.A). Samples were cut into 1 cm long sections prior to positioning in the receiver vessel of the Aqualab. Determinations were carried out for a minimum of 30 min or until at least 5 consecutive identical readings were obtained. Water potentials were calculated according to the equation:

$$Wp = \frac{RT}{MW} \times \ln(A_w)$$

R = Universal Gas constant (8.314 J. mol⁻¹.K⁻¹)

T = Absolute Temperature (K)

A_w = Measured Water Activity

MW = Molecular weight of H₂O (18.0 g.mol⁻¹)

For both water contents and water potentials, the means were represented graphically as functions of dehydration or rehydration time.

2.2.3 Microscopy

i. Scanning electron microscopy (SEM):

Hydrated and desiccated roots were removed from the plants, flash frozen in liquid nitrogen slush and transferred to a Fissons LT 5400 cryo-stage. The roots were fractured with a cold blade and the ice sublimated from the surfaces. Samples were viewed in a Leica SS440 scanning electron microscope (Leica, U.K.).

ii. Transmission electron microscopy (TEM):

Hydrated samples were excised from the parent plants and small (~ 0.5 mm) pieces fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer containing 0.5% caffeine. Post fixation was in phosphate buffer containing 1% osmium tetroxide. These samples were taken through an ethanol dehydration series and embedded in Spurr's epoxy resin (Spurr, 1969). Sections were cut at 95 nm on a Reichert Ultracut-S microtome and stained with uranyl acetate and lead citrate (Reynolds, 1963). Sections were visualized on a Zeiss EM109 transmission electron microscope (Zeiss, U.S.A). At least 3 sections from 5 different specimens were observed.

Dry specimens were fixed non-aqueously to prevent artifactual rehydration in the chemical fixatives (described above). Dehydrated roots were cut at an acute angle with a scapel blade into 0.5 mm sections. These sections were plunge frozen into liquid propane and freeze substituted (Parthasarathy, 1995; Thomson and Pratt; 1997) as described by van der Willigen (2002). Frozen sections were maintained at -80°C and transferred sequentially into dry acetone for 6 h, 0.1 % tannic acid in dry acetone for 24 h, and 2 % uranyl acetate and 2 % anhydrous glutaraldehyde in dry acetone for 48 h. Samples were warmed to 0°C at 1°C/ h, and placed in 2 % osmium tetroxide, 2 % glutaraldehyde and 2 % uranyl acetate in dry acetone for 24 h at 0°C. After rinsing for 12 h at 0°C in fresh dry acetone, 2 % uranyl acetate and 2 % glutaraldehyde, samples were warmed to 20°C at 1°C/ h and embedded in Spurr's epoxy resin (Spurr, 1969). Sections were cut with a diamond knife at 95 nm, and visualized using a Joel 200 CX TEM without post-staining. At least 10 sections from 5 roots and 2 independent freeze substitutions were analyzed.

2.2.4 Electrolytic leakage

Electrolytic leakage of dehydrated and partially rehydrated roots and leaves were determined by placing tissue segments directly into 3 ml of Milli-Q (Millipore, U.S.A) water in a CM100 conductivity meter. A minimum of 5 leaf samples and 10 root replicates were used. 100 measurements were taken, one every 90 s during the first

determination and one every 20 s during the second determination. The rate of electrolytic leakage for each sample was calculated and expressed relative to the dry mass of tissue used. The electrolytic leakage of hydrated leaf and root tissue that had not been desiccated was used as a control.

2.2.5 Sugar analysis

Sucrose, glucose and fructose levels in hydrated, desiccated and rehydrated roots and leaves were determined using the Boehringer Mannheim sugar food kit according to the manufacturer's instructions. Triplicate root samples were used and leaf samples were determined simultaneously as controls.

2.2.6 Proline analysis

Proline levels in hydrated, desiccated and rehydrated root and leaf samples were determined by colourimetry according to a modified method based on that of Magne and Larher (1992). This protocol eliminates the interference of high sample sugar levels on the determination. Approximately 0.2 g of tissue was ground under liquid nitrogen and placed in 2 ml sterile water. Samples were boiled for 30 min to extract the free proline. 250 µl of the cooled sample was removed and combined with 1 ml Ninhydrin reagent (1 % Ninhydrin in 60 % glacial acetic acid). These were boiled for 30 min prior to extraction against 2.5 ml toluene. The absorbance of the resulting organic phase was determined at 520 nm and the proline content calculated via reference to a standard regression curve generated via the same method. Identical determinations for the desiccated inner leaves of *Eragrostis nindensis* – for which the proline levels are known – were used as a control.

2.3 Results

2.3.1 Gross morphology

X. humilis specimens were found to grow in large mats or clumps, and individuals exhibited a creeping habit. The roots originated from the pseudostem, which was

covered with fibrous leaf scales. The roots of *X. humilis* were observed to be adventitious, though not extensively ramified (Figure 2.1). The annual pattern of growth was apparent from the two types of roots present in the clumps. Older roots had a distinct darker colour and were fragile compared to the younger white roots, which were extremely resilient. The temporal and spatial separation of the two seasons' growth is illustrated in Figure 2.1. Main roots were up to 20 cm in length and 0.1-1 mm thick. No significant differences were observed at the morphological level between hydrated and dry roots, except for a slight crumpling of the root tips in dry specimens.

2.3.2 Anatomy

SEM confirmed the presence of the *velamen radicum* in *X. humilis*, though this was composed of only approximately 1-3 layers of dead cells (Figure 2.2 A). The *velamen* was bordered internally by a single cell layered epidermis and a large exodermis consisting of 10-15 layers of tightly packed schlerenchyma cells (Figure 2.2 B). These exhibited a spiral pattern of thickening and formed a significant portion of the total root volume. The root cortex consisted of large, undifferentiated parenchyma cells surrounding the vascular sheath (Figures 2.2 C-D) that contained a radial array of pronounced metaxylem cells. Large metaxylem vessels (10–20 μm in diameter), protoxylem and phloem elements formed the bulk of the stelar region.

TEM of the hydrated roots indicated that the cortical cells contained one or two large vacuoles that surrounded the remaining organelles (Figure 2.3 A). The nuclei were large, but otherwise unremarkable (Figure 2.3 B). Few mitochondria were observed, and these were generally appressed to the plasmalemma. Cell walls were observed to be regular and intact (Figure 2.3). Apart from the possession of a *velamen* and the extent of cell wall thickening throughout, the root anatomy was found to be consistent with typical monocotyledon anatomy.

A.



B



Figure 2.1: Photographs showing the gross morphology of *X. humilis*. (A) The intact plant, and (B) separation of the living and older (dead) roots.

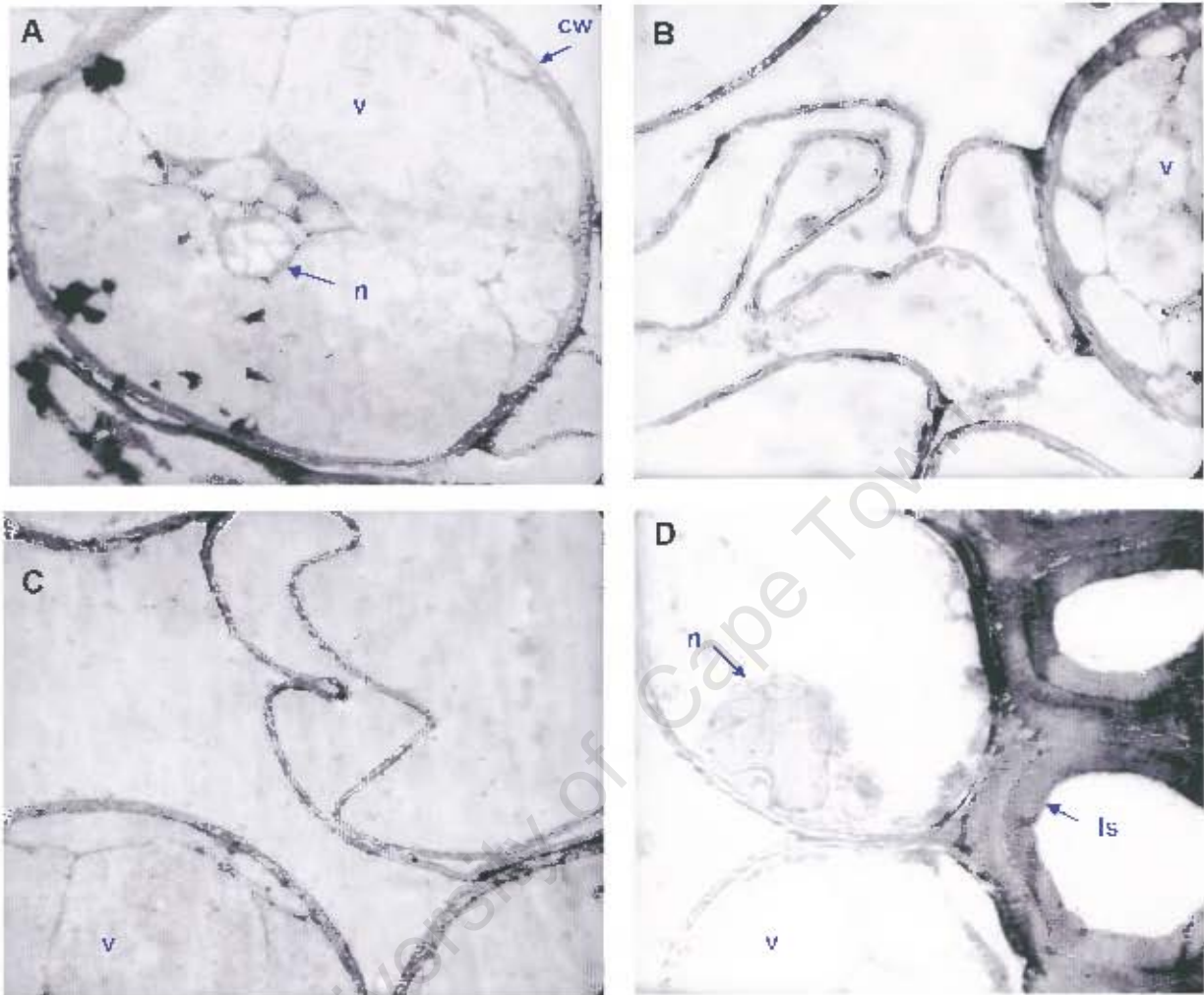


Figure 2.3: Transmission electron micrographs showing cross sections through cells of the cortex of a hydrated *X. humilis* root. (A-C) Cortical parenchyma cells [7 000 x] and (D) the cortical cells bordering the lignified stele [8 000 x]. cw = cell wall, ls = lignified stellar cell, n = nucleus, v = vacuole.

SEM of the older, darker roots revealed that these had undergone extensive collapse, with the cortical region shearing away from the exodermis (Figure 2.4 A). Except for the larger xylem vessels and the lignified stele, the cells of the cortical region were flattened and folded. This indicated that the cortical parenchyma was the only part of the root vulnerable to structural alteration.

Obtaining SEM images of dry roots was problematic due to large-scale shattering that was not observed in either hydrated or dead roots (Figures 2.4 C-D). This precluded identification or observation of any changes undergone during desiccation and was ascribed to the vitrification of the cytoplasm of the root cells during this process. The glass-like nature of the root shards was clearly visible (Figures 2.4 C-D).

Similar problems with root shattering were experienced in the cutting of dry roots prior to sectioning, though this could be avoided by slicing the roots at an angle to the surface. Non-aqueous fixation and TEM of these sections revealed that the cortical parenchyma cells had undergone extensive subdivision of vacuoles (Figure 2.5 C). The vacuoles were extremely small and appeared to contain an electron dense substance (Figure 2.5 D). In this regard the roots appeared to undergo similar ultrastructural changes described for the leaves (Sherwin and Farrant, 1998). Other organelles were not distinguishable from the vacuoles (Figures 2.5 C-D). Some apparent cell wall folding occurred in the cortical region, but this was variable and may have arisen during the sectioning of the roots for fixation. However, collapse of the root tips was visible prior to fixation, indicating that cell wall adjustment may occur in certain regions. As would be expected for dead tissue, the lignified stellar region was not visibly affected by desiccation, although occasional metaxylem vessel lumen blockage was observed (Figure 2.5 B). In general, analysis of root sections was extremely difficult due to the density of the cytoplasmic contents and the complexity of the arrangement of cellular components.

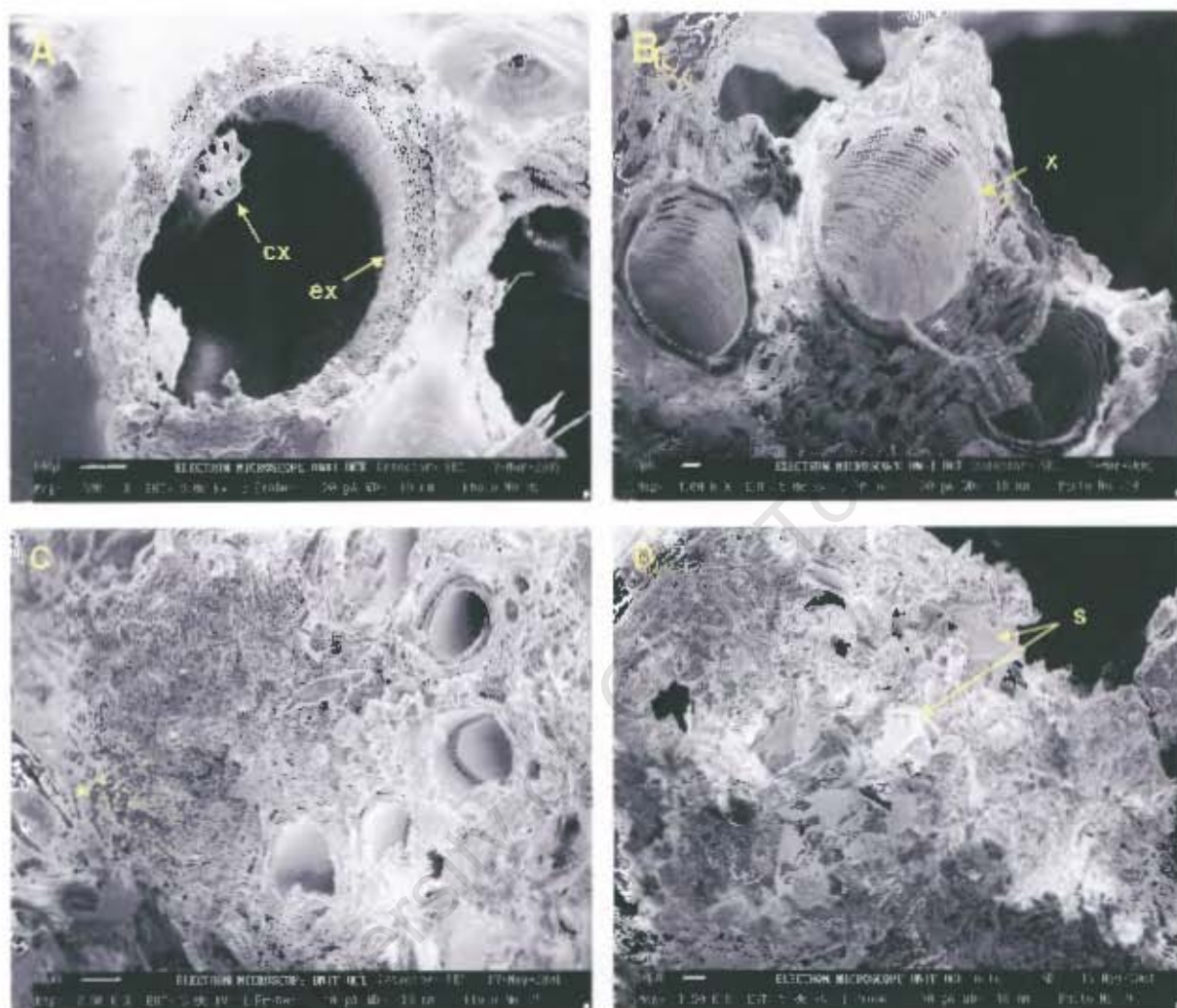


Figure 2. 4: Scanning electron micrographs of freeze-fractured (A-B) older (dead) and (C-D) desiccated *X. humilis* roots. Shattering of the dry roots is evident from the presence of the glass-like shards (s). cx = cortex, ex = exodermis, x = xylem. Magnifications and scale bars are indicated on the images.

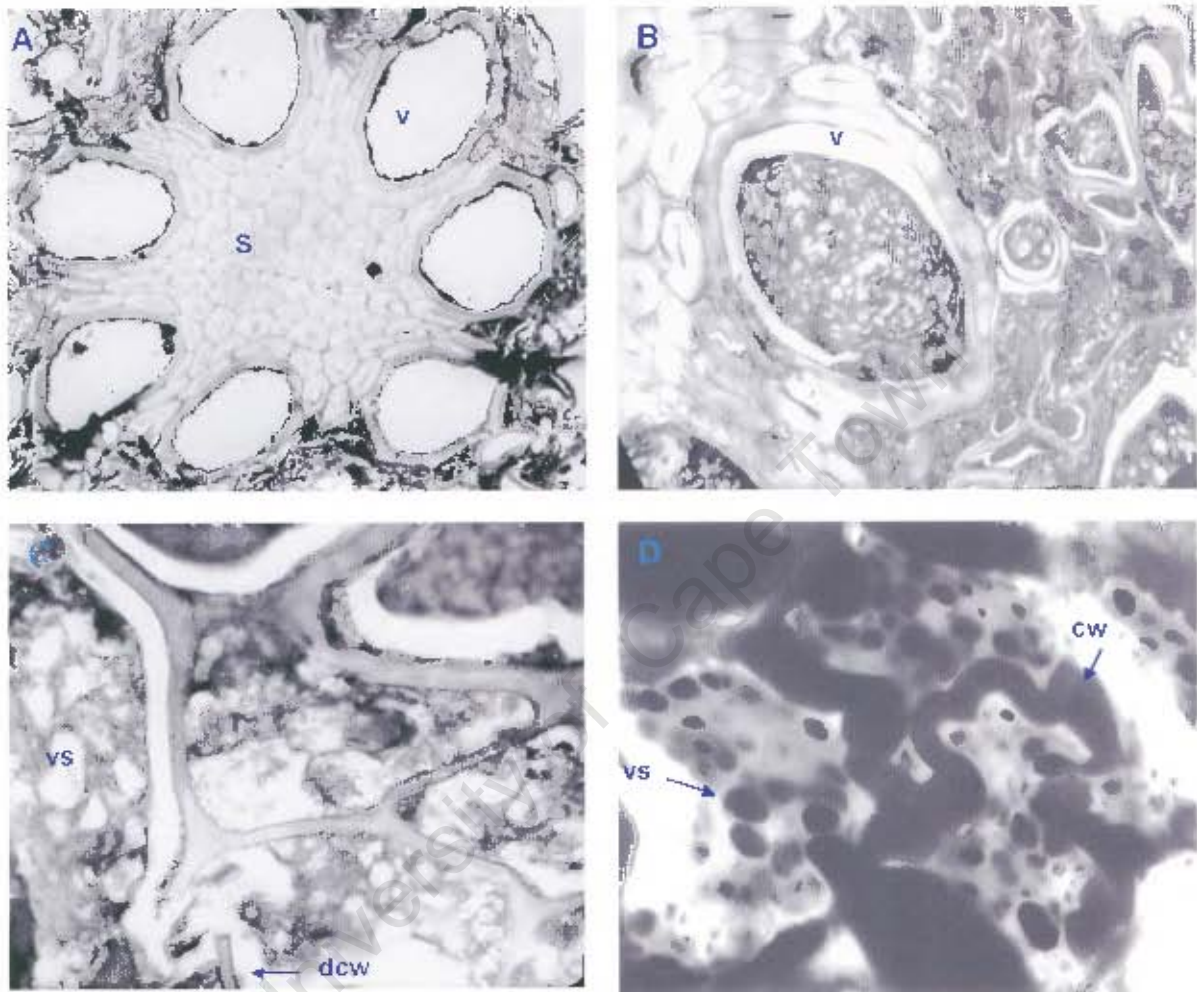


Figure 2.5: Transmission electron micrographs of cross sections through dehydrated *X. humilis* roots. (A) The intact stele [1 000 x] (B) metaxylem vessels [2 000 x] (C) cortical cells filled with vesicles [10 000 x] (D) young vacuolated cortical cells (*image inverted for clarity) [60 000 x]. cw = cell wall, dcw = damaged cell wall, s = stele, v = vessel, vs = vesicle containing electron dense substance.

2.3.3 Water contents and water potentials

Figure 2.6 A indicates that despite a small delay in the drop in RWC (between 0-7 days), roots of *X. humilis* were found to lose water at very similar rates to the leaves. The roots typically attained slightly lower RWCs than the leaves (approximately 3%). In all cases, the AWC of roots was significantly lower than that of the leaves (data not shown).

Roots were found to rehydrate extremely rapidly, attaining RWCs of 60-80 % within 3 h, and full hydration within 6-12 h after the application of water. This was several fold faster than the leaves, which only rehydrated within 24-48 h (Figure 2.6 B). This rapid rehydration was confirmed by the corresponding shifts in the water potentials of the roots and leaves (Figure 2.6 C). The discrepant rehydration of the roots was attributed to the absorption of the water by the *velamen* and exodermis. The leaves underwent re-greening during rehydration while no visible metabolic processes were evident for the roots. Attempts to monitor translation initiation with ^{35}S labeled methionine and leucine were unsuccessful due to low protein levels (data not shown), and as a result, the relative time frame for metabolic recovery could not be determined. Whether or not the rapid absorption of water by the roots corresponded to a 'sponge effect' rather than cellular re-activation was unclear.

2.3.4 Electrolytic leakage

Electrolytic leakage rates for leaves and roots did not differ significantly between hydrated controls and dehydrated or partially rehydrated samples (Figure 2.7) suggesting that structural integrity was maintained. Although rehydrated roots had 20 % higher leakage rates compared to controls, this was not considered to be indicative of extensive damage. For roots, the absolute values of the electrolytic leakage were observed to be very high during the first 30 min of analysis. This was conserved between controls and test samples, suggesting that it corresponds to release of ions from the surface of the roots rather than from membrane leakage.

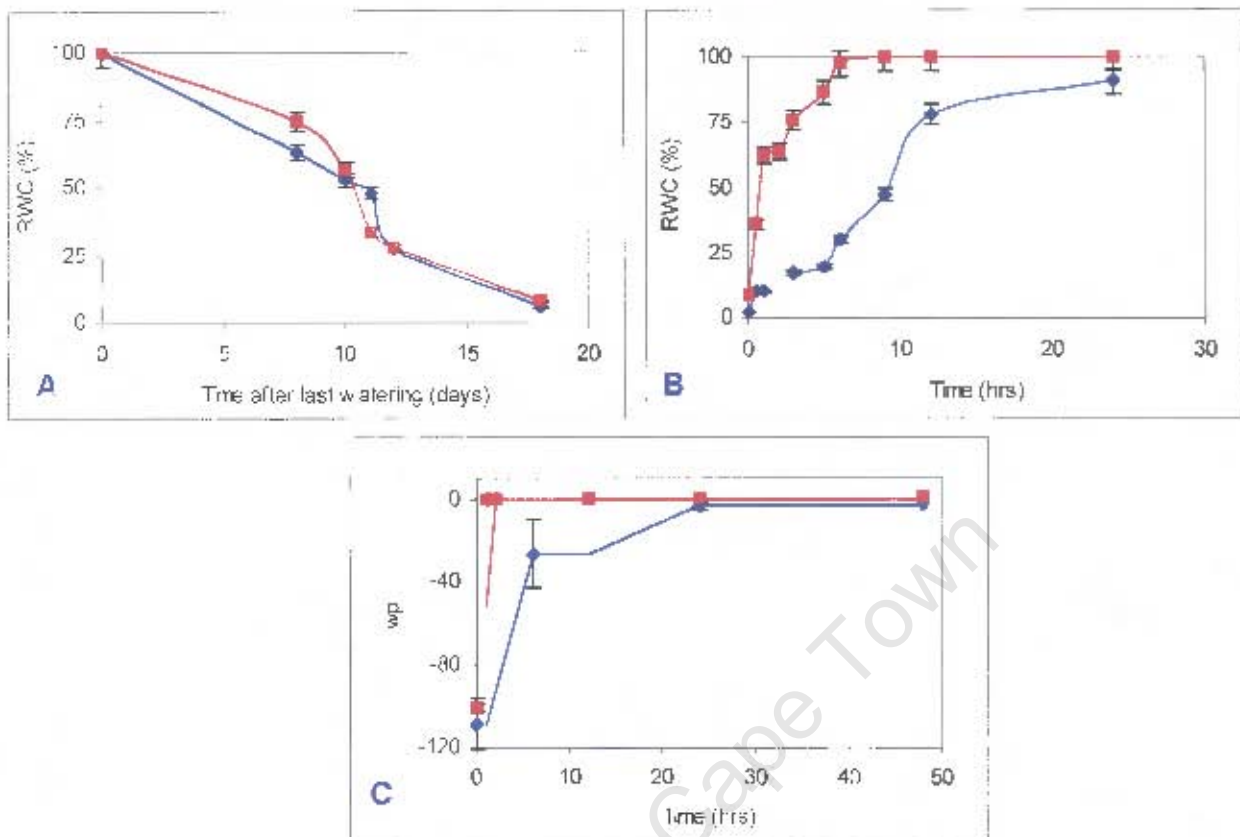


Figure 2.6: Changes in the water status of the roots and leaves of *X. humilis*. Relative water contents during (A) dehydration and (B) rehydration. (C) Water potentials during rehydration. Vertical bars represent standard deviations of the means.

[↑ roots; ♦ leaves].

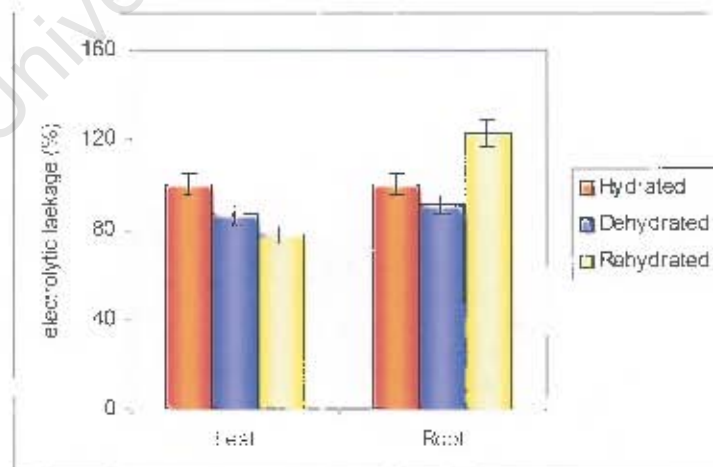


Figure 2.7: Electrolytic leakage of the roots and leaves of *X. humilis*. Values are expressed as percentages of the hydrated controls. Standard deviations of the means were set at 5 %.

2.3.5 Proline and sugars

In order to establish the nature of the substances accumulated in the vesicles and those responsible for the brittleness of the dehydrated roots, sugar and proline levels were investigated.

Dehydrated roots had approximately 3 fold higher levels of sucrose than hydrated samples (Figure 2.8). This accumulation of sucrose was considered significant enough to imply that sucrose could play a role in stabilization of the dehydrated cells. Glucose and fructose levels decreased, but this decrease combined with their levels in the hydrated tissue did not suggest that they were the primary carbohydrate source for sucrose synthesis. Sucrose levels decreased during rehydration, and this occurred in-line with a slight increase in glucose or fructose levels (Figure 2.8). The absence of a large increase in monosaccharide levels suggested that sucrose is utilized immediately as an energy source during recovery. The ratio of fructose to glucose in the root samples (approximately 6:1) was distinct from that that observed in leaf samples (approximately 2:1; Figure 2.8). Sucrose levels for the hydrated leaf controls were similar to those reported by Cooper (2001) indicating that the method used was sufficiently accurate.

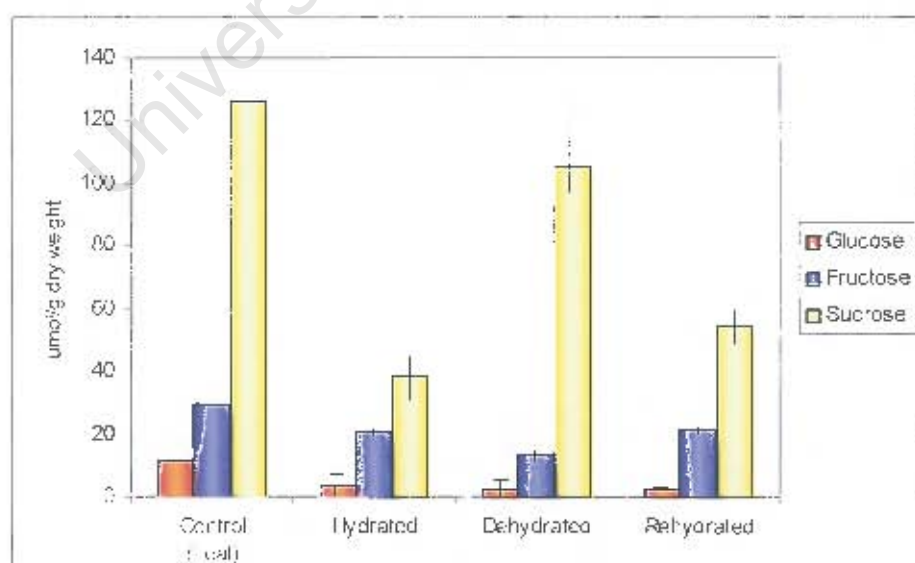


Figure 2.8: Glucose, fructose and sucrose levels in roots of *X. humilis*. Changes in glucose levels are non-significant ($P = 0.95$). Standard deviations are represented by vertical bars. The leaf data is presented as an internal control.

The levels of proline in the *E. nindensis* controls (Figure 2.9) corresponded exactly with those reported in the literature (Tymms and Gaff, 1979) indicating that the method employed was both accurate and free from interference by high sugar levels. Proline levels in the dry leaves of *X. humilis* were approximately double those in the hydrated samples and 3 times those in the rehydrated samples (Figure 2.9). This suggested that proline accumulated during desiccation and was actively removed during rehydration, possibly through its use as an energy source. Proline levels in the roots could not be determined by this method since a substance present in the samples caused the organic solvent fraction to mix with the aqueous fraction. Nevertheless, no colour development was observed in these samples, which could indicate that proline levels in these organs remain low.

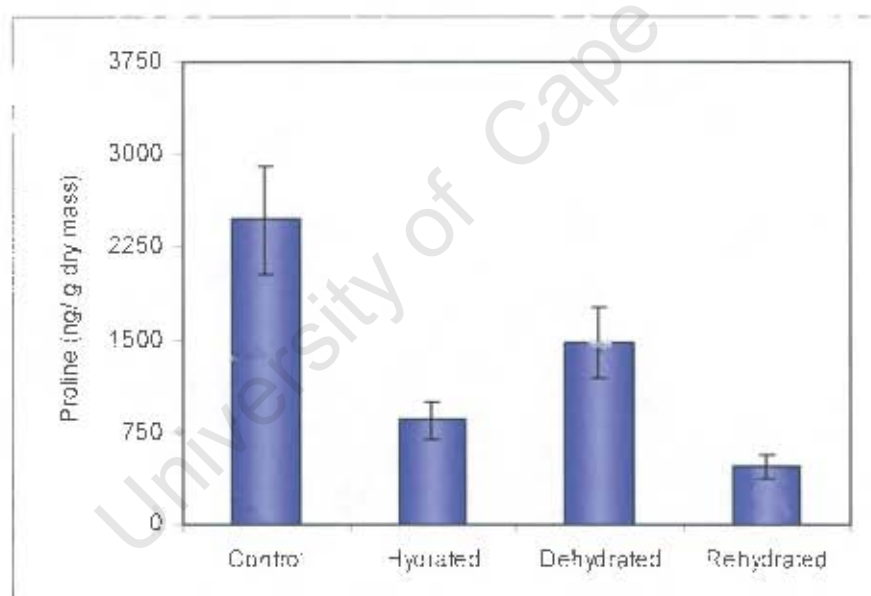


Figure 2.9: Proline levels in the leaves of *X. humilis* as determined by reference to a standard proline curve constructed under the same experimental conditions. Dehydrated inner leaves of *E. nindensis* were used as a control.

2.4 Discussion

The results provide interesting insights into the morphological and physiological mechanisms of desiccation tolerance in the roots of *X. humilis*. Morphologically, *X. humilis* is typical of plants adapted to inselbergs and hyper-variable habitats. The formation of clumps, which brings the fibrous leaf bases into close proximity, may assist in retaining water and may protect the meristems from fire damage (Porembski and Barthlott, 2000). They may also reduce the rate of water loss to enable protection mechanisms to be implemented (Farrant *et al.*, 1999). The creeping habit is not unusual within the family Velloziaceae which encompasses unusually extensive morphological variation (Kubitzki, 1998; Porembski and Barthlott, 2000). The pseudostems of *X. humilis* have not been studied in detail, but it has been suggested that they remain living at the apical portions only, an observation that is supported by the findings in this study (Figure 2.1). This pattern of growth is mirrored by the roots – a fact that suggests that the older tissues, like the older leaves of *C. plantagineum*, can only survive a limited number of desiccation and rehydration events (Norwood *et al.*, 1999).

From SEM of hydrated roots it was clear that although the *velamen radicum* is present in *X. humilis*, it is not as prominent as in other species in this family (eg: *Vellozia variiegata*; Porembski and Barthlott, 1995). In the Orchidaceae the *velamen* has a recognized role in water absorption, and the ability of members of the Velloziaceae and Cyperaceae to absorb water extremely rapidly has been attributed to its presence in their roots (Porembski and Barthlott, 1995; Porembski and Barthlott, 2000). However, from the size of the *velamen* in *X. humilis* (1-3 cell layers; Figure 2.2) it seems unlikely that this structure contributes significantly to the water absorbing capabilities of the roots. It is possible that the extensive exodermis (Figure 2.2 A-B) may assist in the process. From the SEM images of the older, presumably dead roots (Figure 2.4 A-B), it is clear that the exodermis plays a central role in maintaining the structural order of the roots.

In the hydrated state, the cortical cells form the largest living component of the roots. These cells appeared relatively unremarkable, with a large central vacuole occluding most of the cytoplasm (Figure 2.3). The transition to the desiccated state appears to involve extensive subdivision of the vacuoles (Figure 2.5), as has been reported for leaf cells of this and other desiccation tolerant systems (Vertucci and Farrant, 1995; Farrant and Sherwin, 1998; Farrant *et al.*, 1999). These vacuoles were similar in appearance to those observed in the leaf cells by Farrant and Sherwin (1998) and Farrant *et al.*, 1999). The nature of the electron dense substance is uncertain. Inorganic ions reportedly localize to the vacuoles during osmotic stress in some species (Rathinasabapathi, 2000) and this is a likely possibility in the roots. The subdivision of large vacuoles during desiccation is thought to be a mechanism of minimizing membrane damage and of minimizing mechanical stress by maintaining pressure against the cell wall (Farrant *et al.*, 1999). Limitation of physical strain is accomplished by cell wall folding in *C. wilmsii* (Farrant and Sherwin, 1998), but in *X. humilis* roots only limited (and possibly artifactual) wall folding was observed. In the root tips wall folding may occur due to the lack of extensive secondary thickening. Difficulties with TEM analysis of root sections is a common problem and is often related to the presence of high levels of carbohydrates (Buschman *et al.*, 2002).

The dense, lignified stele of *X. humilis* is typical of slow growing species, which invest in robust tissues with long life spans. It is associated with high tensile strength and ability to survive mechanical stresses (Wahl and Ryser, 2000). The metaxylem vessels in this region were extremely large (10-20 μm) relative to overall size of the roots (100-1000 μm). This may be a functional requirement for achieving high root pressures that assist in the refilling of embolised vessels - a mechanism that is thought to operate in *Myrothamnus flabellifolius* (Sherwin *et al.*, 1998; Tyree, 2001).

The rate of water loss from the roots and leaves were extremely similar (Figure 2.6 A) suggesting that both sets of organs lose their cellular water in-line with the removal of the water from the soil, as has been reported for *M. flabellifolius* by Sherwin *et al.* (1998). The rapid rehydration of the Velloziaceae is well documented (Porembski and Barthlott, 1995). However, the extent to which the roots supercede the aerial portions

in their rehydration (Figure 2.6 B) has not been described. This may be a 'sponge effect' facilitated by the anatomical structures described above, and metabolic recover may not occur in conjunction with water absorption. The water may be withheld at the exodermis or endodermis in order to facilitate slower (and presumably less damaging) cellular rehydration. Similar phases of rehydration have been observed by electrolytic leakage analysis of soybean axes (Senaratna and McKersie, 1986).

Investigation of electrolytic leakage rates confirmed that the desiccated and rehydrated roots experienced little membrane damage (Figure 2.7). The slight increase in the rate of leakage associated with root rehydration is not likely to be significant. It is probably associated with the release of endogenous amphiphiles from the plasma membrane (Hoekstra *et al.*, 2001) and can be readily distinguished from large-scale damage, which is often reflected by electrolytic leakage rates of 400-500 times higher than controls (Senaratna and McKersie, 1986; Sherwin and Farrant, 1998). The preservation of membrane integrity has been reported for the leaves of *X. humilis* and for other desiccation tolerant plants (Sherwin and Farrant 1998; Farrant *et al.*, 1999).

The shattering of the dry roots during preparation for SEM (Figures 2.4 C-D) suggested that glass formation is involved in the stabilization of the cellular environment in the roots. The levels of sucrose the roots were slightly lower than those reported for the hydrated leaves of *X. humilis* (Figure 2.8, Cooper, 2001). This may indicate that other carbohydrates play a more significant role than sucrose in the roots. Indeed, raffinose-series oligosaccharides have been correlated with seed longevity and in *X. villosa* make up roughly 10 % of the total sugar content of the dry plant (Ghasempour *et al.*, 1998). The roots of *C. plantagineum*, which do not accumulate sucrose during desiccation, contain high levels of stachyose (Bartels and Salamini, 2001). However, the lower sucrose levels may merely be a reflection of the fact that the volume of living cells in the roots is much lower than in the leaves. The effective concentration of sucrose per cell could be much higher than in the leaves if it were calculated on a living cell-for-cell basis. In this case, the resultant sucrose levels would be approximately 4-5 fold higher and would indicate that this compound does

form a key component of cytoplasmic vitrification. This speculation is consistent with the hypothesis that the roots act as a substantial source of carbohydrates for the aerial portions of resurrection plants (Norwood *et al.*, 2001).

It has been proposed that the use of 2-octulose a carbon source for sucrose synthesis by *C. plantagineum* allows it to accumulate sucrose during dehydration independently of photosynthesis (Ingram and Bartels, 1996; Scott, 1990). Whether *X. humilis* also makes use of related carbohydrates has not been determined. However, it seems unlikely that tolerance would rest entirely on *de novo* carbon fixation during the onset of desiccation – especially given that high levels of alternative carbohydrates (stachyose or trehalose) are probably required simultaneously for the prevention of sucrose crystallization (Ghasempour *et al.*, 1998; Norwood *et al.*, 2001; Oliver *et al.*, 2001). Nevertheless, this could be a factor underlying the inability of *X. humilis* to survive rapid dehydration (Farrant *et al.*, 1999).

The literature surrounding the relevance of proline to the study of plant stress is varied and often contradictory. Despite better performance under stress, transgenic Tobacco plants over-producing proline do not exhibit the ability to survive dehydration (Bajaj *et al.*, 1999) – a fact that contributes to the hypothesis of Gibon *et al.* (2000) that proline itself has no protective effect against the consequences of dehydration. While proline may be an important component of stress resistance in some plants, it certainly does not appear to be a ubiquitous response. Several desiccation tolerant plants accumulate proline during the onset of drying, but others, such as *C. plantagineum*, do not (Tymms and Gaff, 1979). It is suggested that the variation may be explained by a link between poikilochlorophylly and proline accumulation. However, although it is true that proline accumulated in the dry leaves of *X. humilis*, the levels attained were not as high as in other species (such as *E. nindensis*, Figure 2.9). This is consistent with the findings of Tymms and Gaff (1979) for *X. villosa* and implies that proline may have a limited role in desiccation responses of this genus. Despite the failure of the analysis of proline levels in the roots, the substance involved in the mixing of the hydrophobic and hydrophilic phases may be interesting. Among the proteins related to desiccation tolerance, dehydrins (LEA, D-11 family) have been reported to have detergent-like properties (Close, 1996) and it is possible that one of these proteins may be involved.

From the data presented above, it is evident that the roots undergo some changes similar to those recorded for the leaves, and are able to maintain structural and functional integrity through anatomical and metabolic compensation. The exact importance and nature of the compounds accumulated in the cytoplasm and in the vacuoles during desiccation remain to be investigated, but the findings provide the first insights into the functional anatomy of the roots of *X. humilis* during desiccation and rehydration. The data also provide some physiological context for the molecular techniques described in later chapters.

University of Cape Town

Targeting Candidate Genes

Stress sensing and signaling systems

3.1 Introduction

One benefit of research into the responses of non-tolerant model organisms to water deficit is that it provides potential candidate genes that can be investigated in desiccation tolerant organisms. One such candidate gene is *Arabidopsis thaliana* histidine kinase1 (*AtHK1*), which was isolated by degenerate PCR and library screening by Urao and co-workers (Urao *et al.*, 1999). When introduced into yeast mutants lacking the *sln1* and *sho1p* osmosensors, *AtHK1* suppressed lethality in highly saline media. It is abundantly expressed in the roots of *A. thaliana* where it is thought to function as an osmosensor. Both *Athk1* and *Sln1* belong to the histidine kinase superfamily, of which 350 members have been described. These histidine kinases form an integral part of two-component signaling systems that are involved in the sensing of environmental stimuli.

In eukaryotes, protein kinases that target specific threonine, serine or tyrosine residues usually predominate. In prokaryotes, however, two-component systems are the primary signaling systems. They constitute up to 1 % of the encoded proteins and regulate a vast range of responses (West and Stock, 2001). Yet, it was only recently that two component systems were identified in plants and in yeast (Chang *et al.*, 1993; Maeda *et al.*, 1994). However, they are significantly less well represented in the genomes of eukaryotes (Urao *et al.*, 2000).

Two component systems are typically composed of a histidine kinase receptor and its cognate response regulator. The histidine kinases are membrane-bound, homodimeric proteins that contain a periplasmic N-terminal input domain and a cytoplasmic C-terminal kinase (output) domain with an invariant histidine residue. The structure of the kinases varies in relation to the conditions to which they respond, but they possess several conserved domains - designated the H, N, G1, F and G2 boxes (Urao *et al.*, 2000; West and Stock, 2001). The response regulators are generally found toward the end of the phosphorelay system where they act as phosphorylation-dependent transcription factors. They consist of a C-terminal effector (or output) domain and a conserved N-terminal regulatory (or input) domain with an invariant aspartate residue. The regulatory domains may be located within the histidine kinases or may be separate phosphorelay proteins (Lorhmann and Harter, 2002).

The signaling via two-component systems operates when detection of an altered state results in an ATP-dependent trans-phosphorylation of the conserved histidine residue in one subunit of the core kinase by the other subunit. The phosphoimidazole formed reacts with and transfers the phosphate group to the aspartate residue of the regulatory domain of the cognate response regulator. This activates the effector domain that regulates the specific response (often DNA binding leading to altered gene expression; Figure 3.1 A). The response regulators have autophosphatase activity that limits the duration of the phosphorylated state - giving rise to systems that have half-lives of several seconds to several hours (Urao *et al.*, 2000b; West and Stock, 2001; Lorhmann and Harter, 2002).

More complex versions of the simple two-component system exist, since some make use of phosphorelay intermediates that may or may not be located within the histidine kinase protein (Figure 3.1 B). These hybrid type histidine kinases contain additional phosphotransfer (HPT) or internal response regulator domains. Although it is estimated that up to 20 % of histidine kinases are of the hybrid type, signaling via these proteins is thought to be predominantly a eukaryotic feature (West and Stock, 2001). The signaling is further complicated by interaction with downstream mitogen activated

protein kinase (MAPK) cascades or with other two component systems (West and Stock, 2001; Lohmann and Harter, 2002).

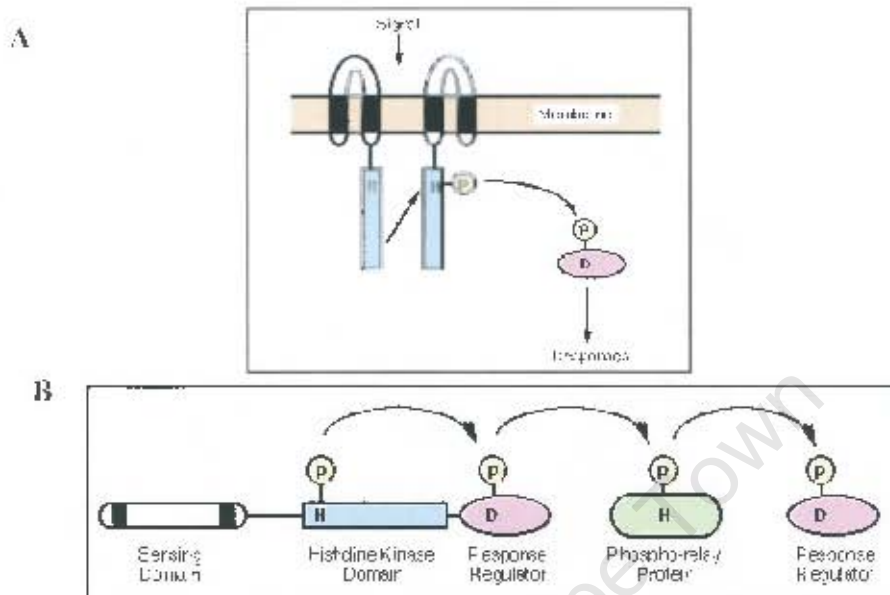


Figure 3.1: The functioning of two component systems (A) A simple two-component system in which a phosphate group is transferred directly from the histidine kinase to the response regulator. (B) A multiple phosphorelay system involving a hybrid type histidine kinase (images from Schaller *et al.*, 2001).

The *Arabidopsis* genome encodes for 17 histidine or hybrid histidine kinases and 23 response regulators (Schaller *et al.*, 2001; Lohmann and Harter, 2002). The response regulators have been classified as either types A or B depending on their domain structure and responsiveness to cytokinins (Imamura *et al.*, 1999; Lohmann and Harter, 2002). To date, ethylene and cytokinin perception and signaling are the most common functions ascribed to the *Arabidopsis* two-component systems (D'Agostino *et al.*, 2000; Sakakibara *et al.*, 2000; West and Stock, 2001; Lohmann and Harter, 2002).

A number of the *Arabidopsis* genes are expressed primarily in the roots, namely, AHK4 (Ueguchi *et al.*, 2001), AThK1 (Urao *et al.*, 1999) and ARR5 (D'Agostino *et al.*, 2000). Although other mechanisms of signaling in response to water deficit have been

identified - notably via phospholipase D and diacylglycerol (Frank *et al.*, 2000; Munnik *et al.*, 2000; Munnik and Meijer, 2001) and serine-threonine kinase activation (Hwang and Goodman, 1995), the two component systems have more clearly defined roles in sensing altered environmental conditions. Thus, *ATHK1* homologues, and potentially novel two component system elements, are considered to be promising candidate genes for the characterization of root responses to desiccation and rehydration. This chapter describes an attempt to clone such target genes as a preliminary step in investigating their role(s) in the desiccation tolerance mechanisms of *X. humilis*.

3.2 Materials and Methods

3.2.1 Probe preparation

The *ATHK1* clone was obtained from Kazuo Shinozaki (R.I.K.E.N, Japan). The insert was amplified by PCR by combining 1 μ l of the DNA with 2 μ l dNTPs (10 mM), 5 μ l PCR buffer (10 x), 4 μ l $MgCl_2$ (25 mM), 0.5 μ l SuperTherm *Taq* (Southern Cross Biotech, SA) and 2 μ l each of the T7 and T3 primers (10 μ M). PCR conditions were 94 °C (2 min), [94 °C (30 s), 55 °C (1 min), 72 °C (3 min)] x 35, 72 °C (5 min). The product was purified directly from the reaction using a Qiagen Quick PCR Purification Column (Qiagen, U.S.A) according to the manufacturer's specifications. Purified product was checked by agarose gel electrophoresis (Chapter 4) and quantified using the GeneQuant DNA calculator. 40 μ g of the DNA was labeled with [32 P]-dCTP (Amersham, U.S.A) using the Megaprime DNA labeling Kit (USB, U.S.A) as per product instructions. 1 μ l aliquots were set aside before and after Sigma spin column (Sigma, U.S.A) purification for scintillation counting and probe quantification.

3.2.2 Southern and northern blot analysis

Genomic DNA was extracted from the roots of *X. humilis* using the Qiagen Plant DNA Extraction Kit (Qiagen, U.S.A) as per manufacturer's instructions. The DNA was eluted in 50 μ l molecular grade water (Sigma, U.S.A), quantified by spectrophotometry and

checked by agarose gel electrophoresis. 10 µg samples were digested overnight at 37 °C with *Eco*R1 or *Bam*H1 (Amersham, U.S.A) and electrophoresed on a 0.7 % agarose gel. RNA from dehydrating and rehydrating roots and leaves was prepared as described (Chapter 4) and electrophoresed on a formaldehyde agarose gel (Ausubel *et al.*, 1988). Southern and northern transfer onto Hybond N+ membrane (Amersham, U.S.A) was performed according to standard methodology (Ausubel *et al.*, 1988). 1 x 10⁶ dpm of labeled AThK1 DNA was added per ml of hybridization buffer, and hybridizations performed overnight at 42 °C. Signals were detected by autoradiography (Ausubel *et al.*, 1988).

3.2.3 Primer design

The internet-based Blast facility (<http://www.ncbi.nlm.nih.gov/>) was used to search for plant histidine kinases and response regulators. Identified sequences were translated in the DNAMAN programme, and aligned by sequence similarity using DNAssist. The maximum number of sequences available from monocotyledonous plants was used. Degenerate nucleotide sequences of conserved regions were obtained by reverse translation in DNAMAN. The melting temperatures (T_m) of the primers were determined using DNAMAN. Primers were also aligned to check internal and mutual complementarity. The primers were checked for specificity by running blast searches on the sequences and by comparing them to published primers and conserved regions (Urao *et al.*, 1999). Once the primer designs had been verified, the nucleotides were synthesized in the Department of Molecular and Cell Biology Oligonucleotide Synthesis Unit.

3.2.4 Degenerate primer PCR

Total RNA and poly(A)⁺ were prepared as described (Chapter 4). Reverse translation was performed by heating of 2.5 µg of RNA to 65°C for 10 min in the presence of 0.5 µl random hexamer primer and 0.5 µl RNase Inhibitor (Promega, U.S.A). The reaction was equilibrated to 37°C for 10 min prior to the addition of 2.0 µl dNTPs; 4 µl RT buffer

and 1 μl MMLV-RT (Promega, U.S.A). Reactions were incubated at 40°C for one hour. The cDNA was checked by electrophoresis on denaturing agarose gels and used as the template for PCR.

All PCR reactions were made up in molecular grade water (Sigma, U.S.A) in 100 μl volumes containing 2-3 μl dNTPs (10 mM), 10 μl PCR buffer (10 X) and 0.5 μl enzyme. DNA, primer and MgCl_2 concentrations were varied as follows:

- DNA (1 ng/ μl ; 2 ng/ μl or 5 ng/ μl)
- Primers (0.5 μM , 1.0 μM or 1.5 μM each)
- MgCl_2 (2 mM, 2.5 mM or 3 mM)

The following PCR conditions were tested:

Table 3.1: Parameters tested for degenerate PCR optimization

Polymerase	Annealing Temperature (°C)						DMSO (%)			Additives		
	42	45	48	50	55	58	2	3	5	BSA	TX	DOC
<i>Tth</i>	+	+	+	+	-	-	-	-	-	+/-	+/-	+/-
Promega <i>Taq</i>	-	+	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-
SuperTherm	+	+	+	+	+	+	+/-	+/-	+/-	-	-	-

BSA = Bovine Serum Albumin (5mg/ml), TX= Triton X-100 (0.1 %) and DOC = deoxycholate (0.1 %).

The PCR cycles were: 94°C (2 min), [94°C (30 s), annealing temp (1 min), 72°C (90 s)] x 35, 72°C (5 min). Controls with no primer, no template or no enzyme were used. 10 μl of the reactions were electrophoresed on agarose gels alongside appropriate markers to determine the success of the reactions.

3.2.5 Cloning

Where highly specific banding patterns were observed, 50 μ l of the reaction was electrophoresed on a 1 % low melting point agarose gel alongside a 100 bp ladder (Promega, U.S.A). Selected bands were cut from the gels under long wavelength UV light with a sterile scalpel. The agarose was melted at 70°C for 10 min and equilibrated to 37°C for a further 10 min. 3 μ l of the sample was used for cloning of the fragment into the pGEM-T Easy vector (Promega, U.S.A) essentially as described by the manufacturer. The ligation reactions were used to transform competent XL1-Blue cells according to the method of Chung and Miller (1988). Transformants were plated onto LB agar plates containing 100 μ g/ ml ampicillin, 10 μ g/ml IPTG and 5 μ g/ml X-Gal and incubated at 37°C overnight.

3.2.6 Screening and sequencing

Colony PCR was performed according to the method of Roschier *et al.* (2000) as described (Chapter 4). 10 μ l of each PCR reaction was electrophoresed on an agarose gel to check for inserts. Colonies containing inserts were miniprepmed as described (Chapter 4; Isch-Horowitz and Burke, 1981). Inserts were sequenced on a MegaBACE 500 sequencer (Molecular Dynamics, U.S.A) using the M13 forward primer.

Flanking vector regions were trimmed from the insert sequences using DNAMAN. Database searches were carried out using the BLASTn and BLASTx algorithms (Altschul *et al.*, 1997). Multiple sequence alignments were performed using DNAssist.

3.3 Results

3.3.1 Northern and Southern Analysis using *AThK1*

To investigate whether histidine kinase homologues are present in *X. humilis* and whether they are differentially regulated in response to desiccation and rehydration, Southern (Southern, 1975) and northern Blots were carried out. The *AThK1* insert was amplified by PCR and used to probe genomic DNA blots. This analysis revealed that sequences with a high level of similarity to the full-length *AThK1* probe were present in the genomic DNA of *X. humilis* (Figure 3.2). Single bands were obtained for both the *Bam*H1 and *Eco*R1 digests, but low signal intensity prevented the identification of other potential bands.

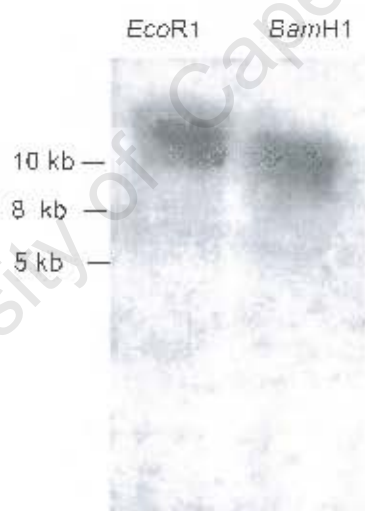


Figure 3.2: Southern blot analysis showing the representation of *AThK1*-related sequences in genomic DNA of *X. humilis*.

Although bands were detected on the northern blots, the signal intensity was too low (even after 2 week exposures) for accurate conclusions about the expression of potential homologues to be drawn. This was thought to be a consequence of the low levels of expression of the endogenous transcript and the use of a heterologous probe.

3.3.2 Primer design

Plant-specific histidine kinases and response regulator sequences were obtained from the GeneBank database and aligned in order to identify conserved domains. The alignments are shown in Figures 3.3 and 3.4.

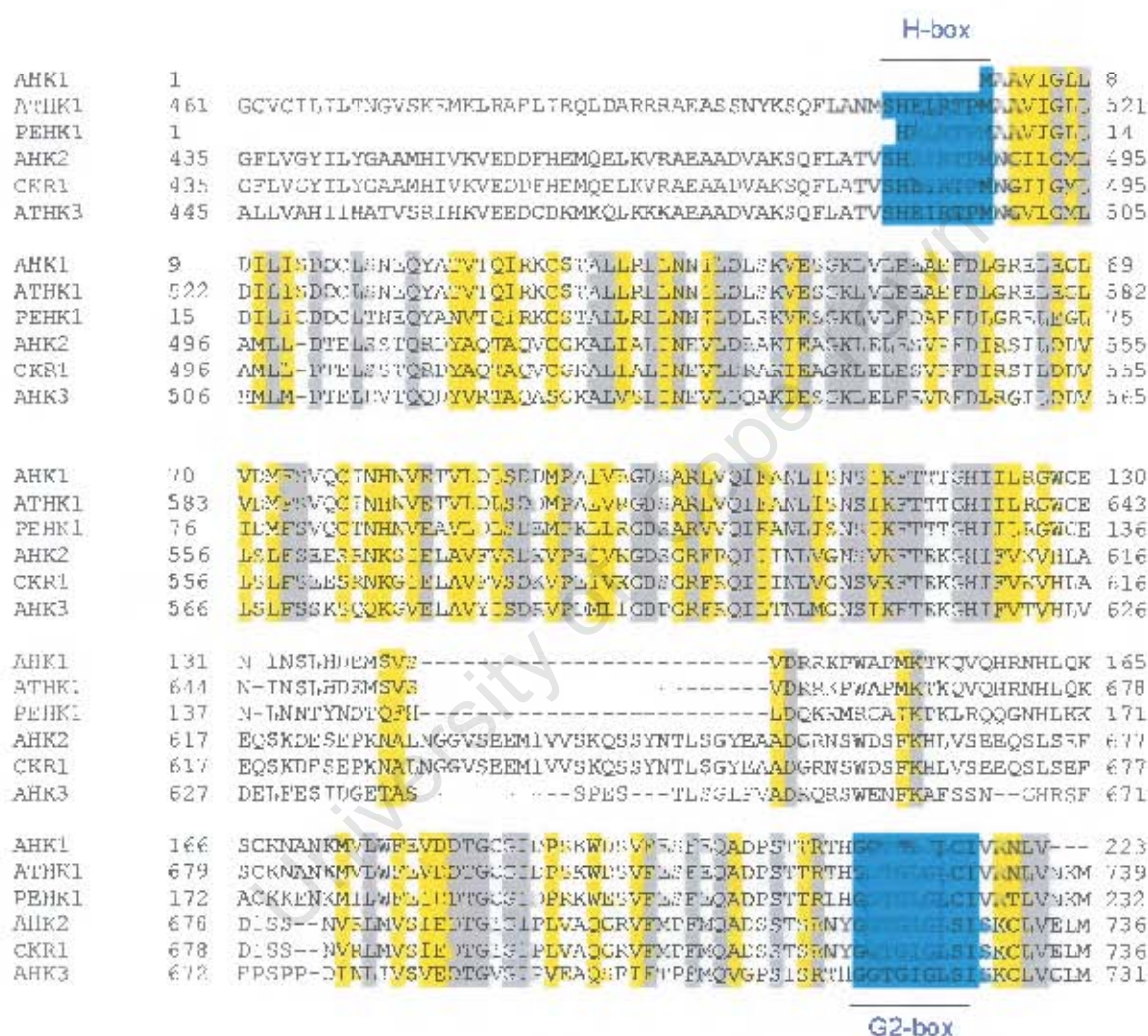


Figure 3.3: Multiple sequence alignments of selected histidine kinases

*AHK1-3: *Arabidopsis* histidine kinase; ATHK1: *Arabidopsis thaliana* histidine kinase 1; PEHK: *Populus* ATHK1 homologue; CKR1: Cytokinin receptor 1.

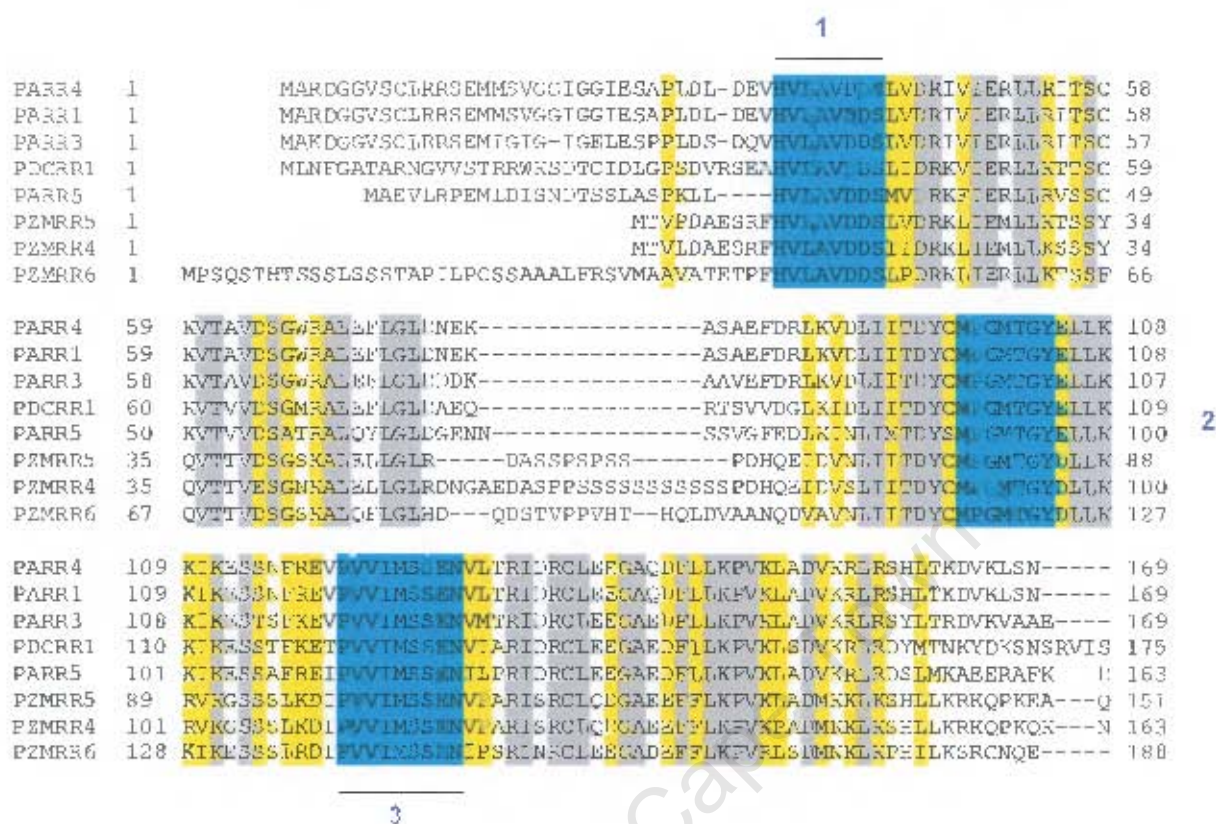


Figure 3. 4: Multiple sequence alignments of response regulators.

*PARR = *Arabidopsis* response regulators 1-5; PZMRR = *Zea mays* response regulators 1-6, PDCRR = *Daucus carota* response regulator.

Primers to the nucleotide sequences of the conserved regions of both the histidine kinases and the response regulators were synthesized. A number of the primers were excessively degenerate, so extensive use was made of inosine bases to reduce this to acceptable levels. Poly(G) tracts were also added to the 5' ends to increase the Tms where necessary. The primers exhibited no significant self-complementarity. When the primers were used to search the GeneBank database, the first 100 matches for each were either the targeted histidine kinases or response regulators. Conserved domain detection (CDD) facility recognized both the HTPase and response regulator subunit domains targeted by the primers. The details of the primers designed are summarized in Table 3.2.

Table 3.2: Degenerate primers for two component system genes.

Primer	Target	Sequence (5'-3')	Length	T _m *	Degen**	Expected Product
HK	F	H-box CCCCA(TC)GA(gA)(CTA)TI(CA)GIACICC	20	58	24	600-800 bp
	R	G2-box CCA(gA)NCCIA(gAT)ICCI(gC)(TA)ICCICC	22	68	96	
RR	F 1	Box 1 GGGGCA(CT)GTN(CT)TIGCIGTICA	21	60	16	F1/R3 = ~ 400 bp
	F 2	Box 2 GCGATGCCNGGIATGACIGG	20	65	4	F2/R3 = ~ 100 bp
	R 2	Box 2 GGGCCNGTCATCCIGGCAT	20	65	4	F1/R2 = 200 - 300 bp
	R 3	Box 3 GCGGGCAT(TgA)ATNACIACIGG	20	62	12	

*T_m = thermodynamic melting temperature (°C). **Degen. = degeneracy estimate.

3.3.3 Optimization of degenerate RT-PCR and cloning

A large range of PCR conditions were tested, starting with the use of *Tth* DNA polymerase according to the protocol used by Urao *et al.* (1999) in the cloning of *ATHK1*. This method did not yield defined bands, and variation of the types of PCR stabilizers (BSA, Triton, deoxycholate), DNA concentrations and primer concentrations had no effect on the result. The use of Promega Taq DNA polymerase without the BSA, triton and deoxycholate did result in the detection of two bands in the histidine kinase reactions and three bands in the response regulator reactions, but these were faint and variable.

SuperTherm Taq yielded the most clearly defined banding patterns. Both histidine kinase and response regulator reactions were optimal with 1 ng/μl DNA template, 1 μM of each primer, 3 mM MgCl₂ and 5 % DMSO. No BSA, triton or deoxycholate was necessary. The best primer combination for the response regulators was found to be F1/ R3 (refer to Table 3.2) and these were routinely used. Alterations in the annealing temperatures resulted in increased specificity for the 500 bp and 600 bp products in the histidine kinase PCR reactions, and for the 400 and 450 bp products in the response regulator reactions (Figure 3.5 A-C). Optimal annealing temperatures were found to be 55 °C for the histidine kinases (Figure 3.5 C) and 50 °C for the response

regulators (Figure 3.6 B). The bands obtained at these temperatures were considered to be within the acceptable range for the expected products (~700 bp and ~ 300 bp respectively).

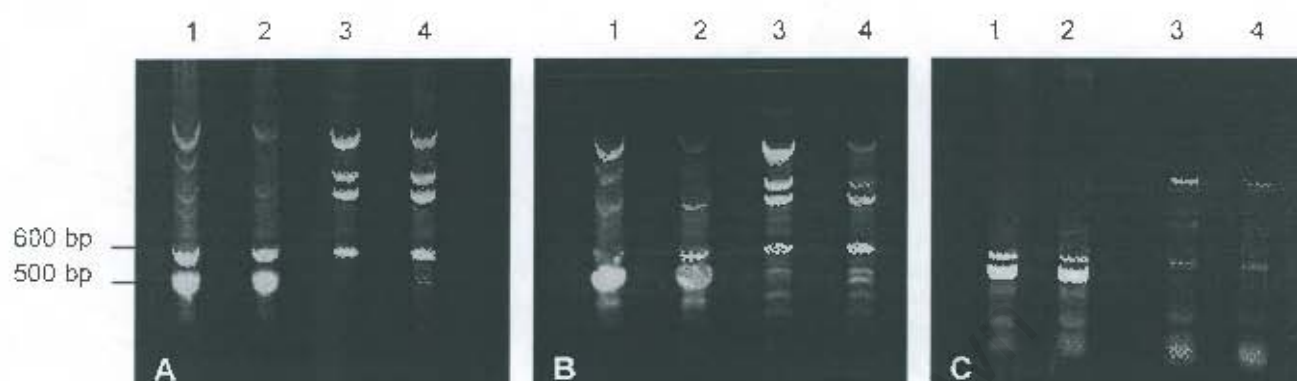


Figure 3.5: Effect of annealing temperatures and DMSO on product yield from RT-PCR. (A) 45°C. (B) 50°C and (C) 55°C. 1 = histidine kinase primers, no DMSO, 2 = histidine kinase primers, 5 % DMSO, 3 = response regulator primers, no DMSO, 4 = response regulator primers, 5% DMSO. Optimized PCR conditions were used.

The 500 bp and the 600 bp bands from the histidine kinase PCR at 55°C were cloned into the pGEM-T Easy vector, the inserts confirmed by colony PCR (Figure 3.6) and sequenced.

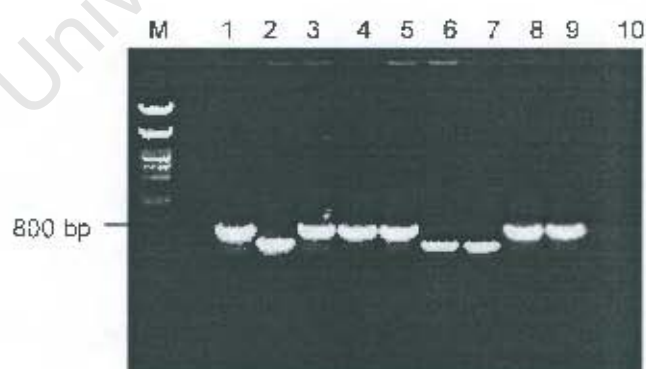


Figure 3.6: Amplification of cloned inserts by colony PCR. Lanes 1-9 = products from selected colonies, lane 10 = negative control, M = marker (*Pst*II digested lambda DNA).

3.3.4 Sequence analysis

BLAST results and multiple sequence alignments revealed the presence of the conserved histidine kinase domains in both sequences. However, both the sequences displayed high similarity to bacterial genes, with the 500 bp product being putatively identified as a *RstA* homologue and the 600 bp product as a *PhoR* homologue (data not shown). Overall, the products displayed > 80 % sequence identity to the corresponding *Psuedomonas aeruginosa* and *E. coli* genes. It was concluded that both products were of bacterial origin. Nevertheless the *RstA* sequence displayed 18 % sequence similarity to *Athk1* at the amino acid level and contained the distinctive H-box at the 5' end (Figure 3.7) indicating that the primers were specific for histidine kinases.



Figure 3.7: Characterization of the *RstA* homologue. (A) Conserved domain detection. (B) Partial Amino acid sequence alignment of *RstA* homologue and *AthK1*.

In an attempt to remove the interference of the bacterial cDNAs, leaf and root poly(A) RNA was purified, converted to cDNA using poly(dT) priming and used in the optimized PCR reactions described above. Annealing temperatures were lowered to 45°C. This

approach yielded a variety of different bands in the two reactions (Figure 3.8). The 700 bp and 800 bp products detected in the histidine kinases reactions were closer to the expected size of ~ 700 bp. The 400 bp and 450 bp products were brighter and sharper for the response regulators (Figure 3.8). Due to time constraints for the project, these bands were not cloned and sequenced but were excised and stored at 4°C for later cloning.

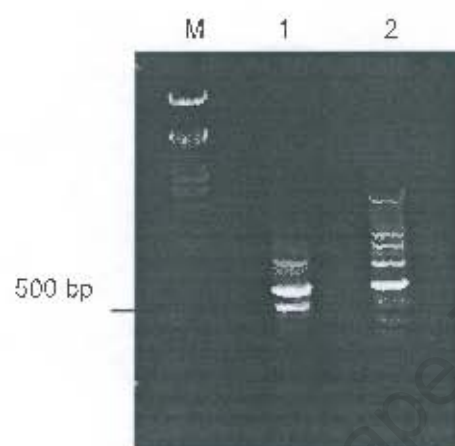


Figure 3. 8: PCR from cDNA prepared by RT from poly(A) RNA. Lane 1 = histidine kinase primers + DMSO. 2 = response regulator primers + DMSO. M= marker.

3.4 Discussion

Although incomplete, the data presented above suggest that there are two-component system homologues present in *X. humilis*. The Southern blot analysis (Figure 3. 2) indicates that there are sequences related to *ATHk1* present in the genomic DNA of *X. humilis*. As was the case for Urao *et al.* (1999) a single band was detected in the *Bam*H1 and *Eco*R1 lanes, suggesting that only one copy of a potential homologue is present in the genome. However, it should be borne in mind that these bands may merely represent hybridization of the heterologous probe to incompletely digested (ie: large) DNA fragments.

Transcripts encoding kinases are generally expressed at very low levels in the cell. This combined with the lower levels of hybridization achieved with sequences that are not identical, means that the weak signal intensities for the northern blots are to be expected. Urao *et al.* (1999) experienced similar problems with northern blot analysis, despite using a fully homologous *ATHK1* sequence.

The primers were specific for histidine kinases and response regulators (Figures 3.3 and 3.4). The histidine kinase primers were similar to those designed by Urao *et al.* (1999) except that the reverse primer differed at two nucleotides. These were resolved through the use of degenerate bases. The differences may have arisen as a consequence of the heavy reliance on bacterial sequences by Urao *et al.* (1999) in contrast to the use of plant sequences in this study (Figure 3.3). The response regulator primers were significantly more degenerate than the histidine kinase primers – hence the need for several different combinations (Table 3.2). Nevertheless, the primers were shown to be effective in targeting histidine kinases (Figure 3.7).

The process of optimizing the PCR conditions was extensive and time-consuming. *Tth* DNA polymerase is considered to be a better choice of polymerase than *Taq* DNA polymerase which lacks 3'-5' proof reading capabilities (Eckert and Kunkel, 1990). However, it is more sensitive to temperature fluctuations than the *Taq* polymerases (*Tth* polymerase specification sheet, Promega, U.S.A) and this may account for the failure of the reactions in which it was used. Urao *et al.* (1999) employed BSA, triton and deoxycholate in conjunction with low annealing temperatures (42°C) in order to obtain specific products with this enzyme, but the approach was not successful with *X. humilis* cDNA. The SuperTherm *Taq* resulted in significantly higher yields of product than the Promega *Taq* – a result that could be due to either higher activity or to lower template specificity of the SuperTherm *Taq*. Since the sequenced bands were, however, all histidine kinases template specificity may not account for the increased product yield.

RT-PCR using degenerate primers is acknowledged to be problematic, particularly when primers have high degeneracy levels and different Tms (Loffert *et al.*, 1998; Linhart and Shamir, 2002), as was the case in this study (Table 3.1). Ideally primers should have a maximum degeneracy of 16 (Loffert *et al.*, 1998), although 128-fold degeneracy in each primer has been successfully employed for the amplification of single copy gene from the human genome (Girgis *et al.*, 1988). Degeneracy at the 3' end of a primer is generally more problematic as it may inhibit efficient extension, but degenerate bases decrease the efficiency of a PCR reaction regardless of their position (Loffert *et al.*, 1998; Linhart and Shamir, 2002). This can be counteracted to some extent by increasing the concentration of the primers at the expense of template specificity. In this study relatively high primer concentrations (1 μ M) were used, but did not appear to affect the primer specificity for histidine kinases (Figure 3.7).

The use of random hexamers for first strand synthesis was unfortunate, as it appears to have lead to the reverse transcription of bacterial sequences, possibly derived from soil/ bacteria. This may prove to be a limitation to the approach used. Poly(dT) priming from purified poly(A) RNA may be a means of reducing or overcoming this problem, although bacterial RNAs may still have short poly(A) tracts at their 3' ends (Ausubel *et al.*, 1988). The fact that higher bands were obtained for the RT-PCR from poly(A) RNA (Figure 3.8) may be due to this alteration, but may also be a result of the increase in the concentration of the template. Leaf RNA was not routinely used because potential osmosensing kinases are unlikely to be expressed at high levels in these tissues – a contention that is supported by the northern blot results of Urao *et al.* (1999).

Both sets of primers designed in this study will be useful resources for the cloning and characterization of signaling factors that may have roles in the responses of *X. humilis* to desiccation and rehydration. Apart from osmosensors, the cytokinin receptors (CKI1 and CRE1) and their cognate response regulators (eg: ARR1 and ARR5; D'Agostino *et al.*, 2000; Sakai *et al.*, 2001) are particularly interesting two component system elements that may be involved in the regreening of plants during rehydration. The

involvement of alternate two component systems in rehydration of resurrection plants remains to be explored.

In this study, Southern blot analysis indicated the presence of a putative homologue of *ATHK1* in the genome of *X. humilis*. Degenerate primers designed to the histidine kinase and response regulators were shown to be highly specific under optimized PCR conditions. Attempts to clone the correct sequences are underway and will be used in conjunction with screening of the normalized libraries described in Chapter 5 to characterize these signaling systems in *X. humilis*.

University of Cape Town

cDNA Library Construction

A molecular entry into desiccation tolerance

4.1 Introduction

cDNA libraries remain the basic currency in genome and transcriptome analysis. In many cases they are the primary means of gaining significant sequence and expression data, and underlie more advanced techniques such as functional characterization, microarray analysis and promoter studies (Clark *et al.*, 1999; Das *et al.*, 2001; Seki *et al.*, 2001).

Even in instances where the entire genome of an organism has been sequenced, there is still a heavy reliance on the characterization of ESTs present in cDNA libraries (Clark *et al.*, 1999; Nam *et al.*, 2002). One of the main reasons for this is that current algorithms cannot correctly predict all ORFs in a genome because of difficulties in defining functional codons from identical, but non-functional ones (Das *et al.*, 2001; Haas *et al.*, 2002). A clear example was provided by Reymond *et al.* (2001) who characterized human chromosome 21 genes predicted by exon mapping programmes. Only 20 % of these predicted genes (PREDS) were found to encode real transcripts, in contrast to the highly accurate predictions based on the existence of ESTs. Gene prediction is largely limited by the lack of understanding of the importance of the context of sequences, and of the lack of understanding of the impact of secondary structure in defining biologically relevant ORFs (Hannehalli and Levy, 2001). Some progress has been made in this area through the use of CpG islands, transcription factor binding sites and first splice-donor sites to increase the stringency of the algorithms (Davuluri *et al.*, 2001).

In addition cDNA libraries are critical in the further study of the regulation of temporal and spatial gene expression. To date *in silico* studies can only identify promoters for about 50 % of genes with roughly 80-90 % accuracy (Fujibuchi and Kanehishi, 1997; Pederson *et al.*, 1999; Lui and States, 2002) and mapping of co-regulated genes on the basis of these promoters is restricted (Terai *et al.*, 2001). The heavy reliance of promoter prediction algorithms on the presence of CpG islands also means that the regulation of tissue-specific expression is likely to remain dependent on biological studies (Hannehalli and Levy, 2001).

For non-model organisms such as *X. humilis*, *in silico* studies are not possible. Large-scale insight into molecular features that characterize its growth and development will initially depend heavily on the use of cDNA libraries in traditional 'forward' genetic studies. Consequently, the value of the information that will be gained about desiccation tolerance and the relationship between tolerant and non-tolerant plants will be directly related to the quality of the sequences contained in such libraries.

4.1.1 The production of high quality libraries

In order to maximize the amount of information generated by the production of a cDNA library, the sequence length and representation should be as great as possible. Ideally, a library should contain full-length sequences representing all the genes expressed in the organism or tissue from which they are derived (Draper *et al.*, 2002). In practice, extremely high cloning efficiencies are required to clone all the represented sequences. The probability that a given mRNA will be represented in a cDNA library is calculated as:

$$p(x) = (1 - [1 - f]_n)$$

Where '*n*' is the number of recombinants and '*f*' is the frequency of a particular mRNA. Thus, assuming that the average size a transcript is 2 kb and that a typical cell contains 0.6 pg of mRNA (or 500 000 transcripts), there is a probability of 99.9 % that a library of 5×10^6 recombinants will contain the rarest transcript (1 copy per cell).

However, it is expected that libraries containing above 1×10^6 recombinants will contain the majority of transcripts (Soares and Bonaldo, 1998).

To achieve the cloning efficiencies described above, the choice of vector is critical. Although plasmid vectors are significantly easier to manipulate, and can be electroporated with efficiencies up to 1×10^{10} transformants per μg of DNA (Dower *et al.*, 1988; Soares and Bonaldo, 1998), bacteriophage vectors have two important advantages over the plasmid vectors. Firstly, the number of recombinants generated in a typical ligation is about 3-fold higher, and secondly, a higher number of phages can be screened, usually with much lower background levels (Soares and Bonaldo, 1998). Derived vectors such as Lambda ZAP (Stratagene, U.S.A) are particularly popular since they combine the advantages of both. This is due to the fact that Lambda ZAP contains a plasmid (pBluescript) component which has an f1 origin of replication, allowing for excision of a plasmid vector without sub-cloning. One additional advantage of the ZAP system is the inclusion of the *LacZ'* gene which allows for colour selection recombinants (Soares and Bonaldo, 1998; Lambda ZAP Manual, Stratagene, 2001). The general features of this system are illustrated below (Figure 4.1).

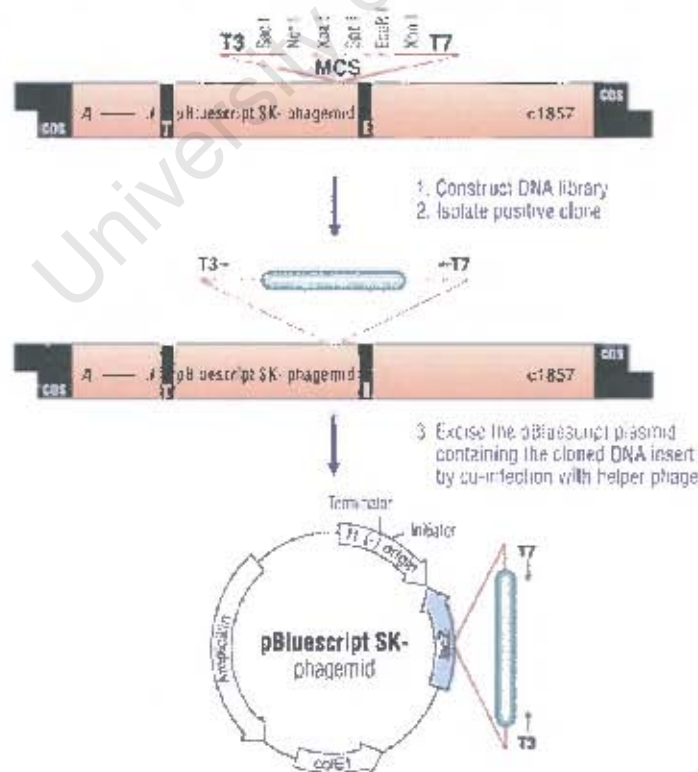


Figure 4.1: The Lambda-Zap vector (Image from the Uni-Zap Manual Stratagene).

Yet, even in instances where the highest cloning efficiencies are achieved, not all transcripts may be usefully represented in a library. This is due to the fact that cDNA libraries routinely contain high levels of inserts that are (1) chimaeric, (2) truncated, (3) comprised of poly(A) tails alone, (4) comprised of the 3' UTRs and (v) contaminants (Adams *et al.*, 1991; Adams *et al.*, 1992; Soares and Bonaldo, 1998).

The most serious of these problems is the occurrence of chimaeric inserts, since these cannot be easily identified and may generate misleading information (Soares and Bonaldo, 1998). It has been estimated that at least 30-35 % of EST sequences deposited in the public domain contain errors (Haas *et al.*, 2002). The majority of these are due to sequencing errors and the inclusion of vector sequences in the EST data, but some arise through the deposition of chimaeric sequences. Chimaeric inserts arise primarily during the ligation of the adapter ends, to the cDNA and during the ligation of the cDNA into the cloning vector. Their occurrence can be limited by the inclusion of a large excess of adapters relative to cDNA, by the elimination of small inserts and by the directional cloning of the inserts through the use of linker-primers.

Failure of the reverse transcriptase enzyme, the existence of extensive secondary structure in the template and frequent internal priming result in the inclusion of small inserts and inserts consisting of the 3' UTRs or of poly(A) tracts (Soares and Bonaldo, 1998; Seki *et al.*, 2001; Nam *et al.*, 2002). Nam *et al.* (2002) have shown how the general use of poly(A) primers during the first strand synthesis reaction can lead to the cloning of truncated transcripts through priming from internal poly(A) tracts in gene sequences. This has a serious impact on the quality of sequences available in the public domain. In fact these authors estimate that up to 12 % of the sequences available in the dbEST database are the result of this phenomenon (Nam *et al.*, 2002).

Even without such internal priming, most reverse transcriptases fail to generate full-length sequences. The use of trehalose, RNA chaperones and compounds that eliminate secondary structure have not compensated for this failure (Das *et al.*, 2002). Several researchers have attempted to overcome this problem through the use of 5'

biotin cap trapper or 7-methyl guanosine (m^7G) antibody selection methods, which retain only full-length sequences (Das *et al.*, 2001; Seki *et al.*, 2001). However, the problem with these methods is that they result in very low cloning efficiencies (libraries tend to consist of < 10 000 clones) due to the low trapping efficiency and the continued problems with the RT step (Das *et al.*, 2001). Consequently, high levels of starting mRNA are required, and up to 20 libraries need to be generated from a single source in order to gain representation of the full transcriptome. Size fractionation of the cDNA or mRNA is a more inexpensive and technically simpler process that may yield high frequencies of full-length or almost full-length sequences (Draper *et al.*, 2002). However, this tends to eliminate short full-length sequences and therefore biases the data against small genes. Consequently, small ORFs are excluded from annotation routines (Rabinowicz and Byrne, 2001).

Clearly, although cDNA library construction is one of the most frequently employed methods in molecular biology, there is as yet no unequivocal physical approach to ensure that libraries of sufficiently high quality are obtained. This has led to the use of large-scale sequencing of multiple libraries coupled to powerful bio-informatic analyses to produce high quality, non-redundant genesets. This approach uses the sheer quantity of sequence data to increase the probability of all transcripts being usefully represented, but is both expensive and labour-intensive.

4.1.2 cDNA libraries in water stress research

One of the largest contributions to the understanding of the types of genes employed during water stress responses stems from the work carried out on *Arabidopsis* at the RIKEN institute by Shinozaki and colleagues (Seki *et al.*, 2001; Seki *et al.*, 2001b; Seki *et al.*, 2002). They have constructed and screened several full-length cDNA libraries, assembled non-redundant databases of *Arabidopsis* genes, and screened these libraries for water, cold and heat responsive expression. From their results it is clear that there is some overlap in the utilization of genes in the amelioration of the effects of different stresses, but that the bulk of the genes are transcribed in relation to specific stresses (Seki *et al.*, 2001; Seki *et al.*, 2001b; Seki *et al.*, 2002). This suggests that

condition-specific libraries should be constructed in order to maximize the characterization of elements involved in the response in question.

Libraries of several desiccation tolerant plants, including *X. viscosa* (Mundree and Farrant, 2000), *C. plantagineum* (Bartels *et al.*, 1990), *T. ruralis* (Scott and Oliver, 1994) and *S. stapfianus* (Neale *et al.*, 2000) have been constructed. These have been successfully used to isolated a range of genes from desiccation tolerant plants and have provided an invaluable insights into the mechanisms of desiccation tolerance. Interestingly, problems with reverse transcription, ligation efficiency, insert size and sequence recovery have been reported (Koonjul *et al.*, 1999) – indicating the need for quality control steps to be implemented prior to utilization of the libraries. This is especially true for libraries derived from recalcitrant plant material. None of the libraries constructed to date have been use to map the levels of commonality or divergence between the transcriptomes of tolerant and non-tolerant species – potentially one of the more interesting spin-offs of an in depth characterization of a library.

This chapter describes the production and evaluation of root- and stress-specific cDNA libraries from *X. humilis*. It is envisioned that these will complement related leaf libraries and constitute a basic resource for addressing questions about the molecular foundations of desiccation tolerance in this species.

4.2 Materials and methods

4.2.1 Plant material

Plants were maintained and harvested as described in Chapter 2. For desiccation library construction, roots were harvested at RWCs of 95%, 60%, 50%, 30% and 5%. For the rehydration libraries roots were harvested at 0, 6, 12, 24, 48 and 72 h after rewetting of the soil (ie: RWCs of 5 %, 60 % and 100 %).

4.2.2 RNA extraction and analysis

RNA isolation was based on the modified method of Chomczynski and Sacchi (1987) as outlined in the Tri-reagent specification sheet (Molecular Research, UK). Roots were ground under liquid nitrogen in a mortar and pestle and transferred while frozen to a Falcon tube containing 10 times the volume of Tri-Reagent (ie: 10 ml per 1 g of root material). Samples were vortexed for 30 s and aliquotted into 2 ml micro-centrifuge tubes. These were left at room temperature for 10 min to allow cell lysis to occur. All centrifugation steps employed a Savant Super-Speed desktop refrigerated centrifuge (Savant, U.S.A) and were performed at 12 000 RCF and 4°C for 15 min. The basic protocol was modified to allow removal of polysaccharides and phenolics common in plant tissues (Chomczynski and Mackey, 1995). RNA was precipitated overnight at -70°C by the addition of 0.1 volume of high salt solution (0.8 M sodium citrate, 1.2 M sodium chloride, 0.1 % DEPC) and 1 volume of isopropanol (Sigma, U.S.A). After recovery and drying of the pellets, RNA was resuspended in 20 µl nuclease-free water (Sigma, U.S.A) and incubated at 65°C for 10 min. Identical samples were pooled and then transferred to -70°C until analysis and further use.

The concentration, yield and purity of the RNA were determined from the absorbance at 260 nm and 280 nm. Pure RNA when re-suspended in water or TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA) typically has a ratio of between 1.8 and 2.1 (Chomczynski and Sacchi, 1987). RNA samples were run on agarose gels in the presence of formamide (Sigma, U.S.A) as described elsewhere (Chomczynski and Mackey, 1995). Images were used to visually confirm the relative concentrations, as well as the overall quality, of the RNA. RNA was taken to be intact if the 28S RNA band (~3.9 kb) was approximately twice the intensity of the 18S RNA band (~1.8 Kb).

The quality of the RNA and its suitability for cDNA synthesis was confirmed by RT. 2.5 µg of RNA was heated to 65°C for 10 min in the presence of 0.5 µl oligo(dT)₁₅ primer and 0.5 µl RNase Inhibitor (Promega, U.S.A). The reaction was equilibrated to 37°C for 10 min prior to the addition of 1.0 µl [³²P] dATP (Amersham, U.S.A), 1.6 µl of each of dCTP, dGTP and dTTP (Megaprime Kit, USB, U.S.A), 4 µl RT buffer and 1 µl

MMLV-RT (Promega, U.S.A). Reactions were incubated at 40°C for one hour and electrophoresed on denaturing agarose gels. Gels were sealed in plastic and placed in a autoradiographic cassette containing a phosphor screen (Kodak, U.S.A). Images were processed on a Bio-Rad Molecular Imager (Bio-Rad, U.S.A).

4.2.3 mRNA isolation

Equal masses of RNA from each of the dehydration and rehydration samples were pooled to yield 800 µg of total 'dehydration RNA' and 700 µg of 'rehydration RNA'. PolyAtract mRNA isolation kits (Promega, U.S.A) were used to extract the poly(A)⁺ RNA (Promega PolyAtract Manual, 2002). The final mRNA samples were resuspended in ~10 µl nuclease-free water. 1 µl of each sample was electrophoresed on a 1.5 % agarose gel for analysis. The remainder was stored at -80°C until use in the cDNA synthesis reaction.

4.2.4 Library construction

cDNA synthesis was performed using the ZAP-cDNA[®] synthesis kit (catalogue number 200400; Stratagene, U.S.A.). For the first-strand synthesis, a minimum of 5 µg of purified poly(A) RNA was resuspended in 37.5 µl nuclease-free water and heated to 65°C for 10 min to eliminate interfering secondary structures. This was carried out in preference over treatment with the methyl-mercury hydroxide suggested by the Stratagene protocol.

Aliquots (typically 1-2 µl) of the first- and second-strand reactions were analyzed by alkaline-agarose gel electrophoresis in order to establish the success of the reactions, to check for the presence of hairpinning, and to determine the size range of the newly synthesized cDNA (ZAP cDNA Synthesis Kit, Stratagene). cDNA modification was only continued if adequate amounts of cDNA, as given by comparison with Lambda phage DNA markers, were detected.

i. Size fractionation and cloning

Two methods of size fractionation were employed during the course of this study. The first was the Sepharose® CL-2B gel-filtration method recommended for the ZAP-cDNA® synthesis, while the second was the more unorthodox agarose gel fractionation method (Pennica *et al.*, 1983).

For column fractionation, 3.5 µl of column loading dye was added to the sample which was loaded directly onto the Sepharose® medium packed into a 1 ml nuclease-free pipette. Fractions of 100 µl were collected from the base of the column starting from the moment the dye front reached the - 0.2 ml mark and ending when the dye front reached the base of the column. On average 10-12 fractions were collected. These were processed by phenol-chloroform [1:1 (v/v)] and chloroform extraction before being precipitated overnight at -20°C by the addition of 2 volumes of 100 % (v/v) ethanol.

For the gel-based fractionation, the sample was loaded onto a 1 % low melting point agarose (FMC Bioproducts, U.S.A) gel containing 1 µg/ml ethidium bromide. A 100 bp DNA marker (Promega, U.S.A) was loaded alongside the sample, which was electrophoresed for 1 hr at 100 V. The gel was visualized under long-wavelength (365 nm) UV light and all cDNA running at positions higher than 500 bp was excised. The excised gel band was transferred to a sterile micro-centrifuge tube and stored at 4°C until further use. DNA was recovered by β -agarase (New England Bio-labs, U.S.A) digestion – as outlined in the manufacturer's protocol. cDNA was precipitated overnight at -20°C by the addition of 0.1 volume sodium acetate (3M) and 2.5 volumes 100 % (v/v) ethanol, and resuspended in 3 µl molecular grade water.

Where the concentration of the DNA was sufficient to allow efficient cloning, 2.5 µl of the cDNA (typically ~ 50–100 ng) was ligated into the Uni-ZAP® vector as per manufacturer's specifications. Ligation reactions were incubated at 4°C for 48 hrs.

ii. Packaging, titering and amplification

4 μ l of the ligation reaction was added directly to an unthawed Gigapack® Gold packaging extract (Catalogue number 200450; Stratagene, U.S.A) and packaging carried out as per the manufacturer's specifications. 1 μ l of the phage suspension was immediately used for titrating, while the remainder was stored at 4°C until used for amplification.

For the titrating, 1 μ l of the primary library phage library was added to 200 μ l XL1-Blue MRF' cells (OD 0.5 in 10 mM MgCl₂) and incubated in a water bath for 15 min at 37°C without shaking. The mixture was plated in NZY top agar onto cooled (30 mm) NZY agar plates containing 100 μ g/ml ampicillin. This process was repeated with the control vector provided by the manufacturer. Plates were incubated upside down at 37°C overnight.

The number of plaque forming units (pfu) and the ratio of blue (non-recombinant plaques) to white (recombinant) plaques were determined. The total number of plaques was used to estimate the titre of the primary library and the efficiency of the packaging. In all cases, the 'ideal' number of plaques was considered to be 1×10^6 pfu in order to ensure that as many of the expressed genes were cloned. In cases where this was not achieved, an additional synthesis was attempted.

Since primary phage libraries tend to be susceptible to loss of titre over long periods of time, the libraries were amplified within two days of the initial titrating. The host preparation was essentially the same as described for the titrating. However, for the plating, 600 μ l of XL1-Blue cells were combined with ~ 50 000 pfu of the primary library and the transfected cells plated in 18 ml NZY top agar onto 280 mm x 280 mm NZY agar plates supplemented with 100 μ g/ ml ampicillin. These plates were incubated at 37°C until the plaques were just touching (typically approximately 8 hrs).

The plaques were overlaid with 10 -15 ml SM buffer and maintained with gentle rocking at 4°C overnight. The SM buffer phage suspensions were recovered from the plates and pooled in sterile 50 ml Sterilin® tubes. The plates were rinsed with an additional 5 ml SM buffer. Chloroform (5 % (v/v)) was added to the suspensions which were left to incubate for 15 min. Cell debris was removed by centrifugation at 1 000 RCF for 10 min.

The titre of these amplified libraries was determined as described (section 2.7.8). However, 1×10^{-4} , 1×10^{-5} , 1×10^{-6} and 1×10^{-7} dilutions of the phage in SM buffer were used. Amplification was considered successful if the titre of the amplified library was greater than 1×10^9 pfu/ml. Aliquots of the libraries were stored in 7 % (v/v) DMSO at -70°C for future use.

4.2.5 Evaluation of libraries

Excision was carried out using 10^7 pfu of the amplified library, 10^8 XL1-Blue MRF' cells and 10^9 pfu ExAssist helper phage as described by the manufacturer. Colonies were plated out onto LB agar plates containing 100 µg/ml ampicillin, 75 µl of IPTG (0.1 M; USB, U.S.A) and 250 µl X-gal (25 mg/ml; USB, U.S.A). Although not specified in the Stratagene manual, colour selection was used to evaluate the success of the excision and to assist in the selection of recombinants.

Individual colonies were inoculated into 5 ml LB containing 100 µg/ml ampicillin and cultured overnight at 37°C. Cultures were centrifuged for 5 min in 2 ml micro-centrifuge tubes to pellet the cells. The supernatant was removed and the plasmid DNA prepared as described by Isch-Horowitz and Burke (1981). The DNA was lyophilized after the isopropanol precipitation and resuspended in 20-50 µl TE buffer containing 20 µg/ml RNase A.

For restriction enzyme digestions, 8 µl of plasmid DNA was combined with 1 µl buffer H (10 x, Amersham), 0.5 µl *EcoRI* (10 U/µl, Amersham) and 0.5 µl *XhoI* (10 U/µl,

Amersham). The reactions were digested for a minimum of 2 hrs at 37°C and electrophoresed on a 1 % agarose gel.

Additional screening was accomplished by colony PCR performed according to the protocol of Roschier *et al.* (2000). Colonies were picked using a micropipette tip and placed directly into the 50 µl reactions. PCR conditions were: 94°C (2 min), [94°C (30 s); 56°C (30 s); 72°C (1 min)] x 10, [94°C (30 s); 51°C (30 s); 72°C (1 min)] x 20, 72°C (5 min). Appropriate controls were employed. Aliquots (10 µl) of the PCR reactions were loaded onto a 1 % agarose gel and electrophoresed under standard conditions. Products were visualized and photographed for future reference.

For both restriction enzyme and colony PCR analyses the percentages of colonies containing inserts were recorded. Insert sizes, taking into account the 180 bp of flanking vector sequence, were calculated from comparison to a DNA marker.

4.2.6 Screening with β -tubulin

30 000 pfu from each of the amplified libraries were plated onto NZY plates as described above. Plaques were lifted in duplicate onto Hybond-N membranes (Amersham Pharmacia Biotech, U.S.A) and prepared as outlined in the Uni-ZAP manual (Stratagene, U.S.A). DNA was fixed to the nylon membranes by UV cross-linking at 70 000 $\mu\text{J}.\text{cm}^{-2}$ in an UV cross-linker (Amersham, U.S.A). The membranes were dried and wrapped in cling-film until use.

Bacterial clones carrying the *Arabidopsis thaliana* β -tubulin isoforms I, II and III were obtained from Mark Knight (Oxford, UK). Clones were cultured overnight at 37°C in 5 ml LB supplemented with 100 µg/ml ampicillin. Plasmid DNA was prepared as described above and digested with *NotI* and *SalI* (Amersham). Inserts were excised from the gel under long wavelength UV light using sterile scalpel blades. DNA was recovered from the gel using the Gene Clean® II kit (Bio101, U.S.A) as per the manufacturer's specifications.

5 μ l (25 ng) of the purified product was used in a Megaprime (USB, U.S.A) labeling reaction as described by the manufacturer. Labeling reactions were carried out for a maximum of 30 min. The unincorporated nucleotides and primers were removed by purification on Sigma post-reaction spin columns (Sigma, U.S.A) according to the product specifications. Scintillation counting in a Beckman LS 5 000 TD liquid scintillation system (Beckman, U.S.A) was employed to determine the percentage incorporation and the total disintegrations per minute (dpm) present in the reaction volume.

Hybridizations were performed as outlined in the Lambda Library User's Manual (Clontech, U.S.A). At least 2×10^6 dpm per ml of buffer was used. Positive plaques were detected by autoradiography at -70°C for 24 hrs followed by developing of the film on an Okamoto x-ray film developer (Okamoto, JP). The number of positive signals was determined and the expected numbers of rare transcripts calculated by dividing the total number of tubulin isoforms by 1000. This was compared to the theoretical probability of finding the rarest transcript (1 in 500 000), calculated according to the following equation derived from that presented in section 2.1.1:

$$n = \frac{\ln(1 - P)}{\ln(1 - F)}$$

Where n = the number of recombinants, P = the probability of finding an insert
 F = the frequency of the sequence in the population (0.000002).

4.2.7 Northern blot analysis

To investigate the regulation of β -tubulin II in the roots and leaves to further assess the suitability of this gene for library evaluation, radio-labeled β -tubulin II probes were prepared as described above (section 4.2.6). These were used to probe duplicate northern blots of leaf and root RNA obtained from plants at various water contents. Blots were prepared according to standard methodology (Ausubel *et al.*, 1989). The *Arabidopsis thaliana* actin (Mendel Biotech Clone set, Mendel Biotech, U.S.A) and *X. humilis* 18S RNA genes were employed as loading controls.

4.2.8 Data analysis and management

All images were digitized using an HP ScanPro 5200C scanner and imported into Adobe Photoshop® where they were collated and modified for presentation.

4.3 Results

4.3.1 RNA and mRNA isolation and evaluation

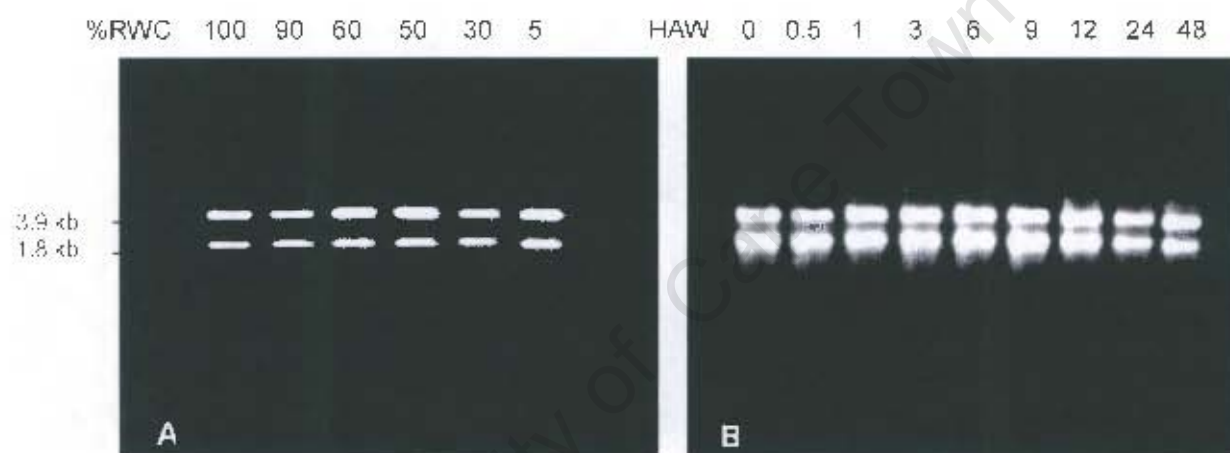


Figure 4.2: RNA isolated at varying water contents from roots of (A) dehydrating and (B) rehydrating *X. humilis* plants. 1-2 μg of RNA was loaded per lane. The sizes and positions of the 18S and 28S ribosomal subunits are indicated. RWC = relative water content, HAW = hours after watering.

Figure 4.2 indicates that high quality RNA was isolated from the roots of *X. humilis* at specified water contents or hours after watering. The yield of RNA per dry mass of root tissue ranged from 0.005 % to 0.05 %, making the routine isolation of mg quantities of RNA possible. RNA pellets were dark brown in colour, implying contamination by phenolic compounds. Nevertheless, the 18S and 28S RNA bands were clear and intact. The 28S band was approximately double the intensity of the lower 18S band, indicating that degradation of RNA had not occurred during the harvesting and

extraction. Bands corresponding to chloroplast RNA were not observed, suggesting that the root samples were relatively free of leaf contamination. The lack of small and degraded RNA also implied that debris and bacterial contamination was limited.

This RNA was used in the isolation of poly(A)⁺ RNA. In an initial test of dT-cellulose chromatography and the Promega PolyAtract system, it was found that the Promega system gave higher yields of poly(A)⁺ RNA (data not shown). These yields ranged from between 1 % and 3 % of total RNA (ie: 5-10 µg of poly(A)⁺ RNA) as determined by ethidium bromide fluorescence assays. This contained little observable ribosomal RNA contamination. The average size of the transcripts was ~2kb with the maximum and minimum sizes being ~6kb and ~0.2 kb respectively (Figure 4.3). The quality of the RNA was confirmed by the successful incorporation of [³²P] dATP into cDNA via RT (Figure 4.3). Poly(A)⁺ RNA from both hydrated and dehydrated root and leaf samples was reverse transcribed, although high levels of variation in the success of incorporation were observed in samples from intermediate water contents (data not shown).

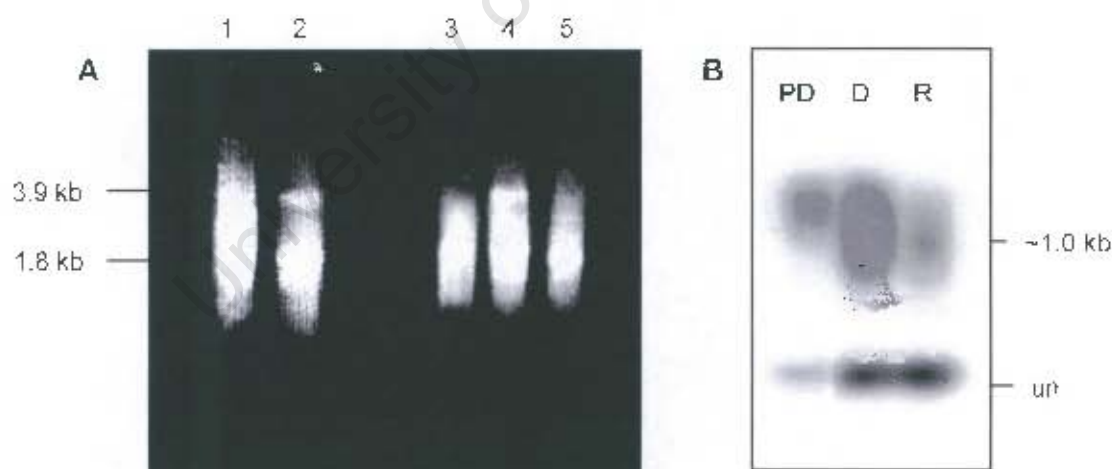


Figure 4.3: (A) Poly(A)⁺ RNA derived from pooled dehydration (lanes 1-2) and rehydration samples (lanes 3-5). Approximately 0.5 µg RNA from several separate isolations was loaded per lane. (B) Autoradiograms of [³²P] dATP labeled cDNA derived from root RNA at different water contents. PD = partially dehydrated, D = dehydrated, PR = partially rehydrated, un = unincorporated nucleotides.

4.3.2 cDNA synthesis and cloning

In line with the observations about the availability of the poly(A)⁺ RNA for RT, both first- and second-strand reactions generated cDNA of the expected size range (Figure 4.5). Since these reactions contained pooled 'dehydration' or 'rehydration' samples, differential conversion of poly(A)⁺ RNA from separate time points could not be assessed. Nevertheless, the yields of cDNA were within expected ranges, suggesting that efficient RT of the bulk of poly(A)⁺ RNA occurred. The resulting cDNA ranged in size from ~300 bp to ~3 kb, with the average size being approximately 1-1.5 kb (Figure 4.4).

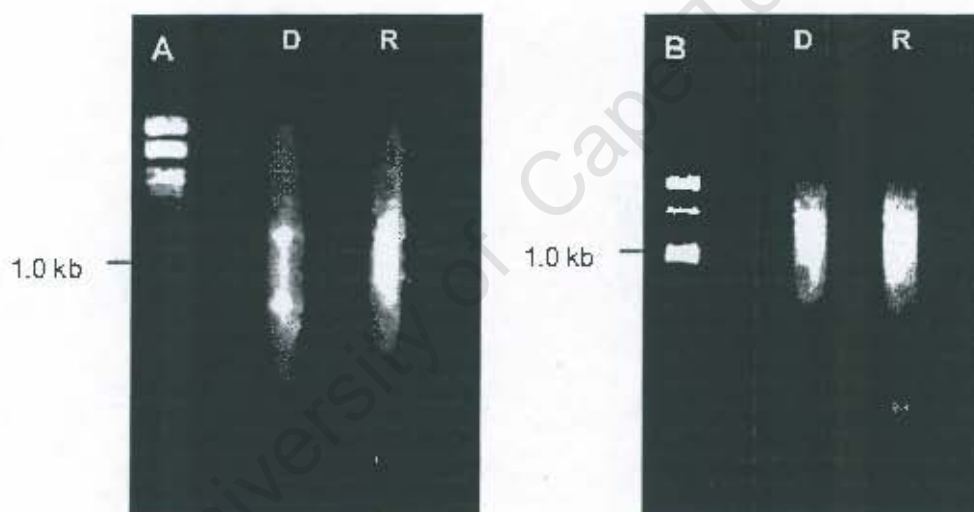


Figure 4.4: Photographs of (A) first-strand and (B) second-strand synthesis reactions. 1-2 μ l of DNA was loaded per lane and electrophoresed on alkaline agarose gels in the presence of ethidium bromide. D = pooled dehydration samples, R = pooled rehydration samples.

This cDNA was fractionated using the Stratagene Sepharose columns provided with the ZAP-cDNA synthesis kit and using a standard low-melting point agarose gel fractionation method. The total amount of cDNA recovered in six fractions from the columns averaged 250-300 ng in four separate fractionation attempts. Because of the limited amounts of DNA and the requirement for 100 ng of DNA per ligation, no

assessment of the size range of the fractionated DNA could be gained. Moreover, fractionation of 3 sets of 'rehydration' cDNA failed due to binding of the cDNA to the columns. On the other hand, gel fractionation yielded at least 600 ng of DNA of 500 bp and above per fractionation.

Cloning of the fractionated DNA generated several libraries. In many cases the amount of cDNA proved limiting, resulting in libraries of primary titres lower than 500 000 pfu. Two successful libraries were selected for further manipulation and characterization. The details of the fractionation, ligation and primary titres of these are represented below (Table 4.1). A clear difference in the efficiency of the ligation and packaging of the two libraries is evident. The primary influence appeared to be the amount of cDNA available per ligation (Table 4.1). While the number of pfu in the primary dehydration library was not ideal, this was the highest obtainable using the column-fractionated cDNA. The titre of this primary library is also made more acceptable by the fact that the levels of non-recombinant clones were low (Table 4.1). Both libraries were successfully amplified to titres $> 1 \times 10^9$ pfu/ml. Generally, 100-200 ml of library were stored at -70°C for future use. Relitering of the libraries at intervals during the course of a year indicated that the amplified libraries were not subject to significant loss of titre under these conditions.

Table 4.1: Summary of key library data.

Library	Fractionation	cDNA ligated	Primary titre (pfu)	% non-recombinants	Amplified titre (pfu)
Dehydration	column	60 ng	0.4×10^6	5.0	1×10^{11}
Rehydration	gel	120 ng	1.5×10^6	1.0	1×10^{10}

4.3.3 Library evaluation

The presence and features of the inserts in the libraries were determined through excision, restriction analysis and colony PCR. Excision of the pBluescript plasmid from both libraries resulted in high numbers of colonies, the vast majority of which

contained inserts (Figure 4.5; Table 4.2). Colony PCR was found to be easier and significantly faster than restriction analysis for the routine screening colonies for inserts. The results of the colony PCRs were confirmed by digestion of the plasmid DNA from the total libraries and selected clones (Figure 4.5). The two methods generated identical information, indicating that the sizes of the inserts in both libraries ranged from approximately 0.5-3.0 kb with the average size being approximately 0.9-1.0 kb. No significant difference between statistics for the inserts from the different libraries was observed, suggesting that the two methods of size fractionation were equally efficient. These statistics are summarized in table 4.2.

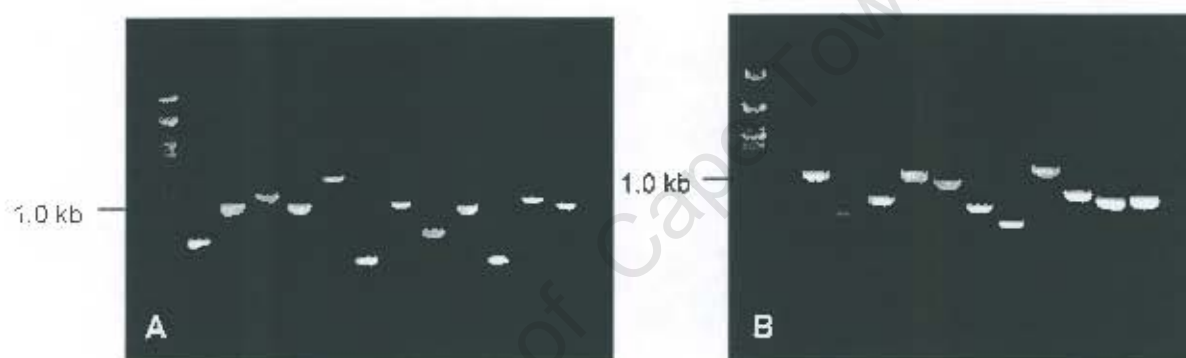


Figure 4.5: Evaluation of library inserts. Agarose gels of colony PCR reactions indicating the presence of inserts in 12 randomly selected clones from the (A) dehydration and (B) rehydration libraries.

Table 4.2: Average insert sizes and insert size ranges for dehydration and rehydration libraries.

Library	Ave. size (kb)	Std. dev.	Range
Dehydration	1.1	0.51	0.5-3.0
Rehydration	1.0	0.25	0.5-3.2

The most alarming change in the libraries during excision was the dramatic shift in the ratio of blue to white clones. For both libraries this ratio increased several fold from 5 % and 1 % to approximately 74 % and 69 % of the dehydration and rehydration libraries respectively (Figure 4.6).

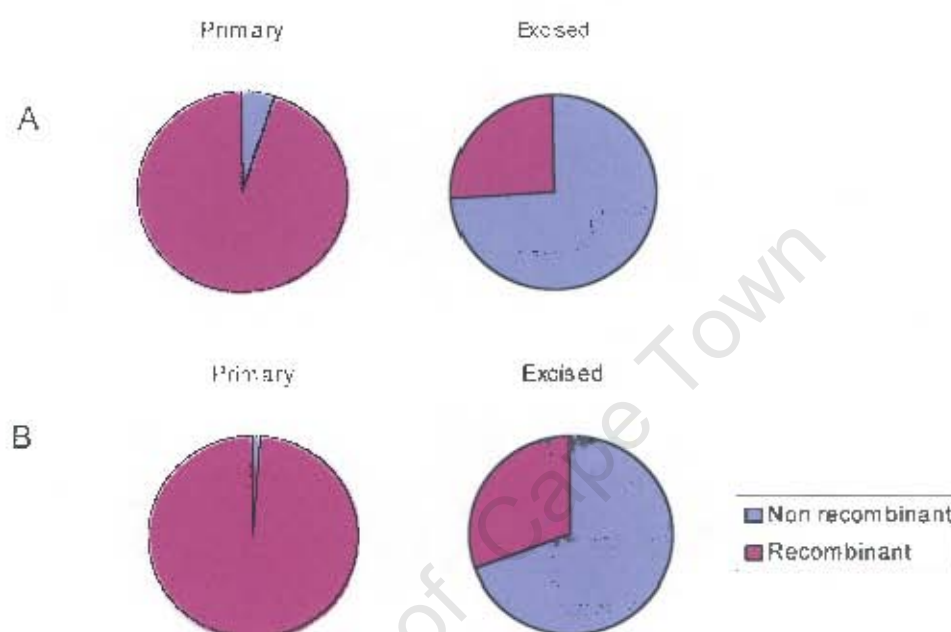


Figure 4.6: Pie charts indicating the shift in the percentage of blue and white clones in (A) dehydration and (B) rehydration libraries during the excision procedure.

Since the representation of genes in down-stream applications could be severely compromised by such a shift, routine screening of the colour selected libraries was undertaken. Both light blue and blue colonies contained a significant percentage of inserts (80-100%; Figure 4.7 A), indicating that this was not a true increase in the number of non-recombinants in the libraries. However, the average size of the inserts was observed to be smaller in the light blue and dark blue colonies (Figure 4.7 B). Because of the correlation between the decrease in insert size and the shift in the representation of the clones was taken to be due to the failure of the *lacZ* colour

selection system. It was concluded that the insert representation of both libraries was of a suitable standard and was not significantly affected by the excision process.

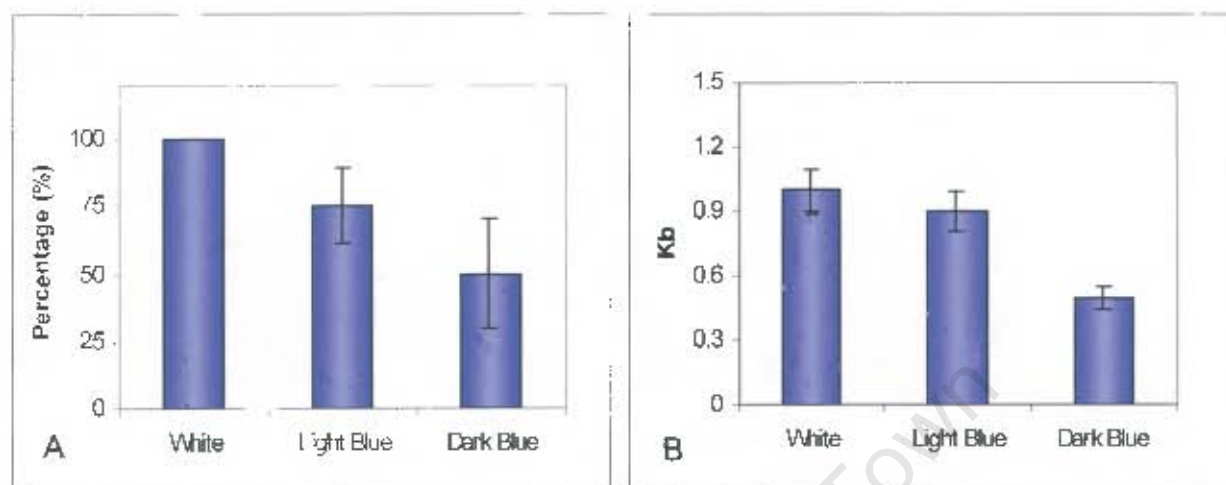


Figure 4.7: Graphs indicating (A) the percentage of colonies containing inserts and (B) the average sizes of inserts in colour selected colonies.

4.3.4 β -tubulin screens

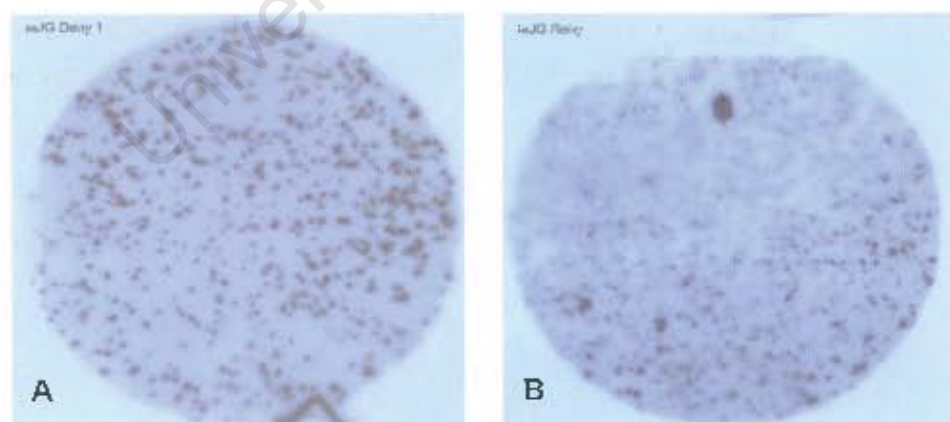


Figure 4.8: Representative autoradiographs indicating the representation of β -tubulin isoforms in the (A) dehydration and (B) rehydration libraries. Approximately 30 000 pfu were screened per membrane.

Table 4.3: Summary of the statistics relating to the percentage of positive plaques detected in the β -tubulin screen.

Library	% β -tubulin (total)	% β -tubulin (per isoform)	TNRT*	P**
Dehydration	3.06	1.02	2	0.45
Rehydration	0.18	0.06	1.5	0.95

TNRT* = theoretical number of rare transcripts, P** = probability of finding the rarest transcript

Figure 4.8 and Table 4.3 summarize the results of the library screens. Both libraries exhibited an extremely high number of positives. The percentage of positives per β -tubulin isoform was approximately 1.0 % and 0.06 % for the dehydration and rehydration libraries respectively (Figure 4.8; Table 4.3).

The theoretical ratio (1: 1000) of rare transcripts to common transcripts (such as β -tubulin) in a typical cell was used to determine the potential numbers of any rare transcript in the libraries. Although the number of pfu in the libraries differed by more than 3 fold, this value was observed to be similar for both (Table 4.3). However, when this was compared to the probability of a library containing the rarest transcript, there was a large discrepancy between the two values. The probability that the rarest sequence will be represented in the libraries was calculated to be approximately 45 % and 95 % in the dehydration and rehydration libraries, respectively. This was attributed to the fact that the theoretical values for the complexity of the transcriptome were unavailable. Overall the tubulin representation confirmed the presence of plant-derived inserts and indicated that the rehydration library was the better quality library.

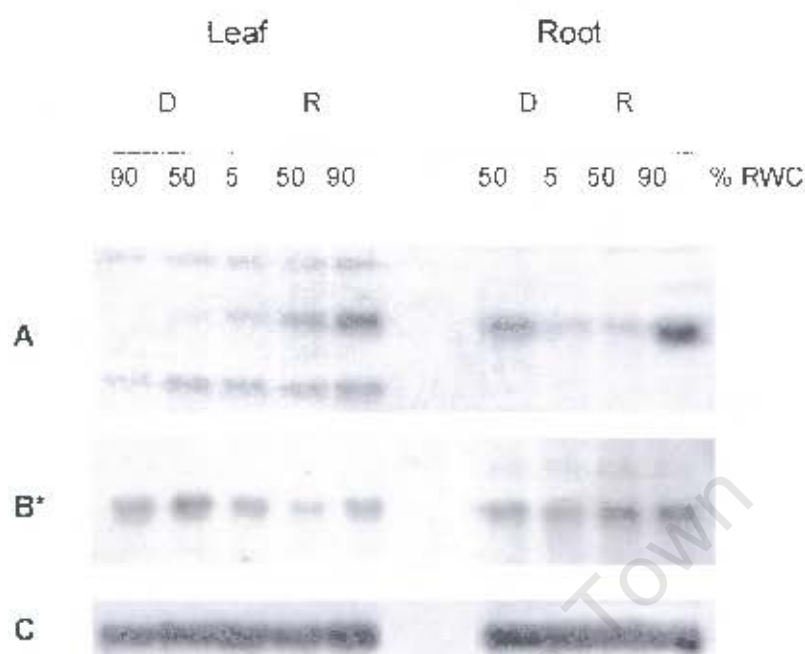


Figure 4.9: Northern blot analysis of (A) β -tubulin II (B) β -actin and (C) 18S RNA expression in the leaves and roots of *X. humilis*. 10 μ g of total RNA was loaded per lane. Controls indicated sensitivity to the pg level. * Actin blot carried out once only.

Figure 4.9 illustrates the northern blot analysis of β -tubulin levels in *X. humilis* tissues at various water contents. These results indicated that the levels of β -tubulin expression during dehydration and rehydration were variable. Levels of tubulin II were higher in the roots than in the leaves. In the roots the expression of tubulin II was high in the partially hydrated and rehydrated samples, but little or no expression was observed in the dehydrated samples (Figure 4.9 A). Multiple bands were detected in the leaf RNA. The uppermost band was represented consistently, but the lower two bands appeared to increase in intensity during the rehydration period (Figure 4.9 A). Whether these bands were due to alternate splicing or due to cross-hybridization to related β -tubulin isoforms was not empirically determined. The variability in the tubulin expression patterns was considered to cast some doubt on the validity of the conclusions drawn from the library screening, since it indicates that direct comparisons

between rehydration and dehydration (or root and leaf) libraries could not be drawn. Northern analysis of the expression of the 'house-keeping' gene, β -actin indicated that the levels of this gene also fluctuated extensively during desiccation and rehydration (Figure 4.9 B). In root samples, actin was expressed at low levels during dehydration, but at slightly higher levels during rehydration. In the leaves, actin expression was higher in the partially dry and rehydrated samples than in either the hydrated or dry samples (Figure 4.9 B). Although high levels of cross-hybridization were observed in the 18S RNA blots, the primary 18S RNA band intensity did not change significantly, indicating that the amounts of RNA loaded were consistent.

4.4 Discussion

The data presented above imply that libraries of a suitably high standard, in terms of the length and representation of cDNAs, were obtained. These libraries represent significant resources for the future study of mechanisms of desiccation tolerance in *X. humilis*.

It is likely that two separate sets of transcripts are mobilized during dehydration and rehydration in order to ameliorate the distinct stresses experienced during these periods. The limits to cloning efficiencies and the abundance of house-keeping genes mean that combining RNA samples tends to reduce the frequency of rare transcripts in mixed pools of RNA. For this reason, two separate libraries – one dehydration and one rehydration - were constructed. Although this will lead to increased redundancy of common genes between the two libraries, it may also increase the possibility of cloning transcripts involved in the stress responses.

Despite the fact that the RNA utilized for the construction of the libraries appeared to be of high quality (Figure 4.2) a number of researchers have experienced difficulties with the RT of RNA from resurrection plants (eg: Koonjul *et al.*, 1999) and from roots in general. This is thought to be as a result of the inclusion of polyphenolics and oligosaccharides in the RNA (Chomckzynski and Mackey, 1995; Koonjul *et al.*, 1999) – a suggestion that is supported by the colouration of the RNA and the variability of the

RT observed in this study. The problems experienced with the fractionation of the cDNA produced from the pooled rehydration RNA also imply that there could be unusual structure associated with the RNA template. Both these possibilities suggest role for RNA stabilization in desiccation tolerance.

The fact that *X. humilis* relies heavily on translation rather than transcription during the initial phases of rehydration (Dace *et al.*, 1999) means that mRNAs must be stored in a stable form in the desiccated plant. The results of O'Mahony and Oliver (2001) indicated that this was true for the fdt tolerant moss *Tortula ruralis* but not for the mdt grass *Sporobolus stapfianus* which underwent ordered breakdown of the ribosomal RNA. However, these authors only studied the ribosomal RNA - the breakdown of which may be a mechanism of terminating translation during the later phases of desiccation. The remaining RNA may be stabilized or stored in some way. Wood and Oliver (1999) demonstrated that RNA is packaged into messenger ribonucleoprotein particles in response to desiccation in *T. ruralis* gametophytes. They have suggested that this is a common mechanism of protection in resurrection plants. It is likely, however, that several mechanisms, including poly(A) tail extension (Kusov *et al.*, 2001) and cap binding (Hugouvieux *et al.*, 2001) operate in conjunction to bring about this stabilization. The finding that the condensation of commonly occurring isoprenoid moieties to adenine residues results in increased thermal stability of RNA (Kierzek and Kierzek, 2001) also suggests that chemical modification may be involved. Whether all transcripts are targeted for storage, or whether key transcripts are selected is an interesting question that remains to be explored.

The difficulties related to the manipulation of angiosperm resurrection plant RNA highlight the need for the consistent quality control measures employed in the course of this study. The failure of the RT of RNA from a single time point within the pools of RNA could dramatically alter the representation of transcripts within the libraries.

Although the cloning of the cDNA generated from the poly(A)⁺ RNA pools only yielded 0.4×10^6 pfu (Table 4.1) for the dehydration library, this was considered acceptable, largely because given the small number of living cells, and the lack of photosynthetic

functions, the population of transcripts is expected to be less complex than in other tissues. Reddy *et al.* (2002) demonstrated that primary libraries of water stressed rice roots consisting of only 5 000 clones were sufficient to identify several novel genes. Even if roots do have more complex gene expression patterns than the anatomy suggests the high titre of the rehydration library (1.5×10^6 pfu; Table 4.1) means that the bulk of the transcripts expressed in the roots during water stress are likely to be represented in this library.

The higher titre of the rehydration library is also an indication of the importance of the amount of cDNA used in the ligation reaction, and of the success of the gel fractionation method in generating sufficient cDNA to make this possible (Table 4.2). The efficiency of this method was confirmed by the fact that there were no significant differences in the average size and overall range of the inserts in the two libraries (Figure 4.5). The fact that the average insert size was approximately 1 kb suggests that the majority of the inserts are not full length. However, the wide range of insert sizes, and the exclusion of small inserts makes it likely that most inserts will be large enough to allow sufficient sequence information to be gained.

The shifts in the numbers of blue colonies during the excision procedure (Figure 4.6) was alarming as the levels of non-recombinants in a library should preferably be as low as possible (Soares and Bonaldo, 1998). Analysis of these clones revealed that this apparent shift was due to the failure of the colour selection method. This has been reported by several authors and is thought to arise when the sequences inserted into the multiple cloning sites are too small to disrupt the functioning of the *lacZ'* product (Promega Technical Manual 042, 1999; Ali *et al.*, 2001). The decrease in the average size of inserts between white and blue colonies (Figure 4.7) indicates that this is likely to be the primary cause. However, it has been reported in the literature that DNA fragments up to 2 kb have been cloned and produced blue colonies. Such fragments are usually cloned in-frame do not contain stop codons (Promega Technical Manual 042, 1999).

Although screening with marker genes is a traditional means of evaluating libraries (Hagen *et al.*, 1988; Soares *et al.*, 1994), the results of the screening conducted in this study were difficult to interpret. The percentage of tubulin per library suggests that the chances of locating a particular rare gene are better for the dehydration library (Table 4.3). However, the probability values indicate a very low chance of finding a very rare transcript in this library. This apparent contradiction arises from the fact that the ratio of common genes to rare genes has not been determined for plants. The figure of 1000 is a theoretical figure drawn primarily from animal studies (Soares *et al.*, 1994). Additionally it does not take into account the total number of pfu in the primary library, and therefore does not relate the probability of finding a transcript to the complexity of the libraries. On the other hand, the probability estimate takes into account the effect of the titres on the range of genes originally cloned. But this figure does not factor in fluctuations or deviation in the expression patterns and ratios of genes in the source material and assumes that the gene of interest will be represented only once in 500 000 transcripts. Nevertheless, the bulk of the data, combined with qualitative observation, support the idea that the dehydration library contains a less complex array of rare genes. This goes against the findings of Hagen *et al.* (1988) that of 25 libraries examined, those containing less than 0.1 % actin did not yield any clones of interest (low abundance sequences).

β -tubulin and β -actin are traditionally the house-keeping genes of choice for the evaluation of libraries (Hagen *et al.*, 1988; Soares *et al.*, 1994) but this is only true for systems in which their expression is stable. The northern blot analyses were conducted to explore the validity of using tubulin representation as a means of comparison between libraries. They indicated that neither actin nor tubulin II exhibited stable expression during desiccation or rehydration (Figure 4.9). The significance of this is unknown and could not be pursued due to time constraints. Although the 18S RNA expression was found to be consistent, it is not expected that this will be present in libraries constructed from purified poly(A)⁺ RNA. More effective screening procedures should rely on genes for which the expression is known to be relatively invariable. However, such a gene will have to be characterized empirically as little is known about what constitutes a housekeeping gene in a resurrection plant. Clearly,

the basic cellular machinery of *X. humilis* is also affected by the onset of water flux – at least at the transcriptional level.

This problem has received some attention from recent microarray experiments which indicate that of the 30 genes exhibiting the most stable expression patterns across a wide range of conditions, none corresponded to those traditionally assigned house-keeping functions (Wu *et al.*, 2001). These experiments also indicated that the large use of genes from large families is also problematic because cross hybridization occurs for sequences with greater than 80 % sequence identity (Wu *et al.*, 2001). Such cross hybridization tends to mask the true expression patterns of a single representative.

Cross hybridization may explain the range of bands observed in the tubulin blot (Figure 4.9 A). In *Arabidopsis* there are 9 members of the β -tubulin family all of which share extensive sequence homology (Snustad *et al.*, 1992). Given the conservation of tubulin sequences (despite the lack of conservation of expression patterns) it is probable that a similar relationship between tubulin isoforms exists in *X. humilis* and thus results in cross hybridization. The lack of multiple bands in the root samples suggests that either β -tubulin II or III (these are different isoforms of the same gene – Snustad *et al.*, 1992) is not expressed in this tissue or that alternate splicing of introns occurs only in the leaves. The question of cross-hybridization raises additional problems for the library evaluation based on tubulin representation, as the number of isoforms effectively detected is unknown.

In this study two stress- and tissue-specific libraries were constructed from recalcitrant material. The analysis of these libraries highlighted several issues that may have relevance for ongoing resurrection plant research. The evidence supports the idea that the libraries generated in the course of this study are of a suitably high standard. However, insight into the quality and complexity of the libraries relies either on extensive screening with a variety of genes, or on large-scale sequencing. Both of these are expensive procedures. Methods of reducing the redundancy of the libraries,

and of increasing their quality and usefulness to make such procedures cost effective are addressed in the next chapter.

University of Cape Town

Normalization of cDNA Libraries

A rational approach to gene discovery

5.1 Introduction

The representation of genes in cDNA libraries presumably reflects that in the population of mRNAs from which they are derived (Soares *et al.*, 1994; Bonaldo *et al.*, 1996; Soares and Bonaldo, 1998). The mRNAs in a typical somatic cell tend to be distributed in three frequency classes: (I) supervalent (or abundant), (II) intermediate and (III) rare (Bishop *et al.*, 1974; Bonaldo *et al.*, 1996). The superavalent class consists of between 10 to 15 mRNAs that are found in frequencies of ~5000 copies per cell – therefore making up about 10–20 % of the total mRNA mass (Bonaldo *et al.*, 1996). The intermediate class typically consists of 1000–2000 mRNAs that make up 40–45% of the mRNA mass; while the complex or rare class is made up of approximately 15 000–20 000 transcripts. This last class of transcripts only makes up 40–45 % of the total mRNA mass, yet contains 90 % of the expressed sequences (Bonaldo *et al.*, 1996).

This implies that once the common sequences in a typical cDNA library have been identified, expected redundancy levels are at least 60% (Soares and Bonaldo, 1998) and that the rarest gene will only be represented once in 500 000 clones. In some libraries derived from complex tissues the frequency of the occurrence of rare transcripts may even be as low as 10^{-7} (Soares *et al.*, 1994; Soares and Bonaldo, 1998). This means that large-scale sequencing of standard libraries is an extremely inefficient and expensive way of discovering novel genes. Moreover, it restricts microarray experiments to cases where either entire libraries have been sequenced and the redundant clones removed, or to where a small, highly redundant gene set is screened.

To a large extent, these problems can be overcome by the use of normalized libraries in which the frequency of all clones is in a narrow range, and which therefore exhibit very low redundancy levels (Bonaldo *et al.*, 1996). Normalization generally results in libraries in which supervalent and intermediate class transcripts represent only ~ 5% of the total sequences, while the rare transcript representation is increased to 95%. This corresponds to a 13.5 fold decrease in common sequences and a 2.5 fold increase in rare transcripts (Soares and Bonaldo, 1998). The process also results in a decrease in the total number of clones to 10 000–100 000 clones. This enables the relatively easy production of complete microarrays. Normalization is thought to be a more efficient approach than subtraction (Diatchenko *et al.*, 1996) to the characterization of genetic responses because it retains in high and intermediate abundance sequences that may be of interest in differential or functional studies. Subtraction, on the other hand, may be more effective in improving the representation of novel sequences alone. Both methods serve to address the dramatic fall-off in the rate of EST discovery (Wang *et al.*, 2000).

Two general approaches to normalization have been described. The first utilizes the hybridization of cDNA to genomic DNA (Weissman, 1987; Soares and Bonaldo, 1998). Using this approach, the frequency of each cDNA in the normalized library should be proportional to its representation in the genome. However, the feasibility of this method, particularly in light of the difficulty in obtaining sufficient rare cDNA for the hybridization, has not been established (Soares *et al.* 1994; Soares and Bonaldo, 1998). More successful approaches have been based on the principles of re-association kinetics.

5.1.1 Re-association kinetics in normalization

If a population of DNA duplexes is dissociated and then re-associated, the reaction will follow second order kinetics with respect to the concentration of single stranded DNA remaining unhybridized. This can be expressed as:

$$C_0 t_{1/2} = 1/k$$

for the limits C_0 ($t = 0$) and C ($t = t$) and where $t = t_{1/2}$ and $C/C_0 = 1/2$. k represents the rate constant of the reaction and $C_0 t_{1/2}$ the product of the total DNA concentration and the time (Soares and Bonaldo, 1998). Such a dynamic is represented graphically in a $C_0 t$ curve (Figure 5.1).

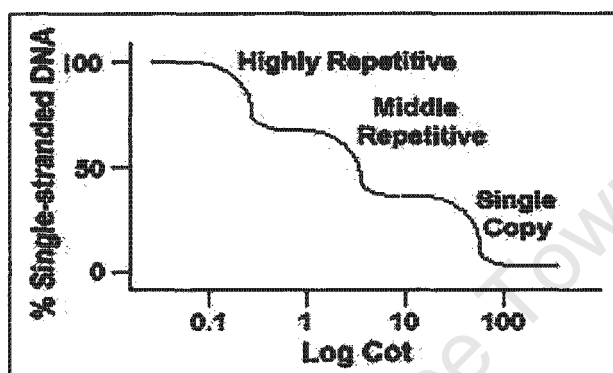


Figure 5.1: A typical $C_0 t$ curve of a complex DNA solution.

Since the re-association time is inversely proportional to the concentration of the DNA, the rarer the DNA sequence in the original reaction, the longer it will take to find its reaction partner and re-anneal (Ko, 1990; Sasaki *et al.*, 1994; Soares *et al.*, 1994; Bonaldo *et al.*, 1996). Therefore, as the reaction proceeds, the bulk of the common sequences in the population will form double stranded duplexes, while the rare sequences will remain single stranded.

The elimination of the double stranded duplexes (or common sequences) generates a single stranded DNA population in which the frequencies of all remaining sequences are in a very narrow range (preferably 1-2) or 'normalized'. Note that in practice it is impossible to achieve a library of complete parity. Yet, at the same time, complete loss of any insert, regardless of its abundance, does not occur (Ko, 1990; Soares and Bonaldo, 1998). To balance redundancy against enrichment of rare sequences, a suitable $C_0 t$ time should be chosen. This is however dependent on the complexity of the sequence population, and on the GC content and average lengths of the

sequences represented. In general C_0t times of between 5 and 20 are chosen. C_0t times of less than 1 result in an enrichment of class I (common) sequences, while C_0t times greater than 50 lead to a less significant representation of this class (Bonaldo *et al.*, 1996). In short, the annealing times have to be optimized for the material at hand, and/or the specific application required.

5.1.2 Kinetic methods of normalization

At least four independent methods have been developed on the basis of re-association. Ko's (1990) method relies on the re-association of sheared, linker-primed 200-400 bp cDNA fragments corresponding to the 3' ends of genes. His rationale for constructing libraries consisting entirely of 3' ends of genes was that, since the non-coding ends are almost always unique to a particular gene, this prevents the hybridization of related members of a gene family. In turn, this reduces the likelihood of the elimination of rare genes from the cDNA pool (Ko, 1990; Soares and Bonaldo, 1998). Patanjali and co-workers utilized a similar approach, though the hybridizing cDNA consisted of both coding and non-coding regions generated by PCR from cloned λ gt10 inserts (Patanjali *et al.*, 1991). The drawback of this method is that cDNA inserts must be short and relatively homogenous in length in order for the PCR to work efficiently (Patanjali *et al.*, 1991; Soares and Bonaldo, 1998).

A slightly different approach was employed by Sasaki *et al.* (1994). Instead of HAP chromatography, this procedure uses immobilized cDNA as the means of separating single and double stranded DNA populations. Sample mRNA is converted to cDNA and immobilized on a latex bead support. This is used in four rounds of hybridization against an identical total mRNA sample, which is gradually depleted in class I and II transcripts. The final population of mRNA is converted to cDNA and cloned into an appropriate vector (Sasaki *et al.*, 1994). Although this is a highly effective method, it is limited by the requirement for large amounts of pure mRNA (Sasaki *et al.*, 1994; Soares and Bonaldo, 1998).

Perhaps the most successful method of normalization to date is that developed by Soares *et al.* (1994) and modified by Bonaldo *et al.* (1996). This method utilizes single stranded DNA template generated either *in vivo* or *in vitro* from cDNA libraries. The template is hybridized to the complementary inserts generated from the same source libraries by PCR or plasmid digestion (Soares *et al.*, 1994; Bonaldo *et al.*, 1996). The use of almost full length cDNA overcomes many of the problems of cross-hybridization inherent in other normalization methods, while the use of library derived template means that source material is not limiting. Soares' method is also perhaps the only normalization method to have found widespread commercial application. The method was patented (Bonaldo and Soares, 1997) and has been a contributing factor in the success of the Integrated Molecular Analysis of Genomes and their Expression (I.M.A.G.E.) Consortium's gene discovery programme. It is discussed in greater detail in the sections below.

5.1.3 The Soares method

The defining characteristic of Soares' method of normalization is the use of full length, or almost full length, single stranded template DNA derived from cDNA libraries. This approach includes a number of possible methods of generating both the template (or 'tracer') and the annealing DNA (or 'driver').

i. tracer

Bonaldo *et al.* (1996) tested the production of single stranded tracer via the combined action of Gene II and exonuclease III (Hoheisel, 1993; Johnston *et al.*, 1995), via *in vitro* transcription using T3 or T7 RNA polymerases, and via super-infection of bacterial libraries with M13K07 helper phage. The M13K07 helper phage system relies on the ability of the phage to excise a faithful single stranded copy of a bacterial plasmid containing the f1 origin of replication. This occurs without lysis of the host cells – a fact that can be exploited to produce relatively pure single stranded DNA without the need for complex extraction procedures (Viera and Messing, 1987; Short *et al.*, 1988).

While the Gene II/ Exo III system (Life Technologies) is beneficial in that it circumvents distortions in the representation of genes arising from unequal growth of clones with different size inserts, it does not result in complete conversion of all plasmids to single stranded circles – making an additional HAP purification prior to normalization necessary (Bonaldo *et al.*, 1996). This, combined with the much higher yields of the M13K07 helper phage system, makes *in vivo* tracer production the method of choice (Bonaldo *et al.*, 1996; Soares and Bonaldo, 1998; Ali *et al.*, 2000; Reddy *et al.*, 2002).

ii. driver

The original method employed by Soares *et al.* (1994) relied on a controlled oligo(dT) primer extension to generate short (200-300 bp) complementary fragments of driver DNA. The rationale behind the use of fragments corresponding only to the 3' ends was to address the problem of cross-hybridization first recognized by Ko (1990). However, this method does not take into account the fact that long poly(A) sequences present in most expressed mRNAs (in Alu tails, for example) tend to hybridize to oligo(dT) tracts in the template cDNA (Bonaldo *et al.*, 1996; Wang *et al.*, 2002). This does not influence the representation of common sequences, which hybridize regardless of the poly(A) tracts, but does have a serious effect on rare sequences. The hybridization of these regions of homology leads to the selective loss of rare genes because of the generation of short double stranded segments (Martin and Pardee, 2002; Wang *et al.*, 2002).

Bonaldo *et al.* (1996) produced driver DNA by restriction digestion and purification of the inserts from the plasmid library. Although this limits problems associated with the use of oligo(dT) primers, it is problematic in that it biases the reaction against inserts with internal restriction enzyme sites. As a result most workers favour the use of driver DNA derived from PCRs using the T3/ T7 primers. This necessitates the use of vector- and strand-specific blocking oligonucleotides to eliminate hybridization of inserts to the vector sequences flanking the inserts (Bonaldo *et al.*, 1996; Ali *et al.*, 2000).

The various methods are summarized diagrammatically below.

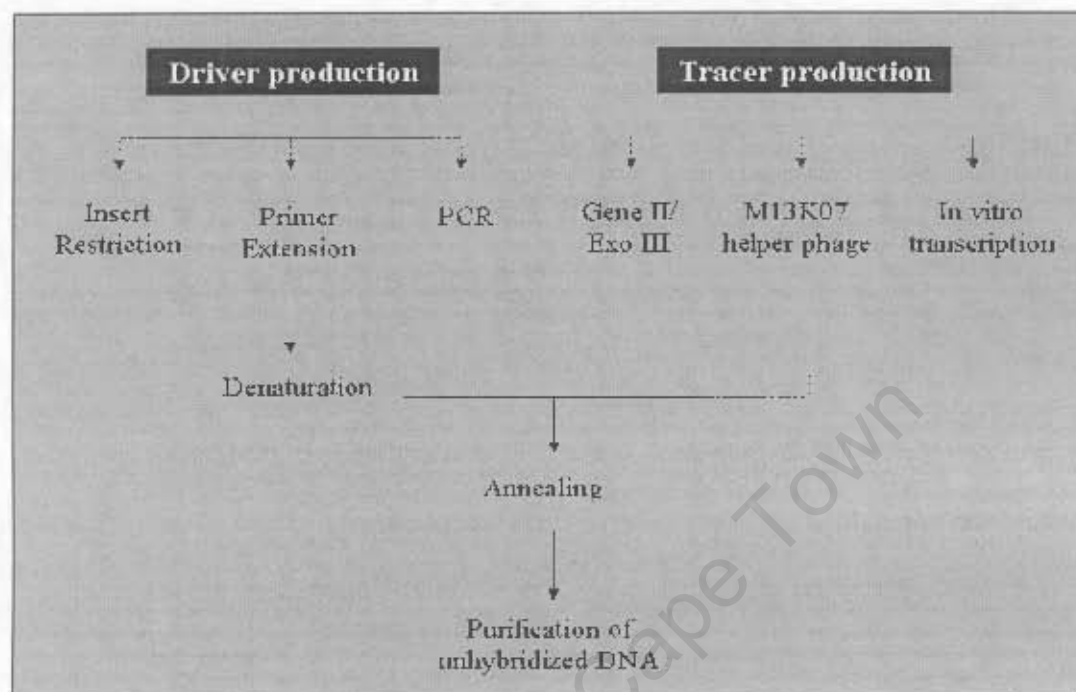


Figure 5.2: An outline of the available methods of normalization. The driver DNA is denatured and annealed to the tracer in the presence of blocking oligonucleotides to achieve the required $C_{0,t}$ time (adapted from Bonaldo *et al.*, 1996).

A population can only be said to be normalized if it is separated from the mass of DNA duplexes that constitute the common sequences. The most effective method of doing so is via hydroxyapatite (HAP) column chromatography.

5.1.4 HAP column chromatography

HAP consists of calcium phosphate crystals that bind nucleic acids by virtue of the interaction between the phosphate backbone of the nucleic acid and the calcium residues in the resin (Britten *et al.*, 1974; Soares and Bonaldo, 1998). Normalization makes use of the different affinity of HAP for single and double stranded DNA. At low concentration of phosphate ions (10–30 mM) HAP binds both single and double

stranded DNA. At intermediate concentrations of phosphate ions (120–150 mM), HAP binds only double stranded DNA and allows the release of single stranded DNA., whereas at high concentrations of phosphate ions (400-500 mM) HAP binds neither double nor single stranded DNA (Britten *et al.*, 1974; Soares and Bonaldo, 1998). In its simplest form, therefore, separation of double stranded duplexes from the single stranded normalized DNA population involves binding of the double stranded DNA to HAP in 120 mM sodium phosphate and collection of the single stranded eluate.

The definition of the binding capacity of HAP is somewhat arbitrary as the binding of DNA is higher for duplexes that have no mismatched bases. It is also higher for linear double stranded DNA than for circular plasmid DNA (for this reason, DNA is routinely linearized with *PvuII* prior to loading on HAP columns). However, for most applications the capacity is approximately 0.5 µg of DNA per mg of HAP (Britten *et al.*, 1974). It is this high binding capacity, combined with the selectivity of HAP, that makes it suitable for the normalization of re-associated DNA populations.

In most methods, the single stranded DNA eluate from the HAP columns is converted to double stranded plasmid DNA and electroporated into an appropriate host in order to complete the normalization process.

5.1.5 The effectiveness of normalization

As noted earlier, normalization tends to reduce the frequency of class I (common) and class II (intermediate) sequences and increase the representation of class III (rare) sequences. Representation of class I and II transcripts is reduced from approximately 60 % to approximately 5% while the representation of rare transcripts increases from 40 % to 95 %. Ali *et al.* (2000), normalizing to C_{ot} values of 10, were able to demonstrate a 10-fold decrease in the frequency of the supervalent *c-hordein* transcript, and a corresponding 10-fold increase in the frequency of the rare *fatty acid desaturase 3* gene. The representation of these two genes was similar in the normalized library, despite the *hordein* gene being represented in 50-fold higher frequency in the starting non-normalized library (Ali *et al.*, 2002). Essentially,

therefore, the percentage of the library that the class makes up is directly proportional to the number of independent sequences in that class (Soares and Bonaldo, 1998).

It is interesting that the representation of Alu repeat-containing cDNAs tends to be preserved - suggesting that imperfectly matched hybrids do not bind to the HAP columns, or that they melt during the elution. This implies that related, cross-hybridizing sequences will not be eliminated from the normalized libraries and that gene families are preserved in the final library (Bonaldo *et al.*, 1996). The process does not appear to discriminate against sequences on the basis of size if full length inserts are used for the driver. If however truncated driver fragments are used (eg; driver derived from primer extension reactions) then the normalization tends to result in a higher representation of truncated sequences and sequences lacking the 3' UTRs.

Such is the success of normalization in gene discovery that it is estimated that over 87.5 % of all human ESTs have been derived from normalized libraries (Soares and Bonaldo, 1998). The procedure has also been applied to libraries from specific tissues of mouse and rat amongst others (Bonaldo *et al.*, 1996). However, there are few normalized libraries derived from plant material. This is largely related to the extent to which plant research lags behind mammalian molecular biology, and to the lack of information about the sequence complexity of most plant genes. To date normalized libraries exist for only four plant species, namely *Arabidopsis thaliana* (Asamizu *et al.*, 2000a), *Lotus japonicus* (Asamizu *et al.*, 2000b), *Triticum aestivum* (All *et al.*, 2000) and *Oryza sativa* (Reddy *et al.*, 2002). In each of these libraries, the typical percentage of novel sequences was 20-30 %, at least 5 to 10-fold higher than in their non-normalized counterparts. Clearly therefore, the power of this technique in gene discovery programmes is considerable.

5.1.6 Normalization and plant desiccation tolerance

In terms of the broader context of this thesis, the most interesting of the plant libraries are those derived from rice (Reddy *et al.*, 2002). In a typical year, the yield of rice is

decreased by about 15 % due to the effects of abiotic stress, a figure that is almost double that attributed to biotic stresses. Drought stress in particular is the most detrimental of these stresses, causing wide-scale yield instability across Asia (Reddy *et al.*, 2002). Given the complexity of the plant responses to desiccation and drought outlined in chapter 1, it is clear that it would be difficult to breed for drought tolerance in this or any other species. Large-scale EST projects offer a progressive means of unraveling the complex biology underlying increased resistance to stresses, and make possible the lateral transfer of such traits in a way that the study of single genes cannot. In sequencing only 1000 of the 25 000 clones represented in a normalized leaf library from rice grown at 50 % RWC, Reddy *et al.* (2002) demonstrated that approximately 29 % corresponded to completely novel ESTs, while the redundancy amongst these clones was only 3.5 % (as opposed to 10 % in the non-normalized library).

The report of Reddy *et al.* (2002) is also the first published attempt to normalize libraries generated from water-stressed roots. However, the authors were unable to produce either standard or normalized libraries of acceptable quality (the standard root library contained only 5000 cfu; Reddy *et al.*, 2002).

Genes related to metabolism, metallothionein-like genes, ABA- and DREB-responsive genes, transcription factors and anti-oxidant genes were found to be the most prevalent classes in the normalized library. Many of these have already been reported as abiotic stress response factors in rice and *Arabidopsis* microarray analyses (Kawasaki *et al.*, 2001; Seki *et al.*, 2001, Seki *et al.*, 2002), thus confirming the usefulness of normalization in the understanding of complex biological responses.

It is clear that normalization of cDNA libraries enables large-scale study of complex traits, and is especially beneficial in cases where little funding or priority is attached to the sequencing of the genome of the organism in question. However, few researchers have focused on the production of plant libraries. Only one group (Reddy *et al.*, 2002) has used the technology to study plant stress, and none have applied it to desiccation tolerant species or to the role of the roots in the process.

This chapter describes the production of normalized libraries from root libraries of desiccating and rehydrating *X. humilis*. It is anticipated that this will generate a deeper understanding of the specificity of root gene expression in desiccation tolerance through facilitation of large-scale sequencing and microarray analysis.

5.2 Methods

5.2.1 Mass excision of pBluescript phagemid

Mass excision of the dehydration and rehydration cDNA libraries described in Chapter 4 was performed with the ExAssist helper phage system employing a protocol modified from that described by Invitrogen (Invitrogen Tech-Online, 2002). XL1-Blue MRF' cells (Stratagene, U.S.A) were cultured at 37°C until OD 0.5. 50 ml of these cells were centrifuged at 2 000 g for 10 min in a Beckman JA 2-21 Refrigerated centrifuge (Beckman, U.S.A). The pellet was resuspended in 10 ml MgSO₄ (10 mM). Cells were incubated at 37°C for 15 min without shaking in order to regrow pili. Transfection with 10⁹ pfu of the amplified Lambda Zap library and 10¹¹ pfu of ExAssist helper phage (Stratagene, U.S.A) was carried out at 37°C for 15 min without shaking, and then for 130 min with shaking at 200 rpm. The culture was heated to 65°C for 15 min and centrifuged for 15 min at 4 000 g. The supernatant containing the excised phagemids was transferred to a sterile conical tube and stored at 4°C until further use.

For recovery of the plasmid, SOLR cells (Stratagene, U.S.A) were cultured with kanamycin (75 µg/ml) to OD 0.5, centrifuged at 2 000 g for 10 min and resuspended in 100 ml MgSO₄ (10 mM). After incubation at 37°C without shaking for 15 min, the cells were infected with the entire 37 ml phagemid supernatant. The mixture was incubated without shaking at 37°C before being inoculated into 400 ml 2 x YT medium and cultured at 30°C with shaking at 200 rpm. Ampicillin (100 µg/ ml) was added after 2 h and the incubation continued for 14 h. Cells were centrifuged at 4 000 g for 10 min and the pellets resuspended in 6 ml LB medium. The entire 6 ml was plated out on 28

cm x 28 cm LB agar plates containing 100 µg/ml ampicillin. Bacteria were cultured overnight at 37°C.

5.2.2 Isolation of single stranded library DNA

Single stranded DNA was prepared *in vivo* using the M13K07 system supplied with the Lambda Zap® packaging kit (Stratagene, U.S.A). Although a protocol described by Invitrogen (Invitrogen Tech-Online, 2002) formed the basis of the isolation, a number of methods and factors affecting the amount of single stranded DNA recovered were tested. These included adjustments of the:

- host strain (XL1-Blue MRF', SOLR)
- type of helper phage (VCSM13, ExAssist, M13K07)
- MOI (10, 100, 1000, 10 000)
- culture age (ODs 0.1, 0.3, 0.5, 0.6)
- cell recovery (plate scrape or liquid culture)
- precipitation conditions:
 - PEG concentrations (3%, 5%, 10%, 15%)
 - duration (2 h, 24 h, 48 h 1 week)

In the optimized protocol employed in this study, SOLR cells containing the excised library DNA were scraped from the LB plates and resuspended in 30 ml LB medium. Aliquots (1/50th or ~ 600 µl) were stored in 50 % glycerol at -70°C or immediately inoculated into 1 l 2 x YT broth and cultured to OD 0.1. The culture was super-infected with M13K07 helper phage at a multiplicity of infection (MOI) of 10 and incubated for 1 h at 37°C. Kanamycin (75 µg/ml) was added and the culture incubated at 37°C with vigorous shaking for 16 h. Cells were pelleted by centrifugation at 16 000 g for 20 min and the supernatant recovered. Phages were precipitated at -20°C for 1 week by the addition of 0.1 volume of 20 % PEG 6000 (Sigma, U.S.A) and 2.5 M NaCl, and by centrifugation at 16 000 g for 1 h.

The phage pellet was resuspended in 2 ml sterile water and extracted sequentially with 1 volume phenol and 1 volume phenol: chloroform (1:1 v/v). The single stranded DNA was precipitated at -20°C for 24 hrs with 2.5 volumes ethanol (100 %), 0.1 volume sodium acetate (3M) and 0.5 μl glycogen (20 mg/ml) as described by Soares and Bonaldo (1998). The DNA was recovered by centrifugation at 16 000 g for 1 hr. The pellet was resuspended in 200 μl water. Several μl aliquots were set aside for quantification and electrophoresis (as described in Chapter 3) and the remainder stored at -20°C until further purification. S1 nuclease digestion (Ausubel *et al.*, 1988) was performed to check that the DNA extracted was single stranded.

5.2.3 Purification of single stranded tracer

Single stranded DNA was purified by HAP chromatography on DNA Grade Bio-Gel[®] HTP HAP (Bio-Rad, U.S.A) after *PvuII* digestion as described by Ali *et al.* (2000). The sole modification was that the 0.14 M sodium phosphate elution buffer of Soares and Bonaldo (1998) was used as this included SDS and EDTA. For each purification five fractions of 1 ml each were collected and extracted 6-10 times against anhydrous n-butanol to reduce the total sample volume to less than 500 μl .

The following methods of desalting the recovered DNA were tested:

- Promega DNA Clean-up columns (Promega, U.S.A)
- Sigma Post-reaction purification columns (Sigma, U.S.A)
- dialysis (Ausubel *et al.*, 1988)
- gel purification followed by Gene Clean (BIO 101, USA) or Qiagen Quick column (Qiagen, U.S.A) extraction.

Gel purification (Chapter 3) followed by Qiagen Column gel extraction as outlined by the manufacturer was most effectively employed. DNA was eluted in 30 μl molecular grade water (Sigma, U.S.A).

Because of the small volumes and low concentrations, the DNA was quantified using an ethidium bromide plate assay (Uni-Zap[®] CDNA synthesis kit, Stratagene) or GeneQuant DNA calculator (Amersham Pharmacia Biotech, U.S.A). The remaining DNA was checked by agarose gel electrophoresis and stored at -20°C until use.

5.2.4 Generation of the driver DNA

Driver DNA was generated from 100 ng of the tracer by PCR (Ali *et al.*, 2000). PCR conditions were as follows: 94°C (1 min); [94°C (30 s); 55°C (30 s); 72°C (3 min)] x 30; 72°C (5 min). After confirmation of the success of the PCR by running 1/10th of the reaction on a standard agarose gel, the driver was purified directly from the PCR reaction using Promega PCR columns (Promega, U.S.A) or Qiagen PCR Clean-up Columns (Qiagen, U.S.A). DNA was eluted in 30 µl water and quantified by ethidium bromide plate assay or spectrophotometry on a GeneQuant DNA calculator (Amersham Pharmacia Biotech, U.S.A).

5.2.5 Hybridization

Hybridizations were carried out according to the protocol of Ali *et al.* (2000). Hybridization reactions contained 80 µg tracer DNA, 1.68 µg driver, 50 % (v/v) formamide (Sigma), 10 µl hybridization buffer (5 M NaCl, 100 mM Tris-HCl pH 8.0, 5 mM EDTA) and 10 µg of each of the blocking oligonucleotides. These oligonucleotides were designed to complement the positive sense strands of the vector regions flanking the inserts. Their sequences are given below.

5' A: 5' CCT CGT GCC GAA TTC CTG CAG CCC GGG GGA TCC ACT AGT TCT AGA GCG GC

5' B: 5' CGC CAC CGC GGT GGA CCA GCT TTT GTT CCC TTT AGT GAG GGT TAA T 3'

3' A: 5' TAA TAC GAC TCA CTA TAG GGC GAA TTG GGT ACC GGG CCC CCC CTC GAG
TTT TTT TTT TTT TTT TTT TT 3'

Reactions were incubated at 42°C for time periods selected to achieve C_0t values of 10 or 15 s/mol.dm³. These times were calculated according to the equation:

$$t = C_0t / C$$

Where t = time in seconds and

C = total DNA concentration in mol.dm⁻³

At the end of the hybridization period, reactions were stored at -20°C until purification of the normalized population of DNA.

5.2.6 Purification of the normalized DNA population

Single stranded DNA was purified as described in section 5.2.3. However samples were directly desalted on Qiagen PCR purification columns (Qiagen, U.S.A) without gel purification. The DNA was eluted from the Qiagen column in 30 µl of water. The single stranded template was converted to double stranded plasmid through use of the Sequenase kit (USB, U.S.A) as per manufacturer's directions, or via a single-step (ie: non-amplifying) PCR reaction. To the template was added 0.5 µl SuperTherm Taq (Southern Cross Biotech, S.A), 1 µl M13 forward primer (50 µM), 4 µl PCR buffer (10 x), 1.6 µl MgCl₂ (25 mM) and 2 µl dNTPs (10 mM). PCR conditions were: [80°C (2 min), 50°C (3 min), 72°C (30 min)] x 1. The reactions were purified by sequential phenol and phenol: chloroform (1: 1 v/v) extraction and precipitated overnight. DNA was recovered by centrifugation at 16 000 g for 30 min. The pellet was washed twice with ethanol (70 %) and resuspended in 12 µl molecular grade water.

As a control measure, selected normalization reactions were used to produce radio-labeled DNA via the single-step PCR reaction described above. 4 µl [³²P] dCTP (Amersham, USA) and 2 µl of each of dATP, dGTP and dTTP (Megaprime Labeling Kit, USB, U.S.A) were used in place of the dNTPs. 1 µl of the reaction was set aside before and after purification on a Sigma post-reaction purification column (Sigma, U.S.A). The percentage incorporation was determined from scintillation values obtained on a Beckman LS 5000 TD liquid scintillation system (Beckman, U.S.A).

5.2.7 Electroporation

For construction of the normalized libraries, plasmid DNA was electroporated into ElectroMAX™ DH10B™ Cells (Life Technologies, U.S.A). In general, the DNA was electroporated in 1.0 µl – 2.5 µl units into 25 µl aliquots of DH10B™ Cells using a Micro-pulser™ (Bio-Rad, U.S.A) and 0.1 cm electrode gap Gene Pulser® cuvettes (Bio-Rad, U.S.A). Immediately after electroporation, 1 ml SOC medium was added to the cells, which were incubated for 1 h at 37°C. Cells were centrifuged at 1 000 g for 5 min before being plated onto 28 cm x 28 cm LB agar plates containing ampicillin (100 µg/ ml). The plates were incubated overnight at 37°C.

Initial optimization of the electroporation conditions was carried out using the PUC19 control DNA supplied by the manufacturer. A range of voltages (1.8 kV, 2.1 kV and 2.5 kV) were tested for maximum electroporation efficiency as determined by the numbers of colonies present on the plates after overnight incubation.

5.2.8 Preparation of libraries for storage and arraying

If a suitable number of colonies (> 1000) were obtained, these were individually picked and inoculated into 96 well microtitre plates (Nunc Products, U.S.A) containing 75 µl LB supplemented with ampicillin (100 µg/ ml). Plates were incubated overnight at 37°C with shaking. To each well was added an equal volume of 50 % glycerol. The plates were labeled and stored at -70°C for future use.

5.2.9 Evaluation of the normalized libraries

Insert sizes were evaluated by amplifying inserts from 12 randomly selected colonies by colony PCR (Chapter 4, Roschier *et al.*, 2000). For library screening, the same β -tubulin isoforms used for the evaluation of the un-normalized libraries were employed. 600 cfu were spotted using a 96 pin replicator (Nunc, U.S.A) onto LB agar plates containing 100 µg/ml ampicillin. Colonies were incubated overnight at 37°C and lifted

onto Hybond-N nitrocellulose membranes (Amersham, U.S.A). The colony hybridizations were carried out exactly as outlined by Soares and Bonaldo (1998) using the radio-labeled β -tubulin as a probe (Chapter 4). The percentage of positives was compared to those obtained in the screening of the un-normalized libraries. Verification of these results using Southern blotting of plasmid DNA from normalized and un-normalized libraries was also attempted.

5.3 Results

5.3.1 Preparation of tracer and driver DNA

Preparation of single stranded DNA from the excised libraries was extremely problematic. Of all the traditional methods tested, none yielded sufficient amounts of DNA. The results were unaffected by the adjustment of MOIs, host strains, helper phage or culture conditions. A representative result is illustrated in Figure 5.3.

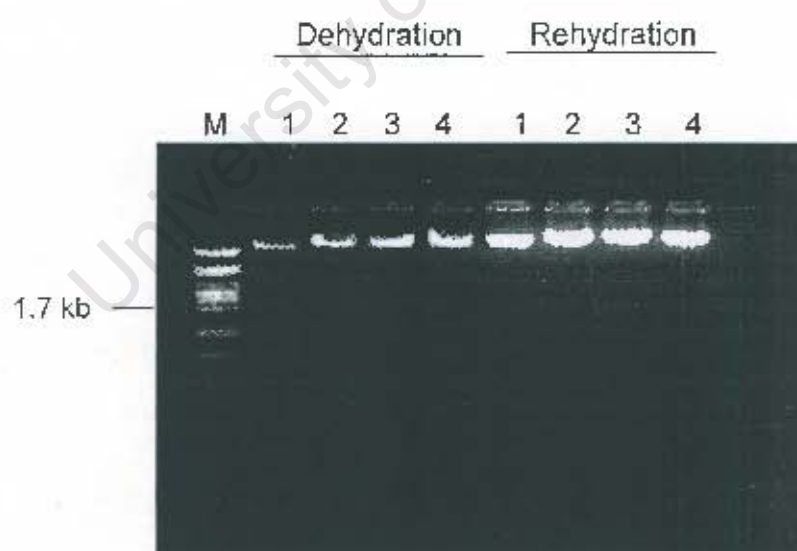


Figure 5.3: Effect of the helper phage multiplicity of infection (MOI) level on the recovery of single stranded DNA from the root dehydration and rehydration cDNA libraries. Lane numbers 1–4 indicate a MOI of 1×10^4 , M = marker (*Pst*II digested lambda DNA).

This indicated that the bulk of the DNA isolated by these methods was high molecular weight double stranded DNA. Extensive testing of the factors affecting the production of single stranded DNA *in vivo* indicated that the supernatant had to be stored for at least a week at -20°C in order to precipitate the phages. This was contrary to the 1 h precipitations recommended in all protocols.

Nevertheless, large amounts ($\sim 200\ \mu\text{g}$) of single stranded DNA were isolated using the extended precipitation method (Figure 5.4). The single stranded DNA generally ran on agarose gels at the same speed as the 1.7 kb marker, but this varied according to the exact buffering conditions. This accorded with the theoretical prediction of between 0.5 kb and 2.0 kb. The dehydration library DNA was relatively free of contamination by single stranded helper phage DNA, but the rehydration library DNA contained a large amount of the helper phage DNA (Figure 5.4). The exact nature of the two bands was unclear.

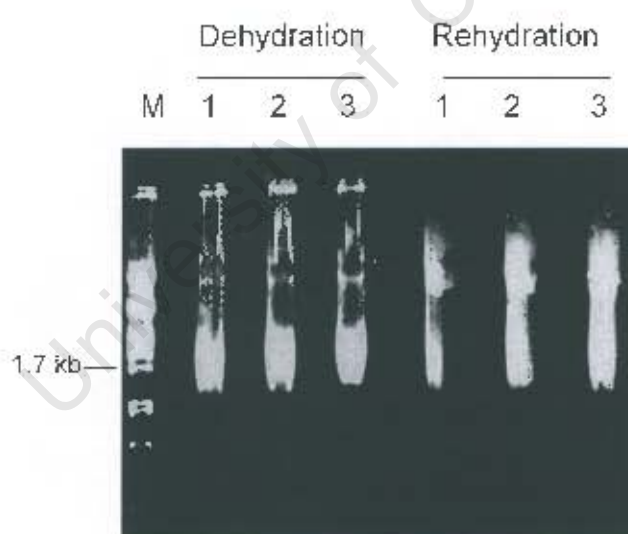


Figure 5.4: Single stranded DNA recovered from the dehydration and rehydration cDNA libraries. Lane numbers indicate the amount (in μl) of DNA loaded. M = marker (*Pst*II digested lambda DNA).

Digestion with S1 nuclease confirmed that the library DNA was single stranded and that there was little or no double stranded DNA contamination (Figure 5.5).

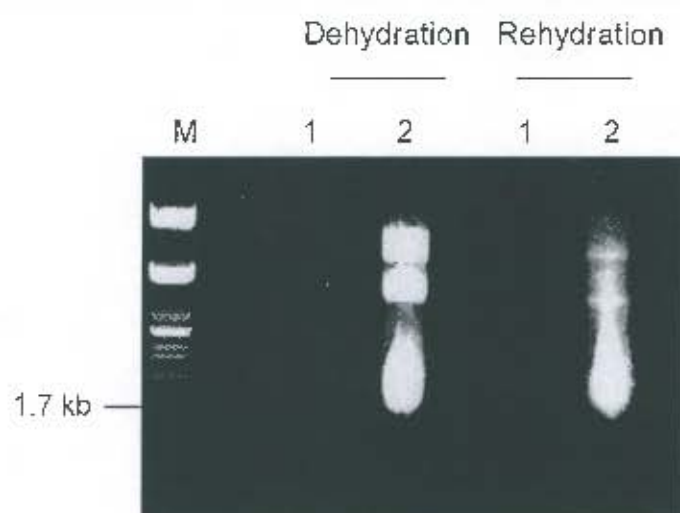


Figure 5.5: S1 nuclease digests of the single stranded dehydration and rehydration library DNA. Lanes: 1 = S1 nuclease digested sample, 2 = original sample, M = marker (PstII digested lambda DNA).

Single stranded DNA was not affected by the *PvuII* digestion used to linearize contaminating double stranded DNA prior HAP column chromatography. Although the yields of single stranded DNA were low, HAP column chromatography was successful (Figure 5.6). The bulk of the DNA eluted from the column between fractions 2 and 3, and appeared to be relatively pure (Figure 5.6).

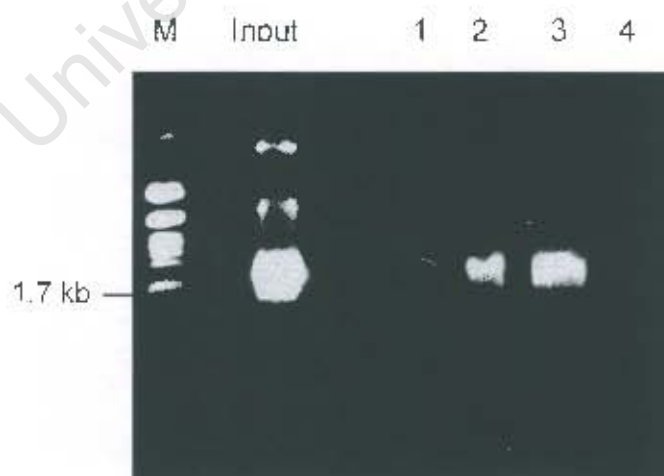


Figure 5.6: Recovery of purified single stranded DNA from HAP columns. Input = 10 % of original amount of DNA loaded. Lane numbers indicate fraction numbers. M = marker (PstII digested lambda DNA).

Recovery of the single stranded DNA from the elution buffer was problematic as the butanol extractions resulted in salt concentrations of $> 5M$ in the $500 \mu l$ volume. Purification on Sigma spin columns or by gel fractionation followed by DNA recovery using Qiagen gel extraction columns was found to yield suitable amounts of DNA and to ensure removal of the contaminating helper phage DNA (Figure 5.7). Approximately $10-20 \mu g$ of tracer was recovered per $100 \mu g$ of *PvuII* digested DNA.

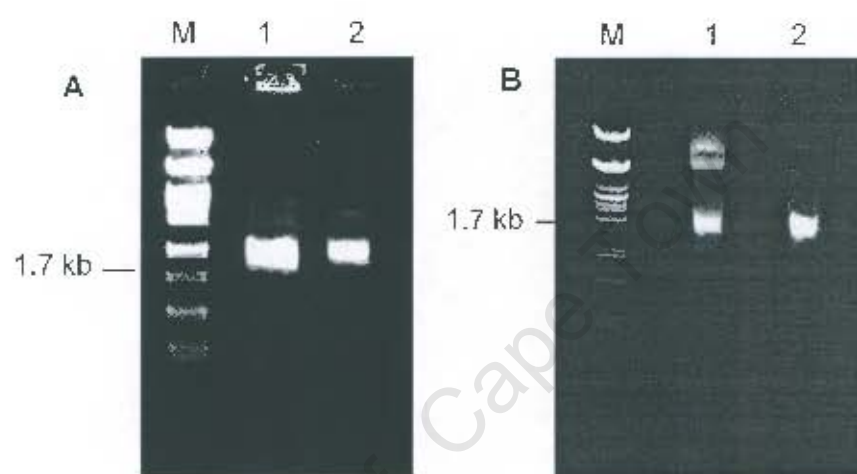


Figure 5.7: Representative images of the desalting and gel purification of HAP purified single stranded DNA. (A) Sigma spin column desalting of dehydration library DNA (B) gel and Qiagen column purification of rehydration library DNA. Lanes: 1 = DNA before purification, 2 = tracer DNA after purification, M = marker

PCR of the driver using the tracer as a template was extremely efficient (Figure 5.8) and yielded in excess of $2 \mu g$ DNA per $100 \mu l$ reaction. A wide size range of inserts was generated – typically $0.4 kb$ to $5 kb$ with an average of $1 kb- 1.5 kb$ (Figure 5.8).

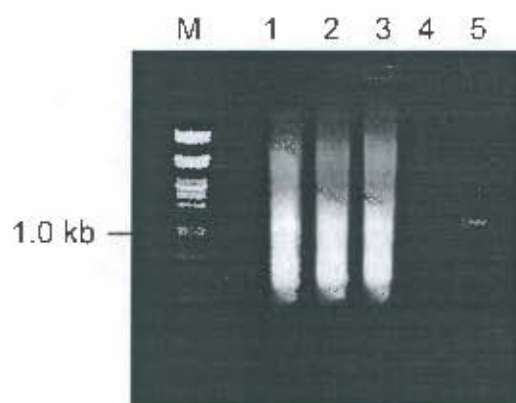


Figure 5.8: Driver DNA produced by PCR from the dehydration and rehydration tracer DNA. Lanes: 1 = dehydration driver DNA, 2 and 3 = rehydration driver DNA, 4 = negative (no primer) control, 5 = template DNA, M = marker.

Quantification of the driver and tracer DNA using the ethidium bromide assay and the GeneQuant DNA calculator was found to yield discrepant results. The difference between the two methods was 30-50 % on average, with the ethidium bromide assay yielding the higher values. Where available, the GeneQuant data was used.

5.3.2 Hybridization and purification

Ten normalized DNA populations were generated using the methods described. These included 4 dehydration and 4 rehydration libraries with C_{ot} values of 10 or 15 s/mol.dm³ and one dehydration and one rehydration library of C_{ot} 1.5 and 2.5 s/mol.dm³ respectively. The lower C_{ot} values for the latter two were a consequence of the over-estimation of DNA concentrations by the ethidium bromide plate assay. Times required to achieve C_{ot} values of 10 and 15 for the DNA concentrations used were calculated to be 56 h and 83 h respectively.

Single stranded DNA recovery from the HAP column after the hybridizations was complicated by the fact that the starting amount of DNA used in the reactions was only 80 ng. Nevertheless, Qiagen PCR columns facilitated the recovery of the single stranded DNA. This was confirmed by PCR from a selected purified reaction. The gel

indicated that plasmid DNA containing inserts of the expected size range were recovered from the HAP columns (Figure 5.9).

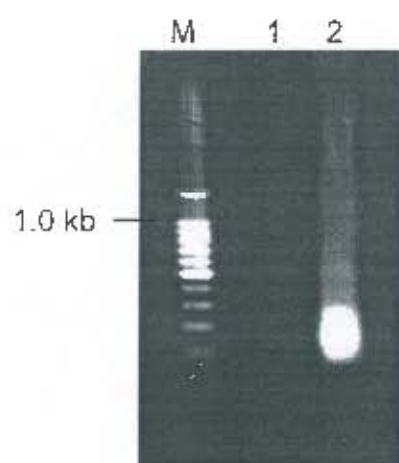


Figure 5.9: PCR of inserts from normalized single stranded DNA samples. Lanes: 1 = negative (no primer) control, 2 = 10 % of PCR reaction using R_{15A} library DNA as a template, M = marker (Promega 100 bp ladder).

The bright band in Figure 5.9 was interpreted to be flanking vector sequence amplified preferentially from non-recombinant clones, combined with primer dimers. To test the efficiency of the conversion of recovered single strand DNA to double stranded plasmid, the percentage incorporation of [32 P]-dCTP was monitored. The percentage incorporation into double stranded DNA using the Sequenase kit was found to be only 0.8 %. This was taken to explain the failure of initial attempts to normalize the libraries. The single-step PCR reaction was considered to be more reliable than the Sequenase method and was routinely and effectively employed.

5.3.3 Electroporation

Although the manufacturer's instructions specified an electroporation voltage of 2.5 kV for the DH10BTM cells, optimization using the pUC19 DNA provided indicated that 5-10 fold more colonies were obtained with voltages between 1.8 kV and 2.1 kV. A voltage of 1.8 kV was therefore employed for subsequent electroporations.

Of the 10 normalized DNA populations generated, 8 were electroporated. The purity of the library DNA was found to be crucial to the success of the electroporation. Low time constants (< 1.0 ms) were obtained for libraries that had not been extracted against phenol: chloroform. Only three of the electroporated libraries yielded more than 100 colonies. The details of these normalized libraries are summarized in table 5.2. Because of the adequate number of colonies obtained for the D_{10C} and R_{10C} libraries, the C_{0t} 15 libraries generated at the same time were not electroporated, but were stored at -20°C for possible future use.

Table 5.1: Summary of the normalized libraries generated by electroporation into DH10BTM cells

Library	Name	C_{0t} s/mol.dm ³	Ave. time constant	Colonies
Rehydration	R_2	2.5	0.9 ms	800
Dehydration	D_{10C}	15	3.5 ms	4 000
Rehydration	R_{10C}	15	2.0 ms	6 000

5.3.4 Screening

Due to time constraints only R_2 was completely screened. This library consisted of approximately 90 % recombinant clones with inserts ranging from 0.4 kb to 3 kb. The average insert size determined from the colony PCR was approximately 1.0 kb (Figure 5.10). This indicated that no significant selection against the larger inserts had taken place during the normalization procedure. However, analysis of the D_{10C} library indicated that a reduction in the average insert size of the order of approximately 0.2-0.3 kb had occurred (results not shown). Whether this was due to the extended C_{0t} time or due to preferential incorporation of smaller plasmids during electroporation was not determined.

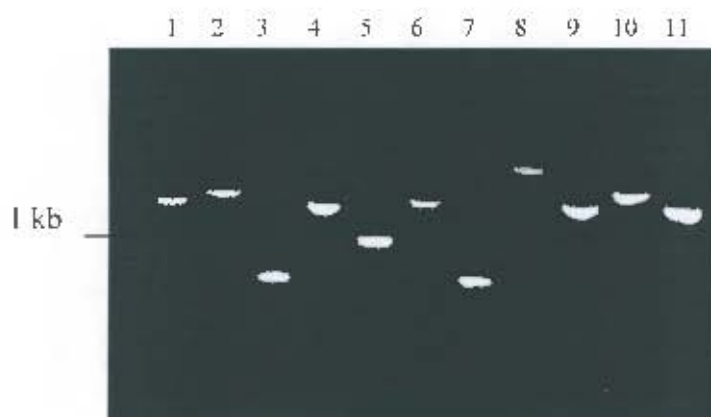


Figure 5.10: Amplification of cloned inserts by colony PCR. Lanes 1- 11 represent inserts from selected colonies. Marker and controls not shown.

Colony hybridization with β -tubulin revealed that the R_2 library had not undergone significant normalization as tubulin made up approximately 1.5 % of the library (Figure 5.11). Preliminary analysis of the D_{10c} library indicated a possible maximum 3-fold reduction in tubulin levels (data not shown). However, the background levels of hybridization were high, making it difficult to distinguish between true and false positive signals. Attempts to verify the results using the Southern blot procedure employed by Bonaldo *et al.* (1996) were not successful.

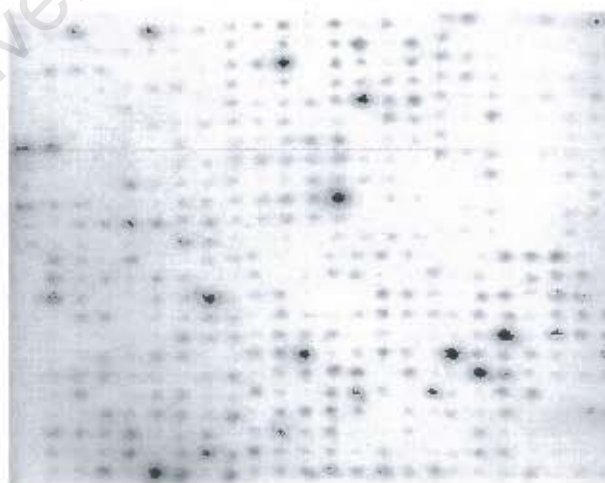


Figure 5.11: Autoradiograph of a cloneset (~600 clones) of the R_2 library probed with β -tubulin I-III by colony hybridization.

5.4 Discussion

The generation of large amounts of single stranded DNA (Figure 5.4) enabled the testing of several C_0t times and the production of a number of normalized libraries. The factors tested during the attempt to generate single stranded DNA *in vivo* (eg: MOI, helper phage and host) may impact on the amount of DNA recovered. However, the failure of the short precipitation step negated the direct observation of these effects (Figure 5.3). No reason for the difficulties experienced could be discerned, but this modification to the traditional protocol should be borne in mind.

Bonaldo *et al.* (1998) have suggested that HAP purification of single stranded DNA produced *in vivo* is unnecessary since only single stranded phagemids are released into the supernatant. The lack of double stranded DNA remaining after S1 nuclease digest (Figure 5.5) suggests that no double stranded DNA was present in the preparations. However, HAP column chromatography was employed as a quality control measure and resulted in the recovery of sufficient DNA for a large number of normalizations (Figure 5.6).

The desalting of the fractions after HAP chromatography may be one of the most problematic steps in the normalization procedure. It is estimated that salt concentrations in the butanol extracted fractions may be as high as 10 M, and that single stranded DNA may bind a number of surfaces irreversibly under such conditions. The columns used by Soares *et al.* (1994) and Soares and Bonaldo (1998) are no longer commercially available (Soares pers. comm., 2001) meaning that a suitable replacement had to be determined empirically. Figure 5.7 indicates that the Qiagen Quick columns (Qiagen, U.S.A) may be used effectively for this purpose.

Because the method of normalization employed in this study makes use of driver generated by PCR (Figure 5.8) rather than by digestion of plasmid from the un-normalized library (Soares *et al.*, 1994) blocking oligonucleotides were designed to the flanking vector sequences. A (dT)₂₀ tract was included to eliminate potential hybridization between the poly(A) tails expected to be located at the 3' ends of the

inserts. The use of poly(dT) priming may lead to downstream difficulties with the libraries since it has been shown that internal priming may selectively eliminate rare transcripts during the hybridization (Martin and Pardee, 2002; Wang *et al.*, 2002). Wang *et al.* (2002) have shown that significantly shorter poly(A) primers overcomes this flaw in current subtraction methods.

Although it was difficult to obtain conclusive results from the screening procedures, the generation of a small library normalized to a C_0t value of 2.5 allows for comparison of the effects of the hybridization time on the abundance of various genes. Ali *et al.* (2001) used a similar approach. For the cereal EST library normalized to C_0t 2.5 they detected no significant reduction in the frequency (or redundancy) of common genes. This is similar to the result obtained for the screening of library R_2 in this study (Figure 5.11). For the libraries normalized to C_0t 10 Ali *et al.* (2001) obtained 18 000 independent colonies which displayed a 10 fold reduction in certain common genes and a 10 fold increase in specific rare genes. Of the inserts sequenced, 24 % were novel. It is anticipated that the D_{10c} and R_{10c} libraries generated in this study will display similar levels of normalization, though – given the tissue and plant source - with a potentially higher occurrence of novel genes. Future characterization of the extent of normalization will depend on alternate methods of screening being employed, since the statistical relevance of colony hybridization using small numbers of colonies is questionable and the use of Southern blots is problematic.

However, direct comparison of the *X. humilis* and cereal data is not a completely valid approach since the extent of normalization of a population of sequences depends largely on the re-association rates achieved. This re-association rate varies according to a number of parameters that are summarized in Table 5.2.

Table 5.2: Factors affecting the reaction rate (data from Soares and Bonaldo, 1998).

1	Temperature	Temps above or below T_m decrease k .
2	Salt	High salt increases the reaction rate.
	GC content	High GC content increases the reaction rate.
	Solvents	Increasing concentrations decrease the rate.
	Complexity	The greater the complexity, the lower the reaction rate.
	length	K proportional to root of the concentration of shorter strand.

While conditions such as temperature, salt concentration and solvents can be adjusted, the DNA length, GC content and complexity are endogenous factors and cannot be controlled in individual DNA samples. Neither the complexity or average GC content of the genome of *X. humilis* is known, meaning that these cannot be compensated for in the normalization procedure. Nevertheless, these factors should not differ too considerably between cereals used by All *et al.* (2001) and Reddy *et al.* (2002) and the monocotyledonous *X. humilis*. Consequently, by comparison to the cereal data it is expected that the libraries reported in this dissertation will contain a significant percentage of the rarer genes expressed in the roots of *X. humilis*.

A criticism that could be leveled against libraries normalized to the relatively high C_{ot} values of 10 -15 is that the genes likely to be important in desiccation may be upregulated and will therefore be comparatively abundant in the starting libraries. Yet, up-regulated genes remain relatively rare compared to the super-abundant genes such as tubulin and actin that the normalization aimed to reduce or eliminate. Secondly, the expression of a transcript during stress may not correlate with its relative importance in the response mechanisms, largely due to the existence of translational and post-translational control mechanisms.

Three methods are routinely used to answer the questions raised above about the success of normalization, namely colony hybridization (Grunstein and Hogness, 1975), Southern blot analysis (Southern, 1975) and large-scale single pass

sequencing (Bonaldo *et al.*, 1996; Ali *et al.*, 2001). While single pass sequencing is the ideal approach, it remains expensive. The traditional screening techniques provide initial estimates of the quality of the data likely to be obtained. Preliminary screening of R₂ by colony PCR showed no significant bias against large inserts (Figure 5.10). This library contained inserts having the same average insert size (~1kb), and a similar size range (0.4 kb- 3 kb) as that determined for the non-normalized libraries (Chapter 3). The preservation of insert length accords with the findings of Bonaldo *et al.* (1996), Ali *et al.* (2001) and Reddy *et al.* (2002) that truncated inserts are not selectively retained. This is largely due to the fact that the driver used in the hybridization was used in vast excess over the tracer, and that this DNA generally spanned the entire length of the inserts (Bonaldo *et al.*, 1996). The D_{10c} and R_{10c} libraries remain to be fully screened to assess the extent of normalization, but initial sequencing results (data not shown) suggest that the process of normalization effectively increased the proportions of class II and III transcripts.

The libraries generated in this dissertation - to our knowledge, the first from a resurrection plant - will serve to provide both functional and sequence data through their use in large-scale sequencing and microarray projects. It is anticipated that they will contribute significantly to the understanding of the molecular features that characterize the responses of the roots of *X. humilis*, and perhaps other desiccation tolerant plants, to severe water stress.

Conclusions

There is little doubt that the extensive molecular resources available for *Arabidopsis* research have had a considerable impact on biological studies in recent years. These resources allow, through comparison, the identification of features that are unique to the biology of other plants. This may be accomplished at the level of single target genes, or gene families, or at the whole transcriptome or genome levels. Either way, the characterization of divergent elements may be of great significance in the coming years as the rate of discovery of novel sequences continues to decline (Wang *et al.*, 2000).

The unique features that enable resurrection plants such as *X. humilis* to survive desiccation and rehydration have particular significance to biotechnology. It is thought that whatever elements confer the ability to survive anhydrobiosis may be gainfully employed in improving the drought resistance of crops in an increasingly arid landscape. In this regard, several papers have shown that the use of *cis*-acting elements or over-expressed genes is a feasible approach to increasing survival and yields (Pilon-Smits *et al.*, 1995; Alvim *et al.*, 2001). However, such high aims rest on a sound understanding of the biology of the acquired trait.

For these reasons, this dissertation aimed to generate information and resources that would enable the elucidation of the biology of desiccation tolerance mechanisms of *X. humilis*. Particular attention was given to the roots because of their roles in the early sensing and amelioration of water deficit, and because the information about these organs currently lags far behind that for the leaves. This study revealed several features of the functional anatomy and physiology of the roots during desiccation and rehydration

that were previously not described. Highly specific primers were developed to target two-component systems that may be involved in the regulation of the physiological responses. Lastly, un-normalized and normalized (or low redundancy) stress- and tissue-specific cDNA libraries were constructed and screened with several housekeeping genes. The normalized libraries represent the first described for a resurrection plant and will enable the large-scale sequence and microarray analysis of the transcriptome of these organs to be completed. It is hoped that they contribute in some way to the further understanding of plant responses to water deficit.

University of Cape Town

References

Adams M D, Kelley J M, Gocayne J D, Dubnick M H, Polymeropoulos M H, Xiao H, Merrill C R, Wu A, Olde B, Moreno R F, Kerlavage A R, McComble W R and Venter, J C. (1991). Complementary DNA sequencing and the Human Genome Project. *Science*. 252, 1651-1656.

Adams M D, Dubnick A R, Kerlavage R, Moreno R, Kelley J M, Utterback T R, Nagle J W, Fields C and Venter J C. (1992). Sequence identification of 2 375 human brain genes. *Nature*. 355, 632-634.

Ali M, Jensen C R, Morgensen V O, Andersen M N and Henson I E. (1999). Root signaling and osmotic adjustment during intermittent soil drying sustain grain yield of field grown wheat. *Field Crops Research*. 62 (1), 35-52.

Ali S, Holloway B and Taylor W C. (2000). Normalization of cereal endosperm EST libraries for structural and functional genomics. *Plant Molecular Biology Reporter*. 18, 123-132.

Alpert P. (2000). The discovery, scope and puzzle of desiccation tolerance in plants. *Plant Ecology*. 151, 5-17.

Altschul S F, Madden T L, Schaffer A A, Zhang J, Zhang Z, Miller W and Lipman D J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*. 25, 3389-3402.

- Alvim F C, Carolino S M B, Cascardo J C M, Nunes C C, Martinez C A, Otoni W C and Fontes E P B. (2001).** Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. *Plant Physiology*. 126, 1042-1054.
- Asamizu E, Nakamura Y, Sato S and Tabata S. (2000a).** A large scale analysis of cDNA in *Arabidopsis thaliana*: generation of 12 028 non-redundant expressed sequence tags from normalized and size selected cDNA libraries. *DNA Research*. 7, 175-180.
- Asamizu E, Nakamura Y, Sato S and Tabata S. (2000b).** Generation of 7 137 non-redundant expressed sequence tags from a legume, *Lotus japonicus*. *DNA Research*. 7, 127-130.
- Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A and Struhl K. (1988).** *Current Protocols in Molecular Biology*. Green-Wiley Interscience, New York.
- Aziz A, Martin-Tanguy J, and Larher F. (1999).** Salt stress-induced proline accumulation and changes in tyramine and polyamine levels are linked to ionic adjustment in tomato leaf discs. *Plant Science*. 145, 83-91.
- Bajaj S, Targoff J, Liu L F, Ho T H D and Wu R. (1999).** Transgenic approaches to increase dehydration-stress tolerance in plants. *Plant Molecular Biology*. 5, 493-503.
- Bartels D, Schneider K, Terstappen G, Platkowski D, and Salamini F. (1990).** Molecular cloning of abscisic acid-modulated genes which are induced during desiccation of the resurrection plant *Craterostigma plantagineum*. *Planta*. 181, 27-34.
- Bartels D, Englehardt K, Roncarati R, Schneider K, Rotter M, and Salamini F. (1991).** An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein. *The EMBO Journal*. 10, 1037-1043.

- Bartels D, Hanke C, Schnelder K, Michel D and Salamini F. (1992).** A desiccation-related ELIP-like gene from the resurrection plant *Craterostigma plantagineum* is regulated by light and ABA. *The EMBO Journal*. 11(8):2771-8.
- Bartels D and Salamini F. (2001).** Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level. *Plant Physiology*. 127, 1346-1353.
- Bernacchia G, Schwall G, Lottspelch F, Salamini F and Bartels D. (1995).** The transketolase gene family of the resurrection plant *Craterostigma plantagineum*: differential expression during the rehydration phase. *The EMBO Journal*. 14 (3), 610-618.
- Bewley J D. (1979).** Physiological aspects of desiccation tolerance. *Annual Review of Plant Physiology*. 30, 195-238
- Bewley J D and Oliver M J. (1992).** Desiccation tolerance in vegetative plant tissues and seeds: protein synthesis in relation to desiccation and a potential role for protection and repair mechanisms. *Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular and Molecular Levels*. G N Somero, C B Osmond and C L Bolis (eds.). Springer-Verlag, Berlin. 141-159.
- Bishop J O, Morton J G, Rosbash M and Richardson M. (1974).** Three abundance classes in HeLa cell messenger RNA. *Nature*. 250, 199-204.
- Blomstedt C K. (1998).** Drought-stimulated genes correlated with desiccation tolerance of the resurrection grass *Sporobolus stapfianus*. *Plant Growth Regulation*. 24, 153-161.

- Bonaldo M D F, Lennon G and Soares M B. (1996).** Normalization and Subtraction: two approaches to facilitate gene discovery. *Genome Research*. 6, 791-806.
- Bonaldo M D F and Soares M B. (1997).** Procedure for normalization of cDNA libraries. *US Patent 5702898*.
- Bonhert H J, Nelson D E and Jensen R G. (1995).** Adaptions to environmental stresses. *The Plant Cell*. 7, 1099-1111.
- Borsani O, Diaz P, Agius MF, Valpuesta V and Monza J. (2001).** Water stress generates an oxidative stress through the induction of a specific Cu/ Zn superoxide dismutase in *Lotus corniculatus* leaves. *Plant Science*. 161, 757-763.
- Bowler C and Fluhr R. (2000).** The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends in Plant Science*. 5, 241-246.
- Bray E A. (1997).** Plant responses to water deficit. *Trends in Plant Science*. 2(2), 48-54.
- Bray E A. (2002).** Classification of genes differentially expressed during water-deficit stress in *Arabidopsis thaliana*: an analysis using microarray and differential expression data. *Annals of Botany*. 89: 803-11.
- Britten R J, Graham D E and Neufeld BR. (1974).** Analysis of repeating DNA sequences by reassociation. *Methods in Enzymology*. 29, 363-441.
- Browse J and Xin Z. (2001).** Temperature sensing and cold acclimation. *Current Opinion in Plant Biology*. 4, 241-246.
- Buschmann H, Potter U and Beeching J. (2002)** Ultrastructure of cassava root studied by TEM and SEM. *Microscopy and Analysis*. 25, 9-11.

- Carvajal M, Cerda A, and Martinez V. (2000).** Does calcium ameliorate the negative effect of NaCl on melon root water transport by regulating aquaporin activity? *New Phytologist*. 145 (3), 439-447.
- Chandler J W and Bartels D. (1997).** Structure and function of the vp1 gene homologue from the resurrection plant *Craterostigma plantagineum* Hochst. *Molecular and General Genetics*. 256(5): 539-546.
- Chang C, Kwok S F, bleecker A B and Meyerowitz E M. (1993).** *Arabidopsis* ethylene response gene ETR1: similarity of product to two-component regulators. *Science*. 262, 539-544.
- Chen W, Provart N J, Glazebrook J, Katagiri F, Chang H S, Eulgem T, Mauch F, Luan S, Zou G, Whitham S A, Budworth P R, Tao Y, Xie Z, Chen X, Lam S, Kreps J A, Harper J F, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X and Zhu T. (2002).** Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell*. 14(3):559-74.
- Cheong Y H, Chang H S, Gupta R, Wang X, Zhu T and Luan S. (2002).** Transcriptional Profiling Reveals Novel Interactions between Wounding, Pathogen, Abiotic Stress, and Hormonal Responses in *Arabidopsis*. *Plant Physiology*. 129(2):661-77.
- Chomczynski P and Sacchi N. (1987).** Single-step method of RNA isolation by acid guanidium thiocyanate – phenol – chloroform extraction. *Analytical Biochemistry*. 162, 156-159.
- Chomczynski P and Mackey K. (1995).** Modification of the Tri-Reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. *Biotechniques*. 19, 942-945.

- Chung C T and Miller R H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Research*. 16 (8), 3580.
- Clark M D, Panopoulou G D, Cahill D J, Bussow K and Lehrach H. (1999). Construction and analysis of arrayed cDNA libraries. *Methods in Enzymology*. 303, 205-233.
- Clarkson D T, Carvajal M, Henzler T, Waterhouse R N, Smyth A J, Cooke D T and Steudle E. (2000). Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress. *Journal of Experimental Botany*. 51, 61-70.
- Close T J. (1996). Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum*. 97, 795-803.
- Colmenero-Flores J M, Moreno L P, Smith C E and Covarrubias A A. (1999). Pvlea-18, a member of a new late-embryogenesis-abundant protein family that accumulates during water stress and in the growing regions of well-irrigated bean seedlings. *Plant Physiology*. 120, 93-103.
- Conley T R, Peng H P and Shih M C. (1999). Mutations affecting induction of glycolytic and fermentative genes during germination and environmental stresses in *Arabidopsis*. *Plant Physiology*. 119(2), 599-607.
- Cooper K. (2001). The effect of drying rate on the resurrection species *Craterostigma wilmsii* (homoiochlorophyllous) and *Xerophyta humilis* (poikilochlorophyllous). *MSc. Thesis, University of Cape Town*.
- Crocker J L, Witte W T and Auge R M. (1998). Stomatal sensitivity of six temperate, deciduous tree species to non-hydraulic root-to-shoot signalling of partial soil drying. *Journal of Experimental Botany*. 49 (321), 761-774.

Crowe J H and Crowe L M. (1984). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science*. 223, 701-703.

Crowe J H, Crowe L M and Chapman D. (1984). Presevation of membranes in anhydrobiotic organisms: the role of trehalose. *Science*, 223, 701-703.

Dace H, Sherwin H, Illing N and Farrant J M. (1999). Use of metabolic inhibitors to elucidate mechanisms of recovery from desiccation stress in the resurrection plant *Xerophyta humilis*. *Plant Growth Regulation*. 24, 171-177.

D'Agostino I B, Deruere J and Kleber J. (2000). Characterization of the response of the *Arabidopsis* response regulator gene family to cytoinin. *Plant Physiology*. 124, 1706-1717.

Das M, Harvey I, Chu L, Sinha M and Pelletier J. (2001). Full-length cDNAs: more than just reaching the ends. *Physiological Genetics*. 6, 57-80.

Davies W J and Zhang J. (1991). Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Physiology and Plant Molecular Biology*. 42, 55-76.

Davuluri R V, Grosse I and Zhang M Q. (2001). Computational identification of promoters and first exons in the human genome. *Nature Genetics*. 29(4):412-7.

Degenhardt B and Glimler H. (2000). Cell wall adaptions to multiple environmental stresses in maize roots. *Journal of Experimental Botany*. 51, 595-603.

Delrot S, Atanassova R and Maurousset L. (2000). Regulation of sugar, amino acid and peptide plant membrane transporters. *Biochimica Et Biophysica Acta Biomembranes*. 1, 1-2.

- Deng X, Phillips J, Meijer AH, Salamini F and Bartels D. 2002. Characterization of five novel dehydration-responsive homeodomain leucine zipper genes from the resurrection plant *Craterostigma plantagineum*. *Plant Molecular Biology*. 49(6):601-10.
- Desikan R, Mackerness S A-H, Hancock J T and Neill S J. (2001). Regulation of the *Arabidopsis* transcriptome by oxidative stress. *Plant Physiology*. 127, 159-172.
- Diatchenko L; Lau Y-F C, Campbell A P, Chechik A, Maqadam F, Huang B, Lukyanov S, Lukyanov C, Gurskaya N, Sverdlov E D and Diebert PD. (1996). Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences, USA*. 93, 6025-6030.
- Draper M P, August P R, Connolly T, Packard B, Call K M. 2002. Efficient cloning of full-length cDNAs based on cDNA size fractionation. *Genomics*. 79(4):603-7.
- Drmanac R and Drmanac S. (1999). cDNA screening by array hybridisation. *Methods in Enzymology*. 303, 165-179.
- Duff R J, Olivier M J and Wood A J. (1999). A *Tortula ruralis* cDNA encoding small-subunit ribosomal protein S3a: polysomal retention of transcript in response to desiccation and rehydration. *The Bryologist*. 102, 418-425.
- Dure L. (1993). Structural motifs in LEA proteins. *Plant Response to Cellular Dehydration during Environmental Stress*. T J Close and E A Bray (eds.). ASPB, Rockville. 91-103.
- Eckert K A and Kunkel T A. (1990). High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acid Research*. 18, 3739-3744.

- Egerton-Warburton L M, Balsamo R A, and Close T J. (1997).** Temporal accumulation and ultrastructural localization of dehydrins in *Zea mays*. *Physiologia Plantarum*. 101, 545-555.
- Eisen M B and Brown P O. (1999).** DNA arrays for analysis of gene expression. *Methods in Enzymology*. 303, 179-205.
- Epstein C B and Butow R A. (2000).** Microarray technology - enhanced versatility, persistent challenge. *Current Opinion in Biotechnology*. 11, 36-41.
- Farrant J M, Cooper K, Kruger L A and Sherwin H W. (1999).** The effect of drying rate on the survival of three desiccation tolerant angiosperm species. *Annals of Botany*. 84, 371-379.
- Farrant J M. (2000).** A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species. *Plant Ecology*. 151, 29-39.
- Felix G, Regenass M and Boller T Q. (2000).** Sensing of osmotic pressure changes in tomato cells. *Plant Physiology*. 124, 1169-1179.
- Fowler S and Thomashow M F. 2002.** *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*. 14(8):1675-90.
- Franco O L and Melo F R. (2000).** Osmoprotectants - a plant strategy in response to osmotic stress. *Russian Journal of Plant Physiology*. 47(1), 137-144.
- Frank W, Munnik T, Kerkmann K, Salamini F, Bartels D. 2000.** Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. *Plant Cell*. 12 (1), 111-24.

- Fujibuchi W and Kanehisa M.** 1997. Prediction of gene expression specificity by promoter sequence patterns. *DNA Research*. 4(2):81-90
- Furini A, Koncz C, Salamini F and Bartels D.** (1997). High level transcription of a member of a repeated gene family confers dehydration tolerance to callus tissue of *Craterostigma plantagineum*. *The EMBO Journal*. 16, 3599-3608.
- Gaff D.** (1971). Desiccation-tolerant flowering plants in Southern Africa. *Science*. 174, 1033-1034.
- Gaff D.** (1977). Desiccation tolerant vascular plants of Southern Africa. *Oecologia*. 31, 95-109.
- Gaff D F.** (1980). Protoplasmic tolerance of extreme water stress. *Adaptions of plants to water and high temperature stress*. N C Turner and P J Kramer (eds.). John Wiley and Sons, New York. 207-230
- Gaff D.** (1989). Responses of desiccation tolerant 'resurrection' plants to water stress. *Structural and Functional Responses to Environmental Stresses: Water Shortage*. K H Kreeb, H Richter and T M Hinckley (eds.). SPB Academic Publishing, The Hague. 254-267.
- Gaff D.** (1997). Mechanisms of desiccation tolerance in resurrection vascular plants. *Mechanisms of Environmental Stress Resistance in Plants*. A S Basra and R K Basra (eds.). Harwood Academic Publishers, Netherlands. 43-57.
- Gaff D F, Bartels D and Gaff J L.** (1997). Changes in gene expression during drying in a desiccation-tolerant grass *Sporobolus stapfianus* and a desiccation-sensitive grass *Sporobolus pyramidalis*. *Australian Journal of Plant Physiology*. 24, 617-622.

Ghasempour H R, Gaff D F, Williams R P W and Gianello R D. (1998). Contents of sugars in leaves of drying desiccation tolerant flowering plants, particularly grasses. *Plant Growth Regulation*. 24, 185-191.

Gibon Y, Sulpice R and Larher F. (2000). Proline accumulation in canola leaf discs subjected to osmotic stress is related to loss of chlorophylls and to the decrease of mitochondrial activity. *Physiologia Plantarum*. 110, 469-476.

Gilmour S J, Sebolt A M, Salazaar M P, Everard J D and Thomashow, M F. (2000). Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiology*. 124, 1854-1856.

Girgis S I, Alevizaki M, Denny P, Ferrier G J M and Legon S. (1988). Generation of DNA probes for peptides with highly degenerate codons using mixed primer PCR. *Nucleic Acids Research*. 16, 1037-1041.

Girke T, Todd J, Ruuska S, White J, Benning C and ohlrogge, J. (2000). Microarray analysis of developing *Arabidopsis* seeds. *Plant Physiology*. 124, 1570-1581.

Godoy J A, Luna R, Torres-Schumann S, Moreno J, Rodrigo R M and Pintor-Torro. (1994). Expression, tissue distribution and subcellular localization of dehydrin TAS14 in salt-stressed tomato plants. *Plant Molecular Biology*. 26, 1921-1934.

Gowing D J G, Davies W J and Jones H G. (1993). Xylem-transported abscisic acid: the relative importance of its mass and its concentration in the control of stomatal aperture. *Plant, Cell and Environment*. 16, 453-459.

Griffiths A and Bray EA. (1996). Shoot induction of ABA-requiring genes in response to soil drying. *Journal of Experimental Botany*. 47, 1525-1531.

- Grunstein M and Hogness D S. (1975). Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proceedings of the National Academy of Science, USA*. 72, 3961-3965.
- Gutierrez R A, Macintosh G C and Green P J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. *Trends in Plant Science*. 4, 429-438.
- Haas B J, Volfovsky N, Town C D, Troukhan M, Alexandrov N, Feldmann K A, Flavell R B, White O and Salzberg S L. (2002). Full-length messenger RNA sequences greatly improve genome annotation. *Genome Biology*. 3 (6), 29.1-29.12.
- Hagen F S, Gray C L and Kuijper J L. 1988. Assaying the quality of cDNA libraries. *Biotechniques*. 6 (4), 340-5.
- Hallam N D and Luff S E. (1980). Fine structural changes in the mesophyll tissue of the leaves of *Xerophyta villosa* during desiccation. *Botanical Gazette*. 141(2), 173-179.
- Hannenhalli S and Levy S. 2001. Promoter prediction in the human genome. *Bioinformatics*. 17 (1s), 90-96.
- Hanson L, McMahon K A, Johnson M A T and Bennett M D. (2001). First nuclear DNA C-values for 25 angiosperm families. *Annals of Botany*. 87, 251-258.
- Hare P D and Cress W A (1997). Metabolic implications of stress induced proline accumulation in plants. *Plant Growth Regulation*. 21, 79-102.
- Harmer S L, Hogenesch J B, Straume M, Chang H-S, Han B H, Zhu T, Wang X, Kreps J A and Kay S A. (1995) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science*. 290, 2110-2113.

- Harmer S L and Kay S A. (2000). Microarrays: Determining the balance of cellular transcription. *The Plant Cell*. 12, 613-615.
- He J X, An L Z, Lin H H and Llang H G. (1999). Evidence for transcriptional and post-transcriptional control of protein synthesis in water-stressed wheat leaves: A quantitative analysis of messenger and ribosomal RNA. *Journal of Plant Physiology*. 155(1), 63-69.
- Hilbricht T, Salamini F and Bartels D. (2002). CpR18, a novel SAP-domain plant transcription factor, binds to a promoter region necessary for ABA mediated expression of the CDeT27-45 gene from the resurrection plant *Craterostigma plantagineum* Hochst. *The Plant Journal*. 31(3), 293-303.
- Hilhorst H W M. (1998) The regulation of seed dormancy. The membrane hypothesis revisited. *Seed Science Research*. 8, 77-90.
- Hirt H. (1997). Multiple roles of MAP kinases in plant signal transduction. *Trends in Plant Science*. 2(1), 11-15.
- Hoekstra F A, Wolkers W F, Bultink J, Golovina E A, Crowe J H and Crowe L M. (1997). Membrane stabilization in the dry state. *Comparative Biochemistry and Physiology*. 117 A, 335-341.
- Hoekstra F A, Golovina E A and Bultink J. (2001). Mechanisms of plant desiccation tolerance. *Trends in Plant Science*. 6(9).
- Hohelsel J D. (1993). On the activities of *Escherichia coli* exonuclease III. *Analytical Biochemistry*. 209 (2), 238-46.
- Hose E, Steudle E and Hartung W. (2000). Absciscic acid and hydraulic conductivity of maize roots; a study using cell- and root-pressure probes. *Planta*. 211, 874-882.

- Hugovieux V, Kwak J M and Schroeder J I. (2001). An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis*. *Cell*. 106, 477-487.
- Hwang I and Goodman H. (1995). An *Arabidopsis thaliana* root-specific kinase homolog is induced by dehydration, ABA and NaCl. *The Plant Journal*. 8 (1), 37-43.
- Imamura A, Hanaki N, nakamura A, Suzuki T, Taniguchi M, Kiba T, Ueguchi C, Sugiyama T and Mizuno T. (1999). Compilation and characterization of *Arabidopsis thaliana* response regulators implicated in His-Asp phosphorelay signal transduction. *Plant Cell Physiology*. 40 (7) 733-742.
- Ingram J and Bartels D. (1996). The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*. 47, 377-403.
- Ingram J, Chandler JW, Gallagher L, Salamini F and Bartels D. 1997. Analysis of cDNA clones encoding sucrose-phosphate synthase in relation to sugar interconversions associated with dehydration in the resurrection plant *Craterostigma plantagineum* Hochst. *Plant Physiology*. 115(1): 113-21.
- Ish-Horowitz D and Burke J F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Research*. 9, 2989-2998.
- Ishitani M, Xiong L, Stevenson B and Zhu J-K. (1997). Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *The Plant Cell*. 9, 1935-1949.
- Iturriaga G, Gaff D F and Zentella R. (2000). New desiccation-tolerant plants, including a grass, in the central highlands of Mexico, accumulate trehalose. *Australian Journal Of Botany*. 48(2), 153-158.

- Iyer S and Caplan A. (1998). Products of proline catabolism can induce osmotically regulated genes in rice. *Plant Physiology*. 116, 203-211.
- Jackson M. (1997). Hormones from roots as signals for the shoots of stressed plants. *Trends in Plant Science*. 2(1), 22-28.
- Jarbeau J A, Ewers F W and Davis S D. (1995). The mechanism of water stress-induced embolism in two species of chaparral shrubs. *Plant Cell and Environment*. 18, 1-8.
- Jeschke W D and Hartung W. (2000). Root-shoot interactions in mineral nutrition. *Plant and Soil*. 226, 57-69.
- Jia W and Zhang J (2000). Water stress-induced abscisic acid accumulation in relation to reducing agents and sulfhydryl modifiers in maize plants. *Plant, Cell and Environment*. 23, 1389-1395.
- Johnston S, Lee J H and Ray D S. (1985). High level expression of M13 gene II protein from an inducible polycistronic messenger RNA. *Gene*. 34, 137-145.
- Klerzek E and Klerzek R. (2001). Influence of N⁶-isopentenyladenosine (i⁶A) on thermal stability of RNA duplexes. *Biophysical Chemistry*, 91, 135-140.
- Kirch H H, Vera-Estrella R, Gollack D, Quigley F, Michalowski C B, Barkla B J and Bohnert H J. (2000). Expression of water channel proteins in *Mesembryanthemum crystallinum*. *Plant Physiology*. 123(1), 111-124.
- Kirch H H, Nair A and Bartels D. 2001. Novel ABA- and dehydration-inducible aldehyde dehydrogenase genes isolated from the resurrection plant *Craterostigma plantagineum* and *Arabidopsis thaliana*. *Plant Journal*. 28(5), 555-67.

- Kizis D, Lumberras V and pages M. (2001) Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. *FEBS letters*. 498, 187-189.
- Kjellbom P, Larsson C, Johansson I, Karlsson M and Johanson U. (1999). Aquaporins and water homeostasis in plants. *Trends in Plant Science*. 4(8), 308-314.
- Kleines M, Elster R C, Rodrigo M J, Blervacq A S, Salamini F and Bartels D. (1999). Isolation and expression analysis of two stress-responsive sucrose-synthase genes from the resurrection plant *Craterostigma plantagineum* (Hochst.). *Planta*. 209(1):13-24.
- Ko M S H. (1990). An equalized cDNA library by the reassociation of short double stranded cDNAs. *Nucleic Acids Research*. 18, 5705-5711.
- Koonjul P K, Brandt W E, Lindsey G G and Farrant J M. (2000). Isolation and characterisation of chloroplasts from *Myrothamnus flabellifolius* Welw. *Journal of Plant Physiology*. 156, 584-594.
- Kubitzki, K. (1998) Velloziaceae. *The Families and Genera of Vascular Plants*. K Kubitzki (ed.). Springer-Verlag, Berlin. 459-467.
- Kuhlemeier C. (1992). Transcriptional and post-transcriptional regulation of gene expression in plants. *Plant Molecular Biology*. 19, 1-14.
- Kusov Y Y, Shatirishvili G, Dzagurov G, Gauss-Muller V. (2001) A new G-tailing method for the determination of the poly(A) tail length applied to hepatitis A virus RNA. *Nucleic Acids Research*. 29 (12), 57-67.
- Lander E S. (1999). Array of hope. *Nature Genetics Supp.*, 21, 3-4.

- Lee K O, Janh H H, Jung B G, Chi Y H, Lee J Y, Choi L Y, Lee J R, Lim C O, Cho M J and Lee S Y. (2000). Rice 1cys- peroxiredoxin over-expressed in transgenic does not maintain dormancy but enhances antioxidant activity. *FEBS Letters*. 486, 103-106.
- Leprince O, Harren F J M, Bultink J, Alberda M and Hoekstra F A. (2000). Metabolic dysfunction and unabated respiration precede the loss of membrane integrity during dehydration of germinating radicles. *Plant Physiology*, 122, 597-608.
- Liu R and States DJ. (2002). Consensus promoter identification in the human genome utilizing expressed gene markers and gene modelling. *Genome Research*. 12(3):462-9.
- Linhart C and Shamir R. (2002). The degenerate primer design problem. *Bioinformatics*. 18 (1s) 172-181.
- Loffert D, Seip N, Karger S and Kang J. (1998). PCR optimization: degenerate primers. *Qiagen News*. 2, 3-6.
- Lopez-Bucio J, Nieto-Jacoba M F, Ramirez-Rodriguez V and Herrera-Estrella L. (2000). Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. *Plant Science*. 160 (1), 1-13.
- Lorhmann J and Harter K. (2002). Plant two-component signaling systems and the role of the response regulators. *Plant Physiology*. 128, 363-369.
- Ludlow, M. (1989). Strategies of response to water stress. *Structural and Functional Responses to Environmental Stresses: Water Shortage*. K H Kreeb, H Richter and T M Hinckley (eds.). SPB Academic Publishing, The Hague. 269-281.
- Maeda T, Wurgler-Murphy S M and Salto H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature*. 369, 554-558.

- Magne C and Larher F. (1992).** High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry*. 200, 115-118.
- Marlaux J-B, Bockel C, Salamini F and Bartels D. (1998).** Desiccation- and abscisic acid-responsive genes encoding major intrinsic proteins (MIPs) from the resurrection plant *Crateostigma plantagineum*. *Plant Molecular Biology*. 38, 1089-1099.
- Martin K J and Pardee A B. (2000).** Identifying expressed genes. *Proceedings of the National Academy of Sciences, USA*. 97 (8), 3789-3791.
- Maurel C and Chrispeels M J. (2001).** Aquaporins. A molecular entry into plant water relations. *Plant Physiology*. 125, 135-138.
- May M J, Vernoux T, Leaver C, Montagu M V and Inze D. (1998).** Glutathione homeostasis in plants: implications for environmental sensing and plant development. *Journal of Experimental Botany*. 49, 649-667.
- Mizoguchi T, Ichimura K and Shinozaki K. (1997).** Environmental stress response in plants: the role of mitogen activated protein kinases. *Trends in Biotechnology*. 15.
- Mundree S G and Farrant J M. (2000).** Some physiological and molecular insights into the mechanisms of desiccation tolerance in the resurrection plant *Xerophyta viscosa* Baker. *Plant Tolerance to Abiotic Stresses in Agriculture: Role of Genetic Engineering*. J H Cherry (ed). Kluwer Academic Publishers, The Hague. 201-222.
- Mundree S G, Whittaker A, Thomson J A and Farrant J M. (2000).** An aldose reductase homolog from the resurrection plant *Xerophyta viscosa* Baker. *Planta*. 211, 693-700.

- Munnik T, Meijer H J, Ter Riet B, Hirt H, Frank W, Bartels D and Musgrave A. 2000. Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Journal*. 22 (2), 147-54.
- Munnik T and Meijer H J G. (2001). Osmotic stress activates distinct lipid and MAPK signaling pathways in plants. *FEBS letters*. 498, 172-178.
- Nam D K, Lee S, Zhou G, Cao X, Wang C, Clark T, Chen J, Rowley J D and Wang S M. (2002). Oligo(dT) primer generates a high frequency of truncated cDNAs through internal poly(A) priming during reverse transcription. *Proceedings of the National Academy of Science, USA*. 99 (9), 6152-6156.
- Neale A D, Blomstedt C K, Bronson P, Le T-N, Guthridge K, Evans J, Gaff D F and Hamill J D. (2000). The isolation of genes from the resurrection grass *Sporobolus stapfianus* which are induced during severe drought stress. *Plant, Cell and Environment*. 23, 265-277.
- Netting A G. (2000). pH, abscisic acid and the integration of metabolism in plants under stressed and non-stressed conditions: cellular responses to stress and their implication for plant water relations. *Journal of Experimental Botany*. 51, 147-158.
- Ndimia T, Farrant J, Thomson J and Mundree S. (2001). Molecular characterization of XVT8, a stress-responsive gene from the resurrection plant *Xerophyta viscosa* Baker. *Plant Growth Regulation*. 35 (2), 137-145.
- Norwood M, Truesdale M R, Richter A and Scott P. (1999). Metabolic changes in the leaves and roots during dehydration of the resurrection plant *Craterostigma plantagineum* (Hochst). *South African Journal of Botany*. 65(5 & 6), 421-427.

- Norwood M, Truesdale M R, Richter A and Scott P. (2000). Photosynthetic carbohydrate metabolism in the resurrection plant *Craterostigma plantagineum*. *Journal of Experimental Botany*. 51(343), 159-165.
- Okayama H and Berg P. (1982). High efficiency cloning of full-length cDNA. *Molecular Cell Biology*. 2, 161-170.
- Oliver M J, Wood A and Velten J. (1995). Rehydrins TR288 and TR155 - gene isolation and characterization. *Plant Physiology*. 108 (2), 107.
- Oliver M J, Wood A J and O'Mahony P. (1997). How some plants recover from vegetative desiccation: A repair based strategy. *Acta Physiologia Plantarum*. 19(4), 419-425.
- Oliver M J and Bewley J D. (1997). Desiccation-tolerance of plant tissues: A mechanistic overview. *Horticultural Reviews*. 18, 171-213.
- Oliver M J, Wood A J and O'Mahony P. (1998). "To dryness and beyond" - preparation for the dried state and rehydration in vegetative desiccation-tolerant plants. *Plant Growth Regulation*. 24, 193-201.
- Oliver M J, Tuba Z and Mishler B D. (2000). The evolution of vegetative desiccation tolerance in land plants. *Plant Ecology*. 151(1), 85-100.
- Oliver A E, Hincha D K and Crowe J H. (2001). Looking beyond sugars: the role of amphiphilic solutes in preventing adventitious reactions in anhydrobiotes at low water contents. *Comparative Biochemistry and Physiology*. 131, 515-525.
- O'Mahony P and Oliver M J. (1999). The involvement of ubiquitin in vegetative desiccation tolerance. *Plant Molecular Biology*. 41, 657-667.

- O'Mahony P J and Oliver M J. (1999b).** Characterization of a desiccation-reponsive small GTP-binding protein (Rab2) from the desiccation-tolerant grass *Sporobolus stapfianus*. *Plant Molecular Biology*. 39, 809-821.
- O'Mahony P J and Oliver M J. (2001).** The effect of desiccation and rehydration on the integrity of small subunit ribosomal RNAs in desiccation tolerant and intolerant plants. *Plant Physiology and Biochemistry*. 39, 67-71.
- Oztur Z N, Talame V, Deyholos M, Michalowski C B, Galbraith D W, Gozukirmizi N, Tuberosa R and Bohnert HJ. 2002.** Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Molecular Biology*. 48(5-6), 551-73.
- Parthasarathy M V. (1995).** Freeze-substitution. *Methods in Cell Biology*. 49, 57-69.
- Patanjall S R, Parimoo S and Weissman S M. (1991).** Construction of a uniform-abundance (normalized) cDNA library. *Proceedings of the National Academy of Science, USA*. 88, 1943-1947.
- Pederson A G, Baldi P, Chauvin Y and Brunak S. (1999).** The biology of eukaryote promoter prediction – a review. *Computers and Chemistry*. 23, 191-207.
- Pennica D, Holmes W E, Kohr W J, Harkins R N, Vehar G A, Ward C A, Bennett W F, Yelverton E, Seeburg P H, Heyneker H L, Goeddel D V and Collen D. (1983).** Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature*. 301, 214-221.
- Phillips J R, Hilbricht T, Salamini F and Bartels D. 2002.** A novel abscisic acid- and dehydration-responsive gene family from the resurrection plant *Craterostigma plantagineum* encodes a plastid-targeted protein with DNA-binding activity. *Planta*. 215(2):258-66.

- Phimister B.** (1999). Going global. *Nature Genetics*. 21 (1s), 1.
- Piatkowski D, Schneider K, Salamini F and Bartels, D.** (1990). Characterisation of five abscisic acid-responsive cDNA clones isolated from the desiccation-tolerant plant *Cratogeomys 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, Jouken M J W, Weisbeek P J and Smeekens S C M.** (1995). Improved performance of transgenic fructan accumulating tobacco under drought stress. *Plant Physiology*. 107, 125-130.
- Porembski S and Barthlott W.** (1995). On the occurrence of a velamen radicum in Cyperaceae and Velloziaceae. *Nordic Journal of Botany*. 15(6), 625-629.
- Porembski S and Barthlott W.** (2000). Granitic and gneissic outcrops (inselbergs) as centres of diversity for desiccation-tolerant vascular plants. *Plant Ecology*. 151, 19-28.
- Qin X and Zeevart J A D.** (1999). The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proceedings of the National Academy of Science, USA*. 96(26), 15 354-15 361.
- Rabinowicz P D and Byrne M E.** (2001). Follow that plant! *Genome Biology*. 2 (2), 2.
- Ramsay G.** (1998). DNA chips: State-of-the-art. *Nature Biotechnology*. 16, 40-44.
- Rathinasabapathi B.** (2000) Metabolic engineering for stress tolerance: installing osmoprotectant synthesis pathways. *Annals of Botany*. 86, 709-716.
- Reddy A S N.** (2001). Calcium: silver bullet in signaling. *Plant Science*. 160, 381-404.

Reddy A R, Ramakrishna W, Sekhar A C, Ithal N, Babu P R, Bonaldo M D F, Soares M B and Bennetzen J L. (2002). Novel genes are enriched in normalized cDNA libraries from drought stressed seedlings of rice (*Oryza sativa* L. subsp. *Indica* cv. Nagina 22). *Genome*. 45, 204-211.

Rengel Z. (1992). The role of calcium in salt toxicity. *Plant, Cell and Environment*. 15, 625-632.

Reymond P, Weber H, Damond M and Farmer E E. (2000). Differential gene expression in response to mechanical damage and insect feeding in *Arabidopsis*. *Plant Cell*. 12. 707-719.

Reymond A, Friedli M, Henrichsen C N, Chapot F, Deutsch S, Ucla C, Rossier C, Lyle R, Guipponi M and Antonarakis SE. 2001. From PREDs and open reading frames to cDNA isolation: revisiting the human chromosome 21 transcription map. *Genomics*. 78(1-2):46-54.

Reynolds E S. (1963). The use of lead citrate at high pH as an electron opaque stain for electron microscopy. *Journal of Cell Biology*. 17, 208-212.

Roschier M, Kuusisto E and Salminen A. (2000). A differential display protocol to identify differentially expressed mRNAs in potassium-derived cerebellar granule cells. *Brain Research Protocols*. 5, 121-131.

Sakakibara H, Taniguchi M and Sugiyama T. (2000). His-Asp phosphorelay signaling: a communication avenue between plants and their environment. *Plant Molecular Biology*. 42 (2), 273-278.

- Sasaki Y F, Ayusawa D and Oishi M. (1994).** Construction of a normalized cDNA library by introduction of a semi-solid mRNA-cDNA hybridization system. *Nucleic Acids Research*. 22, 987-992.
- Sauter A and Hartung W. (2000).** Radial transport of abscisic acid conjugates in maize roots: its implication for long-distance stress signals. *Journal of Experimental Botany*. 51 (346), 929-935.
- Sauter A, Davies W J and Hartung W. (2001).** The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *Journal of Experimental Botany*. 52 (363), 1991-1997.
- Schaller G E, Matthews D, Gribskov M and Walker J C. (2001).** Two-component signaling elements and histidyl-to-aspartyl phosphorelays. *The Arabidopsis Book*. C Somerville and E Meyerowitz (eds.). ASPB, New York.
- Schena M, Shalon D, Davis R W and Brown P O. (1995).** Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*. 270, 467-470.
- Schneider K, Wells B, Schmelzer E, Salamini F and Bartels, D. (1993).** Desiccation leads to the rapid accumulation of both cytosolic and chloroplastic proteins in the resurrection plant *Craterostigma plantagineum* Hochst. *Planta*. 189, 120-131.
- Schroda M, Vallon O, Wollman F A and Beck C F. (1999).** A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. *Plant Cell*. 11(6), 1165-1178.
- Schwab K B and Gaff D F. (1986).** Sugar and ion content in leaf tissues of several drought tolerant plants under water stress. *Journal of Plant Physiology*, 125, 257-265.

- Scott H and Oliver M J. (1994).** Accumulation and polysomal recruitment of transcripts in response to desiccation and rehydration of the moss *Tortula ruralis*. *Journal of Experimental Botany*. 45, 577-583.
- Scott P. (2000).** Resurrection plants and the secrets of eternal leaf. *Annals of Botany*, 85, 129-166.
- Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozak, K, Carninci P, Hayashizaki Y and Shinozaki K. (2001).** Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *The Plant Cell*. 13, 61-72.
- Seki M, Narusaka M, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y and Shinozaki K. (2001b).** *Arabidopsis* encyclopedia using full-length cDNA and its application. *Plant Physiology and Biochemistry*. 39, 211-220.
- Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oona Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y and Shinozaki K. (2002).** Monitoring the expression profiles of 7 000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *The Plant Journal*. 31 (3), 279-292.
- Senaratna T and McKersie B D. (1983).** Characterisation of solute efflux from dehydration injured soybean (*Glycine max* L. Merr) seeds. *Plant Physiology*. 72, 911-914.
- Serraj R., Sinclair T R and Purcell L C. (1999).** Symbiotic N₂ fixation in response to drought. *Journal of Experimental Botany*. 50, 143-155.
- Sherwin, H. W., and Farrant, J. M. (1996).** Differences in rehydration of three desiccation-tolerant angiosperm species. *Annals of Botany*. 78, 703-710.

- Sherwin H W, Pammenter N W, February E, Willigen C V and Farrant J M. (1998). Xylem hydraulic characteristics, water relations and wood anatomy of the resurrection plant *Myrothamnus flabellifolius* Welw. *Annals of Botany*. 81, 567-575.
- Sherwin H W and Farrant J M. (1998). Protection mechanisms against excess light in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*. *Plant Growth Regulation*. 24, 203-210.
- Short J M, Fernandez J M, Sorge J A and Huse W D. (1988). Lambda ZAP: A bacteriophage vector with *in vivo* excision properties. *Nucleic Acids Research*. 16: 7583-7600.
- Simonneau T, Barrieu P and Tardieu F. (1998). Accumulation rate of ABA in detached maize roots correlates with root water potential regardless of age and branching order. *Plant, Cell and Environment*. 21, 1113-1122.
- Smirnoff N. (1993). The role of activated oxygen in the response of plants to water deficit and desiccation. *New Phytologist*. 125, 27-58.
- Smirnoff N and Bryant J A. (1999). DREB takes the stress out of growing up. *Nature Biotechnology*. 17(3), 229-230.
- Smith M J and Berjak P. (1995). Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and desiccation-sensitive seeds. *Seed Development and Germination*. J Kigel and G Galili (eds.). Marcel Dekker, New York, 702-746.
- Snustad D P, Haas N A, Kopczak S D and Sliflow C D. (1992). The small genome of *Arabidopsis* contains at least none expressed beta-tubulin genes. *Plant Cell*. 4 (5), 549-556.

Soares M B, Bonaldo M D F, Jelene P, Su L, Lawton L and Efstratiadis A. (1994). Construction and characterization of a normalized cDNA library. *Proceedings of the National Academy of Science, USA*. 91, 9228-9232.

Soares M B and Bonaldo M D F. (1998). Constructing and screening normalized cDNA libraries. *Genome Analysis: A Laboratory Manual*. B Birren, E Green, S Klapholz, R M Meyers and J Roskams (eds.). Cold Spring Harbour Press, USA. 3, 49-157.

Somero G N. (1992). *Adapting to stresses: convergence on common solutions. Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular and Molecular Levels*, G N Somero, C B Osmond and C L Bolis (eds.). Springer-Verlag, Berlin. 3-18.

Southern E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*. 98, 503-517.

Spurr A R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructural Research*. 26, 31-43.

Starck Z, Choluj D and Gawronska H. (1998). The effect of drought hardening and chilling on ABA content in xylem sap and ABA - delivery rate from root of tomato plant. *Acta Physiologia Plantarum*. 20(1), 41-48.

Steudle E. (2000). Water uptake by roots: an integration of views. *Plant and Soil*. 226, 45-56.

- Stockinger E J, Gilmour S J and Thomashow, M F. (1997)** *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/ DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proceedings of the National Academy of Science, USA*. 94, 1035-1040.
- Stockinger E J, Mao Y, Regier M K, Triezenberg SJ and Thomashow M F. (2001).** Transcriptional adaptor and histone acetyltransferase proteins in *Arabidopsis* and their interactions with CBF1, a transcriptional activator involved in cold-regulated gene expression. *Nucleic Acids Research*. 29 (7), 1525-1533.
- Sun W Q and Leopold C A. (1997).** Cytoplasmic vitrification and survival of anhydrobiotic organisms. *Comparative Biochemistry and Physiology*. 117(3), 327-333.
- Teraï G, Takagi T and Nakai K. 2001.** Prediction of co-regulated genes in *Bacillus subtilis* on the basis of upstream elements conserved across three closely related species. *Genome Biology*. 2(11).
- Thomson W W and Platt K A. (1997).** Conservation of cell order in desiccated mesophyll of *Selaginella lepidophylla* ([Hook and Grev.] Spring). *Annals of Botany*. 79, 439-447.
- Tuba Z, Proctor M C F and Csintalan Z. (1998).** Ecophysiological responses of homoichlorophyllous and poikilochlorophyllous desiccation tolerant plants: a comparison and an ecological perspective. *Plant Growth Regulation*. 24, 211-217.
- Tymms M J, Gaff D F and Hallam N D. (1982).** Protein synthesis in the desiccation tolerant angiosperm *Xerophyta villosa* during dehydration. *Journal of Experimental Botany*. 33, 332-343.

- Tymms M J and Gaff D F. (1984).** Recovery of protein synthesis and RNA synthesis after rewetting of desiccated leaves of the angiosperm *Xerophyta villosa*. *Biochemica Physiologia Planzen*. 179, 211-217.
- Tyree M T. (2001).** Capillarity and sap ascent in a resurrection plant: does theory fit the facts? *New Phytologist*. 150, 9-21.
- Ueguchi C, Koizumi H, Suzuki T and Mizuno T. (2001)** Novel family of sensor histidine kinase genes in *Arabidopsis thaliana*. *Plant Cell Physiology*. 42 (2) 231-235.
- Urao T, Yakubov B, Satoh R, Yamaguchi-Shinozaki K, Seki M, Hirayama T and Shinozaki, K. (1999).** A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *The Plant Cell*. 11, 1743-1754.
- Urao T, Yamaguchi-Shinozaki K and Shinozaki K. (2000).** Two-component systems in plant signal transduction. *Trends in Plant Science*. 5 (2), 67-74.
- Urao T, Miyata S, Yamaguchi-Shinozaki K and Shinozaki K. (2000b).** Possible His to Asp phosphorelay signaling in an *Arabidopsis* two-component system. *FEBS letters*. 478, 227-232.
- Vandepoele K, Raes J, Veylder L D, Rouze P, Rombauts S and Inze D. (2002).** Genome-wide analysis of core cell cycle genes in *Arabidopsis*. *The Plant Cell*. 14, 903-916.
- Velasco R, Salamini F and Bartels D. (1994).** Dehydration and ABA increase mRNA levels and enzyme activity of cytosolic GADPH in the resurrection plant *Craterostigma plantagineum*. *Plant Molecular Biology*. 26, 541-546.

- Velasco R, Salamini F and Bartels D. (1998).** Gene structure and expression analysis of the drought- and abscisic acid-responsive CDeT11-24 gene family from the resurrection plant *Craterostigma plantagineum* Hochst. *Planta*. 204(4), 459-471.
- Velten J and Oliver M J. (2001).** Tr288, a rehydrin with a dehydrin twist. *Plant Molecular Biology*. 45, 713-722.
- Velten J and Oliver M J. (2002).** Replacement Histones: A Role in Desiccation Tolerance in Moss? *American Society of Plant Biologists Annual Meeting, 2002*.
- Verslues P E and Sharp R E. (1999).** Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials. II. Metabolic source of increased proline deposition in the elongation zone. *Plant Physiology*. 119, 1349-1360.
- Vertucci C W and Farrant J M. (1995).** Acquisition and loss of desiccation tolerance. *Seed Development and Germination*. J Kigel and G Galili (eds). Marcel Dekker, New York. 237-271.
- Viera J and Messing J. (1987).** Production of single-stranded plasmid DNA. *Methods in Enzymology*. 153, 3-11.
- Wahl S and Ryser P. (2000).** Root structure is linked to ecological strategies of grasses. *New Phytologist*. 148, 459-471.
- Walters C, Farrant JM, Pammenter N W and Berjak P. (2002).** Desiccation stress and damage. *Desiccation and survival in Plants –Drying without Dying*. M Black and H W Pritchard (eds.). CABI Publishing, Oxon. 263-292.
- Wang J, Sheehan M, Brookman H and Timko M P. (2000)** Characterization of cDNAs differentially expressed in roots of tobacco (*Nicotiana tabacum* cv Burley 21) during the early stages of alkaloid biosynthesis. *Plant Science*. 158, 19-32.

- Wang Y H, Garvin D F and Kochian L V. (2001). Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. *Plant Physiology*. 127:345-359
- Weissman SM. (1987). Molecular genetic techniques for mapping the human genome. *Molecular Biological Medicine*. 4, 133-143.
- West A H and Stock A M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends in Biochemical Sciences*. 26 (6) 369-376.
- Willigen C V. (2002). Comparison of the resurrection grass, *Eragrostis nindensis*, with the related desiccation-sensitive species, *Eragrostis curvula*. *PhD Thesis, University of Cape Town*.
- Wood A J and Oliver M J. (1999). Translational control in plant stress: the formation of messenger ribonucleoprotein particles (mRNPs) in response to desiccation of *Tortula ruralis* gametophytes. *The Plant Journal*. 18(4), 359-370.
- Wood A J, Duff R J and Oliver M J. (1999). Expressed sequence tags (ESTs) from desiccated *Tortula ruralis* identify a large number of novel plant genes. *Plant Cell Physiology*. 40(4), 361-368.
- Wood A J, Duff R J and Oliver M J. (2000). The translational apparatus of *Tortula ruralis*: polysomal retention of transcripts encoding the ribosomal proteins RPS14, RPS16 and RPL23 in desiccated and rehydrated gametophytes. *Journal Of Experimental Botany*. 51(351), 1655-1662.
- Wu S-H, Ramonell K, Gollub J and Somerville S. (2001). Plant gene expression profiling with DNA microarrays. *Plant Physiology and Biochemistry*. 39, 917-926.

Yamaguchi-Shinozaki K and Shinozaki K. (1994). A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low temperature, or high-salt stress. *Plant Cell*. 6, 251-264.

Zhu T, Budworth P, Han B, Brown D, Chang H-S, Zou G, and Wang X. (2001). Toward elucidating the global gene expression patterns of developing *Arabidopsis*: parallel analysis of 8 300 genes by a high-density oligonucleotide probe array. *Plant Physiology and Biochemistry*, 39, 221-242.

University of Cape Town