

'6

**INVESTIGATING THE MECHANISMS OF DESICCATION  
TOLERANCE IN THE RESURRECTION PLANT,  
*MYROTHAMNUS flabellifolius* (WELW.)**

**Priyum K. Koonjul**

Thesis presented for the Degree of  
**DOCTOR OF PHILOSOPHY**  
in the Department of Biochemistry

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.

**PREFACE**

Associate Professor George G. Lindsey, Professor W. Brandt and Associate Professor Jill M. Farrant supervised this thesis. It is hereby declared that this thesis, submitted for the degree of Doctor of Philosophy in Biochemistry at the University of Cape Town, is the result of my own investigation, except where the work of others is acknowledged.

signature removed

Priyum K. Koonjul

November, 1999.

---

---

## ACKNOWLEDGEMENTS

I would like to acknowledge the following people:

My Mom, Dad, sister, brother and Dave for their continual enthusiasm, moral support and interest in my work over the years. I would especially like to thank my parents for flying to South Africa to be with me during the last stage of my PhD.

My supervisors, Associate Professor G.G. Lindsey, Professor W. Brandt and especially Associate Professor J.M. Farrant for their guidance during the course of this project. I am also indebted to Dr K. Denby for her help with the molecular work and for her critical reading of my thesis, Dr M. Purves for her input in the chloroplasts work, M. Jaffer and Dr H. Sherwin for their help with the electron microscopy, Dr Michael Smith for doing the lipid analysis, C. Lawrence for doing the cation analysis, N. Eden, T. Anderson and P. Thompson for sharing their photographic skills, Dr S. Mundree for helping me with RACE and K. de Ronde for doing some of the antioxidant analysis for me.

I would also like to thank Professor D. Bartels, Dr V. Abratt, Professor N. Illing, Mr W. Mpoloka, Dr S. Mackerness and Mr A. Shoko for providing the different antibodies and probes I used, P. Ma for synthesising all the oligos, D. James and K. Van Heeren for doing all the automated sequencing for me.

My fellow colleagues and friends: Anne, Anita, Brett, Carnita, Clare, Ethne, Fabrice, Javed, Keren, Jennifer, Kim, Kurt, Laure, Libby, Lynn, Pat, Pauline, Sanjay, Yolande, Yuval and a special thanks to Mantoa for always making me think positive and keeping me going. Mr and Mrs Myburgh for all their support and love.

Finally, I would like to thank everybody in the departments of Biochemistry, Botany and Microbiology who willingly provided assistance wherever possible.

---

**LIST OF ABBREVIATIONS**

A <sub>260</sub>	Absorbance reading at 260 nm
A <sub>280</sub>	Absorbance reading at 280 nm
A <sub>600</sub>	Absorbance reading at 600 nm
aa	Amino acid
AAP	Abridged anchor primer
ABA	Abscisic acid
AFLP	Amplified restriction fragment polymorphism
AMPS	Ammonium peroxodisulphite
AP	Ascorbate peroxidase
ATP	Adenosine triphosphate
β-gal	β-galactosidase protein
bp	base pair (s)
BSA	Bovine serum albumin
C <sub>a</sub>	Chlorophyll a
C <sub>b</sub>	Chlorophyll b
C <sub>a+b</sub>	Chlorophyll (a+b)
C <sub>x+c</sub>	Xanthophylls + carotenes
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary DNA
<sup>3</sup> Chl	Triplet state of chlorophyll
CIP	Calf intestinal phosphatase
COS-1 cells	African green monkey kidney cells
cpm	Counts per minute
<i>C. plantagineum</i>	<i>Craterostigma plantagineum</i>
<i>C. wilmsii</i>	<i>Craterostigma wilmsii</i>
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DD-PCR	Differential display polymerase chain reaction
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine triphosphate
DM	Dry mass
DMF	Dimethylfluoride
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
dpm	Disintegrations per minute
DS	Desiccation-sensitive
ds	Double stranded
dsp	Desiccation stress protein
DT	Desiccation-tolerant
DTT	Dithiothreitol (1-threo-1,4-dimercapto-2,3 butanediol)
dTTP	Deoxythymidine triphosphate
dw	Dry weight

---

<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol-bis( $\beta$ -aminoethyl ether) N, N, N', N'-tetraacetic acid
EST	Expressed sequence tag
EtBr	Ethidium Bromide
fig.	Figure
$F_M/F_V$	Quantum efficiency
Fru-6-P	D-Fructose-6-phosphate
Glc-6-P	D-Glucose-6-phosphate
GR	Glutathione reductase
GSH	Glutathione
GSP	Gene specific primer
GSSG	Oxidised glutathione
HEPES	4-(2-Hydroxyethyl)-1-piperazineethane sulphonic acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HPI	D-hexose-6-phosphate-ketol-isomerase
HSP	Heat shock protein
ICP-AES	Inductively coupled plasma atomic emission spectrometer
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside
kb	kilo base
KCl	Potassium chloride
kDa	kilo Dalton
LB	Luria-Bertani medium
LEA	Late embryogenesis abundant
LHC	Light harvesting complex (chlorophyll a/b binding protein)
$\mu$ Ci	MicroCurie
$\mu$ g	Microgram
$\mu$ l	Microlitre
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation Time of Flight
MCS	Multiple cloning site
MES	(2[N-morpholino] ethanesulfonic acid)
<i>M. flabellifolius</i>	<i>Myrothamnus flabellifolius</i>
MgCl <sub>2</sub>	Magnesium chloride
min	Minute
ml	Millilitre
MMLV	Monkey murine leukemia virus
MOPS	Morpholinopropanesulphonic acid
mRNA	Messenger RNA
MW	Molecular weight
NaCl	Sodium chloride
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)

---

---

NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
nt	nucleotide(s)
NMR	Nuclear magnetic resonance
•OH	hydroxyl radical
O <sub>2</sub> <sup>•-</sup>	superoxide radical
<sup>1</sup> O <sub>2</sub>	singlet oxygen
oligo	Oligodeoxyribonucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PB	Phosphate buffer
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFD	Photon flux density
pfu	plaque forming units
PMSF	Phenylmethylsulphonyl fluoride
PNK	Phosphonucleotide kinase
PSI/II	Photosystem I/II
<i>P. sativum</i>	<i>Pisum sativum</i>
PVP	Poly[1-vinylpyrrolidone-2]
RAB	Responsive to abscisic acid
RACE	Rapid amplification of cDNA ends
RAP-PCR	RNA fingerprinting by arbitrarily primed PCR
<i>rbcL</i>	rubisco large subunit
<i>rbcS</i>	rubisco small subunit
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rATP	Ribosomal ATP
RT	Reverse transcription
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	Relative water content
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecyl sulphate
SM buffer	Saline medium buffer
smHSP	Small heat shock protein
SOD	Superoxide dismutase
ss	single stranded
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TEMED	N,N,N',N' - tetramethylethylene-diamine
T <sub>g</sub>	Glass transition temperature

---

---

T <sub>m</sub>	Melting temperature
TNTC	Too numerous to count
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	Transfer RNA
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet light
W	Watts
X-Gal	5-Bromo-4-chloro-3-indoyl-β-D-galactoside
<i>X. humilis</i>	<i>Xerophyta humilis</i>
<i>X. viscosa</i>	<i>Xerophyta viscosa</i>

---

---

**List of Figures**

- Fig. 1.1: Dry *M. flabellifolius* plant
- Fig. 1.2: Green *M. flabellifolius* plant
- Fig. 2.1: Oxidation of ascorbate to dehydroascorbate
- Fig. 2.2: Reduction of 2-monodehydroascorbate
- Fig. 2.3: Redox reaction of glutathione
- Fig. 2.4: Discontinuous sucrose gradient showing banding pattern of chloroplasts of *P. sativum*, *C. wilmsii* and *M. flabellifolius*
- Fig. 2.5: Light microscopy of chloroplasts isolated from *P. sativum*, *C. wilmsii* and *M. flabellifolius*
- Fig. 2.6: EM of chloroplasts from *P. sativum*
- Fig. 2.7: EM of chloroplasts from *C. wilmsii* and *M. flabellifolius* purified on a sucrose gradient
- Fig. 2.8: Trehalose gradient showing banding pattern of chloroplasts of *P. sativum*, *C. wilmsii* and *M. flabellifolius*
- Fig. 2.9: EM of chloroplasts from *C. wilmsii* and *M. flabellifolius* purified on a trehalose gradient
- Fig. 2.10: EM of chloroplasts from hydrated leaves of *C. wilmsii* and *M. flabellifolius*
- Fig. 2.11: SDS-PAGE of chloroplastic proteins from *P. sativum*, *C. wilmsii* and *M. flabellifolius*
- Fig. 2.12: Chloroplastic gene expression in dry and hydrated leaves of *M. flabellifolius*
- Fig. 2.13: Western blot of chloroplastic proteins from *P. sativum*, *C. wilmsii* and *M. flabellifolius* against Group III LEA protein
- Fig. 2.14: EM of chloroplast from dry leaf of *M. flabellifolius*
- Fig. 3.1: EM of hydrated leaf of *M. flabellifolius* showing polyphenolic bodies
- Fig. 3.2: MALDI-TOF analysis comparing RNA isolated using different methods
- Fig. 3.3: Agarose gel electrophoresis of RNA
-

- 
- Fig. 3.4: Alkaline agarose gel
- Fig. 3.5: PCR of *HSP12* gene in the presence of varying amounts of RNA isolated from *M. flabellifolius*
- Fig. 3.6: Effect of various additives on PCR of *HSP12* gene
- Fig. 3.7: Effect of PVP on PCR of *HSP12* gene
- Fig. 3.8: Effect of green tea polyphenolic on PCR of *HSP12* gene
- Fig. 4.1: Schematic diagram showing steps involved in construction of a cDNA library
- Fig. 4.2: Alkaline agarose gel
- Fig. 4.3: Checking sizes of fragments eluted from spin column
- Fig. 4.4: Expression analysis of clones isolated from differential screening of cDNA library
- Fig. 5.1: General strategy for differential display
- Fig. 5.2: DD-PCR gel comparing RNA isolated from dry and hydrated leaves of *M. flabellifolius* using different primer combinations
- Fig. 5.3: Reamplification of cDNA bands isolated from DD-PCR gel
- Fig. 5.4: Reverse Northern analysis
- Fig. 5.5: Sequence analysis of clone dd-1
- Fig. 5.6: ORF analysis of clone dd-1
- Fig. 5.7: Sequence analysis of clone dd-5
- Fig. 5.8: ORF analysis of clone dd-5
- Fig. 5.9: Sequence analysis of clone dd-6
- Fig. 5.10: ORF analysis of clone dd-6
- Fig. 5.11: Sequence analysis of clone dd-7
- Fig. 5.12: ORF analysis of clone dd-7
- Fig. 5.13: Sequence analysis of clone dd-8
- Fig. 5.14: ORF analysis of clone dd-8
- Fig. 6.1: Schematic diagram showing the steps involved during 5' RACE
- Fig. 6.2: Flow diagram showing controls taken during 5' RACE using control RNA and DNA purchased from Life Technologies.
-

- 
- Fig. 6.3: PCR of controls taken to check various steps involved during the RACE procedure
- Fig. 6.4: RACE products separated by agarose gel electrophoresis
- Fig. 6.5: DNA sequence analysis of fragment DD<sub>1</sub>GSP<sub>1</sub>
- Fig. 6.6: Analysis of the putative open reading frames for fragment DD<sub>1</sub>GSP<sub>1</sub>
- Fig. 6.7: DNA sequence analysis of fragment DD<sub>5a</sub>GSP<sub>1</sub>
- Fig. 6.8: Analysis of the putative open reading frames for fragment DD<sub>5a</sub>GSP<sub>1</sub>
- Fig. 6.9: DNA sequence alignment of fragment DD<sub>5b</sub>GSP<sub>1</sub> and fructokinase isolated from *L. esculentum* .
- Fig. 6.10: Analysis of the putative open reading frames for fragment DD<sub>5b</sub>GSP<sub>1</sub>
- Fig. 6.11: DNA sequence analysis of fragment DD<sub>8</sub>GSP<sub>1</sub>
- Fig. 6.12: Analysis of the putative open reading frames for fragment DD<sub>8</sub>GSP<sub>1</sub>
-

**List of Tables**

- Table 1: Acid-soluble and alkali-soluble polyphenolic contents in *M. flabellifolius*, *Rosa spp*, *X. humilis*, *C. wilmsii*, *Saintpaulia* and *P. sativum*.
- Table 2: Lipid composition of chloroplasts isolated from *P. sativum*, *C. wilmsii* and *M. flabellifolius*
- Table 3: Content of photosynthetic pigments and antioxidants from dry and hydrated leaves of *M. flabellifolius*
- Table 4: Content of polyphenolics and soluble sugars from dry and hydrated chloroplasts of *M. flabellifolius*
- Table 5: Content of cations from dry and hydrated chloroplasts of *M. flabellifolius*
- Table 6: Titration of cDNA library
- Table 7: Results of homology search of DD-PCR fragments with the database
- Table 8: Results of homology search of RACE-PCR fragments with the database
-

---

**ABSTRACT**

Resurrection plants, including *Myrothamnus flabellifolius*, grow in shallow soil upon rocky outcrops where they experience regular periods of water stress. Associated with this is light stress. The presence of light under water limiting conditions can result in photo-oxidation which causes damage to plant tissues. *M. flabellifolius* is a homoichlorophyllous plant and thus retains chlorophyll during desiccation. The mechanisms whereby this plant prevents photo-oxidation damage are not known and thus one of the objectives of this study was to characterise the chloroplasts and the changes they undergo during dehydration. It was shown that chloroplasts from *M. flabellifolius* could only be isolated using trehalose gradients (instead of sucrose gradients) and were found to have a higher buoyant density than chloroplasts isolated from another resurrection plant, *Craterostigma wilmsii*. The latter had the same buoyant density as those isolated from the desiccation sensitive plant *Pisum sativum*. The increased buoyant density in *M. flabellifolius* was ascribed to the unusual ultrastructure of the thylakoid membranes. The latter have a staggered conformation (staircase arrangement) rather than the discrete granal and intergranal conformation found in most plants.

Several strategies, including both physical and biochemical ones, are used by the plant to protect itself from the above mentioned forms of stresses. The physical strategies included reorientation of the leaves under light conditions (thereby shading chlorophyll from light) as well as separation of the thylakoid membranes during depletion of water. The latter was found to be unique to this species and was thought to be brought about by a decrease in cation levels during drying. Separation of the thylakoid membranes (and hence separation of photosystem I and II) might be a mechanism to decrease the rate of photosynthesis and minimize photo-oxidation damage under water stress conditions. The biochemical strategies, on the other hand, involved accumulation of polyphenolics (including anthocyanins), sugars, antioxidants (ascorbate peroxidase, glutathione reductase, superoxide dismutase) and LEA-related proteins. These molecules are all known to confer tolerance to water and/or light stress in plants. Furthermore, this plant

---

accumulated several stromal proteins in the range of 10-15 kDa, which were not found in other plants. These proteins could be implicated in protection of chloroplasts from this species under stress conditions.

Although polyphenolics were shown to protect the plant and its organelles during desiccation, their presence often pose a problem in molecular biology work. Polyphenolics form complexes with proteins (including enzymes), polysaccharides as well as nucleic acids. They are usually stored in vacuoles and organelles but once the latter are damaged (for example, during grinding of tissues), these polyphenolic molecules are released causing potentially damaging interactions with the above mentioned macromolecules. This study has shown that even traces of polyphenolic contamination inhibits several enzymes, for example, Taq. polymerase. Molecular studies carried out on *M. flabellifolius* therefore proved to be a challenge.

Several problems were therefore encountered when standard molecular methodologies were used in order to isolate and characterise genes which are induced upon desiccation in *M. flabellifolius*; every reaction had to be optimised for this system. A number of different strategies were thus used to identify genes which could be implicated in conferring tolerance in this species. Differential expression analysis confirmed that these genes were present only in dry and not in hydrated tissues. Sequence comparison of some of these genes with the public database showed homology to genes/proteins implicated in desiccation tolerance. Two of these clones showed homology to sugar transporter proteins. Biochemical studies carried out on *M. flabellifolius* showed the accumulation of hexose sugars during desiccation in this species. It was thus tempting to speculate about the roles of these sugar transporters during desiccation in *M. flabellifolius*.

Thus it appears that *M. flabellifolius* uses physical, biochemical as well as molecular mechanisms to protect itself from light and/or water stress.

---

---

**TABLE OF CONTENT**

Preface	i
Acknowledgements	ii
List of abbreviations	iii
List of figures	vii
List of tables	x
Abstract	xi
<b>CHAPTER 1: AN OVERVIEW OF DESICCATION TOLERANCE IN ANGIOSPERMS</b>	
<b>1.1 General Introduction</b>	<b>1</b>
<b>1.2 Water: An ideal biological solvent</b>	<b>2</b>
<b>1.3 Evolution of desiccation tolerance</b>	<b>2</b>
1.3.1 Desiccation evasion	3
1.3.2 Desiccation resistance	3
1.3.3 Desiccation tolerance	3
<b>1.4 Theories of desiccation tolerance</b>	<b>5</b>
<b>1.5 Damage resulting from cellular dehydration</b>	<b>6</b>
1.5.1 Damage to the membrane structure	6
1.5.2 Damage to proteins and nucleic acids	8
<b>1.6 Protective mechanisms against water stress and desiccation</b>	<b>9</b>
1.6.1 Minimizing mechanical damage associated with loss of turgor	9
1.6.2 Minimizing metabolic stress	10
1.6.2.1 Accumulation of water stress proteins	10
1.6.2.2 Accumulation of osmolytes	12
1.6.3 Minimizing free radical damage	14
<b>1.7 Resurrection plants as models to study desiccation tolerance</b>	<b>15</b>
1.7.1 <i>Myrothamnus flabellifolius</i> Welw.	16
<b>1.8 Aim of this investigation</b>	<b>19</b>

---

---

**CHAPTER 2: ISOLATION AND CHARACTERISATION OF  
CHLOROPLASTS FROM *M. flabellifolius***

<b>2.1</b>	<b>Introduction</b>	20
<b>2.2</b>	<b>Materials and Methods</b>	27
2.2.1	Plant Material	27
2.2.2	Water content determination	27
2.2.3	Isolation of protoplasts	27
2.2.4	Isolation of chloroplasts and thylakoid membranes	28
2.2.5	Light microscopy	29
2.2.6	Chlorophyll fluorescence studies	29
2.2.7	Transmission electron microscopy	29
2.2.8	Polyphenolic determination	30
2.2.9	Lipid determination from chloroplasts	31
2.2.10	Protein extraction	31
2.2.11	Western blotting	32
2.2.12	RNA isolation	32
2.2.13	Slot blot analysis	33
2.2.14	Chlorophyll and carotenoid content	34
2.2.15	Enzyme assays	34
2.2.15.1	Ascorbate peroxidase assay	34
2.2.15.2	Glutathione reductase	35
2.2.15.3	Cu/Zn Superoxide dismutase	35
2.2.16	Glucose and fructose extraction and measurement	36
2.2.17	Anthocyanin content	36
2.2.18	Determination of content of divalent cations in chloroplasts	37
<b>2.3</b>	<b>Results</b>	38
2.3.1	Isolation, purification and ultrastructural characterisation of chloroplasts	38
2.3.2	Lipid composition of chloroplasts	46
2.3.3	Characterisation of chloroplastic proteins	46
2.3.4	Characterisation of chloroplastic genes	48
2.3.5	Western blot analysis	50

---

---

2.3.6	Photosynthetic pigments and antioxidants of isolated chloroplasts	51
2.3.7	Polyphenolics and sugar content of isolated chloroplasts	53
2.3.8	Divalent cations determination	54
<b>2.4</b>	<b>Discussion</b>	<b>57</b>

### **CHAPTER 3: INHIBITORY EFFECTS OF TRACES OF POLYPHENOLICS DURING PCR; REVERSAL BY THE ADDITION OF POLYVINYLPIRROLIDONE**

<b>3.1</b>	<b>Introduction</b>	<b>63</b>
<b>3.2</b>	<b>Materials and methods</b>	<b>66</b>
3.2.1	Plant material and growth conditions	66
3.2.2	RNA isolation	66
3.2.3	Checking quality of RNA on MALDI-TOF	67
3.2.4	RNA agarose gel electrophoresis	67
3.2.5	Transmission electron microscopy	67
3.2.6	Reverse transcription of <i>M. flabellifolius</i> RNA and amplification of the cDNA by DD-PCR	67
3.2.7	Alkaline agarose gel electrophoresis	68
3.2.8	6% denaturing gel electrophoresis	69
3.2.9	Standard PCR assay	69
<b>3.3</b>	<b>Results</b>	<b>70</b>
3.3.1	Extraction of RNA	70
3.3.2	RT-PCR of extracted RNA samples	73
3.3.3	Reversal of PCR inhibition	75
3.3.4	Confirming the inhibitory effect of polyphenolics on the PCR	77
<b>3.4</b>	<b>Discussion</b>	<b>79</b>

### **CHAPTER 4: CONSTRUCTION AND DIFFERENTIAL SCREENING OF A cDNA LIBRARY FROM DESICCATED LEAVES OF *M. flabellifolius***

<b>4.1</b>	<b>Introduction</b>	<b>81</b>
------------	---------------------	-----------

---

---

<b>4.2</b>	<b>Materials and methods</b>	<b>86</b>
4.2.1	Pedigree of bacterial strains and phage	86
4.2.2	Isolation of RNA	86
4.2.3	Selection of poly A <sup>+</sup> mRNA	86
4.2.4	First strand synthesis	87
4.2.5	Second strand synthesis	88
4.2.6	Blunting cDNA termini	88
4.2.7	Ligating <i>EcoRI</i> adaptors	88
4.2.8	Kinasing <i>EcoRI</i> ends	89
4.2.9	<i>XhoI</i> digestion	89
4.2.10	Preparation of sephacryl S-500 spin column	89
4.2.11	Size fractionation	90
4.2.12	5% Non-denaturing polyacrylamide gel electrophoresis	90
4.2.13	Quantitation of cDNA	90
4.2.14	Ligating cDNA into Unizap XR vector arms	91
4.2.15	Packaging	91
4.2.15.1	Preparation of host bacteria	91
4.2.15.2	Packaging protocol	91
4.2.16	Plating and titering	91
4.2.17	Amplification of Unizap library	92
4.2.18	Labelling of $\beta$ -actin probe using Amersham Megaprime Labelling Kit	92
4.2.19	Reverse prime cDNA labelling	93
4.2.20	Sephadex G-50 chromatography	93
4.2.21	Preparation of nitrocellulose filter replicas	94
4.2.22	Prehybridisation and hybridisation procedures	94
4.2.23	Washes and autoradiography	95
4.2.24	Identification and purification of specific clones	95
4.2.25	<i>In vivo</i> single clone automatic excision process	96
4.2.26	DNA isolation using High Pure plasmid isolation kit (Boehringer Mannheim)	96
4.2.27	PCR using T3/T7 and M13 reverse and forward primers	97
4.2.28	Sequence analysis	97

---

---

4.2.29	Recovery of single stranded DNA from pBluescript	98
<b>4.3</b>	<b>Results</b>	99
4.3.1	Construction of the cDNA library	99
4.3.2	Differential screening of the cDNA library	104
4.3.3	Expression analysis of clones isolated from the library	104
4.3.4	PCR screening of clones expressed during desiccation of <i>M. flabellifolius</i>	105
4.3.5	Sequence analysis of clones expressed during desiccation of <i>M. flabellifolius</i>	106
<b>4.4</b>	<b>Discussion</b>	107

**CHAPTER 5: ANALYSIS OF ALTERED GENE EXPRESSION  
DURING DEHYDRATION IN *M. flabellifolius*  
BY DIFFERENTIAL DISPLAY**

<b>5.1</b>	<b>Introduction</b>	109
<b>5.2</b>	<b>Materials and Methods</b>	113
5.2.1	RNA isolation and DNase treatment	113
5.2.2	Primers	113
5.2.3	Reverse transcription of mRNA	113
5.2.4	DNA polyacrylamide gel	114
5.2.5	Amplification of cDNAs	114
5.2.6	Recovery and reamplification of cDNA bands	114
5.2.7	Purification of PCR product from gel	115
5.2.8	Reverse Northern analysis	115
5.2.9	Cloning of cDNA fragments	115
5.2.10	Transformation of competent cells	116
5.2.11	DNA isolation	116
5.2.12	Sequence analysis	116
5.2.13	End-labelling of DD-PCR products	116
5.2.14	Screening of library	117
<b>5.3</b>	<b>Results</b>	118
5.3.1	Optimization of DD-PCR	118

---

---

5.3.2	Analysis of banding pattern on sequencing gel	119
5.3.3	Reverse Northern analysis	119
5.3.4	Sequence analysis of cloned fragments	121
5.3.5	Screening of cDNA library with DD-PCR products	130
<b>5.4</b>	<b>Discussion</b>	<b>131</b>

## **CHAPTER 6: RAPID AMPLIFICATION OF cDNA ENDS**

<b>6.1</b>	<b>Introduction</b>	<b>134</b>
<b>6.2</b>	<b>Materials and Methods</b>	<b>138</b>
6.2.1	Primers used	138
6.2.2	Isolation of RNA	138
6.2.3	First strand synthesis	138
6.2.4	Purification of cDNA	139
6.2.5	dC Tailing of cDNA	139
6.2.6	PCR of dC-tailed cDNA	140
6.2.7	Agarose gel electrophoresis	142
6.2.8	Purification of bands from agarose gel	142
6.2.9	Sequencing and homology searches	142
<b>6.3</b>	<b>Results</b>	<b>143</b>
6.3.1	Design of primers	143
6.3.2	First strand cDNA synthesis	143
6.3.3	GLASSMAX cDNA isolation spin cartridge purification of cDNA	145
6.3.4	dC tailing of cDNA	145
6.3.5	Amplification of target cDNA	146
6.3.6	Sequencing of 5' RACE products	148
<b>6.4</b>	<b>Discussion</b>	<b>156</b>

## **CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION**

<b>REFERENCES</b>	<b>161</b>
-------------------	------------

---

---

## CHAPTER 1

### AN OVERVIEW OF DESICCATION TOLERANCE IN ANGIOSPERMS

#### 1.1 GENERAL INTRODUCTION

Many parts of the world are subjected to fluctuations in water supply, with approximately one third of the land mass considered to be arid or semi-arid (Ramanathan, 1988). This therefore implies that plants experience water stress at some stage during their life cycle. Some plants cope with this form of stress by being desiccation tolerant. A desiccation tolerant plant is one whose vegetative tissue can survive almost complete dehydration (to approximately 13% relative water content [RWC]) (Gaff, 1971; 1977; Gaff & Ellis, 1974). Based on global climatic and environmental changes, Ramanathan (1988) predicted that crops that are drought tolerant would become an important requirement in the near future. Understanding the mechanisms of desiccation/drought tolerance is therefore of utmost importance for horticultural and agricultural purposes. Much work has been done in this field and several genes that are implicated in water stress tolerance have been isolated from crop plants subjected to water stress. Although much is known about stress tolerance in crop plants, not much progress has been made to improve their drought tolerance. This could be because most crops have a limited capacity for drought tolerance (Oliver & Bewley, 1997). A possible solution is to study the mechanisms of desiccation tolerance in plants that are desiccation tolerant. Genes can be isolated from these plants and ultimately crop plants can be transformed with them.

---

---

## 1.2 WATER: AN IDEAL BIOLOGICAL SOLVENT

The unique physical and chemical properties of water makes it the most important biological solvent. It determines the structure of biomolecules, provides a fluid environment which allows for diffusion of substrates (and hence reactions to occur) as well as providing a milieu which helps maintain the structure of organelles. Being an incompressible fluid, it can fill any gap and space within cells and hence gives cells structural support. Because of its lone pair of electrons and H-atoms, water is able to form hydrophilic interactions with other molecules. However, water is also indirectly involved in hydrophobic interactions since the lattice structure of water is such that one molecule of water is H-bonded to four other water molecules hence allowing the formation of “clathrate molecules” within the water lattice. These interactions in turn stabilise macromolecular conformations and allow for the sequestering of cellular constituents. As a consequence of these strong bonding capabilities, water has a high heat of vaporisation, high heat capacity as well as a high boiling point. These characteristics allow water to remain in the liquid state at physiological temperatures. Water is often a by-product of biological reactions, although in many reactions, it is itself a reactant. Water acts as a protectant of macromolecular structure since it can inhibit deleterious reactions by preventing molecules from interacting with each other. This property makes it a regulator of metabolic reactions occurring in plants (Adams & Rinne, 1980; Clegg, 1986; Hegarty, 1978; Leopold & Vertucci, 1989; McIntyre, 1987; Vertucci & Farrant, 1995).

## 1.3 EVOLUTION OF DESICCATION TOLERANCE

Dehydration has a drastic effect on the physical and biochemical properties of reactions occurring within the cell. Different organisms are able to cope with this form of stress using different strategies. Plants respond to water stress by evasion, resistance or tolerance (Bewley & Krochko, 1981).

---

### 1.3.1 Desiccation evasion

Desiccation evaders, such as the therophytes (summer and winter ephemerals) and geophytes cope with desiccation stress by having a seasonal life cycle. Therophytes produce desiccation tolerant seeds during the dry season whilst geophytes persist as dormant buried corms, rhizomes or bulbs. It is during the moist period that these plants complete their vegetative and reproductive cycles (Bewley, 1979; Bewley & Krochko, 1981).

### 1.3.2 Desiccation resistance

These plants have adaptations that enable them to reduce the loss of water and/or increase the rate of water absorption. Succulent plants, which fall into this category, for example, have developed thick cuticles, dense pubescence, sunken stomata and water storage tissues. In addition, these plants have adopted Crassulacean acid metabolism for photosynthesis. Sclerophyllous xerophytes have xeromorphic shoots and well-developed root systems. Malacophyllous species (soft leaved xerophytes) on the other hand, shed their leaves during periods of drought (Bewley, 1979; Bewley & Krochko, 1981; Oliver *et al.*, 1998).

### 1.3.3 Desiccation tolerance

Desiccation tolerance has been defined as the capacity of an organism or tissue to regain vital metabolism after almost complete dehydration (Gaff, 1971; 1977). This happens when there is prolonged absence of exposure to water resulting in dehydration occurring at a cellular level. Desiccation tolerant species are those that are able to withstand this stress without incurring any permanent damage. They are often referred to as anhydrobiotes (Bewley & Krochko, 1981; Crowe *et al.*, 1992). In plants, desiccation tolerance is more common in the lower order species (algae, bryophytes, ferns) than in higher plants (Bewley, 1979; Stewart & Bewley, 1982). This could be related to the size and complexity of these latter organisms. The lack of vascular tissue in lower plants together with their small size and ability to absorb water through their thalli and gametophytes allow rapid rehydration of their tissues.

---

The majority of higher plants have lost their ability to endure severe water stress since they have a more complex cellular composition with bigger cells, and a vascular system. The vegetative parts of these plants are usually intolerant of even partial dehydration.

Most of the desiccation tolerant angiosperms (termed resurrection plants) are small herbs or shrubs, although some angiosperms can reach up to 1.5 metres, e.g. example *Xerophyta eglandulosa* (Gaff, 1971) and *Myrothamnus flabellifolius* (Sherwin & Farrant, 1996). Desiccation tolerant plants experience different rates of hydration and dehydration depending upon the water status of the environment (Oliver & Bewley, 1997). “Fully desiccation tolerant” plants are those that can withstand total loss of free protoplasmic water at any rate while those that can survive water stress only when the rate of water loss is slow have been termed “modified desiccation tolerant” (Oliver *et al.*, 1998). Hence in algae, bryophytes and lichens (many species of which are fully desiccation tolerant), the rate of water loss can be extremely rapid, with the organisms reaching air dryness within an hour. Angiosperms and ferns, on the other hand, which are modified desiccation tolerant plants achieve slow drying rates by a range of morphological and physiological mechanisms that retard the rate of water loss (Oliver *et al.*, 1998). Desiccation tolerance has been reported in 60-70 species of ferns as well as in at least 60 species of angiosperms (Bewley & Krochko, 1981). Gymnosperms, on the other hand, do not produce any desiccation tolerant species. However, many gymnosperms and angiosperms produce desiccation tolerant propagules in the form of pollen, dormant buds, zygotic embryos and seeds (Bewley, 1979; Bewley & Oliver, 1992; Crowe *et al.*, 1992, 1997a; Hong *et al.*, 1996; Levitt, 1980a; 1980b; Vertucci & Farrant, 1995).

Seeds from various plant species are able to withstand severe desiccation and are often referred to as orthodox seeds (Vertucci & Farrant, 1995). Their longevity is dependent on their storage conditions, amongst which the moisture content and the temperature seem to be the most important factors (Ellis & Roberts, 1980; Ellis *et al.*, 1990; Roberts, 1973). Plant species that grow in temperate climates where there is a period of unfavourable weather are those that produce orthodox seeds. In contrast, plants growing in aquatic or wet tropical habitats produce recalcitrant seeds which are

---

desiccation sensitive (Roberts *et al.*, 1984).

Desiccation tolerance is a feature that is not unique to plants. It also occurs in bacteria as well as in more complex organisms such as fungi, tardigrades and yeast. It is believed that desiccation tolerance in plants has evolved at least on twelve different occasions (Oliver & Bewley, 1997) which would imply that the mechanisms of tolerance differ from species to species. The dry organism can stay in this state for decades or even centuries under cold and dry conditions. However, at the onset of favourable conditions, this state is readily reversed and vital metabolism is resumed. A dormant state during desiccation tolerance might appear disadvantageous for the organism since there is low productivity and no growth. However, this mechanism of protection against water stress does confer several advantages. Since it allows a suspension of metabolic activity, it therefore provides tolerance to extreme temperatures as well (Leopold & Vertucci, 1987).

#### 1.4 RECENT THEORIES OF DESICCATION TOLERANCE

It has been noticed that all desiccation tolerant organisms accumulate large amounts of osmolytes while drying. Large amounts of glycerol, sugars, especially trehalose have been found in dry fungal spores, the cysts of brine shrimps, slime moulds, yeast cells (Crowe *et al.*, 1987) and anhydrobiotic nematodes (Madin & Crowe, 1975). Orthodox seeds, on the other hand, accumulate an abundance of sugars such as raffinose, stachyose and sucrose (Amuti & Pollard, 1977; Caffrey *et al.*, 1988; Horbowicz & Obendorf, 1992; Koster & Leopold, 1988) while sucrose is the major soluble carbohydrate present in pollen (Hoekstra *et al.*, 1992). It is thought that these polyhydroxyl compounds including glycerol, sugar alcohols and other low molecular weight molecules replace the structural water of cellular components thereby preserving integrity of the cell structure during desiccation. They do this by reducing the “proximity effects” and stabilising the dehydrated cell by inhibiting metabolism (Clegg, 1986). In this regard, polyhydroxyl compounds have been found to stabilise membranes and proteins (Crowe *et al.*, 1987). This protective effect of sugars has led to the “*water replacement hypothesis*” (Crowe, 1971; Crowe *et al.*, 1992; Womersley, 1981) that suggests that the hydroxyl groups of sugars substitute for water by

---

hydrophilic interactions upon removal of water in desiccation tolerant cells.

Another hypothesis suggested to account for desiccation tolerance is the “*glass formation hypothesis*”. Glass formation or vitrification is characteristic of concentrated solutions of sugars and has several properties that are relevant to desiccation tolerant organisms (Burke, 1986). Perhaps the most important property is that these “solidified liquids” retain molecular disorder by filling spaces in the dry tissue previously occupied by water. These glasses replace “bound water” and reduce tissue collapse and therefore the damage caused by interactions between the different cell components brought about by a change in solute concentration. Glasses offer resistance to desiccation since they have a lower water vapour pressure than crystalline solids. They also have a higher viscosity which prevents chemical diffusion and promotes metabolic dormancy and hence stability (Burke, 1986). In desiccation tolerant cells, the high viscous or vitreous glassy state may serve to inhibit disruption of cellular membranes, denaturation of cytoplasmic proteins and deterioration caused by the accumulation of free radicals. Glass formation has been shown to occur in the cytoplasm of soybean and *Arabidopsis thaliana* seeds (Williams & Leopold, 1989; Wolkers *et al.*, 1998a), in pollen from *Typha latifolia* (Buitink *et al.*, 1996), and in leaves of *Craterostigma plantagineum* (Wolkers *et al.*, 1998b).

## 1.5 DAMAGE RESULTING FROM CELLULAR DEHYDRATION

### 1.5.1 Damage to the membrane structure

Membranes are very prone to dehydration and hydration damage since the turgor pressure varies with the water content. As the cell shrinks or swells, the membranes are subjected to tension and may rupture, especially when they are attached to or bound by a cell wall (Iljin, 1957). Removal of water also causes an increase in the concentration of cellular solutes and an increased viscosity of the cytoplasm (Vertucci & Farrant, 1995) as well as the production of free radicals. The deleterious effects of free radicals are manifested primarily in cellular membranes where peroxidative reactions result in lower levels of fatty acid saturation and the formation of fatty acids and lipid hydroperoxides (Dhindsa & Matowe, 1981). This leads to malfunction of the

---

membranes and eventually to an extensive leakage of cytoplasmic components either during dehydration or rehydration of the tissue (Senaratna & McKersie, 1983; Senaratna *et al.*, 1985; Tetteroo *et al.*, 1996). Although membranes are a primary site of injury, the cascade of unregulated reactions during free radical attack may also affect proteins and nucleic acids (Dizdaroglu, 1991; Wolff *et al.*, 1986).

The basic structure of a membrane can be described as a bilayer of lipids and proteins with the hydrophobic hydrocarbon chains of the lipids facing inwards while the hydrophilic head groups are positioned on either side of the bilayer. It has been proposed (Simon, 1974) that when the RWC drops to below 20%, the membrane structure becomes disordered and hence the phospholipid bilayer is replaced by a hydrophobic hexagonal II arrangement. This type of organisation was first described by Luzzati & Husson (1962) as consisting of water cylinders enclosed in a lipid matrix with the hydrocarbon chains radially orientated outwards from the central axis of the cylinders. The impermeable membrane becomes porous causing leakage of solutes and a loss of internal compartmentalisation (Bewley & Krochko, 1981). Upon rehydration, the proteins and lipid molecules reconstitute the membrane bilayer (Simon, 1974). Very little evidence exists to prove the presence of a hexagonal configuration in membranes of desiccation tolerant species. Studies carried out on dry seeds (McKersie & Stinson, 1980; Senaratna *et al.*, 1985), on pollen (Priestley & DeKruiff, 1982) and on desiccation tolerant plants, e.g. the moss *Tortula ruralis* (Singh *et al.*, 1984), show that the lipid bilayer still exists at water contents below 18%. However, it has recently been shown that there is a lamellar-to-hexagonal II phase transition in the plasma membrane of *A. thaliana* during freeze-induced dehydration (Steponkus *et al.*, 1998).

Another hypothesis based on lipid phase transitions (Crowe *et al.*, 1983; 1986; 1992; 1996; 1997b) has been postulated when membranes are subjected to water stress. Mixtures of polar lipids and water are considered to be polymorphic in that they can assume various organised structures. Biological membranes are often associated with a lamellar liquid crystalline structure. There is two-dimensional order although the acyl chains exist in a disorderly state. At low temperatures the lamellar phase is obtained. The molecules are packed more tightly and the acyl chains are highly

ordered. A lipid bilayer that comprises only one phospholipid species can be characterised by its  $T_m$ , the transition temperature from the liquid crystalline to the gel phase. In a cell membrane, however, the situation is more complicated since there is a mixture of different lipids, sterols and proteins which therefore cause complex phase transition behaviour.  $^2\text{H-NMR}$  (nuclear magnetic resonance) studies have shown that, in water, the phospholipid headgroups are surrounded by a hydration shell consisting of 11-16 water molecules per lipid (Borle & Seelig, 1983). The hydration shell surrounding the lipid head groups prevents fusion to occur between the different lipid bilayers. Upon dehydration, the hydration shell is removed causing a decrease in the intermolecular distance between the different head groups (Stefanov *et al.*, 1992; Stevanovic *et al.*, 1992). This results in increased Van der Waals interactions between the lipid tails and an increased  $T_m$ .

### 1.5.2 Damage to proteins and nucleic acids

Most enzymatic proteins require the presence of an aqueous medium as water is needed as a donor and/or acceptor of hydrogen bonds. Moreover, water forces apolar amino acids to the interior of a protein and hence has a great influence on the tertiary structure. Removal of the hydration shell of proteins results in extensive conformational changes (Kuntz & Kauzmann, 1974; Labrude *et al.*, 1987; Prestrelski *et al.*, 1993; Sanches *et al.*, 1986) which may be either reversible (Griebenow & Klibanov, 1995) or totally irreversible (Prestrelski *et al.*, 1993). Dehydration followed by subsequent rehydration causes a severe loss of activity for several proteins (Hanafusa, 1969).

Water stress results in the production of free radicals which in turn cause oxidative damages to the cell. The damage ranges from oxidation of the sulphhydryl groups of proteins leading to denaturation of the proteins, lipid peroxidation and free fatty acid accumulation in membranes (McKersie, 1991; Smirnov, 1993) to causing nucleic acids damage (Donaldson *et al.*, 1996, Leopold *et al.*, 1994). A loss in water also causes progressive action of endo and exo-nucleases and the resulting damage, together with other DNA lesions cannot be repaired until water becomes available again (Boubriak *et al.*, 1997; Pammenter & Berjak, 1999).

---

## **1.6 PROTECTIVE MECHANISMS AGAINST WATER STRESS AND DESICCATION**

Dehydration of a living cell has a drastic effect on cellular and macromolecular structure (Crowe *et al.*, 1997b). Both dehydration induced physical and chemical changes cause irreparable changes in desiccation sensitive tissues resulting in cell death. The physical changes include a large change in cellular volume, an increase in the concentration of cellular solutes and an increased viscosity of the cytoplasm (Vertucci & Farrant, 1995). The removal of water from membrane lipids, proteins and nucleic acids causes a physical change in the structure and function in the cell (Crowe *et al.*, 1997b), while chemical changes involve the production of free radicals. Plants which are truly tolerant of desiccation therefore have to overcome several challenges (Sherwin & Farrant, 1996; Vertucci & Farrant, 1995). Since this thesis research will involve an angiosperm resurrection species, the mechanisms of desiccation tolerance in lower order resurrection plants will not be reviewed here. Angiosperm resurrection plants have to cope with desiccation stress by their ability to (1) minimise the mechanical damage associated with loss in turgor; (2) maintain the integrity of macromolecules and membranes via the accumulation of water stress proteins (dehydrins) and osmolytes and (3) minimise toxin accumulation and free radical damage. These criteria can be simplified into two basic components by which desiccation tolerance can be achieved: the ability to cope with mechanical stress and the ability to cope with metabolic stress.

### **1.6.1 Minimizing mechanical damage associated with loss of turgor**

Mechanical stress is the result of shrinkage of the large central vacuole (due to water loss) and the cell contents are thus drawn inwards. This causes tension between the plasmalemma and the more turgid cell wall. Once the plasmalemma is ruptured, cell death occurs (Iljin, 1957). Desiccation tolerant tissues overcome the problem of mechanical stress by a number of strategies which prevent collapse of the plasmalemma. In seeds, vacuoles are filled with storage proteins and vast quantities of insoluble reserves in the form of carbohydrates and lipids are accumulated in their

---

cytoplasm. Farrant and Sherwin (1998) have also observed the replacement of water in vacuoles during dehydration in the resurrection species *Xerophyta viscosa* and *Xerophyta humilis*. The nature of the replacement substance is as yet unknown. Other resurrection species such as *Craterostigma* spp. cope with this mechanical stress by extensive folding and collapse of their cell wall upon dehydration (Farrant & Sherwin, 1998; Sherwin, 1995). This would imply that, instead of rupturing their plasmalemma, the cell wall itself folds inwards. Vicre *et al.* (1999) have shown that there is an increase in the xyloglucan and unesterified pectin levels in *Craterostigma wilmsii* when the RWC drops to below 30% which could contribute to maintenance of the folded cell wall in order to maintain the integrity of the cell during rehydration.

### **1.6.2 Minimizing metabolic stress**

Angiosperm resurrection plants cope with metabolic stress resulting from dehydration using several strategies, all of which help minimise the requirement for cellular repair on rehydration. Desiccation tolerant tissues also switch off metabolism during drying, believed to be a strategy to prevent damage from unregulated metabolism (Farrant & Sherwin, 1998; Pammenter & Berjak, 1999). Metabolic quiescence is achieved by dedifferentiation of organelles and through morphological changes in certain resurrection plants (Farrant, 2000; Farrant & Sherwin, 1998). Protection of the subcellular milieu in these plants is, however, achieved by the accumulation of proteins, osmolytes and antioxidants.

#### ***1.6.2.1 Accumulation of water stress proteins***

LEA (late embryogenesis abundant) proteins are small hydrophilic proteins whose mRNAs accumulate in seeds during maturation and also in vegetative tissues under conditions of water, heat, salt and chilling stress as well as in the presence of the stress response hormone, abscisic acid (ABA) (Bewley *et al.*, 1983; Cai *et al.*, 1995; Close *et al.*, 1989; Cohen & Bray, 1990; Dure *et al.*, 1989; Galau *et al.*, 1986; Galau & Close, 1992; Imai *et al.*, 1996; Ismail *et al.*, 1999; Kiyosue *et al.*, 1994; Mundy & Chua, 1988; Russouw *et al.*, 1995). From the amino acid sequences of these proteins, six different LEA gene families have been defined (Bray, 1993). The LEA group I

---

proteins are Lys-rich and therefore can bind water through dipole-dipole interactions and hence help maintain the shell of hydration during desiccation (Galau *et al.*, 1986; Litts *et al.*, 1991). The LEA group II proteins (dehydrins) have a characteristic 15 amino acid conserved motif (EKKGIMKIKEKLPG) (Bray, 1993) which has a high propensity to form an alpha helix and therefore help maintain structural integrity (Whitsitt *et al.*, 1997). Group III LEA proteins (D-7 family) are noted for their tandem repeat of 11 amino acid motif (TAQAAKEKAGE) (Bray, 1993; Xu *et al.*, 1996) and this family of proteins is thought to help in ion sequestration (Dure, 1993; Dure *et al.*, 1989). Group IV LEA proteins (D-113 family) are thought to be also involved in maintaining membrane integrity via the formation of a shell of hydration just like the group I LEA proteins (Bray, 1993). Group V LEA proteins (D-29 family) are postulated to sequester ions. It is believed that the side chains of these proteins mimic water molecules and hence maintain a shell of hydration around membranes (Bray, 1993; Wolkers *et al.*; unpublished results).

Several small heat shock proteins (smHSPs) have also been classified as LEA Group VI proteins, including HSP 12 in yeast (Mtwisha *et al.*, 1998). These proteins, thought to be involved in the acquisition of thermotolerance (Lee *et al.*, 1995), fall into four different categories depending on the gene sequence. In comparison with the LEA proteins which have been found to be localised in the cytoplasm (Close *et al.*, 1993; Russouw *et al.*, 1997), these smHSPs have been found in the cytoplasm as well as in chloroplasts, the endoplasmic reticulum, in mitochondria and also with other membranous structures (Helm *et al.*, 1993; Lenne & Douce, 1994; Vierling, 1991; Waters *et al.*, 1996). Some LEA proteins function as chaperones to maintain membrane integrity during desiccation and also help in the condensation of chromatin during desiccation (Pammenter & Berjak, 1999). Another recent role for these proteins in their involvement with carbohydrates to form a stable glass (Wolkers *et al.*, unpublished results). However, only three LEA proteins have thusfar been purified. These are the LEA group II dehydrin protein (Ceccardi *et al.*, 1994), the LEA group I Em protein from wheat germ (Espelund *et al.*, 1992) and the group I p11 protein from pea axis (Russouw *et al.*, 1997).

---

### 1.6.2.2 Accumulation of osmolytes

Plants produce large amounts of osmolytes which help maintain structural integrity by stabilising both membranes and proteins. Farrant & Sherwin (1998) have suggested that the accumulation of oligosaccharides may be a result of monosaccharide removal and propose that it is this ability to remove monosaccharides which is vital for the survival of organisms during dehydration. It is also believed that a lowering in the amount of monosaccharides results in a reduction of respiratory substrates which in turn leads to metabolic quiescence and hence protection in the dehydrated state (Farrant & Sherwin, 1998). Furthermore it was suggested that monosaccharides could play a role in the Maillard-type reactions which would be detrimental to protein structure especially at low water levels (Vertucci & Farrant, 1995).

Two properties of sugars are considered to be responsible for their stabilising effect on membranes and proteins. These are (1) the ability of sugars to form a direct interaction with proteins through hydrogen bonding in the dried state (Crowe *et al.*, 1987), replacing the original hydrogen bonds of water (water replacement theory) and (2) via the formation of a highly viscous glassy state of the sugar-protein mixture. Below the characteristic glass transition temperature,  $T_g$ , glasses have solid-like properties. When the viscosity rises to above  $10^{12}$  Pa, there is very low mobility of molecules and hence proteins are immobilised and kept in their native state (Franks *et al.*, 1991; Levine & Slade, 1992). Whereas trehalose seems to be the carbohydrate that is accumulated in anhydrobiotes in the animal kingdom, sucrose is the major saccharide that accumulates in desiccation tolerant higher plants. Pollen grains may contain up to 25% of their dry weight in the form of sucrose (Hoekstra *et al.*, 1992). In seeds, however, besides the presence of sucrose, there is accumulation of other oligosaccharides and cyclitols (Amuti & Pollard, 1977; Brenac *et al.*, 1997; Horbowicz & Obendorf, 1994; Koster & Leopold, 1988). However, sucrose seems to be the predominant sugar in desiccated tissues of higher plants especially in the resurrection species *C. plantagineum* (Alamillo & Bartels, 1996; Ingram *et al.*, 1997) and *Boea hygroskopica* (Bianchi *et al.*, 1993). In *M. flabellifolius*, it has been shown that there is an increase of trehalose and arbutin (a glycosylated hydroquinone) during desiccation (Bianchi *et al.*, 1993). The accumulation of trehalose seems anomalous especially as this sugar is known to be very rare in higher plants (Gussin, 1972) and

has been shown to be toxic to plants (Caffrey *et al.*, 1988; Veluthambi *et al.*, 1981). To date it has been found only in *Selaginella* (Kander, 1967) and in the two primitive ferns *Botrychium lunaria* (L.) Sw. and *Ophioglossum vulgatum* (Forst.) (Kander & Senser, 1965; Kander, 1967).

Studies carried out by various groups of workers have shown that trehalose is a better protectant than sucrose (Crowe *et al.*, 1987; Leslie *et al.*, 1995; Ooms *et al.*, 1994). The “water replacement theory” cannot explain the superior protecting efficiency of trehalose compared with sucrose. However, trehalose has a significantly higher glass transition temperature than most sugars including sucrose. Studies carried out on myoglobin has shown that trehalose prevents protein collapse and could be due to increased viscosity or retention of “internal water molecules”. The surface hydration layer and a few internal water molecules confer stability and flexibility of proteins. Once water molecules are removed, the internal mobility of the protein diminishes. However, in the presence of trehalose, it was demonstrated that there was no myoglobin collapse. This would imply that trehalose prevents the loss of “internal water molecules” (Sastry & Agmon, 1997). These internal water molecules together with the surface hydration water are very important for the preservation of internal mobility of a protein and the integrity of the membrane. It also seems that the geometry of the sugar is very important for the interaction with the phospholipid bilayer and it has been shown that trehalose forms more favourable interactions than sucrose (Chandrasekhar & Gaber, 1988).

An increase in proline content during desiccation has also been noticed in bacteria, algae, crustaceans (Aspinall & Paleg, 1981) and higher plants (Büßis & Heineke, 1998; Schwacke *et al.*, 1999; Tymms & Gaff, 1979; Yoshida *et al.*, 1997). It has been shown, for example, that proline is the most abundant amino acid in pollen from petunia and tomato as well as from many grass species (Bathurst, 1954; Schwacke *et al.*, 1999; Zhang *et al.*, 1984). Proline can be oxidised to glutamate providing a source of energy, nitrogen and amino groups for the re-establishment of metabolic processes. Proline is soluble in water and is thought to act as an osmoticum or as a sink for nitrogenous compounds during desiccation. Many of these compounds, for example, ammonium ions, can inhibit cell metabolism upon rehydration. Proline has also been

---

implicated in the detoxification of free radicals (Aspinall & Paleg, 1981; Saradhi & Saradhi, 1991).

ABA is also implicated in desiccation tolerance in seeds as well as in resurrection plants (Bewley & Oliver, 1992; Chandler & Robertson, 1994; Farrant & Sherwin, 1998; Vertucci & Farrant, 1995). ABA regulates stomatal closure during dehydration and hence has a protective role in drought resistance (Mittelheuser & van Steveninck, 1969). In addition, it plays an important role in the induction of genes (Ingram & Bartels, 1996) including those encoding for LEA proteins (Dure *et al.*, 1989; Kermodé, 1990) which are known to confer protection during desiccation. It has thus been shown that ABA induced genes are expressed during dehydration in *C. plantagineum* (Piatkowski *et al.*, 1990).

Many desiccation tolerant plants are also known to accumulate lipid droplets upon desiccation (Gaff, 1989). These lipids are thought to be storage products and provide energy for metabolic preparations prior to anhydrobiosis. This energy helps maintain metabolism during the desiccated phase while during rehydration, it is used for the revival of the cell (Womersley, 1981). Lipid droplets may also maintain spatial separation of organelles thus preventing damage due to “proximity effects” (Levitt, 1980a; 1980b).

### 1.6.3 Minimizing free radical damage

In hydrated tissues, the production of free radicals is controlled by anti-oxidants such as ascorbic acid, glutathione (GSH), carotenoids and enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (AP) and catalase (Oliver & Bewley, 1997). The levels of these enzymes have been found to increase several-fold during water stress. Thus, in *T. ruralis*, a desiccation tolerant moss, the levels of these enzymes were found to increase during slow dehydration and there was a concurrent decrease in lipid peroxidation. Studies carried out on resurrection angiosperms showed that during dehydration the levels of SOD and glutathione reductase (GR) increased markedly in *C. wilmsii* while in *X. viscosa*, there was an increase in the levels of GR, SOD as well as AP (Sherwin & Farrant, 1998). Polyphenolics (such as anthocyanins

---

and carotenoids) are also implicated in preventing damage caused by the production of free radicals (Larson, 1988; Rice-Evans *et al.*, 1996) and will be discussed in greater detail later in the thesis.

## 1.7 RESURRECTION PLANTS AS MODELS TO STUDY DESICCATION TOLERANCE

Resurrection plants are desiccation tolerant and can tolerate extreme water loss or desiccation. Most resurrection plants grow in shallow soils in rocky outcrops where there is limited water and shade. These plants have therefore evolved different mechanisms which allow them to grow under these conditions. Since these plants have the ability to survive near complete dehydration and revive from an air-dried state (Gaff, 1971; 1989), they therefore provide excellent physiological systems for investigating the mechanisms of desiccation tolerance. Resurrection plants have been divided into two distinct classes, homoiochlorophyllous and poikilochlorophyllous plants which retain and lose their chlorophyll on drying respectively (Hamblen, 1961; Tuba *et al.*, 1993; 1994). Poikilochlorophyllous resurrection plants, besides losing their chlorophyll, also dismantle their thylakoid membranes during dehydration (Dace *et al.*, 1998; Hallam & Luff, 1980; Sherwin & Farrant, 1996; Tuba *et al.*, 1994). It has been postulated (Farrant & Sherwin, 1998; Sherwin & Farrant, 1998) that this could be a protective mechanism to prevent photo-oxidation under conditions when photosynthesis is not possible. During rehydration, the photosynthetic apparatus is fully recovered after a few hours. Such a response could be classified as a repair-based one (Oliver & Bewley, 1997). Homoiochlorophyllous plants, on the other hand, presumably have alternative mechanisms to protect themselves against photo-oxidation or might have repair mechanisms to control any damage caused by photo-oxidation. It has thus been shown in various homoiochlorophyllous resurrection species that, upon dehydration, the leaves fold possibly to reduce light-chlorophyll interactions (Farrant & Sherwin, 1998; Sherwin & Farrant, 1998). A large increase in the anthocyanin level in certain resurrection species has been reported (Sherwin & Farrant, 1996). This might also be a protective mechanism against photo-oxidation. It has been found that an increase in sugar levels correlates with an increase in anthocyanin both of which would help protect the plant against desiccation (Casper *et*

---

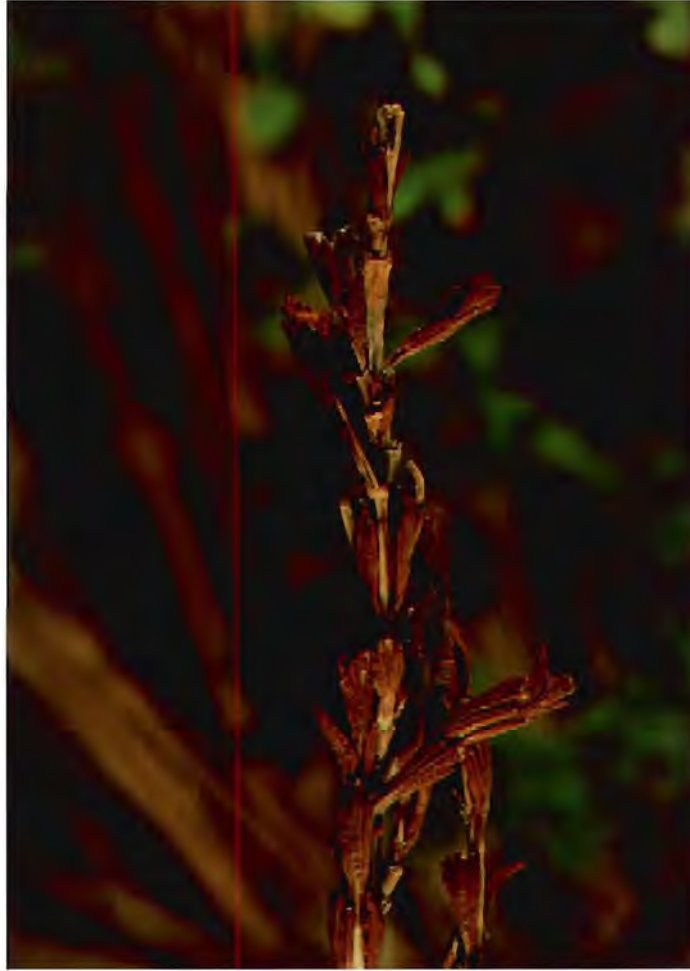
*al.*, 1986).

### 1.7.1 *Myrothamnus flabellifolius* Welw.

*Myrothamnus flabellifolius* Welw. is one of the few desiccation tolerant woody angiosperms which is able to withstand prolonged periods of desiccation without incurring damage (Genckel & Pronina, 1969; Sherwin & Farrant, 1996; Sherwin *et al.*, 1998). This species belongs to the family Myrothamnaceae of which there is a single genus, *Myrothamnus* Welw, but two different species *Myrothamnus flabellifolius* Welw. (Child, 1960) and *Myrothamnus moschatus* Baill. (Mendes, 1978). The latter is indigineous to Madagascar. The distribution, habitat and ecology of *M. flabellifolius* have been described in detail earlier (Child, 1960; Dyer, 1975; Mendes, 1978; Ross, 1972). *M. flabellifolius* Welw. is a multi-stemmed homoiochlorophyllous shrub that grows singly or in clusters throughout the dry summer rainfall regions of Southern Africa. In conditions of limiting water, the plant can lose up to 95% of its RWC. This is accompanied by the loss of approximately 50% of the chlorophyll content whilst the chloroplasts are maintained in an intact state (Sherwin & Farrant, 1996).

The retention of chlorophyll in the dry state may have severe consequences for plants growing under high light conditions since free radical subcellular damage (Navari-Izzo *et al.*, 1995; Seel *et al.*, 1991; Sgherri *et al.*, 1993; 1994a; 1994b; Smirnov, 1993; Van Camp *et al.*, 1996) might be enhanced. This is discussed in more detail in chapter 2. Two protective mechanisms have been described that possibly allow *M. flabellifolius* to reduce this putative free radical mediated damage (Farrant & Sherwin, 1998; Mauve 1966; Wellburn & Wellburn, 1976). Firstly, during desiccation, the leaves fold laterally along the stem so that only the abaxial surfaces are exposed to light (Fig. 1.1). These exposed leaf surfaces become brown due to the accumulation of anthocyanins (Goldsworthy, 1992), which have been proposed to protect against light damage (Quartacci & Navari-Izzo, 1997). Upon rehydration, the leaves unfold displaying their green adaxial surfaces (Fig. 1.2). Secondly, a unique chloroplastic structure has been described (Wellburn & Wellburn, 1976) in which the thylakoid membranes are organised in a staggered conformation,

the “staircase arrangement”, which may be implicated in the protection of the chloroplasts under conditions of water deficit. Other resurrection plants such as *Borya nitida* (Hetherington *et al.*, 1982), *Sporobolus stapfianus* (Quartacci *et al.*, 1997), and *Craterostigma spp* (Schneider *et al.*, 1993; Sherwin & Farrant; 1996; 1998) have a chloroplastic ultrastructural arrangement typical of desiccation sensitive plants.



**Fig. 1.1:** Dry plant showing leaves orientated parallel to stem.



**Fig. 1.2:** Green plant showing leaves orientated perpendicular to stem.

## 1.8 AIM OF THIS INVESTIGATION

Very little is known about the mechanisms of desiccation tolerance in homoiochlorophyllous resurrection plants, especially *M. flabellifolius*. Apart from the report on its unique chloroplast structure (Wellburn & Wellburn, 1976), the only other studies carried out on *M. flabellifolius* have been on the accumulation of compatible solutes during desiccation (Bianchi *et al.*, 1993; Drennan *et al.*, 1993) and its rehydration characteristics (Sherwin & Farrant, 1996). To date all reported work on the chloroplasts from *M. flabellifolius* has been performed using leaf sections (Sherwin & Farrant, 1996; Wellburn & Wellburn, 1976) rather than isolated organelles. In the first part of this thesis, chloroplasts from *M. flabellifolius* were isolated and their properties compared with chloroplasts isolated from the resurrection plant *Craterostigma wilmsii* Engl. (Scrophulariaceae) and the desiccation sensitive plant *Pisum sativum* L. (Fabaceae).

In order to understand the molecular mechanisms underlying desiccation tolerance, it is necessary to isolate and analyse cDNA clones which are expressed under desiccation conditions. Various methodologies can be used to identify cDNA transcripts of interest. Two concurrent strategies were used here to avoid the short comings of each approach. Firstly, a cDNA library was constructed using mRNA isolated from dry leaves of *M. flabellifolius*. This library was used to isolate genes which were present only under desiccation and not rehydration conditions (discussed in detail in chapter 4). The second strategy used was differential display analysis whereby cDNA transcripts from the control and the desiccated plants were compared and transcripts which were differentially expressed in the dry plant were isolated and characterised (described in detail in chapter 5). Both these techniques have been widely used in isolating genes which are differentially expressed in plants (for example Benito *et al.*, 1996; Truesdell & Dickman, 1997; Visioli *et al.*, 1997; Yung *et al.*, 1999) and both methodologies resulted in genes which are putatively involved in conferring desiccation tolerance to *M. flabellifolius*.

---

## CHAPTER 2

### ISOLATION AND CHARACTERISATION OF CHLOROPLASTS FROM *M. flabellifolius*

#### 2.1 INTRODUCTION

The chloroplast is the photosynthetic organelle in green algae and higher plants and being responsible for the capture of light energy and synthesis of carbohydrates, plays a vital role in growth and development of plants. The photosynthetic apparatus has the ability to acclimate to ever-changing environmental stimuli such as light, temperature, availability of water, nutrients and carbon dioxide (Anderson *et al.*, 1995). Nevertheless the changes associated with desiccation (for example, changes in volume and perturbation of the chloroplast envelope), are so great that they are not accommodated by most plants, resurrection plants being the exception. Resurrection plants particularly need to develop efficient strategies to protect their chloroplasts since they are often exposed to water as well as light stress.

Presence of light during periods of dehydration and rehydration can be extremely detrimental to photosynthetically competent tissues. In some species during conditions of limiting water (even mild water stress), stomatal closure occurs. The resulting decrease in the rate of photosynthesis can lead to a high photon flux density (PFD) as the energy absorbed by the chlorophyll molecules cannot be utilized (Demmig-Adams & Adams, 1992). The continuous absorption of light by the photosynthetic apparatus together with the donation of electrons to the wrong electron acceptor can result in generation of reactive oxygen species. This is because the chloroplast electron transport chain operates in a high oxygen environment and hence the propensity of electrons to leak to oxygen forming reactive species is high. At high PFD, accumulation of excitation energy in the light-harvesting chlorophyll antennae of photosystem I and II (PS I and II) causes the formation of the highly reactive singlet excited state of oxygen ( $^1\text{O}_2$ ), which is formed through interaction with the excited triplet state of chlorophyll ( $^3\text{Chl}$ ) (Havaux & Niyogi, 1999). Upon absorption

of a photon, chlorophyll enters the excited singlet state and the excitation energy is dissipated as heat. However, in the presence of excess light, chlorophyll molecules in the singlet state reach the triplet state (Frank *et al.*, 1979). It is estimated that the oxygen concentration inside the chloroplast ranges from 275 to 300 mM, and the formation of superoxide radicals at moderate light intensities ( $350 \mu\text{E m}^{-2} \text{s}^{-1}$ ) can be as high as  $15 \text{ mmol mg}^{-1} \text{ chlorophyll h}^{-1}$  (Navari-Izzo *et al.*, 1996). Superoxide anion radicals ( $\text{O}_2\cdot^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\cdot\text{OH}$ ) are other species formed as a result of direct reduction of PS I (Demmig-Adams & Adams, 1992; Havaux & Niyogi, 1999). If unquenched, these free radicals can cause considerable damage to the tissue (Navari-Izzo *et al.*, 1995; Seel *et al.*, 1991; Sgherri *et al.*, 1993; 1994a; 1994b; Smirnov, 1993; Van Camp *et al.*, 1996). The major types of damages caused by free radicals are oxidation of protein sulfhydryl groups leading to denaturation of proteins, pigment loss and photosystem damage (often termed photoinhibition), lipid peroxidation and free fatty acid accumulation in membranes (McKersie, 1991; Smirnov, 1993). It is therefore very important for chloroplasts to adapt protective mechanisms in order to prevent/reduce photo-damage.

How do resurrection plants cope with light stress under water limiting conditions? In poikilochlorophyllous plants, the degradation of chlorophyll and dismantling of thylakoid membranes during desiccation (Hetherington *et al.*, 1982; Sherwin & Farrant, 1998; Tuba *et al.*, 1993; 1994) are thought to be protection mechanisms to prevent photo-oxidation (Sherwin & Farrant, 1998). Homoiochlorophyllous resurrection species, which retain chlorophyll, have to develop other strategies for protection against light stress. Studies carried out on some of these plants have shown that they have evolved a range of mechanisms to contend with such stress. Among these are physical strategies (changes in leaf orientation to shade chlorophyll from light), accumulation of carotenoids and anthocyanins (mask chlorophyll and act as antioxidants), changes in the chlorophyll a/b ratio and accumulation of antioxidants (eg AP, GR, SOD, catalase and enzymes to regenerate these molecules). For example, *C. wilmsii* (the most extensively studied homoiochlorophyllous plant) folds its leaves to reduce the surface area exposed to light during dehydration and the surfaces that are exposed possess a reflective cuticle to control the amount of light absorbed

(Sherwin & Farrant, 1998). In addition, carotenoids and anthocyanins accumulate in the exposed surfaces of the leaves in *C. wilmsii* and are thought to mask chlorophyll and act as antioxidants (Quartacci & Navari-Izzo, 1997; Sherwin & Farrant, 1996).

Carotenoids are thought to protect against photo-oxidative damage by being able to convert the excited states of oxygen and chlorophyll (especially singlet oxygen and triplet chlorophyll) to their corresponding ground states by forming an excited carotenoid triplet which dissipates its energy as heat (Telfer *et al.*, 1994). It has thus been shown that carotenoids are bound via Van der Waals forces to some of the chlorophylls in purple bacteria (Deisenhofer & Michel, 1991) and to chlorophyll a/b light harvesting complex of PS II (Kühlbrandt *et al.*, 1994) allowing close contact between the two different pigments for efficient electron transfer. Hydroxylation of  $\beta$ -carotene produces the xanthophyll zeaxanthin (Demmig-Adams & Adams, 1992; Pfündel & Bilger, 1994). Zeaxanthin is formed by the reduction of the diepoxide violaxanthin, forming antheraxanthin as an intermediate. Zeaxanthin is reconverted to violaxanthin by epoxidation in the presence of NADPH and  $O_2$ . Synthesis of zeaxanthin hence converts excess exciton energy (excitation energy transferred from one molecule to the next) to heat, thus protecting chloroplasts from photo-damage (Havaux & Niyogi, 1999; Young, 1991).

Anthocyanins have also been implicated in protection against light stress (Chalker-Scott, 1999; Choinski & Johnson, 1993; Drumm-Herrel & Mohr, 1982; Sherwin & Farrant, 1998) as well as other environmental stresses such as cold temperatures (McKown *et al.*, 1996; Parker, 1962), drought stress (Sherwin & Farrant, 1998; Wettstein-Westersheim & Minelli, 1962), osmotic stress (Dutt *et al.*, 1991; Kaliamoorthy & Rao, 1994) as well as having antifungal/antibiotic (Coley & Aide, 1989) and antioxidative properties (Tsuda *et al.*, 1994; 1996). Anthocyanins are photoinduced and they have been postulated to have photoprotective properties against light-induced photooxidation and/or UV damage. Anthocyanins have thus been shown to absorb harmful levels and/or wavelengths of radiation, especially high-energy blue wavelengths that can damage protochlorophyll (in developing leaves) and chlorophyll (in mature leaves) (Chalker-Scott, 1999). Anthocyanins are also

---

implicated in shielding dehydrated plants from photoinhibition (Sherwin & Farrant, 1998). Closely related to the photoprotective property of anthocyanins are their roles in scavenging free radicals. Several studies have shown that anthocyanins are very efficient at reducing the amount of peroxidative damage of the lipid bilayer (Gabrielska *et al.*, 1999). Anthocyanins are located in the lipid bilayer where major oxidative damage is caused by free radicals entering the lipid matrix or those produced by the membranes themselves. Anthocyanins are also thought to play a role in protecting plants against water stress by decreasing the osmotic potential and water potential of leaves which together minimise water loss via evaporation and transpiration (Chalker-Scott, 1999). These pigments also cause a decrease in stomatal conductance which again help reduce water loss (Beeson, 1992; Choinski & Johnson, 1993).

Polyphenolics (including anthocyanins) have been found to afford protection to membranes during dehydration. Golovina *et al.* (1998) and Hoekstra *et al.* (1997) have shown that amphipathic molecules migrate into membranes of desiccation tolerant pollen and seed in conditions of water stress. Partitioning of amphipathic molecules into the bilayer lowers the water content at which the membrane lipids change from the liquid crystalline to the gel phase, hence maintaining integrity of the membrane. The phase change is reversible, an important property for desiccation-tolerance, since the reverse phase change must occur for membranes to be functional during rehydration.

LEA proteins are associated with desiccation tolerance and their functions have already been discussed in detail in sections 1.6.2.1 and will therefore be mentioned briefly in this chapter. Essentially LEA proteins are hydrophilic and are implicated in sequestration of ions and replacement of water molecules (Bray, 1993; Dure, 1993). They are also thought to be involved with carbohydrates to form a stable glass (Wolkers *et al.*, unpublished results). Another role of LEA proteins is their involvement in protection of chromatin in the dry state (Asghar *et al.*, 1994; Goday *et al.*, 1993). Although LEA proteins are mainly associated with seeds, they have also been found in resurrection plants (Bartels *et al.*, 1990; Kuang *et al.*, 1995; Piatkowski

*et al.*, 1990).

Another adaptation used by resurrection plants in the absence of water is the accumulation of sugars which have been shown to confer tolerance by water replacement (Close *et al.*, 1993) and/or by vitrification of the cytoplasm (Burke, 1986). The mechanisms via which sugars protect the plant under desiccated conditions have already been discussed earlier (see section 1.4 and 1.6.2.2). Basically, sugars are able to replace water molecules normally associated with membrane surfaces thereby maintaining proper lipid head-group spacing and hence preventing the lipid membrane to change from the liquid crystalline phase to the gel phase. The second possibility via which sugars exert their protective effect is their ability to form a glassy state (Koster & Leopold, 1988). When water is lost from cells, oligosaccharides (for example sucrose) form high-viscosity, amorphous saturated solutions (glasses). The presence of these glasses causes a stasis on intracellular activity. This also reduces any deleterious effects of deranged metabolism whilst preventing/minimising a phase transition of the lipid bilayer.

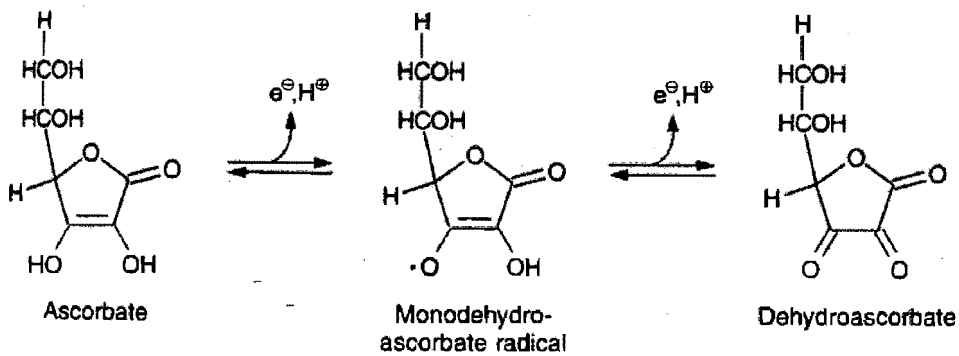
Plants produce various antioxidant enzymes to help cope with the problems associated with photo-damage and water stress. The mechanisms of three of these antioxidants (superoxide dismutase, ascorbate peroxidase and glutathione reductase) involved in protection of chloroplast are outlined below.

Superoxide dismutase (SOD) is classified according to its metal cofactor: Cu/Zn SOD, Mn SOD and Fe SOD. FeSOD is mainly found in prokaryotes while higher organisms have the other types of SOD. In this case emphasis is placed on Cu/Zn SOD as it is the one localised in the chloroplast stroma. However, the functions of all types of SOD are similar. SOD catalyses the dismutation of superoxide into hydrogen peroxide and oxygen using two protons:  $2 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$ .

Superoxide can also be removed by the action of a series of enzymes, including ascorbate peroxidase (AP) and glutathione reductase (GR) (Asada, 1994; 1996; Polle, 1996). Hydrogen peroxide also causes damage to the plant cell and is eliminated by

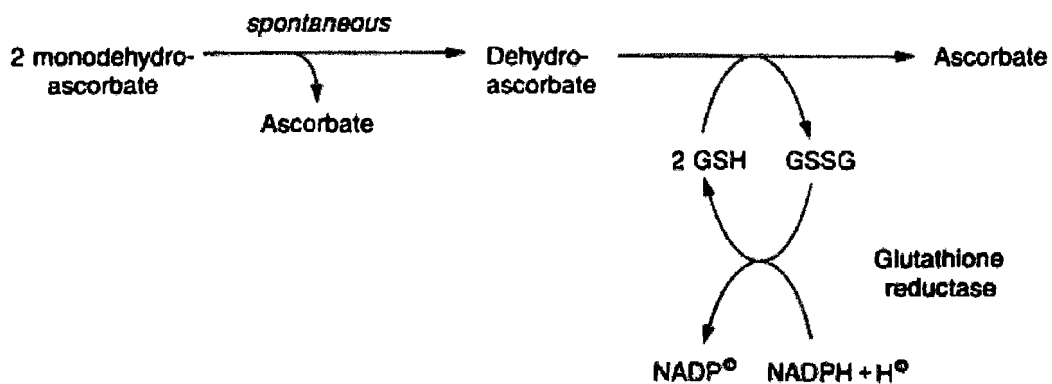
---

AP. Ascorbate, an important antioxidant in plants (located mainly in the chloroplasts), is oxidised by AP forming the monodehydroascorbate radical (Fig. 2.1), which in turn is spontaneously reconverted by PS I to ascorbate via reduced ferredoxin. Monodehydroascorbate can also be reduced to ascorbate by an NAD(P)H dependent monodehydroascorbate reductase.



**Fig 2.1:** The oxidation of ascorbate proceeds via the formation of the monodehydroascorbate radical.

Alternatively, two molecules of monodehydroascorbate can dismutate producing ascorbate and dehydroascorbate. Dehydroascorbate then reacts with glutathione to form ascorbate (Fig. 2.2)



**Fig 2.2:** Dehydroascorbate can be reduced to form ascorbate.

Glutathione (GSH), a  $\gamma$ -glutamyl cysteinyl glycine tripeptide occurs as an antioxidant in animal and plant cells. In plant cells, it has been localised to the cytoplasm,

mitochondria and chloroplasts (Bowler *et al.*, 1992). Oxidation of GSH results in the formation of a disulfide (GSSG) between the cysteine residues of the two glutathione molecules. Reduction of GSSG is catalysed by glutathione reductase using the reducing power of NADPH (Fig. 2.3). Given the destructive nature of free radicals and the fact that they are likely to occur during desiccation, it is therefore not surprising that antioxidant enzymes increase in various resurrection species during dehydration (Seel *et al.*, 1992; Sgherri *et al.*, 1994a; Sherwin & Farrant, 1998).

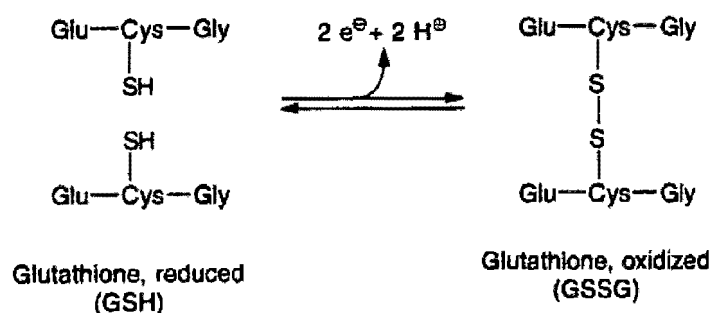


Fig. 2.3: Redox reaction of glutathione.

*M. flabellifolius* is a homoiochlorophyllous plant which would face problems associated with light and water stress. This study aimed at investigating the nature of protection used by this plant in coping with these stresses. The chloroplasts of this species have an unusual thylakoid stacking (Wellburn & Wellburn, 1976), which might be implicated in the protection of the plant under stress conditions. The changes in chloroplast ultrastructure and biochemistry during dehydration were thus investigated. In particular, changes in photosynthetic pigments, anthocyanins, polyphenolics, thylakoid and stromal proteins, sugars and cations and the activities of the antioxidants AP, GR and SOD were examined. This work has been published (Koonjul *et al.*, 1999a).

## 2.2 MATERIALS AND METHODS

### 2.2.1 Plant material

The resurrection plants *M. flabellifolius*, *C. wilmsii* and *X. humilis* were collected from the Buffelskloof Nature Reserve (Mpumalanga, South Africa). They were grown in a mixture of potting soil, river sand and peat and were maintained as described previously (Dace *et al.*, 1998; Sherwin & Farrant, 1996). Only the youngest fully expanded leaves from fully hydrated plants were used for experimental purposes. Dry leaves (5% RWC) of *M. flabellifolius* were used for transmission electron microscopy and for isolation of chloroplasts. Whole plants of this species were dried to an air-dried state by withholding water from the soil. Rose (*Rosa spp*) and African violet (*Saint paulia*) plants were purchased from a local nursery and were used immediately for chloroplast and polyphenolic isolation. Seeds of *P. sativum* were sown in trays of potting soil and were maintained in a greenhouse under the same conditions as the other plants.

### 2.2.2 Water content determination

Water content of leaf material from *M. flabellifolius* was determined gravimetrically by oven drying at 70°C for 48 h. The fresh weight of four fully hydrated leaves were taken individually (fresh weight) before subjecting them to drying (dry weight, dw). The average of the four samples was taken. It was found that the ratio of fresh weight to dry weight was 3.106:1. This ratio was used in all calculations.

### 2.2.3 Isolation of protoplasts

Leaves from *M. flabellifolius*, *C. wilmsii* and *P. sativum* were surface sterilized in 70% (v/v) ethanol for 2 min then in a 1:20 solution of sodium hypochlorite and Tween 20 for another 15 min. The leaves were then washed thoroughly in double distilled water and the waxy outer cuticle scraped off using a sterile scalpel. The leaves were then cut into very fine strips (5 mm<sup>2</sup>) and incubated for 18 h in the dark in an osmoticum containing 0.6 M mannitol, 0.25 g/500 ml MES, pH 5.8 supplemented

---

with 0.2% (w/v) macerozyme (Sigma) and 0.5% (w/v) cellulase (Sigma). The enzyme concentration was increased by 10-fold in the case of *M. flabellifolius* since no apparent digestion was observed. The released protoplasts were isolated from the osmoticum solution by overlaying the latter on a 2 ml cushion of 0.6 M sucrose (per 10 ml osmoticum) and the solution centrifuged at 5000 g for 10 min in a Beckman swing-out SW 65 T<sub>1</sub> rotor. The layer of protoplast was carefully removed and washed 3 times in osmoticum alone. An extract of the alimentary canal of locusts was used as a source of digestive enzyme for *M. flabellifolius* when the cell wall could not be degraded even with an increased concentration of enzyme. The protoplasts were lysed in water to release chloroplasts.

#### 2.2.4 Isolation of chloroplasts and thylakoid membranes

A modification of the protocol described by Kut & Flick, 1986 was used to isolate chloroplasts from fully hydrated leaves of *M. flabellifolius*. Leaves were ground on ice using a mortar and pestle in isolation medium (0.35 M mannitol, 5 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 50 mM Tris-HCl pH 8.0) supplemented with 1% (w/v) caffeine, 1% (w/v) soluble PVP (w/v) poly[1-vinylpyrrolidone-2], 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 5 mM ascorbic acid, 0.1% (v/v) PMSF. Caffeine and PVP are known to bind phenolics and tannins and/or prevent the formation of oxidation products of endogenous phenolics (Anderson, 1968; Koonjul *et al.*, 1998; 1999b; Loomis, 1974; Wilkins & Smart, 1996). Mg<sup>2+</sup> and Mn<sup>2+</sup> were also added to the isolation buffer since it has been reported that these cations are essential for the isolation of intact chloroplasts (Cerovic & Plesnicar, 1984). The ground leaf preparation was filtered through sterile cheesecloth (four layers) and the crude filtrate was then centrifuged at 1000 g to remove cellular debris and nuclei. The supernatant was then loaded onto a sugar gradient which was centrifuged for 1 hour at 81 000 g. A variety of sugar gradients in 25 mM EDTA, 50 mM Tris-HCl pH 8.0 buffer was utilised. Amongst these were discontinuous sucrose gradients consisting of 17 mls 30% (w/v) and 8 mls 52% (w/v) sucrose, discontinuous sucrose gradients consisting of 17 mls 52% (w/v) and 8 mls 80% (w/v) sucrose and linear 30% (w/v) to 100% (w/v) trehalose gradients. Chloroplast bands were carefully removed from the gradient by aspiration and the sugar removed by washing 5 times with 25 mM EDTA, 50 mM Tris-HCl pH 8.0.

Thylakoid membranes were prepared from chloroplasts samples which were lysed for 30 min at 4°C in 10 mM Tris-HCl pH 7.4, 150 mM KCl (Sgherri *et al.*, 1993). The sample was centrifuged at 12 000 g for 20 min and the pellet resuspended in 10 mM Tris-HCl pH 7.4, 150 mM KCl, 1 mM EDTA. The suspension was applied to a 52% (w/v) to 100% (w/v) linear sucrose gradient in this same buffer.

### 2.2.5 Light microscopy

Chloroplasts were routinely checked by light microscopy using a Leitz Diaplan microscope with Hoffman modulating contrast optics.

### 2.2.6 Chlorophyll fluorescence studies

The activity of PS II in isolated chloroplasts was measured using chlorophyll fluorescence techniques using a modulated OS-500 portable fluorometer (Opti-Sciences, USA). Chloroplasts in isolation buffer were dark adapted by maintaining in foil wrapped container for 5 mins. They were then exposed to a saturating light intensity of 4 mmol photons  $m^{-2}s^{-1}$  for 1 s. The initial ( $F_0$ ) and maximum ( $F_M$ ) fluorescence values were recorded.  $F_V$  was obtained by subtracting  $F_0$  from  $F_M$  and  $F_V/F_M$  was calculated. This value corresponds to the quantum efficiency of isolated chloroplasts (Lichtenthaler & Miehe, 1997).

### 2.2.7 Transmission electron microscopy (TEM)

Chloroplasts used for TEM were isolated in chloroplast isolation buffer containing HEPES instead of Tris-HCl. Tris-HCl was replaced with HEPES because of incompatibility with glutaraldehyde fixation. Chloroplasts were centrifuged briefly after isolation and resuspended at 4°C for 16 h in 1 ml fixative which consisted of chloroplast isolation medium containing 3% (v/v) glutaraldehyde. Leaf segments (5  $mm^2$ ) from fully hydrated (100% RWC) leaves from the three species and also dry (5% RWC) leaves (5 different leaves per treatment) of *M. flabellifolius* were also fixed in 3% (v/v) glutaraldehyde. The fixed chloroplast preparation was then carefully transferred to a clean slide onto which some low melting agarose (1% w/v) was

---

added. The agarose/chloroplast mixture was allowed to cool down to 27°C after which the agarose was cut into small blocks (5 mm<sup>2</sup>). The material was post-fixed in 1% (v/v) osmium tetroxide for 1 h. The samples were then washed in buffer for 5 min after which they were dehydrated in a graded ethanol series. The dehydration steps consisted of 10 min in 30% (v/v) ethanol, 10 min in 50% (v/v) ethanol, 10 min in 70% (v/v) ethanol containing 2% (w/v) uranyl acetate, 10 min in 70% (v/v) ethanol, 10 min in 80% (v/v) ethanol, 10 min in 90% (v/v) ethanol, 10 min in 95% (v/v) ethanol, 3 x 10 min in 100% ethanol and finally for 2 x 15 min in 100% acetone. The samples were infiltrated with epoxy resin (Spurr, 1969) as follows: 1:1 acetone (100%): resin for 18 h., 3:1 (resin: 100% acetone) for 8 h, 100% resin for 18 h, 100% resin for another 8 h. The samples were then polymerised for 16 h at 80°C, after which they were sectioned at a gold interference (95 nm) with a Reichert Ultracut-S microtome. Sections (at least 5 per sample) were stained in 2% (w/v) uranyl acetate and 1% (w/v) lead citrate (Reynolds, 1963) and viewed with a Jeol 200 CX transmission electron microscope.

### 2.2.8 Polyphenolic determination

Polyphenolic compounds from isolated chloroplasts (not purified by sugar gradient centrifugation to prevent loss of material) and whole leaves were assayed after thioglycolic acid derivatisation. Leaves were ground in 10 ml methanol and chloroplasts were suspended in methanol. The samples were filtered through Whatman 3MM paper, rinsed with 5 ml methanol and air dried for 1 h. The samples were further dried for 1.5 h at 60°C. 50 mg of each dried sample was boiled for 4 h in 5 ml 2 M HCl containing 0.5 ml thioglycolic acid (Schneiderbauer *et al.*, 1991). Acid-soluble derivatives were qualitatively determined at this stage, the amount of polyphenolic was proportional to the degree of red coloration of the sample. Alkali-soluble derivatives were extracted by centrifuging the thioglycolate extracts at 30 000 g for 10 min after which the supernatant was discarded. The pellet was washed with 5 ml water before being resuspended in 5 ml 0.5 M NaOH. This mixture was agitated gently at 25°C for 18 h before being centrifuged at 30 000 g for 10 min. 1 ml of concentrated HCl was added to the supernatant which was allowed to precipitate at 4°C for 4 h. The pellet obtained after centrifugation was dissolved in 1 ml 0.5 M

NaOH and the amount of alkali-soluble polyphenolics determined spectrophotometrically at 280 nm (Bruce & West, 1989). A commercial polyphenolic extract from green tea (Sigma) was used as a control to quantitate the amount of polyphenolics in the different plant species.

### **2.2.9 Lipid determination from chloroplasts**

Chloroplasts (not purified by sucrose gradient centrifugation) were boiled for 5 min in isopropanol after which lipids were extracted. Lipids were extracted from the chloroplasts by incubation in chloroform: methanol (2:1) for 2 h at 4°C (Sgherri *et al.*, 1993). 0.02% (w/v) butylhydroxytoluene was added as an antioxidant. The bottom chloroform layer, containing the lipids, was evaporated under nitrogen. The lipids were analysed using gas-liquid chromatography (Dr M. Smith, University of Natal, Pietermaritzburg, South Africa).

### **2.2.10 Protein extraction**

Protein extraction from isolated chloroplasts, isolated thylakoids and total leaf tissue was carried out as described by Russouw *et al.*, 1995. The material was ground in insoluble PVP (ratio of material:PVP was 2:1) before proteins were extracted using an isolation medium consisting of 100 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM PMSF pH 7.5 (ratio of material:buffer was 1:5). This medium was supplemented with 1% (w/v) soluble PVP. Extracted proteins were electrophoresed on 20% SDS-PAGE gels containing 0.1% N, N'-methylene bis-acrylamide (Laemmli, 1970). Gels were routinely silver stained (Wray *et al.*, 1981).

### **2.2.11 Western blotting**

The protein gel was transferred to a protein free tray and rinsed in transfer buffer (0.19 M glycine, 20% methanol (v/v), 25 mM Tris-HCl, pH 8.5) before being subjected to capillary transfer (Harlow & Lane, 1988). The gel was laid on pre-soaked nitrocellulose paper (Schleider & Schuell) which was supported by 3 pieces of pre-soaked Whatman 3MM paper cut to the same size as the gel. Transfer was allowed to

---

proceed at 4°C for 18 h at a constant current of 200 mAmps after which the membrane allowed to air dry for at least 15 min. The membrane was then incubated in PBS blocking buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4) containing 5% (w/v) skim milk powder for at least 1 h at room temperature. The primary antibody was added to the buffer, which was left at 4°C for 18 h. The membrane was then washed in PBS buffer containing 1% (v/v) Tween before the secondary antibody (diluted in PBS buffer) was added. The secondary antibody was allowed to react for 30 min, membrane then washed in PBS containing 1% Tween (v/v) before being developed. The washes were as follow: 5 min, 15 min and another 2 x 5 min. The blot was developed using a chemiluminescent detection system (Amersham). Membranes were routinely checked by staining a duplicate blot with 1% (w/v) amido black. Antibodies to the p11 Group I LEA protein were prepared as described (Russouw *et al.*, 1995; 1997). Antibodies to Group II LEA proteins and *C. plantagineum* dsp 21 and dsp 34 proteins were the kind gifts of Prof. T. Close (Department of Botany and Plant Sciences, University of California, USA) and Prof. D. Bartels (Fuer Zuenchtungsforschung, Max-Planck Institute, Koeln, Germany) respectively.

### 2.2.12 RNA isolation

Total RNA from dehydrated and hydrated leaves of *M. flabellifolius* was prepared using several protocols including the TriPure Kit (Boehringer Mannheim), the guanidium thiocyanate protocols (Chomczynski & Sacchi, 1987; Chirgwin *et al.*, 1979). However no RNA could be isolated from *M. flabellifolius* although RNA could be successfully isolated by all methods tested from *P. sativum*. RNA could be isolated using the plant mini Qiagen RNeasy kit; however, the yield was low (Koonjul, 1999). Several protocols were therefore combined (Ainsworth, 1994; Bahloul & Burkard, 1993; Bruce & West, 1989; Soni & Murray, 1994) to give a methodology, which allowed for the extraction of RNA from *M. flabellifolius*. In this modified method, 100 mg fresh weight of leaf tissue was frozen in liquid N<sub>2</sub> and ground to a fine powder in a pre-cooled mortar. The powder was extracted with 0.5 ml extraction buffer, 100 mM Tris-HCl pH 8.6 containing 100 mM NaCl, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 2% PVP and 100 µg/ml proteinase K. PVP was included to interact with released polyphenolics, 2-mercaptoethanol was included to

---

inactivate RNases by disulphide bond disruption (Chirgwin *et al.*, 1979) and to inhibit the free-radical dependent cross-linking of phenolics to nucleic acids (Wilkins & Smart, 1996). SDS was added as a mild denaturing agent as well as to inhibit RNase activity (Soni & Murray, 1994) and proteinase K was added to degrade enzymes involved in the oxidation of polyphenolics and secondary metabolites (Hall *et al.*, 1978; Soni & Murray, 1994; Wan & Wilkins, 1994; Wilkins & Smart, 1996). Following centrifugation at 4°C for 2 min at 10 000 g, the supernatant was extracted twice with an equal volume of phenol:chloroform:isoamylalcohol (24:24:1) and RNA was precipitated at -20°C after the addition of isopropanol supplemented with 0.8 M sodium citrate and 1.2 M NaCl. The RNA pellet was washed with 70% (v/v) ethanol then dissolved in DEPC water.

### 2.2.13 Slot blot analysis

Equal amounts of total RNA (1 µg) from hydrated and dry leaves were blotted onto Hybond-N membrane (Amersham) using a slot blotting apparatus (Hoeffer Scientific Instrument). The blots were cross linked using a UV crosslinker (Amersham). After prehybridisation of the blots in DIG Easy Hybridisation solution (Boehringer Mannheim) at 42°C for 30 min, 25 ng of DIG labelled probe (random priming) was added per ml solution and hybridisation was performed for 20 h at 42°C. Detection and washes were carried out as per the DIG manual (Boehringer Mannheim) using the chemiluminescent detection system. The intensity of the signals were compared using a Macbeth TD 901 densitometer. The oligo probes for the *rbcS* (small subunit of rubisco), *lhc* (chlorophyll a/b binding protein) were the kind gifts of Dr S. Mackerness (Horticulture Research International, Warwick, UK), while the *rbcL* and 18 S rRNA were the kind gifts of Dr V. Abratt and Mr W. Mpoloka (Department of Microbiology, University of Cape Town, South Africa).

### 2.2.14 Chlorophyll and carotenoid content

Photosynthetic pigments were extracted from leaf samples in 100% acetone. The absorbance of the extracts was measured at 470, 644.8 and 661.6 nm. Total

---

chlorophyll (chlorophylls a and b) and total carotenoids (xanthophylls and carotene) content was calculated using the adjusted extinction coefficient values according to Lichtenthaler (1987). The following formulae were used for the quantitation of the different pigments:

$$\text{Chl}_a = 11.24 A_{661.6} - 2.04 A_{644.8}$$

$$\text{Chl}_b = 21.13 A_{644.8} - 4.19 A_{661.6}$$

$$\text{Chl}_{a+b} = 7.05 A_{661.6} + 19.09 A_{644.8}$$

$$C_{x+c} = (1000 A_{470} - 1.90 \text{Chl}_a - 63.14 \text{Chl}_b) / 214$$

where  $\text{Chl}_a$ ,  $\text{Chl}_b$ ,  $\text{Chl}_{a+b}$ ,  $C_{x+c}$  are concentrations of chlorophyll a, chlorophyll b, total chlorophyll (a+b) and total (xanthophylls + carotenes) respectively. Results are presented as mg per gram dry mass.

## 2.2.15 Enzyme assays

### 2.2.15.1 *Ascorbate peroxidase (AP)*

0.05 g of leaf material (dry weight) was ground using a pre-chilled mortar and pestle in the presence of 0.1 g insoluble PVP. Material was extracted in 4-6 ml of 50 mM Tris-HCl pH 7.7 and 2 ml of freshly prepared 2 mM sodium ascorbate. The reaction mixture was centrifuged at 12 000 g for 20 min at 4°C. The activity of the supernatant was assayed at 20°C by measuring the oxidation of ascorbate at 290 nm (Wang *et al.*, 1991) using a Shimadzu UV 1601 spectrophotometer. The reaction mixture of 1 ml contained 25 mM  $\text{K}_2\text{HPO}_4$  buffer (pH 6.1), 1% (w/v) final concentration of soluble PVP, 0.2 mM sodium ascorbate, 0.1 mM  $\text{H}_2\text{O}_2$  and 0.1 ml of the supernatant. The reaction was initiated by the addition of  $\text{H}_2\text{O}_2$ . The assay was performed in triplicate and the activity of the enzyme expressed as units of activity per gram dry weight.

### 2.2.15.2 *Glutathione reductase (GR)*

0.05 g of leaf material (dry weight) was ground with 0.1 g insoluble PVP. Material was extracted in 4 ml of 1 mM  $\text{K}_2\text{HPO}_4$  buffer (pH 7.5) containing 0.4 mM EDTA

and 9.94 mM sodium ascorbate. The extraction mixture was centrifuged for 20 min at 12 000 g at 4°C and the GR activity of the supernatant assayed by measuring the oxidation of NADPH at 340 nm (Dalton *et al.*, 1986) using a Shimadzu UV 1601 spectrophotometer. The reaction mixture (1 ml) contained 0.3 M K<sub>2</sub>HPO<sub>4</sub> buffer pH 7.5, 1% (w/v) soluble PVP, 3 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.15 mM NADPH, 10 mM GSSG and 0.1 ml of the crude enzyme extract. The reaction was started by the addition of GSSG. The assay was repeated four times and activity of the enzyme expressed in units per gram dry weight as well as in nmol min<sup>-1</sup> g dw<sup>-1</sup>.

#### 2.2.15.3 *Cu/Zn Superoxide dismutase (SOD)*

0.02 g (dry weight) of ground leaf tissue was extracted in 50 µl of 0.1 M potassium phosphate buffer pH 7.5, containing 0.1 M mM EDTA, 0.01 g insoluble PVP, 1% (w/v) BSA. The extract was filtered through a Whatman GF/A glass fibre filter (Malan *et al.*, 1990). SOD activity was assayed by measuring the inhibition of nitrite formation from hydroxyl ammonium chloride oxidation at 530 nm (Elstner & Heupel, 1976). Units of activity were calculated from a standard curve obtained by treating a known range of concentrations of SOD from xanthine oxidase as described above. One unit of SOD is defined as the amount needed to cause 50% inhibition of cytochrome c reduction (McKord & Fridovich, 1969). The assay was repeated four times and SOD activity was expressed as units per gram dry weight.

#### 2.2.16 Glucose and Fructose extraction and measurement

Chloroplasts were isolated from the dry and the hydrated leaf of *M. flabellifolius* as described earlier (section 2.2.4), but were not purified by sucrose gradient centrifugation. The chloroplasts were then resuspended in 60 % (v/v) ethanol containing 0.1 M NaOH. The samples were incubated at 95°C for 5 min before being centrifuged for 20 min at 16 000 g. The extracts were neutralised to pH 7-8 by the addition of 100 mM HEPES/100 mM acetic acid. The presence of glucose and fructose was then assayed using the Boehringer Mannheim sugar food kit analysis according to the manufacturer's instructions except that PVP (final concentration of 1% w/v) was also added to the cuvette. The principle of the analysis was based on the

---

methodology described by Bergmeyer & Bernt (1974). Glucose concentration was measured from a coupled assay system which involved the addition of hexokinase (ATP: D-hexose-6-phosphotransferase) to produce Glc-6-P (D-glucose-6-phosphate). The Glc-6-P was then converted to 6-phospho-gluconate following the addition of Glc-6-P dehydrogenase. The NADP present in the reaction was reduced to NADPH and the latter was measured at 340 nm using a Shimadzu UV 1601 spectrophotometer. The production of NADPH was stoichiometrically equivalent to the amount of glucose present. Following the determination of glucose, the amount of fructose present in the chloroplasts samples were assayed by the addition of HPI (D-hexose-6-phosphate-ketol isomerase). This enzyme converts Fru-6-P (D-fructose-6-phosphate) to Glc-6-P. The change in absorbance resulting from the reduction of NADP is equivalent to the amount of fructose present. The amount of glucose/fructose present in the samples were expressed in nmoles per gram dry mass.

### 2.2.17 Anthocyanin content

5 mg (dry mass) of lyophilised leaf material was extracted in 10 ml of acidified methanol (methanol:water:HCl) [79:20:1] for 48 h at 4°C. The extracts were then centrifuged at 10,000 g for 20 minutes and the supernatant was then made up to 13 ml by the addition of acidified methanol. The absorbance was measured at 530 nm and 657 nm and the concentration of anthocyanin was determined using the formula  $A_{530} - 0.33 A_{657}$ . Results are presented as mg per gram dry mass (Sherwin & Farrant, 1998).

Proanthocyanidins (cyanidin and delphinidin) were assayed from chloroplasts isolated from wet and dry *M. flabellifolius* leaves using the protocol of Bate-Smith, 1981. The concentration of cyanidin and delphinidin were quantitated by spectrophotometric readings at 547 nm and 558 nm respectively. The extinction coefficient values were 150 and 300 for cyanidin and delphinidin respectively (Bate-Smith, 1981). Gallotannins from chloroplasts isolated from wet and dry *M. flabellifolius* leaves were qualitatively assayed by adding potassium iodate to the aqueous chloroplast sample. The red coloration was indicative of the presence of gallotannins (personal communication, Professor T. Yoshida, University of Okayama, Japan).

### 2.2.18 Determination of content of divalent cations in chloroplasts

Chloroplasts were isolated from the hydrated and the dehydrated leaves of *M. flabellifolius* (not purified by sucrose gradient centrifugation) and the amount of divalent cations determined using a modified protocol described by Malan & Farrant, 1998. The chloroplast samples were digested in 0.1 M HCl at 180°C for 3-4 h. The solution was then made up to 10 ml of 0.1 M HCl and passed through a 0.45 micron filter to remove any plant debris before the cation analysis was performed. Chloroplastic samples were analysed for total  $Mn^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$ ,  $Fe^{2+}$  and  $Mg^{2+}$  using a Jobin Yvon JY138 Ultra-trace ICP-AES (inductively coupled plasma-atomic emission spectrometer). Extractions were performed in triplicate and internal replicates done.

---

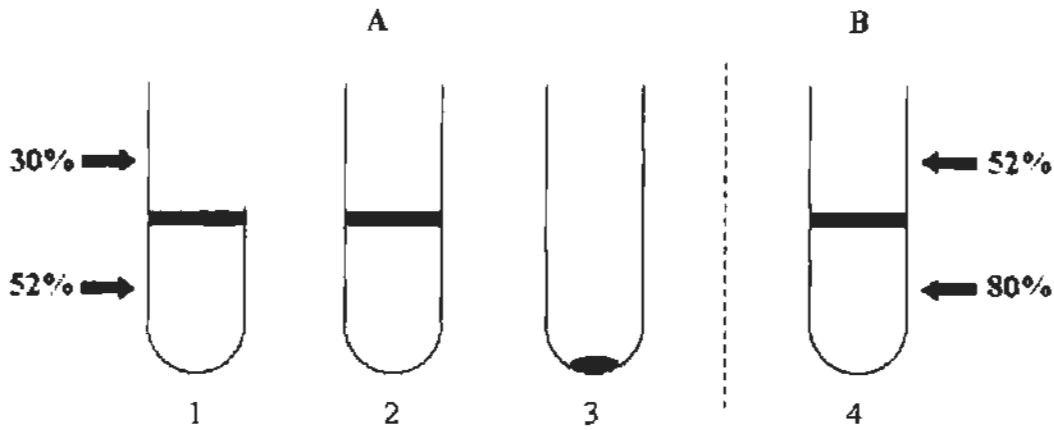
## 2.3 RESULTS

### 2.3.1 Isolation, purification and ultrastructural characterisation of chloroplasts

Initially protoplasts preparations were attempted. No digestion of the leaf tissue from *M. flabellifolius* was obtained even after increasing the cellulase and macerozyme concentrations ten-fold over those normally used and scraping off the waxy cuticle (Johnson *et al.*, 1989). This would suggest that either the cell wall is particularly impervious, possibly a requirement for the plant to survive extreme conditions of drought, or that the cell walls had no substrates for the enzymes used. Since locusts eat a large variety of different indigenous flora, an extract of locust digestive tracts was used as a source of digestive enzymes. No digestion of the leaf was again observed. The control samples from *P. sativum* and *C. wilmsii* yielded viable protoplasts from which chloroplasts were successfully isolated under both digestion conditions.

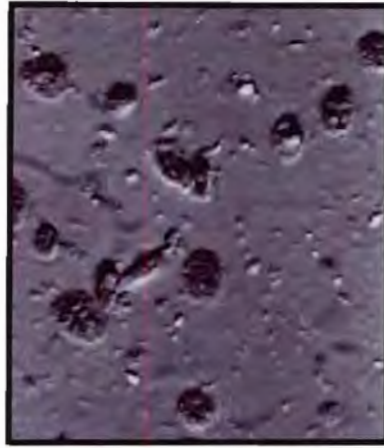
Chloroplasts from hydrated leaves of *M. flabellifolius* could however, be isolated using a modification of the protocol of Kut & Flick (1986) (refer to methods section). Purification of the crude chloroplast preparation from *M. flabellifolius* on a discontinuous 30% (w/v)/ 52% (w/v) sucrose gradient resulted in the chloroplasts sedimenting at the bottom of the tube (Fig. 2.4A). In contrast, chloroplasts from *P. sativum* and *C. wilmsii* banded at the gradient interface. However, purified chloroplasts from *M. flabellifolius* banded at the interface after the gradient was changed to 52% (w/v)/ 80% (w/v) sucrose (Fig. 2.4B). This suggests that either *M. flabellifolius* chloroplasts have a higher buoyant density than those from *P. sativum* and *C. wilmsii* or that artifactual aggregation occurred during their isolation. Aggregation could occur through partial damage of the chloroplastic membrane due to the presence of polyphenolic compounds released from other intracellular compartments (e.g. from vacuoles) during the extraction process resulting in hydrophobic interactions (Vertucci & Farrant, 1995). Alternatively, polyphenolic-mediated cross-linking of membrane components (Anderson, 1968; Loomis, 1974; Wilkins & Smart, 1996) could cause aggregation thus increasing the density of the chloroplasts. Such “di-chloroplasts” or “poly-chloroplasts” would have an increased density – cf. separation of nucleosomal species by density gradient centrifugation (Von Holt *et al.*, 1989).

---

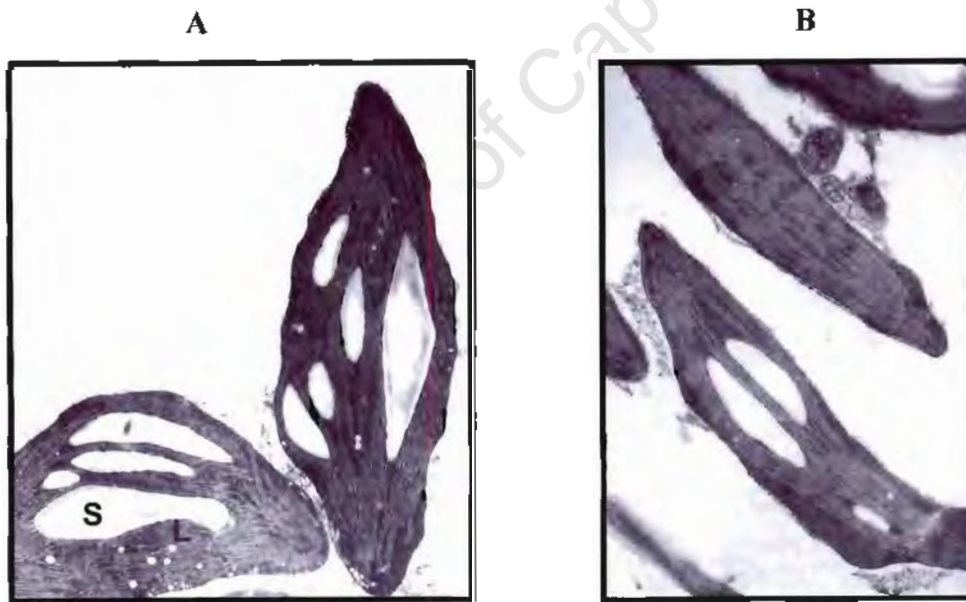


**Fig. 2.4:** A. 30%/52% discontinuous sucrose gradient used for the purification of chloroplasts. Samples 1 & 2 represent *P. sativum* and *C. wilmsii* respectively while samples 3 and 4 both represent *M. flabellifolius*. B. 52%/80% sucrose gradient resulted in banding of chloroplasts of *M. flabellifolius* at the interface.

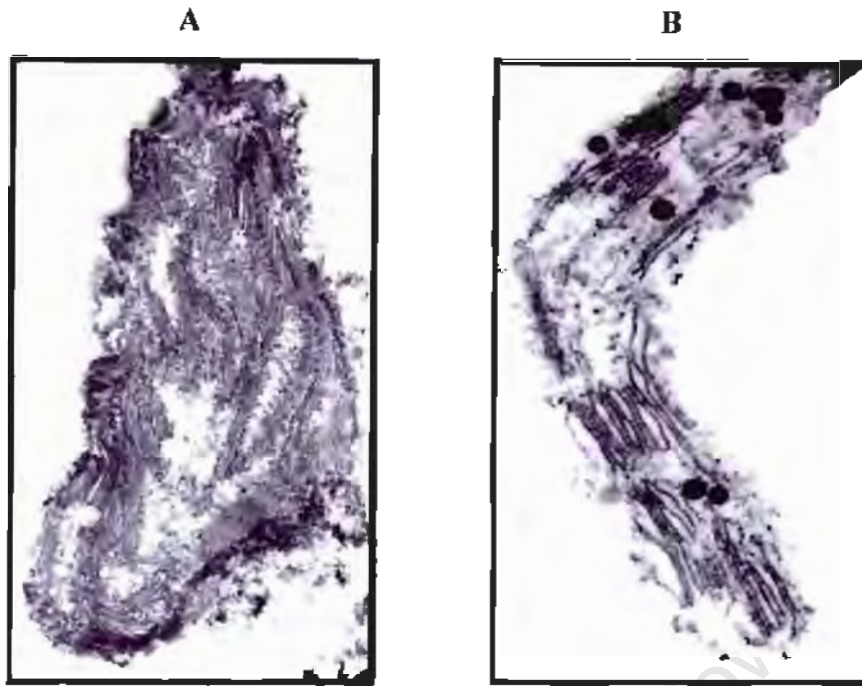
To test the former hypothesis, the material banding at the 52%/ 80% interface after centrifugation was examined by light microscopy and TEM. Material banding at the 30% (w/v)/52% (w/v) interface after centrifugation of *P. sativum* and *C. wilmsii* crude chloroplast preparations, was used as controls. Light microscopy showed that the preparations from all three species were very similar and no aggregation was observed (Fig. 2.5). Ultrastructure studies showed that chloroplasts isolated from *P. sativum* (Fig. 2.6A) appeared identical to those present in intact leaves (Fig. 2.6B). The outer envelope was intact and the thylakoid arrangement was typical of photosynthetically active tissue. In contrast, chloroplasts isolated from both resurrection plants showed considerable damage (loss of outer membrane and blistering of the thylakoid membranes) (Fig. 2.7). No chloroplast aggregation was evident although disruption of aggregates might have occurred during the procedures required for examination under TEM.



**Fig. 2.5:** Light microscopy showing chloroplasts of *M. flabellifolius* (X 10 000) purified by sucrose gradient centrifugation. Chloroplasts isolated from *P. sativum* and *C. wilmsii* were identical (not shown).



**Fig. 2.6:** Electron micrographs showing (A) chloroplasts of *P. sativum* (X 20 000) purified on a discontinuous 30%/52% sucrose gradient and (B) chloroplasts in an intact leaf of *P. sativum* (X 25 000). L, lipophilic bodies; S, starch granules.



**Fig. 2.7:** Electron micrographs showing (A) chloroplasts of *M. flabellifolius* (X 28 000) and (B) *C. wilmsii* (X 22 000) purified on discontinuous sucrose gradients.

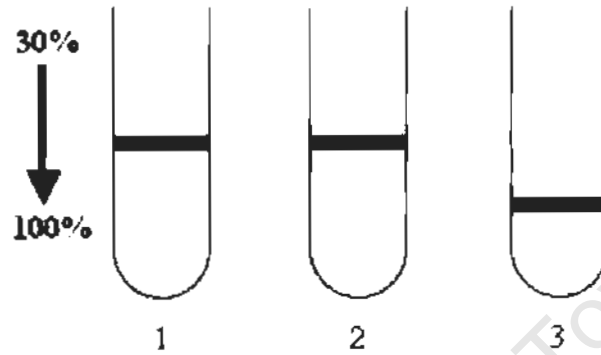
To investigate whether the increased buoyant density observed for chloroplasts isolated from *M. flabellifolius* was due to polyphenolic-mediated cross-linking, the polyphenolic content and buoyant density of chloroplasts from a number of desiccation tolerant and desiccation sensitive species were determined (Table 1). Desiccation sensitive *Rosa spp* and *Saint Paulia ionantha spp* were used because of their taxonomic relationship to *M. flabellifolius* and *C. wilmsii* respectively (Metcalfé & Chalk, 1950). *M. flabellifolius* and *Rosa spp* had the highest amounts of both alkali ( $1.525 \mu\text{g mg wet mass}^{-1}$ ) and acid-soluble polyphenolics, approximately double that of *C. wilmsii*, *S. Paulia* and *P. sativum*. The desiccation tolerant *X. humilis* had intermediate levels of polyphenolics. With the exception of *M. flabellifolius* (banding at the 52%/80% interface), the chloroplasts from the other species banded at the 30%/52% interface. These results suggest that the increased buoyant density of *M. flabellifolius* was not due to polyphenolic-mediated cross linking of chloroplast membranes.

**Table 1:** Acid-soluble polyphenolic (qualitatively based on the degree of red coloration) and alkali soluble polyphenolic contents (determined spectrophotometrically) as described (Schneiderbauer *et al.*, 1991) in various plant species. The sucrose concentrations at which chloroplasts banded are given. DT = desiccation tolerant; DS = desiccation sensitive.

Species	Alkali soluble ( $\mu\text{g.mg}^{-1}$ wet mass <sup>-1</sup> )	Acid soluble (colour intensity)	Sucrose % at interface
<i>M. flabellifolius</i> (DT)	1.525	++++	52%/80%
<i>Rosa</i> (DS)	1.525	++++	30%/52%
<i>X. humilis</i> (DT)	1.325	+++	30%/52%
<i>C. wilmsii</i> (DT)	0.675	++	30 %/52 %
<i>S. paulia</i> (DS)	0.575	+	30%/52%
<i>P. sativum</i> (DS)	0.555	+	30%/52%

Despite the use of various concentrations of PVP and caffeine, substances known to interact with endogeneous polyphenolics (Anderson, 1968; Loomis, 1974), chloroplast membranes of both resurrection species remained damaged (results not shown). A Percoll (polyvinylpyrrolidone coated silica beads) gradient was used in place of sucrose in order to rule out the possibility that the high osmolality of the sucrose gradient caused membrane damage. However, membrane damage was still evident in both resurrection plants (results not shown). Trehalose, which naturally occurs in dry *M. flabellifolius* plants (Bianchi *et al.*, 1993; Drennan *et al.*, 1993; Suau *et al.*, 1991), has been reported to afford better protection than sucrose to membranes during desiccation (Crowe *et al.* 1987; 1988; Leopold & Vertucci, 1987). Trehalose was therefore substituted for sucrose in the gradient system in an attempt to isolate intact chloroplasts from resurrection plants. The crude chloroplast preparations from

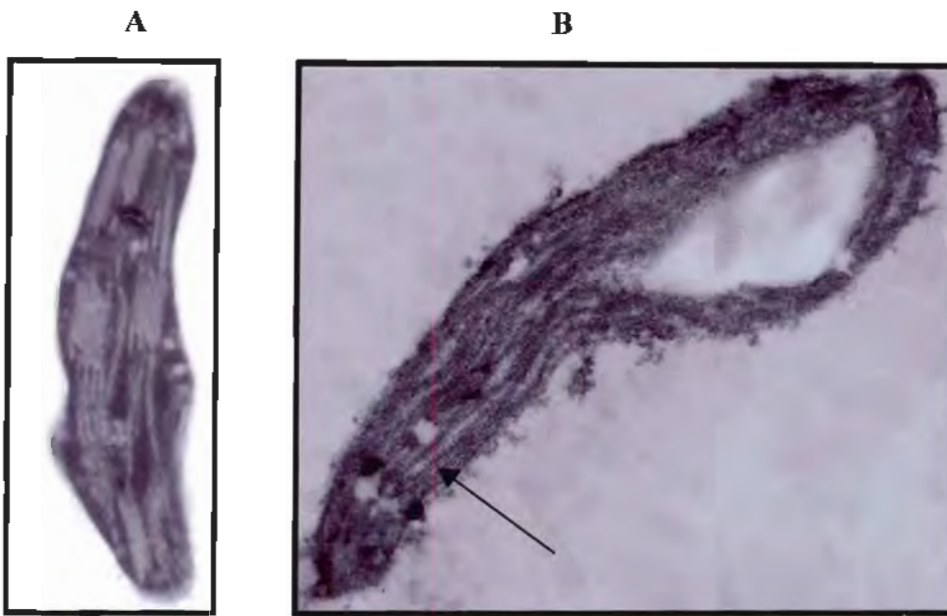
*P. sativum*, *C. wilmsii* and *M. flabellifolius* were centrifuged on 30% (w/v) to 100% (w/v) linear trehalose gradients (Fig 2.8). *M. flabellifolius* chloroplasts again banded at a higher density, approximately 75% compared with 45% for *P. sativum* and *C. wilmsii* chloroplasts confirming that the former chloroplasts indeed had a higher buoyant density.



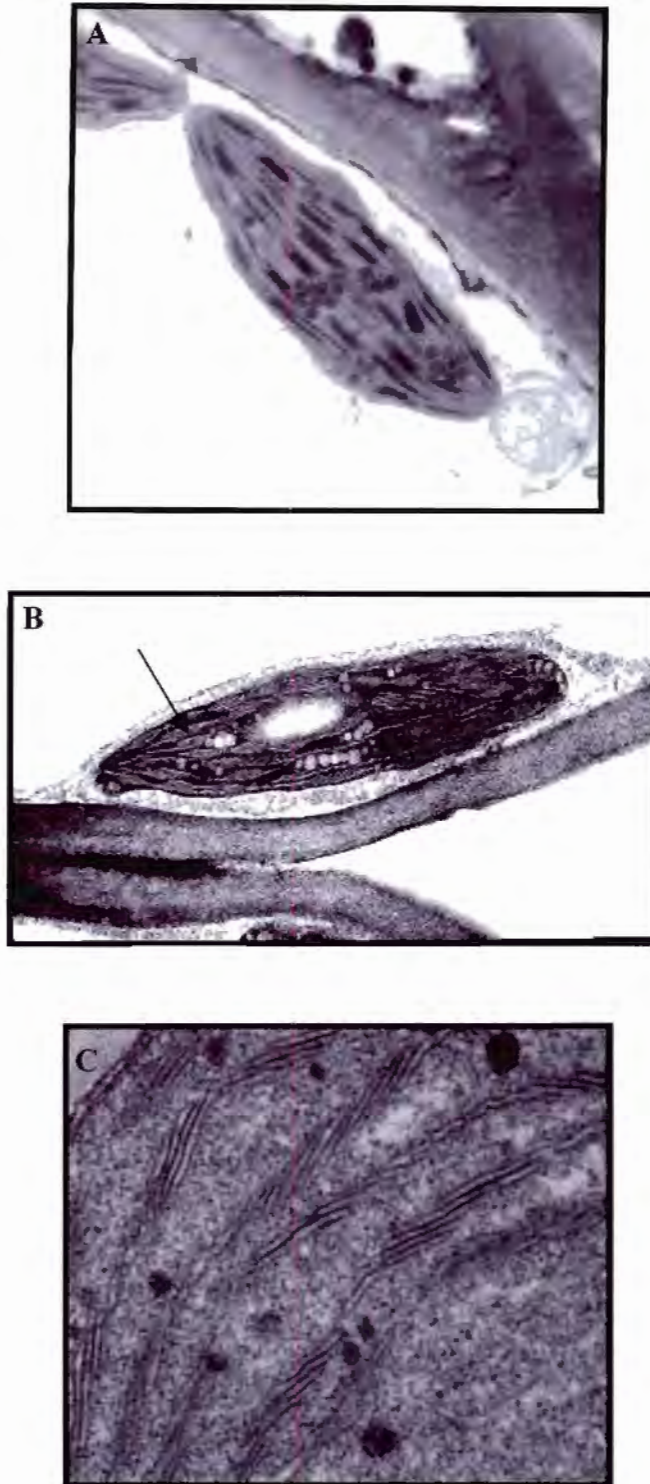
**Fig. 2.8:** Linear trehalose gradient showing the different banding pattern of chloroplasts isolated from *P. sativum* (lane 1), *C. wilmsii* (lane 2) and *M. flabellifolius* (lane 3).

Electron microscopic examination of the chloroplasts isolated from *C. wilmsii* and *M. flabellifolius* (Fig. 2.9A and 2.9B respectively) indicated that there was less damage to the chloroplasts from the resurrection plants when isolated on a trehalose gradient. Although the outer membrane was not clearly defined, thylakoid membranes were intact in both cases. Chloroplasts purified from *C. wilmsii* using trehalose gradients appeared akin to those present in whole leaf sections (Fig. 2.10A). Similarly, chloroplasts from *M. flabellifolius* had an elongated shape typical of those present in whole leaf sections (Fig. 2.10B). In the latter case, there was no evidence of chloroplast clumping which would suggest that the higher buoyant density of chloroplasts from *M. flabellifolius* is likely due to different biochemical properties. The staircase arrangement described by Wellburn & Wellburn (1976) was clearly evident in the chloroplasts (isolated preparation and leaf sections) of *M. flabellifolius*

(arrowed in figures 2.9B & 2.10B). An enlarged picture showing the staircase arrangement is shown in Fig. 2.10C.



**Fig. 2.9:** Electron micrographs showing chloroplasts isolated and purified on a linear 30% to 100% trehalose gradient from *C. wilmsii* (X 32 000) (A) and *M. flabellifolius* (X 28 500) (B).



**Fig. 2.10:** Electron micrographs showing chloroplasts from an intact hydrated leaf of *C. wilmsii* (X 17 200) (A) and *M. flabellifolius* (X 20 000) (B). The staircase arrangement of the thylakoid membranes in *M. flabellifolius* is shown in C.

Chlorophyll fluorescence was used to determine the physiological integrity of isolated chloroplasts from hydrated leaves of *M. flabellifolius*. The parameter  $F_v/F_M$ , which gives an indication of the quantum efficiency of PS II was calculated as 0.63. Although this value was slightly below the normal range of 0.7-0.8, indicative of an intact photosynthetic machinery (Lichtenthaler & Miché, 1997), the integrity of the chloroplasts from *M. flabellifolius* was considered to be essentially intact. It is known that isolated chloroplasts are very susceptible to degradation (Demmig-Adams & Adams, 1992), possibly accounting for the slightly lower value of  $F_v/F_M$  for hydrated chloroplasts.

### 2.3.2 Lipid composition of chloroplasts

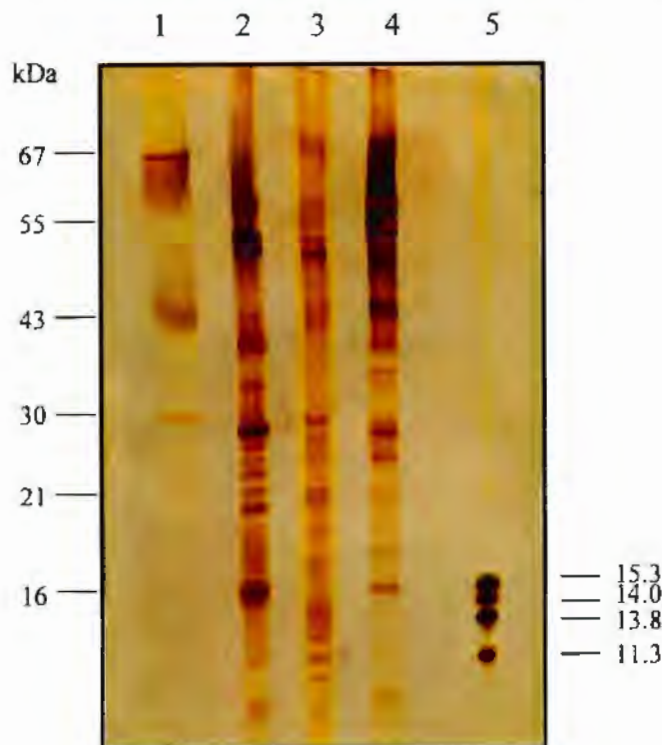
Since an altered membrane lipid composition could alter the buoyant density of chloroplasts, the lipid content of chloroplasts isolated from *M. flabellifolius* was compared with those isolated from *P. sativum* and *C. wilmsii* (Table 2). The lipid composition of chloroplasts from all three plants were similar with unsaturated fatty acids accounting for between 76% and 85% of the total fatty acids present. From this data and the correlation between density and the number of unsaturated bonds in lipid fatty acids, it is unlikely that the increased buoyant density of chloroplasts isolated from *M. flabellifolius* was due to differences in the lipid composition. On the basis of % unsaturated fatty acids/lipid composition, chloroplasts from *P. sativum* would be expected to have the highest buoyant density.

**Table 2:** Lipid composition (% w/w) of chloroplasts isolated from hydrated leaves of *M. flabellifolius*, *P. sativum* and *C. wilmsii*.

Fatty Acid	<i>M. flabellifolius</i>	<i>C. wilmsii</i>	<i>P. sativum</i>
Palmitic (16:0)	17.3	14.7	11.6
Stearic (18:0)	6.0	3.8	2.8
Oleic (18:1)	14.6	17.4	4.8
Linoleic (18:2)	26.4	26.0	18.2
Linolenic (18:3)	35.7	38.0	62.3

### 2.3.3 Characterisation of chloroplastic proteins

Total protein from chloroplasts of *M. flabellifolius*, *P. sativum* and *C. wilmsii* were separated by SDS-PAGE (Fig. 2.11). A wide variety of proteins ranging in size from approximately 10 to 97 kDa were present in all the chloroplast preparations. The banding pattern of the proteins extracted from *C. wilmsii* and *P. sativum* chloroplasts were similar and resembled those reported to occur in other angiosperm species (Kieselbach *et al*, 1998). The protein banding pattern from chloroplasts of *M. flabellifolius* was different to the other species. Prominent polypeptide bands at 55 kDa and 16 kDa (probably representing the large and the small subunits of Rubisco respectively (Keegstra & Yousif, 1986)) and several less prominent bands with molecular weights between 30 kDa and 43 kDa, present in the former two species, were absent or present in very low concentrations in extracts from *M. flabellifolius* chloroplasts. A number of unique proteins, especially in the range of 10 kDa to 15 kDa, were present in *M. flabellifolius*, but not in *C. wilmsii* and *P. sativum*.



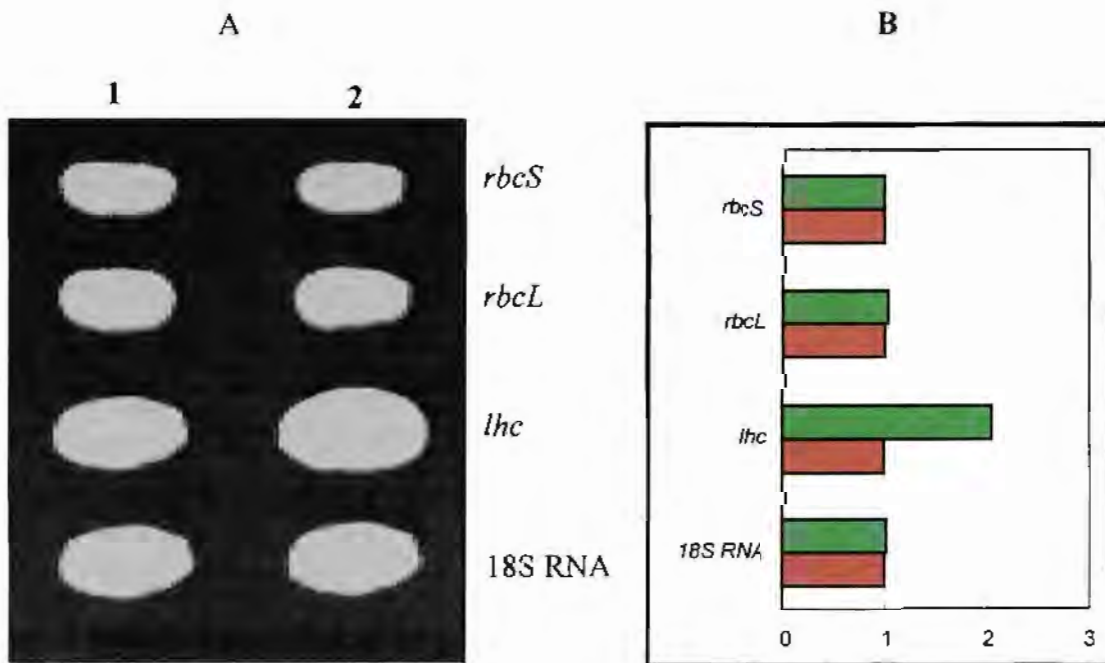
**Fig. 2.11:** Silver-stained SDS-PAGE of chloroplastic proteins. Lanes 2, 3, 4 are samples from *P. sativum*, *M. flabellifolius* and *C. wilmsii* respectively. Lanes 1 and 5 are molecular weight markers.

Thylakoid membranes, isolated by lysis of chloroplasts from the three species, were centrifuged on linear 52% (w/v) to 100% (w/v) sucrose gradients. Whereas thylakoid membranes from *P. sativum* banded at 40% (w/v) sucrose, those from *M. flabellifolius* were found to form significantly more dense bands at 83% (w/v) sucrose. SDS-PAGE separation of thylakoid membrane proteins showed that the only difference was the absence, or the considerable reduction in the quantity of proteins with molecular masses between 30 – 43 kDa in *M. flabellifolius* (results not shown). Since many of the PS II proteins have molecular weights of between 32 kDa and 47 kDa (Kieselbach *et al*, 1998), it was postulated that these proteins were present in low concentrations. The polypeptides with molecular masses from 10 kDa to 15 kDa which were present only in *M. flabellifolius* chloroplast preparations were not present in the thylakoid isolations, suggesting that they were stromal proteins. The different protein complement in *M. flabellifolius* was unlikely to cause an increased density of the thylakoid membranes.

#### 2.3.4 Characterisation of chloroplastic genes

The majority of chloroplast-localised proteins are encoded by nuclear genes. These genes are transcribed by RNA polymerase II and translated in the cytoplasm. Specific precursors target these proteins to the chloroplasts. Two important multigene families are the *rbcS* and the *lhc* genes. The *rbcS* genes encode the small subunit of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) while the *lhc* genes encode the chlorophyll a/b binding proteins associated with PS II. The *rbcL* genes, on the other hand, encoding the large subunit of Rubisco are chloroplast-encoded. The presence of transcripts corresponding to the three mentioned proteins was checked by slot blot analysis. These genes are ubiquitously present in plants but the corresponding proteins could not be seen on SDS-PAGE gels. It was found that transcripts for *rbcS*, *rbcL* and *lhc* were present in both the hydrated and dry chloroplasts (Fig. 2.12A). Densitometric measurements showed that the level of accumulation of the *rbcL* and the *rbcS* genes remained unchanged, while *lhc* declined by a factor of two during drying (Fig. 2.12B). Equal loading of the RNA was confirmed by probing the blots with 18S RNA. Thus it would appear that the proteins encoded by these transcripts

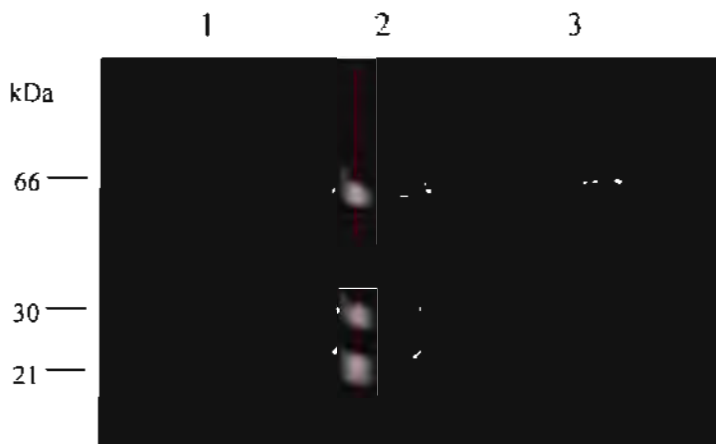
are likely to be present in *M. flabellifolius*, but were either present in low concentrations or were labile, possibly degrading during the extraction procedure. It has been well documented that chloroplastic proteins, especially *lhc* and the large and small subunits of Rubisco are prone to proteolytic degradation (reviewed in Adam, 1996). Although very little is known about the mechanisms of degradation, this might explain the protein profile obtained for *M. flabellifolius*. Although a more comprehensive study is required to test this, it is unlikely that *M. flabellifolius* differs markedly from other  $C_3$  plants in the nature of the key photosynthetic proteins.



**Fig. 2.12:** A. Chloroplastic gene expression in dry (lane 1) and rehydrated leaf tissue (lane 2) of *M. flabellifolius*. Slot blot analysis was prepared using 1  $\mu$ g total RNA per lane. 18S RNA was used to show that equal amount of total RNA per lane were loaded. B. Densitometry analysis of slot blot showing the relative intensity of the different signals. Red bars represent RNA extracted from dry leaves and green bars represent RNA extracted from green leaves.

### 2.3.5 Western blot analysis

Although LEA proteins and desiccation stress proteins (dsp) have been shown to accumulate during seed maturation (Bradford & Chandler, 1992; Close *et al.*, 1993; Finch-Savage *et al.*, 1994; Galau & Close, 1992), they have also been reported to accumulate in response to dehydration in leaf tissues of several species (Mundy & Chua, 1988). To investigate whether LEA and/or dsp proteins were present in chloroplasts isolated from *M. flabellifolius*, the protein gel (Fig. 2.11) was western blotted with a variety of antibodies specific to LEA and dsp proteins. Although antibodies against *P. sativum* LEA group I (prepared according to Russouw *et al.*, 1995; 1997) and II proteins failed to detect any immunopositive bands, an antibody against *C. plantagineum* dsp 21 protein (Bartels *et al.*, 1990; Schneider *et al.*, 1993) gave immunopositive signals in both *C. wilmsii* and *M. flabellifolius* (Fig. 2.13). Proteins of 21, 30 and 66 kDa were detected in the *C. wilmsii* chloroplast protein preparation and a protein of 66 kDa was detected in the *M. flabellifolius* chloroplast protein preparation; no immunopositive material was detected in *P. sativum*. The dsp 21 protein has been described as a nuclear-encoded stromal protein with homology to the group III LEA D29 protein (Baker *et al.*, 1988). It has been reported to be constitutively expressed at low levels in intact chloroplasts and to be induced upon desiccation or abscisic acid treatment (Schneider *et al.*, 1993). Antibodies against the *C. plantagineum* dsp 34 protein, a nuclear-encoded thylakoid membrane protein (Schneider *et al.*, 1993) with homology to the Elip-like protein (Grimm *et al.*, 1989; Grimm & Klopstech, 1987) also gave an immunopositive signal in *C. wilmsii* where a 66 kDa band was detected; no signal was obtained with the *M. flabellifolius* chloroplast preparation (not shown).



**Fig. 2.13:** Western blot of chloroplastic proteins with an antibody raised against the Group III LEA protein (dsp 21) from *C. plantagineum*. Samples are proteins extracted from chloroplasts of *P. sativum* (lane 1); *C. wilmsii* (lane 2) and *M. flabellifolius* (lane 3).

### 2.3.6 Photosynthetic pigments and antioxidants of isolated chloroplasts

Although *M. flabellifolius* has been classified as a homoiochlorophyllous (chlorophyll retaining) desiccation tolerant plant (Hoffman, 1968; Gaff, 1977), it was found that both chlorophyll a and chlorophyll b concentrations in the leaves decreased by 44% and 40% respectively following dehydration (Table 3). These results correlate with those reported earlier for the same species (Sherwin & Farrant, 1998; Kruger, 1998) and other homoiochlorophyllous resurrection plants (Farrant *et al.*, 1999). The levels of total carotenoids remained unchanged ( $0.23 \text{ mg g}^{-1} \text{ dw}$ ) (Table 3); whereas in other resurrection species, these pigments have also been reported to decline (Farrant *et al.*, 1999; Sherwin & Farrant, 1998).

**Table 3:** Content of photosynthetic pigments and antioxidants from hydrated and dry leaves of *M. flabellifolius*. See materials and methods for details on extraction and calculation of results. At least three extracts and several internal replicates were performed per assay.

Compound	unit of measure	Hydrated	Dry
<i>Photosynthetic pigments</i>			
Chlorophyll a	mg. g dw <sup>-1</sup>	1.926 ± 1.001	1.078 ± 0.105
Chlorophyll b	mg. g dw <sup>-1</sup>	0.642 ± 0.002	0.412 ± 0.002
Chlorophyll (a + b)	mg. g dw <sup>-1</sup>	2.568 ± 0.206	1.489 ± 0.907
Total xanthophylls + carotenes	mg. g dw <sup>-1</sup>	0.232 ± 0.004	0.235 ± 0.001
<i>Antioxidants</i>			
Ascorbate peroxidase	μmol min <sup>-1</sup> g dw <sup>-1</sup>	25 ± 1.693	256.91 ± 18.222
Glutathione reductase	nmol min <sup>-1</sup> g dw <sup>-1</sup>	293 ± 32.263	338 ± 26.304
Glutathione reductase	units g dw <sup>-1</sup>	0.3008 ± 0.109	0.3035 ± 0.121
Superoxide dismutase	units g dw <sup>-1</sup>	2.456 ± 0.318	4.718 ± 0.384

It was shown that there was an approximate 10-fold increase in the specific activity of ascorbate peroxidase when the plant was subjected to dehydration (Table 3). The apparently higher level of glutathione reductase activity in extracts from dry plants than from hydrated plants was not statistically significant (Table 3). The level of superoxide dismutase was found to increase two-fold when the plant was subjected to dehydration (Koonjul *et al.*, 1999c). Similar results have been reported for other resurrection species (Sherwin & Farrant, 1998).

### 2.3.7 Polyphenolics and sugar content of isolated chloroplasts

Sugars and amphipathic substances are thought to protect membranes of desiccation tolerant organisms. Sugars are believed to interact with the phosphate of the phospholipid headgroups, replacing water in the dry state (Crowe *et al.*, 1987; 1992; Hoekstra *et al.*, 1991; 1997) while amphiphiles partition readily from the aqueous cytoplasm into the lipid phase of membranes (Golovina *et al.*, 1998; Hoekstra *et al.*, 1997; Terao *et al.*, 1994) facilitating maintenance of fluidity and possibly preventing the elimination of sugars from membranes during drying (Golovina *et al.*, 1998; Hoekstra *et al.*, 1997). The sugar and polyphenolic content of chloroplasts from hydrated and dry leaves are given in Table 4. Sucrose concentration could not be determined, possibly because invertase is sensitive to contaminating substances such as polyphenolics (Koonjul *et al.*, 1998; 1999b; Nishizawa *et al.*, 1989; Singh *et al.*, 1998; Tan *et al.*, 1991). Glucose and fructose contents increased from 39 to 347.2 nM g<sup>-1</sup>dw and 153 to 298 nM g<sup>-1</sup> dw respectively during dehydration. The increase in glucose may be a consequence of starch hydrolysis which occurs during desiccation of most resurrection plants (Dalla Vecchia *et al.*, 1998; Farrant *et al.*, 1999; Gaff, 1989). The increase in fructose is less clear. It could be a consequence of hydrolysis of oligosaccharides or import from the cytoplasm. Glucose has been shown to protect chloroplasts from the chaotropic effects of high ion concentration (Heber & Santarius, 1964), a likely consequence of desiccation. An increased level in glucose upon drying may therefore serve such a function in resurrection plants. On their own, monosaccharides are not as effective as di- or oligosaccharides in water replacement or vitrification (Hoekstra *et al.*, 1997). If they serve such a protective role, it is likely that they do so in combination with other (amphipathic) compounds (Golovina *et al.*, 1998).

There was an increase in alkali-soluble polyphenolic content (from 0.429 to 0.730 µg mg dw<sup>-1</sup>), in total anthocyanin content (59 to 133.38 mg g dw<sup>-1</sup>), in the level of all proanthocyanidins as well as in gallotannin content of chloroplasts during drying (Table 4). Such increases suggest that these polyphenolics may facilitate increased membrane fluidity and, together with sugars, afford protection to membranes during

drying (Golovina *et al.*, 1998; Hoekstra *et al.*, 1997). Polyphenolics, particularly anthocyanins, have been proposed to mask chlorophyll and prevent excess light absorption under limiting conditions of water (Hopkins, 1992; Sherwin & Farrant, 1998). They also act as antioxidants (Larson, 1988; Smirnov, 1993; Terao *et al.*, 1994). Both functions help prevent photo-oxidation during desiccation.

**Table 4:** Content of various polyphenolics and soluble sugars in chloroplasts from hydrated and dry leaves of *M. flabellifolius*. At least three extracts and several internal replicates were performed per assay. Cnd: could not be determined. \* Assay performed once only.

Compound	Unit of measure	Hydrated	Dry
<i>Polyphenolics</i>			
alkali-soluble	$\mu\text{g.g dw}^{-1}$	$0.429 \pm 0.028$	$0.730 \pm 0.037$
acid-soluble		++++	++++
Anthocyanins	$\text{mg.g dw}^{-1}$	$59 \pm 7.540$	$133.38 \pm 14.79$
Cyanidin	$\mu\text{g.g dw}^{-1}$	$13.78 \pm 1.080$	$30.81 \pm 3.006$
Delphinidin	$\mu\text{g.g dw}^{-1}$	$25.89 \pm 2.008$	$61.01 \pm 5.002$
Gallotannin	Colour intensity	+	++++
<i>Sugars</i>			
Sucrose	$\text{nM.g dw}^{-1}$	Cnd	Cnd
Glucose	$\text{nM.g dw}^{-1}$	39*	$347.2 \pm 44.52$
Fructose	$\text{nM.g dw}^{-1}$	153*	$298 \pm 22.472$

### 2.3.8 Divalent cations determination

During dehydration, thylakoid membranes from *M. flabellifolius* became blistered (Fig. 2.14). This was not due to the aqueous fixation used in this study as vapour fixation resulted in a similar ultrastructural appearance (Goldsworthy & Drennan, 1991). The chloroplasts become rounded and the appressed membranes of the thylakoids appeared to have pulled apart. Since it is known that removal of divalent cations from isolated thylakoid membranes causes unfolding of appressed membranes

(Anderson & Aro, 1994), the level of cations was determined in chloroplasts from the rehydrated and the dehydrated plant (Table 5). All the cations measured declined during desiccation with  $\text{Cu}^{2+}$  declining by 95.5%,  $\text{Fe}^{2+}$  by 81%,  $\text{Ca}^{2+}$  by 65%,  $\text{Mn}^{2+}$  by 70% and  $\text{Mg}^{2+}$  by 46%. Presumably these metal cations were exported to other compartments, possibly the vacuoles (which are reduced in size and filled with non-aqueous substances in dry leaves, Farrant & Sherwin, 1998) or to the cell wall.



**Fig. 2.14:** Electron micrograph showing a chloroplast from a dry (5% RWC) intact leaf of *M. flabellifolius* (X 16 500). Note the separation of the thylakoid membranes.

**Table 5:** Content of cations in chloroplasts from hydrated and dry leaves of *M. flabellifolius*. See materials and methods for details on extraction and calculation of results. At least three extracts and several internal replicates were performed per assay.

Compound	Unit of measure	Hydrated	Dry
<i>Cations</i>			
Ca <sup>2+</sup>	µg.g dw <sup>-1</sup>	2102.3 ± 177.46	738.48 ± 50.100
Cu <sup>2+</sup>	µg.g dw <sup>-1</sup>	62.13 ± 6.670	2.7 ± 0.092
Fe <sup>2+</sup>	µg.g dw <sup>-1</sup>	516.38 ± 42.226	97.95 ± 5.913
Mg <sup>2+</sup>	µg.g dw <sup>-1</sup>	219.04 ± 21.852	118.65 ± 8.636
Mn <sup>2+</sup>	µg.g dw <sup>-1</sup>	99.06 ± 8.613	29.65 ± 1.818

## 2.4 DISCUSSION

The results presented in this chapter indicate that *M. flabellifolius* uses both physical and biochemical strategies to evade and protect itself against damage caused by light stress under water limiting conditions. Emphasis was placed on chloroplasts since they are the site of harmful photo-oxidative reactions under water stress (Vertucci & Farrant, 1995).

It was found that chloroplasts from *M. flabellifolius* had an increased buoyant density which was unlikely to be due to the lipid composition and/or protein complement. No correlation was found between the lipid content of chloroplasts isolated from *M. flabellifolius* and the higher buoyant density observed. The lipid compositions of these chloroplasts and chloroplasts isolated from *P. sativum* and *C. wilmsii* were very similar. Since the specific densities of palmitic acid and linolenic acid are 0.85 and 0.91 g ml<sup>-1</sup> respectively, one would anticipate a significant increase in the content of the latter to account for the higher buoyant density. In fact, the linolenic acid content of chloroplasts isolated from *P. sativum* was almost double that of chloroplasts isolated from *M. flabellifolius*. Analysis of chloroplastic proteins from *M. flabellifolius* indicated that this species synthesised various small proteins which were not present in *P. sativum* and *C. wilmsii*. However, it is unlikely that different protein contents would greatly affect the buoyant density as, in general, proteins have a similar buoyant density of approximately 0.75 g ml<sup>-1</sup>.

It was postulated that the increased buoyant density could be due to the unique ultrastructure of the thylakoid membranes (staircase arrangement). This ultrastructure was observed both in the intact leaf and upon isolation of the organelle. Moreover, the buoyant density of both isolated chloroplasts and isolated thylakoid membranes was found to be increased relative to other species studied. Indeed, none of the other resurrection plants studied to date, for example *Borya nitida* (Hetherington *et al.*, 1982), *Craterostigma* spp (Sherwin, 1995; Sherwin & Farrant, 1996; 1998), *S. stapfianus* (Dalla Vecchia *et al.*, 1998) and *Xerophyta* spp (Farrant *et al.*, 1999; Sherwin & Farrant, 1996; 1998; Tuba *et al.*, 1993) have this unusual chloroplastic

ultrastructural arrangement or an increased buoyant density.

Sucrose gradients are commonly used to isolate organelles from various tissues. Use of such gradients to isolate chloroplasts from the resurrection plants used in this study, however, resulted in considerable loss of ultrastructural integrity. The high sucrose concentrations used is akin to partial desiccation of membranes. It has been observed that dehydration and subsequent rehydration leads to structural changes and possibly to membrane breakdown (Navari-Izzo *et al.*, 1995; Sherwin & Farrant, 1996). Active thylakoid membrane preparations could not be prepared from *C. plantagineum* or the fern *Ceterach officinarum* using sucrose gradients (Schwab & Heber, 1984). Although trehalose has been reported to be toxic to many higher plants (Caffrey *et al.*, 1988; Veluthambi *et al.*, 1981), it was found that trehalose was an effective density gradient medium for the isolation of chloroplasts from the two resurrection plants studied. Since intact chloroplasts are routinely purified using sucrose gradients, it would imply that resurrection plant chloroplasts are less robust than those from desiccation sensitive plants, e.g. *P. sativum*. This result appears anomalous as one might expect that organelles from resurrection species to be inherently tolerant of variations in the cytoplasmic milieu which would occur during desiccation. It is interesting that sucrose, the principal sugar present in dry *Craterostigma spp* (Bianchi *et al.*, 1991; Ghasempour *et al.*, 1998) and believed to play an important role in protection against desiccation in plants (Ghasempour *et al.*, 1998; Hoekstra *et al.*, 1991; 1997) did not confer stabilisation of chloroplast membranes in either resurrection plant. Trehalose has previously been shown to be more effective than sucrose at stabilising biological systems against desiccation stress. In particular trehalose, rather than sucrose, has been reported to stabilise both restriction enzymes (Colaco *et al.*, 1992) and membranes (Crowe *et al.*, 1984; 1985; 1987; 1988; Gadd *et al.*, 1987) during lyophilisation and subsequent storage. In the case of membranes it has been suggested that trehalose prevents interactions between adjacent membrane phospholipid head groups (Chandrasekhar & Gaber, 1988) thereby inhibiting the transition from the liquid crystalline to the gel phase (Leslie *et al.*, 1994). Trehalose was more effective in maintaining ultrastructural integrity in the resurrection plants used during this study. Since this sugar occurs only in low

concentrations (30 mg g dw<sup>-1</sup>) in *M. flabellifolius* (Bianchi *et al.*, 1991; Drennan *et al.*, 1993), it is unlikely that it alone, serves to stabilise the chloroplasts membranes *in vivo* during desiccation. It has been shown that trehalose can substitute for HSP 12 in membrane stabilisation of yeasts (Sales *et al.*, 1999) and it is possible that the sugar served a similar role here, substituting for a component(s) which was either removed during the isolation procedure, or was simply not present at high enough concentrations in hydrated chloroplasts.

Despite the apparent fragility of chloroplasts from *M. flabellifolius*, this study has revealed several means by which these chloroplasts might achieve protection against the stresses associated with water loss. Although the protein content of chloroplasts isolated from *M. flabellifolius* was very different from those isolated from *P. sativum* and *C. wilmsii*, a 66 kDa protein recognised by the antibody to the 21 kDa *C. plantagineum* desiccation stress protein was present in isolated chloroplasts from both resurrection plants studied. The dsp 21 protein belongs to the LEA group III family and is likely to play a role in protection against water stress (Baker *et al.*, 1988). A number of small proteins (10-15 kDa) were found to be unique to chloroplasts isolated from *M. flabellifolius*. It is possible that these may help protect the sub-cellular organelle structure during desiccation. It is known that small hydrophilic proteins can limit the production of superoxide radicals (Sgherri *et al.*, 1993) and protect membranes against desiccation (Sales *et al.*, 1999). These proteins need to be isolated and characterised to see whether they are involved in protection of *M. flabellifolius* under limiting water conditions.

The leaves and chloroplasts of *M. flabellifolius* accumulate high levels of acid and alkali-soluble polyphenolics (including anthocyanins) during drying. These polyphenolics could be implicated in the desiccation tolerance of this species in several ways. Firstly, they could protect membranes by partitioning into the bilayer and thus facilitate the maintenance of membrane fluidity and possibly also prevent removal of sugars during drying (Golovina *et al.*, 1998). Amphiphilic polyphenolic molecules increase the permeability of membranes causing a depression in the gel to liquid transition temperature and hence prevent membranes from changing from

liquid crystalline phase into the gel phase during drying. Secondly polyphenolics, such as anthocyanins might play a role in photoprotection.

Starch was no longer evident in the dehydrated chloroplasts which might indicate that carbohydrate metabolism provided an energy source for processes initiated in response to desiccation stress. It was found that there was an accumulation of glucose in the dehydrated chloroplast, which could be a direct result of starch degradation. Although sucrose content could not be measured, it was found that besides the increase in glucose, there was also an increase in the fructose level in dry chloroplasts. These sugars, together with the increased levels of polyphenolics may play a crucial role in protecting membranes during desiccation. Glucose is also known to have a protective role against the chaotropic effects of high ion concentration, which occurs during drying (Heber & Santarius, 1964), although in this case there was a decrease in the cations concentrations during desiccation.

Water stress, coupled with light stress can be very detrimental to vegetative tissues. *M. flabellifolius* appears to utilise several strategies to cope with excess light under limiting water conditions. As water is lost, chlorophyll degradation occurs accompanied by an accumulation in anthocyanins (Table 4). Anthocyanin accumulates in the sub-epidermal layers of the abaxial surface, as reported previously (Goldsworthy, 1992). The increase in anthocyanins could act as a “sunscreen” minimising absorption of light by chlorophyll molecules. Furthermore since anthocyanins are antioxidants, they could quench free radical activity (Larson, 1988; Sherwin & Farrant, 1998; Smirnoff, 1993; Swain, 1965). Under extreme light conditions, the physical strategies used by *M. flabellifolius* might become insufficient resulting in the production of antioxidants to help prevent photo-damage. There was an increase in AP and SOD activity in *M. flabellifolius* during dehydration, while the change in GR was insignificant. Although there is no general trend in the antioxidant response used by resurrection plants, in most cases, the level of these enzymes do increase in response to water and/or light stress. For example, it has been demonstrated that during desiccation of *S. stapfianus*, GR activity increased (Sgherri *et al.*, 1994a). In *Boea hygrosopica* there is a decrease in GR activity while AP

activity remained constant when the plant was subjected to desiccation (Sgherri *et al.*, 1994b). In *T. ruralis*, AP activity was higher in plants grown under high light conditions but activity decreased during dehydration; on the other hand, SOD activity remained constant (Seel *et al.*, 1992). In *X. viscosa*, there is an increase in SOD, AP and GR activities when the plant undergoes dehydration (Sherwin & Farrant, 1998). Since there appears to be considerable variation in antioxidant responses of resurrection plants to desiccation stress, interpretations of these results is complex. It would seem that different resurrection plants use different antioxidant responses for protection against light-induced free radical damage which would occur when water becomes limiting and components of the photosynthetic apparatus are still intact.

It could be postulated that the staircase arrangement of thylakoid membranes in *M. flabellifolius* may more easily accommodate regular separation and reaggregation during drying and rehydration. Work carried out in this chapter showed that separation of thylakoid membranes did occur during drying (Fig. 2.14) and could play a role in the prevention of photo-oxidation. Separation of membranes (and hence separating PS I from PS II) significantly reduces the efficiency of light capture and the transfer of electrons through the photosynthetic pathway. This in turn, could reduce the potential for the production of superoxide radicals at PS I and PS II. The reduction in metal cations in chloroplasts during drying might facilitate membrane separation, since a reduction in divalent cations has been reported to cause disaggregation of thylakoid membranes (Anderson & Aro, 1994). This membrane separation could also be a result of degradation of lhc proteins which were shown to decrease two-fold during dehydration. The loss in lhc proteins could be related to the loss in chlorophyll b and the loss in  $Mg^{2+}$  (which decreased by about 50%) which both are required during the synthesis of these proteins. It is thought that membrane stacking is maintained primarily by the balance of Van der Waals' attractive interactions between the lhc proteins which surround the PS II core complexes, and to a lesser extent, by the screening of repulsive negative charges by positively-charged cations (Chow *et al.*, 1991). Together, the decline in lhc protein and the reduction in divalent cations might therefore cause this separation of thylakoid membranes observed during drying in *M. flabellifolius*. Furthermore, cations such as  $Cu^{2+}$ ,  $Fe^{2+}$ ,

$Mg^{2+}$  and  $Mn^{2+}$  are integral components and/or cofactors of proteins and enzymes of the photosynthetic pathway; metal removal would therefore suspend their activity and also their involvement in free radical formation. Thus ferredoxin reduces triplet oxygen to superoxide while the metallo-enzyme involved in water splitting uses  $Mn^{2+}$  as cofactor. Indeed, this could be a mechanism to switch off photosynthesis before water becomes a limiting factor and hence preventing or minimising the formation of free radicals. Farrant *et al.* (1999) have shown that PS II activity (as measured by chlorophyll fluorescence) declines at relatively high water contents (1.25 g  $H_2O$  g  $dw^{-1}$  or 55% RWC), below which metabolism would be water limited (Vertucci & Farrant, 1995).

From this study it can be concluded that there are several possible cellular mechanisms to protect chloroplasts of *M. flabellifolius* against light stress in water limiting conditions. These mechanisms can be classified as either physical or chemical. Physical protective mechanisms used by *M. flabellifolius* during dehydration include leaf orientation and the separation of thylakoid membranes. The chemical mechanisms, on the other hand, include production of anthocyanin, antioxidant enzymes and proteins implicated in desiccation and depletion of metal ions. Some of these strategies are unique to this species (for example, separation of the thylakoid membranes). Thus it appears that several mechanisms are induced to promote desiccation tolerance in *M. flabellifolius*.

## CHAPTER 3

### INHIBITORY EFFECTS OF TRACES OF POLYPHENOLICS DURING PCR; REVERSAL BY THE ADDITION OF POLYVINYLPIRROLIDONE

#### 3.1 INTRODUCTION

Polyphenolics are polyhydroxylated aromatic molecules that occur at different concentrations in the leaves, bark and fruit of most higher plants (Baxter *et al.*, 1997; Bruce & West, 1989; Dry & Robinson, 1994; Martin *et al.*, 1993; Peterson *et al.*, 1997; Rice-Evans, 1995; Rice-Evans *et al.*, 1996; Salunkhe *et al.*, 1982). They encompass a wide variety of such molecules and include tannins, flavonoids, anthocyanins, alkaloids, carotenoids as well as the phenolic acids (Larson, 1988). Polyphenolics have been isolated from tea leaves, grape berries, castor beans, tomatoes as well as cereals and leguminous plants. Each plant species has a distinct family of polyphenolics associated with it, the amount of which changes with the age and environmental conditions of the plant (Salunkhe *et al.*, 1982; Schneiderhauer *et al.*, 1991). The functions of polyphenolics have been reported to be diverse. It has been postulated that some polyphenolics have anti-oxidant properties and therefore act as free-radical scavengers (Larson, 1988; Rice-Evans, 1995; Rice-Evans *et al.*, 1996; Sherwin & Farrant, 1996; Yu *et al.*, 1997). This property has been directly attributed to the vitamins A, C and E and indirectly to the anthocyanins, which have been reported to increase in concentration in certain plants on exposure to stress (Sherwin & Farrant, 1998). Polyphenolics such as phenolic acid esters and chlorogenic acids have been shown to protect lipids from peroxidation (Rice-Evans & Miller, 1997). Many plants also synthesise and accumulate a broad spectrum of phenolic compounds in response to physiological stimuli and stress (Dixon & Paiva, 1995). It has also been reported that polyphenolics act as plant growth regulators known to inhibit growth. It has been shown that acidic polyphenolics such as tannic, genticic, ferulic, caffeic, hydroxy-benzoic and syringic acids inhibit the growth of hypocotyl in *Amaranthus caudatus* (Ray *et al.*, 1980). Polyphenolic compounds also antagonise the action of plant hormones (Ray *et al.*, 1980; Tamagnone *et al.*, 1998).

---

They also play a role in the defence mechanism of plants by making the leaves taste bitter and hence unpalatable (Frey *et al.*, 1997). Furthermore polyphenolic compounds such as lignin and hydroxycinnamates which occur within the plant cell wall, are known to form molecular bridges, which strengthen the cell wall. It has thus been shown that 4-coumaric acid and ferulic acid link lignin to polysaccharide polymers and hence render the cell wall highly resistant to mechanical and enzymatic disruption (Wallace & Fry, 1994). Polyphenolics have been found to play an important role in keeping fluidity in membranes during dehydration by partitioning from the aqueous cytoplasm into the lipid phase (Golovina *et al.*, 1998; Hoekstra *et al.*, 1997). This role of polyphenolics is particularly important during desiccation of anhydrobiotes. It was thus postulated that polyphenolics might play a role in the protection of organelles, such as chloroplasts during desiccation (see chapter 2).

An important characteristic of many polyphenolics is their propensity to form complexes with proteins (Baxter *et al.*, 1997; Huh *et al.*, 1996; Loomis, 1974), polysaccharides (Logemann *et al.*, 1987; Loomis, 1974; Pich & Schubert, 1993; Staub *et al.*, 1995; Su & Gihor, 1988), alkaloids (Baxter *et al.*, 1997), and nucleic acids (Baker *et al.*, 1990; John, 1992; Kim *et al.*, 1997; Lessard *et al.*, 1997; Peterson *et al.*, 1997; Schneiderbauer *et al.*, 1991; Soni & Murray, 1994; Staub *et al.*, 1995; Su & Gibor, 1988; Wan & Wilkins, 1994; Wilkins & Smart, 1996). The plant cell protoplasm is protected from these potentially damaging interactions by compartmentalisation, as the polyphenolics are stored in vacuoles and organelles (John, 1992; Loomis, 1974; Wilkins & Smart, 1996). Once cells are ruptured, however, these polyphenolics are released to form non-covalent complexes with the afore-mentioned macromolecules. Released polyphenolics, which absorb light in the 260 to 280 nm region (Loomis, 1974; Wilkins & Smart, 1996), are known to copurify with proteins and nucleic acids (Baxter *et al.*, 1997; John, 1992; Kim *et al.*, 1997; Loomis, 1974; Peterson *et al.*, 1997; Schneiderbauer *et al.*, 1991; Soni & Murray, 1994; Staub *et al.*, 1995; Wan & Wilkins, 1994; Wilkins & Smart, 1996) yielding reddish-brown complexes. In addition, the released polyphenolics have been reported to inactivate enzymes such as reverse transcriptase (Nishizawa *et al.*, 1989; Singh *et al.*, 1998; Tan *et al.*, 1991), DNA polymerase (Nakane *et al.*, 1990),

topoisomerase II (Kashiwada *et al.*, 1993), precipitate proteins (Baxter *et al.*, 1997; Haslam, 1974; Salunkhe *et al.*, 1982) and, under oxidising conditions, form covalent cross-links with nucleic acids (Katterman & Shattuck, 1983; Su & Gibor, 1988; Wilkins & Smart, 1996). These latter interactions reduce the recovery of nucleic acids and of specific proteins.

The known co-extraction of polyphenolics together with RNA has resulted in the development of a variety of protocols to avoid inhibition of molecular biological reactions (Baker *et al.*, 1990; Dabo *et al.*, 1993; John, 1992; Kim *et al.*, 1997; Logemann *et al.*, 1987; Peterson *et al.*, 1997; Schneiderbauer *et al.*, 1991; Soni & Murray, 1994; Staub *et al.*, 1995; Verwoerd *et al.*, 1989; Wan & Wilkins, 1994). These methods are based on the removal of polyphenolics by differential solvent precipitation (Manning, 1991), hydrogen bonding to PVP (Loomis, 1974; Pich & Schubert, 1993; Wilkins & Smart, 1996) or by separation of the RNA from the polyphenolics by caesium chloride gradient centrifugation (Glisin *et al.*, 1974), DEAE-cellulose (Mozer, 1980; Staub *et al.*, 1995), CF-11 cellulose (Colpan *et al.*, 1983) or polyamide column chromatography (Collins *et al.*, 1998). These latter chromatographic methods, however, are problematic from a molecular biological perspective, as large amounts of starting material are required due to nucleic acid loss during the removal procedure.

Essentially this chapter deals with optimisation of conditions for RNA extraction, reverse transcription and PCR which are used in the later chapters. In the work presented here, it was demonstrated that RNA extracted from *M. flabellifolius* (of which only very limited quantities of leaf material were available) could be reverse transcribed but not amplified by PCR although the  $A_{260}:A_{280}$  ratio was in the range of 1.9-2.0. These results suggested that traces of polyphenolic contamination of the isolated RNA prevented further molecular manipulations; this could be reversed by omitting chaotropic salts from the isolation buffer together with the inclusion of PVP in the PCR mixture. A condensed version of the work presented in this chapter has been published (Koonjul *et al.*, 1998; 1999b).

---

## 3.2 MATERIALS AND METHODS

All the glassware used in this section were autoclaved then baked at 260°C for 4 h. Plastic ware was soaked in a solution containing 3% H<sub>2</sub>O<sub>2</sub> for 10 min. and washed thoroughly with RNase-free water, unless sterile disposable plasticware was available. RNase-free water was double-distilled Milli-Q water (passed through carbon, ion exchange and organic scavenger cartridges), then treated with DEPC before being autoclaved. All tips were autoclaved and gloves were worn throughout all procedures.

### 3.2.1 Plant material and growth conditions

*M. flabellifolius* plants were collected and maintained as described in section 2.2.1. Only young expanding leaves were used for RNA extraction. As a control, young leaves from *P. sativum* were used for RNA extraction.

### 3.2.2 RNA isolation

Total RNA from wet leaves of *M. flabellifolius* and *P. sativum* was prepared using the protocol described in section 2.2.12. The RNA recovered was further purified on a PEG gradient, which allows for the extraction of any contaminating polyphenolic compounds (Bruce & West, 1989). The RNA pellet was resuspended in 0.3 ml DEPC water to which 0.7 ml of phosphate buffer (0.8M KH<sub>2</sub>PO<sub>4</sub>, 0.8M K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O) and 0.4 ml of 30% (w/v) PEG 8000 was added. The mixture was vortexed vigorously for 1 min. and centrifuged at 3900 g for 10 min. at 4°C. The aqueous phase containing the RNA was carefully removed without disturbing the upper PEG phase or the pellet and the RNA recovered as previously described (section 2.2.12). Soluble PVP was added to a final concentration of 1% (w/v) and the quality and quantity of the RNA was determined from the absorption spectrum from 200 nm to 300 nm. An aliquot of the sample was applied to MALDI-TOF mass spectrometer in order to check the quality of RNA (section 3.2.3).

### 3.2.3 Checking quality of RNA on MALDI-TOF

The purity of the RNA extracted was determined using a Voyager DE<sup>TM</sup>-Pro (Perseptive Biosystems) Matrix Assisted Laser Desorption/Ionisation Time of Flight (MALDI-TOF) mass spectrometer. Approximately 1 picomole of sample (1  $\mu$ l) was added to 1  $\mu$ l sinapinic matrix (10 mg 3,5-dimethoxy-4-hydroxycinnamic acid in 1 ml 50% (v/v) acetonitrile and 0.03% (v/v) trifluoroacetic acid in water) and used for the analysis.

### 3.2.4 RNA agarose gel electrophoresis

A 1% (w/v) formaldehyde agarose gel was prepared by dissolving 0.6 g of agarose in 44 ml of milli-Q water and 6 ml of 10 x MOPS buffer (200 mM MOPS, 50 mM Na-acetate, 10 mM EDTA) at 100°C. The solution was cooled to 55°C at which stage 10 ml of formaldehyde was added. 1 x MOPS was used as the running buffer. The samples were prepared by taking 1-2  $\mu$ l of RNA and adding 8  $\mu$ l of RNA cocktail (67% (v/v) formamide, 17% (v/v) formaldehyde, 13% (w/v) 10 x MOPS and 10 mg/ml EtBr) and the mixture heated for 5 min. at 65°C. Finally loading buffer (50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 1 mM EDTA, pH 8) was added to the sample. The gel was electrophoresed for 1.5-2 hours at 50 V.

### 3.2.5 Transmission electron microscopy

This was carried out as described in section 2.2.7.

### 3.2.6 Reverse transcription of *M. flabellifolius* RNA and amplification of the cDNA by DD-PCR

First strand cDNA synthesis was performed using 200 ng of freshly diluted DNase I treated total leaf RNA from *M. flabellifolius*. An identical amount of *P. sativum* total leaf RNA was used as a control. The reverse transcription mixture consisted of 1  $\mu$ M

of anchored HT<sub>11</sub>G primer (5'AAGCTTTTTTTTTTTTG 3'), 20  $\mu$ M dNTPs, 20 U of RNasin, first strand buffer (25 mM Tris-HCl, pH 8.3, 37.6 mM KCl, 1.5 mM MgCl<sub>2</sub>, 5 mM DTT) and 200 units of MMLV reverse transcriptase in a total reaction volume of 20  $\mu$ l. This mixture was divided into two aliquots of 5  $\mu$ l and 15  $\mu$ l. 0.5  $\mu$ l <sup>32</sup>P  $\alpha$ -dATP (800 Ci/mmol) was added to the 5  $\mu$ l aliquot and both aliquots were incubated for 1 hour at 60°C. First stand cDNA synthesis was checked by electrophoresis of the radioactive aliquot on a 1% alkaline agarose gel (1% (w/v) agarose, 0.187 M NaOH, 12.5 mM EDTA). 2  $\mu$ l of the non-radioactive aliquot was used in a differential display PCR (DD-PCR) which was carried out for 40 cycles in a Hybaid thermal cycler (30 s at 94°C, 1 min. at 55°C, 30 s at 72°C) as described previously (Liang & Pardee, 1992; Liang *et al.*, 1993) except that the MgCl<sub>2</sub> concentration was increased from 1.5 to 4.5  $\mu$ M and the anchored HT<sub>11</sub>G primer concentration was increased from 2 to 10  $\mu$ M. 4  $\mu$ l of the product was electrophoresed on a 6% denaturing polyacrylamide gel for 3.5 hours at 60 W and visualised by autoradiography.

### 3.2.7 Alkaline agarose gel electrophoresis

Alkaline agarose gels denature DNA samples and hence can detect any secondary structure which might occur during first or second strand cDNA synthesis if the newly synthesised strand folds back onto itself and forms an antiparallel double helix. This gel also gives an indication on the size of the cDNA synthesised.

The gel was made by dissolving 0.8 g of agarose in 72 ml of water at 100°C. The mixture was cooled to 50°C before 8 ml of 10 x alkaline buffer (3 ml of 5 M NaOH, 2 ml of 0.5 M EDTA, 45 ml of water) was added and the gel was poured. Thin gels were poured because of later drying and exposure for autoradiography. An aliquot of the cDNA strand was loaded in an equal volume of 2 x loading buffer (200  $\mu$ l of glycerol, 46  $\mu$ l of saturated bromophenol, 750  $\mu$ l of water and 5  $\mu$ l of 5M NaOH). The gel was run for 1.5 hours at 100 mA. After the samples were run for 1.5 h, the gel was dried and exposed for autoradiography at -70°C for 18 h. During the drying process of the gel, multiple layers of Whatman 3MM were placed under the gel, so as to prevent contamination of the dryer with the free nucleotides.

### 3.2.8 6% denaturing gel electrophoresis

6% (w/v) sequencing gels (5.7 g acrylamide, 0.3 g bisacrylamide, 48 g urea, 10 ml of 10 x TBE, 40 ml water, 450  $\mu$ l 10% (w/v) AMPS and 45  $\mu$ l TEMED) were pre-electrophoresed for 30 min. at 90W. Samples were electrophoresed for 3-4 h or until the xylene cyanol dye front reached the bottom of the gel.

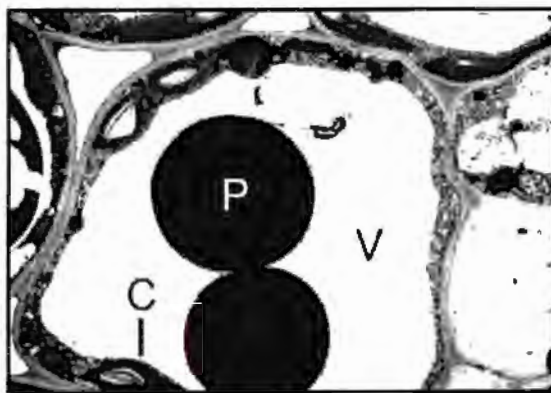
### 3.2.9 Standard PCR Assay

Chromosomal DNA from wild type *Saccharomyces cerevisiae*, the primers LMF1 and LMR1 and the PCR conditions described below were kindly provided by Dr E. Rumbak (Department of Biochemistry, University of Cape Town, South Africa). The forward (5' GATGGATCCATGTCTGACGCAGGTAGAAA 3') and reverse (5' GGTTCTTACTTTAGAAAGCCTTAAGTAT 3') primers were designed to amplify the *HSP12* gene. PCR was performed using 1 unit of Taq polymerase (Qiagen) in a reaction containing 130 ng of template, 20 pmoles of each primer in 0.1 mM dNTPs, 1 mM MgCl<sub>2</sub>, 0.005% BSA (bovine serum albumin), 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.001% gelatin. The yeast DNA was amplified by hot start PCR (300 s at 95°C followed by 120 s at 80°C) after which the Taq polymerase was added. The amplification cycles used were as follows: 5 cycles of 93°C for 45 s, 50°C for 30 s, 72°C for 60 s, 25 cycles of 93°C for 30 s, 65°C for 30 s, 72°C for 60 s; a final extension step of 5 min. at 72°C was used to terminate the reaction. 5  $\mu$ l of the product was analysed on a 1% (w/v) agarose gel which was visualised by ethidium bromide staining. Positive and negative controls were always included to ensure that product formation was not due to contamination.

### 3.3 RESULTS

#### 3.3.1 Extraction of RNA

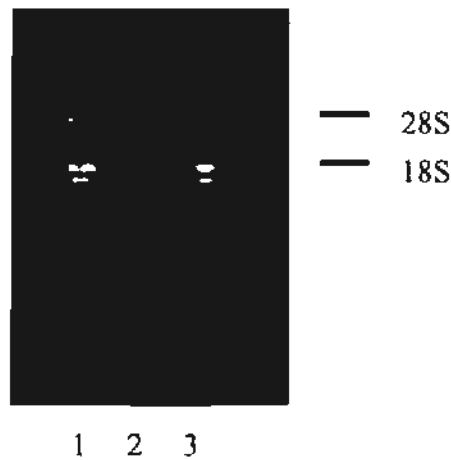
Extraction of RNA from tissue generally involves homogenisation of the material in the presence of chaotropic guanidinium salts (Chirgwin *et al.*, 1979; Chomczynski & Sacchi, 1987; Logemann *et al.*, 1987; MacDonald *et al.*, 1987; Su & Gibor, 1988). These salts effectively deproteinize RNA (Chomczynski & Sacchi, 1987; Cox, 1968), cause the inhibition of endonucleases (Chirgwin *et al.*, 1979) as well as physically separate the RNA from other components in the homogenate (MacDonald *et al.*, 1987). RNA is then recovered from the homogenate by organic solvent precipitation (Manning, 1991; Wallace, 1987), caesium chloride centrifugation (Glisin *et al.*, 1974) or by binding to and elution from specific matrices (Colpan *et al.*, 1983; Mozer, 1980). Homogenisation of *M. flabellifolius* leaves in solutions containing either guanidinium thiocyanate or the less chaotropic guanidinium hydrochloride resulted, after ethanol precipitation, in a reddish-brown pellet that was only partially soluble in water. Although the solution had a high absorbance value in the range of 260 to 320 nm and displayed an  $A_{260}:A_{280}$  ratio in the range 1.2:1.0, no RNA was found to be present when an aliquot was electrophoresed on a formaldehyde gel. Similar results (Baker *et al.*, 1990; Rice-Evans, 1995; Wan & Wilkins, 1994; Wilkins & Smart, 1996) were attributed to co-isolation of polyphenolics together with the RNA. It was found earlier that *M. flabellifolius* leaves contain a large amount of polyphenolics (section 2.3.1) and microscopical examination of a *M. flabellifolius* leaf section showed the presence of distinct electron-dense bodies within the vacuoles (Fig. 3.1). This is in agreement with previous reports that synthesis of the phenolic pigment derived from 3-chlorobenzoate by *Pseudomonas fluorescens* resulted in the accumulation of many electron-dense granules inside the cell (Bernardini & Cersosimo, 1979; Fava *et al.*, 1993; Pellegrini, 1980).



**Fig. 3.1:** Electron micrograph (magnification X 19 500) showing a mesophyll cell from a leaf of *M. flabellifolius*. A chloroplast (C) and an electron-dense polyphenolic body (P) are shown.

These results together with the reddish-brown appearance of the precipitate showed that loss of RNA might have been brought about by polyphenolic-mediated precipitation of nucleic acid after disruption of the leaf cells. Since guanidinium salts disrupt the organisation of the cell thereby releasing compartmentalised polyphenolics into the leaf extract, a number of existing methodologies (Ainsworth, 1994; Bahloul & Burkard, 1993; Bruce & West, 1989; Soni & Murray, 1994) were combined to produce an RNA-containing homogenate with a markedly reduced polyphenolic content (results shown later). The PVP/SDS homogenisation buffer finally used was 100 mM Tris-HCl pH 8.6 containing 100 mM NaCl, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 2% (w/v) PVP and 100  $\mu$ g/ml proteinase K. Homogenisation of *M. flabellifolius* leaves in this buffer (see methods) followed by purification using PEG 8000 resulted, after ethanol precipitation, in a fully water-soluble pellet that was no longer reddish-brown in colour. The  $A_{260} : A_{280}$  ratio was routinely in the range 1.6:1 - 1.7:1 but on addition of PVP at a final concentration of 1% (w/v) to the sample prior to checking the absorbance readings, the ratio increased to be in the range of 1.9-2.0, characteristic of good quality RNA (Lessard *et al.*, 1997; Wilkins & Smart, 1996). When an aliquot of the RNA extracted using this methodology was compared by MALDI-TOF mass analysis with that obtained using the Chomczynski & Sacchi (1987) method, it could clearly be seen that there were many more bands corresponding to impurities (possibly polyphenolics) when the latter method had been



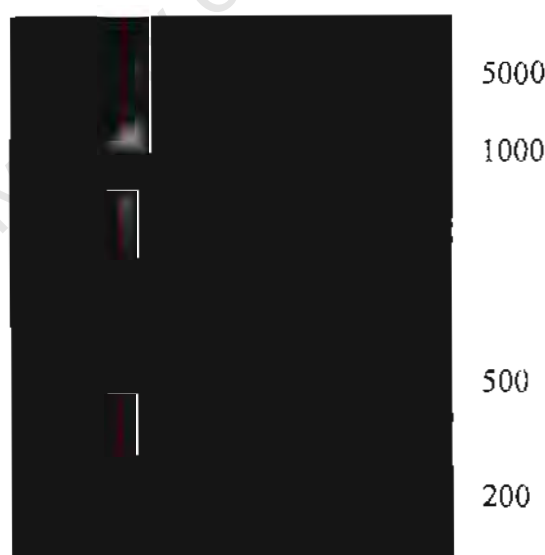


**Fig 3.3:** Agarose electrophoresis in the presence of 17% (v/v) formaldehyde of total RNA isolated from *P. sativum* (lane 1), from *M. flabellifolius* using the method of Chomczynski & Sacchi, 1987 (lane 2) and by the methodology reported here (lane 3). The migration of 28S rRNA and 18S rRNA are shown. 10  $\mu$ g of RNA, determined from the 260 nm absorbance, was loaded in each lane.

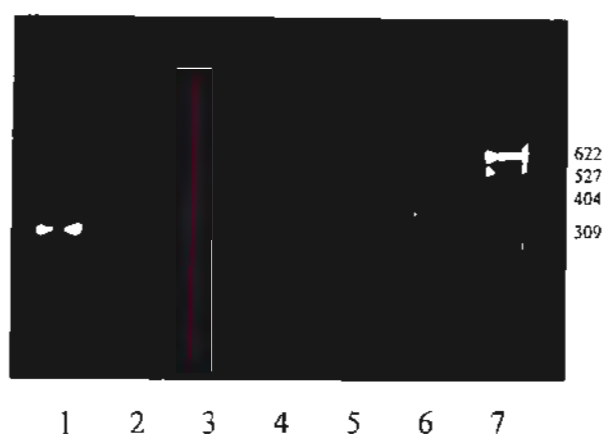
### 3.3.2 RT-PCR of extracted RNA samples

It has been reported that even traces of contamination of an RNA sample with polyphenolics and/or polysaccharides inhibit further manipulations, for example *in vitro* translation (Su & Gibor, 1988), and RT-PCR (Singh *et al.*, 1998; Staub *et al.*, 1995). DD-PCR (see chapter 5) using 200 ng RNA extracted using the PVP/SDS buffer above resulted in no cDNA fragments observed on the sequencing gel (not shown). To determine whether the reverse transcription step of the DD-PCR was successful, 50 ng RNA was reverse transcribed in the presence of  $^{32}$ P- $\alpha$ [dATP]. Autoradiography of the alkaline gel (Fig. 3.4) demonstrated that first strand cDNA synthesis indeed occurred. Radioactivity was incorporated into a wide variety of transcripts migrating with a size in the range 0.1 to over 2 kbp suggesting that failure of the DD-PCR was due to inhibition of the PCR step. To investigate the effect of the *M. flabellifolius* RNA extract on the PCR step, a standard PCR assay was set up using chromosomal DNA extracted from *S. cerevisiae* as a template. The primers used recognised the *HSP 12* gene sequence yielding a product of 355 bp after

amplification. A secondary minor product of 310 bp was also observed at high loading. Addition of 2 ng RNA, determined from the 260 nm absorption, of the *M. flabellifolius* RNA extract to the PCR mixture resulted in an amplification of the target DNA equivalent to that of the control reaction with the production of a fragment of the anticipated size (Fig. 3.5). The yield of the PCR product declined substantially, however, when the amount of input *M. flabellifolius* RNA was increased to 20 ng; no PCR product was observed when this input was further increased to 200 ng. It was therefore concluded that the RNA purified from the *M. flabellifolius* leaf extract still contained impurities that inhibited the PCR step of the DD-PCR despite the  $A_{260}:A_{280}$  ratio of the RNA suggesting that it was of good quality. A number of different procedures were then carried out to further purify the RNA. These included dialysis, re-precipitation of the RNA in the presence of lithium chloride (Su & Gibor, 1988), washing the pellet in the presence of excess potassium acetate (Ainsworth, 1994; Wan & Wilkins, 1994) and purification of the total RNA onto a Qiagen column. None of these methods resulted in an appreciable improvement in the amount of the PCR product.



**Fig. 3.4:** Autoradiograph of alkaline agarose electrophoresis of cDNA produced by reverse transcription of 50 ng RNA isolated from *P. sativum* (lane 1), and *M. flabellifolius* (lane 2). The migration of fragments of bacteriophage lambda digested with *Pst*I is depicted on the right of the gel.

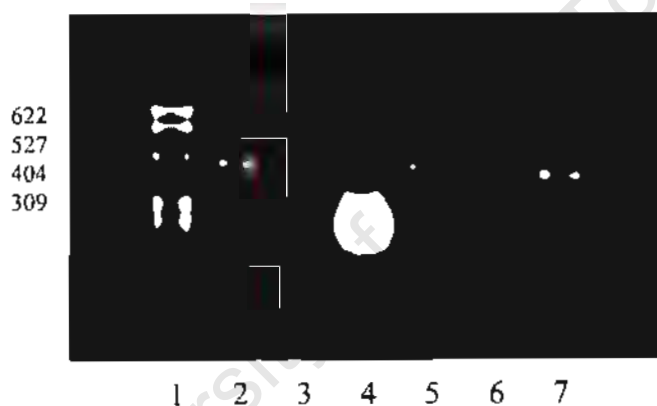


**Fig 3.5:** PCR of the *HSP12* gene from yeast chromosomal DNA in the presence of RNA isolated from *M. flabellifolius*. Lane 1: 2 ng *M. flabellifolius* RNA; lane 2: 20 ng; lane 3: 200 ng; lane 4: 2  $\mu$ g; lane 5: negative control (no template DNA); lane 6: positive control (no *M. flabellifolius* RNA added); lane 7: molecular size marker (*Hpa*II digest of pBR322).

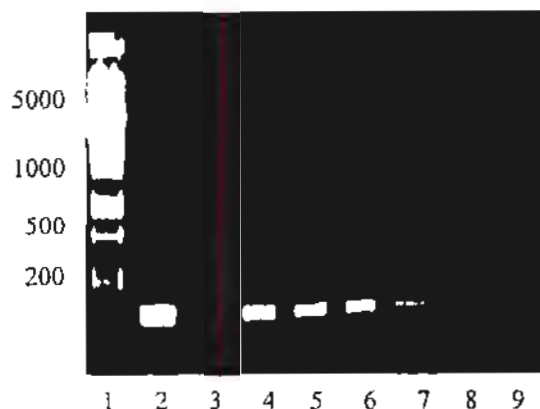
### 3.3.3 Reversal of PCR inhibition

Since polyphenolics interact with both nucleic acids and proteins, random DNA and albumin were added in excess to the PCR mixture to see if they might act as a sink to bind the contaminants in the RNA sample. 200 ng of RNA purified from *M. flabellifolius* leaf extracts was added to the PCR as a negative control. The co-addition of chicken DNA at a 100-fold concentration compared with that of the yeast template resulted in no product being formed (Fig. 3.6). A higher concentration of albumin, 0.025% (w/v) compared with the 0.005% (w/v) used previously, however, resulted in reduced product formation compared with the positive control suggesting that the contaminants in the RNA sample were interacting with the Taq polymerase. This was confirmed by observing that product formation was also achieved when the amount of Taq polymerase used was increased from 1 unit to 2.5 units. Since increasing the amount of albumin or increasing the amount of Taq polymerase both resulted in reversal of inhibition of the PCR by the contaminants present in the RNA sample, DD-PCR was carried out using either increased albumin or increased Taq

polymerase or a combination of both. It was found, however, that despite these modifications, only very faint bands were visible after DD-PCR. Since the addition of PVP to the extraction buffer resulted in a more pure preparation of RNA, the PCR was repeated in the presence of PVP. PVP is known to bind to polyphenolics and the rationale behind adding it to the PCR mixture was to check whether the inhibitory effects of contaminants in the *M. flabellifolius* leaf RNA preparation could be reversed. The standard PCR was therefore carried out in the presence of different final concentrations of PVP together with 200 ng of *M. flabellifolius* leaf RNA. Product formation was observed in the presence of 0.5% to 2% PVP (Fig. 3.7). The product yield, however, was less than that observed with the control PCR. Increasing the PVP concentration above 2% resulted in a markedly reduced product yield.



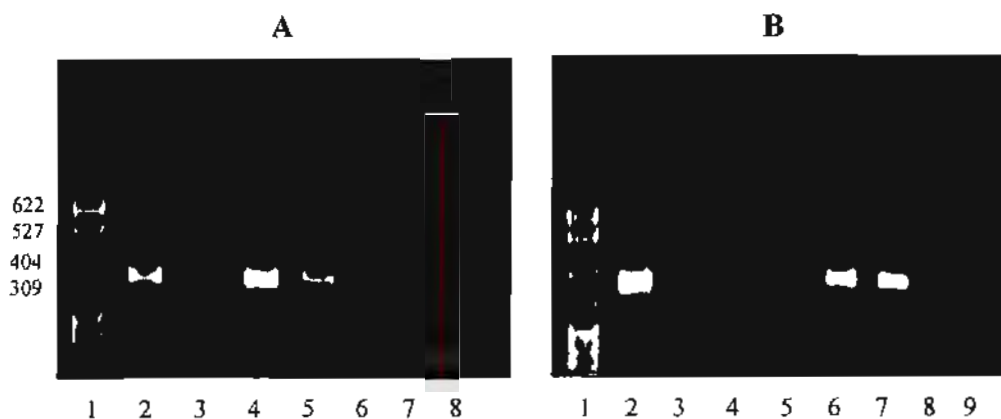
**Fig. 3.6:** Effect of various additives on PCR of the *HSP12* gene from yeast chromosomal DNA using 1 U of Taq polymerase in the presence of 200 ng of *M. flabellifolius* RNA. Lane 1: molecular size marker (*HpaII* digest of pBR322); lane 2: positive control (no *M. flabellifolius* RNA added); lane 3: negative control (no template DNA); lane 4: 20 µg chicken erythrocyte DNA added; lane 5: 0.025% BSA) lane 6: no addition (c.f. lane 3, Fig. 3); lane 7: Taq polymerase increased to 2.5 U.



**Fig 3.7:** Effect of PVP addition to PCR of the *HSP12* gene (1 U of Taq polymerase) in the presence of 200 ng of *M. flabellifolius* RNA. Lane 1: molecular size marker (*Pst* I digest of bacteriophage lambda); lane 2: positive control (no *M. flabellifolius* RNA added); lane 3: negative control (no template DNA); lane 4: 0.5 % final concentration PVP added; lane 5: 1%; lane 6: 1.5% ; lane 7: 2%; lane 8: 3%; lane 9: 5%.

### 3.3.4 Confirming the inhibitory effect of polyphenolics on the PCR

To confirm the assumption that PCR inhibition was caused by polyphenolic contamination of the *M. flabellifolius* RNA, the standard PCR assay was repeated in the presence of a commercial extract of polyphenolics from green tea (Sigma). Although product inhibition was not observed when up to 50 ng polyphenolics was added to the reaction, no product was formed in the presence of 500 ng polyphenolics (Fig. 3.8A). To investigate whether the inhibitory effects of polyphenolic addition could be reversed by the simultaneous addition of PVP, PCR was carried out in the presence of 500 ng polyphenolics together with different concentrations of PVP. It was found (Fig. 3.8B) that the inhibitory effects of polyphenolic addition could indeed be reversed and that product formation occurred in the presence of between 1% and 2% PVP.



**Fig. 3.8** A: Effect of green tea polyphenolic addition to PCR of the *HSP12* gene. Lane 1: molecular size marker (*HpaII* digest of pBR322); lane 2: positive control (no polyphenolic addition); lane 3: negative control (no template DNA); lane 4: 5 ng polyphenolics added; lane 5: 50 ng; lane 6: 500 ng; lane 7: 1 µg; lane 8: 2 µg. B: Effect of PVP addition to PCR of the *HSP 12* in the presence of 500 ng of green tea polyphenolics. Lane 1: molecular size marker (*HpaII* digest of pBR322); lane 2: positive control (no polyphenolic addition); lane 3: negative control (no template DNA); lane 4: 0.5% final concentration PVP added; lane 5: 1%; lane 6: 1.5% ; lane 7: 2%; lane 8: 3%; lane 9: 5%.

### 3.4 DISCUSSION

In this chapter it was demonstrated that RNA could be isolated from milligram quantities of leaves from the polyphenolic-containing resurrection plant *M. flabellifolius*. This was achieved only when the polyphenolic-binding compound, PVP, was included and when guanidium thiocyanate or the less chaotropic salt guanidium hydrochloride was omitted from the isolation medium. Similar results (Baker *et al.*, 1990; Rice-Evans, 1995; Wan & Wilkins, 1994; Wilkins & Smart, 1996) have been attributed to the co-isolation of polyphenolics together with the RNA. Guanidium salts, being extremely effective as protein denaturants (Chomczynski & Sacchi, 1987) presumably causes the release of polyphenolics from vacuoles and organelles (John, 1992; Loomis, 1974; Wilkins & Smart, 1996) resulting in complex formation with both proteins (Baxter *et al.*, 1997; Huh *et al.*, 1996; Loomis, 1974) and nucleic acids (Baxter *et al.*, 1997; John, 1992; Kim *et al.*, 1997; Loomis, 1974; Peterson *et al.*, 1997; Schneiderbauer *et al.*, 1991; Soni & Murray, 1994; Staub *et al.*, 1995; Wan & Wilkins, 1994; Wilkins & Smart, 1996). RNA isolated by the procedure described here, despite having an  $A_{260} : A_{280}$  ratio in the range 1.9 - 2.0 after DNase 1 treatment, was still unsuitable for PCR although it was a substrate for reverse transcription. This may be due to the larger amount of enzyme used for reverse transcription (200 U) than for PCR (1 U) as increasing the amount of enzyme used in the latter procedure resulted in product formation. This result is in agreement with the results obtained by Singh *et al.* (1998) where it was shown that reverse transcription was inhibited by the presence of 6 to 7.5 mg of chlorogenic acid while PCR was inhibited at much lower concentrations (1.2 to 1.8 mg). However no product was obtained using isolated *M. flabellifolius* RNA during DD-PCR. This might be due to the lower annealing temperature used during DD-PCR compared with PCR allowing a more favourable interaction between contaminating polyphenolics and the enzyme used.

This work demonstrates support for the theory that traces of polyphenolics inhibit molecular manipulations involving RNA. Although *M. flabellifolius* was found to have significantly higher levels of polyphenolics present compared with *P. sativum*

---

(see section 2.3.1), this inhibition is probably caused by specific polyphenolics rather than the overall amount. Thus 50 ng of green tea polyphenolics, an amount that would have readily detected in the isolated RNA sample by absorption spectroscopy, had no effect on PCR product formation. Inclusion of PVP in both the RNA isolation medium and the PCR mixture allowed successful amplification of the cDNA suggesting that the contaminants were indeed polyphenolics. This was confirmed by the inhibition of PCR product formation after the addition of green tea polyphenolics to the reaction mixture and the subsequent rescue by the inclusion of PVP. These data suggest that the polyphenolics co-isolated together with the *M. flabellifolius* RNA inhibited subsequent molecular manipulations by binding to the enzymes used for these procedures rather than the nucleic acids present. Evidence for this was that the addition of random chicken DNA had no effect on reversal of inhibition whereas addition of extra protein in the form of either extra enzyme or albumin allowed product formation. The protection of plant organelles by albumin during their isolation has been attributed to hydrogen bond formation between albumin and any polyphenolics present (Loomis, 1974).

In conclusion, these results suggest that RNA isolation from polyphenolic-containing plants should be carried out without the addition of chaotropic salts to the isolation medium and that any subsequent molecular manipulation be performed in the presence of PVP.

---

## CHAPTER 4

### CONSTRUCTION AND DIFFERENTIAL SCREENING OF A CDNA LIBRARY FROM DESICCATED LEAVES OF *M. flabellifolius*

#### 4.1 INTRODUCTION

One method utilised to understand the molecular basis of desiccation tolerance is to identify genes expressed during the desiccation process. To date no molecular biological data has been reported on the desiccation characteristics of *M. flabellifolius*. Most of the molecular biological work on resurrection plants in the field of desiccation tolerance has been concentrated on *C. plantagineum* (Alamillo & Bartels, 1996; Bernacchia *et al.*, 1995; Frank *et al.*, 1998; Furini *et al.*, 1997; Ingram *et al.*, 1997; Iturriaga *et al.*, 1996; Mariaux *et al.*, 1998; Velasco *et al.*, 1998), *Xerophyta spp.* (Dace *et al.*, 1998; Mundree & Farrant, 1999), *S. stapfianus* (O'Mahony & Oliver, 1999) and the moss *T. ruralis* (Wood *et al.*, 1999).

Several cDNA clones which are differentially expressed in dry *C. plantagineum* show homology to LEA proteins, RAB proteins (responsive-to ABA), and dehydrins, which are all known to confer desiccation tolerance (Bartels *et al.*, 1993; Ingram & Bartels, 1996). Transcripts related to sucrose synthetase, sucrose phosphate synthetase (Ingram & Bartels, 1996; Ingram *et al.*, 1997) and glyceraldehyde-3-phosphate dehydrogenase (Velasco *et al.*, 1994) have also been isolated from *C. plantagineum* and have been postulated to be key enzymes involved in cellular protection (Ingram & Bartels, 1996). Molecular studies on *Xerophyta spp* have indicated the upregulation of mRNAs coding for aldose reductase (Mundree *et al.*, unpublished results) and vacuolar ATPases (Mundree & Farrant, 1999). The vacuolar ATPases are thought to be implicated in the induction of protection and other tolerance mechanisms (e.g. sequestration of substances into vacuoles to minimise mechanical damage) (Farrant & Sherwin, 1998). Dace *et al.*

(1998) have shown that *X. humilis* transcribes and stores mRNAs required for the resynthesis of the photosynthetic apparatus during early rehydration, while the plant undergoes dehydration. This enables the plant to prepare for recovery and is one of the many strategies used by resurrection plants to cope with desiccation stress. In *S. stapfianus*, transcripts encoding a thiol protease have been isolated (Blomstedt *et al.*, 1998) and could be involved in the removal of damaged and unwanted proteins during drying, as well as helping in the release of amino acids needed for synthesis of novel proteins (Ingram & Bartels, 1996).

Several strategies can be used to identify differentially expressed genes. One of the initial strategies used was the random selection of probes from a cDNA library (Milner & Sutcliffe, 1983). This method is extremely labour-intensive and requires large amounts of RNA. Other methods include subtractive hybridisation (Sargent, 1987) or screening a library with a subtracted cDNA probe (Watson *et al.*, 1990). More recent methods of detecting differentially expressed genes are RAP-PCR (RNA fingerprinting by arbitrarily primed PCR) (Cherath *et al.*, 1996), RNA fingerprinting based on AFLP (amplified restriction fragment polymorphism) (Bachem *et al.*, 1996) and the differential display technique (Liang & Pardee, 1992).

The conventional method for obtaining several clones simultaneously is by constructing and then screening a cDNA library. What are the advantages of this methodology? A cDNA library contains all the information encoded in the mRNAs at the time of harvest. Since RNA molecules are labile and difficult to amplify, it is simpler to convert the information encoded into a stable DNA duplex (cDNA) and insert this duplex into a self-replicating  $\lambda$ -vector. Figure 4.1 shows the steps involved in the construction of a cDNA library. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and characterised. A representative cDNA library should contain full-length copies of the entire original population of mRNA.

---

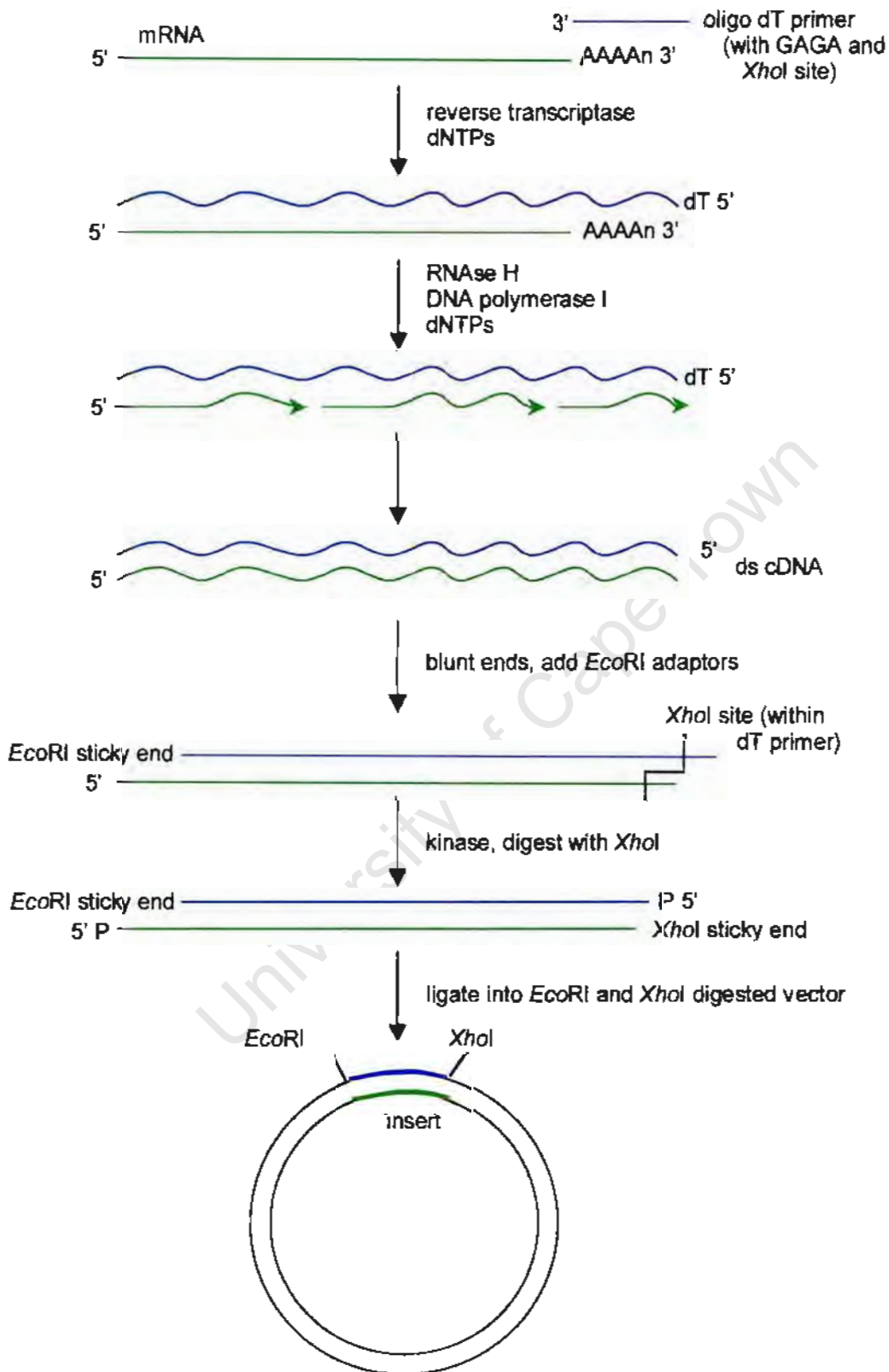


Fig. 4.1: Schematic diagram showing the steps involved in the construction of a cDNA library.

After construction of the cDNA library, clones can be obtained using several screening methods. These range from simply sequencing several clones until the one of interest is identified, through hybridisation methods (e.g heterologous probe) to the more complex methods of expression screening (e.g antibody screening). The chosen method depends on each individual case, and is determined by the type of recombinant library as well as the frequency of a particular cDNA clone in the library (Ausubel *et al.*, 1987). The advantage of using a recombinant library is that a large number of clones can be screened at one time which enables rapid identification of a clone of interest within that population. This is usually achieved by spreading a library on agar plates and lifting replica copies of the population (colony/phage) onto either nylon or nitrocellulose membranes. These membranes can then be probed using methods such as *in situ* hybridisation or probing with antibodies or oligonucleotides. More recently the method of “complementation by functional sufficiency” has been developed which allows for selection of clones of interest with a similar function in a prokaryote (Mundree, 1996).

Another widely and successfully used technique for screening libraries is the differential screening methodology (for example O’Mahony & Oliver, 1999). Essentially, differential screening is done using *in situ* plaque hybridisation. The phage particles are multiplied at high density within the host bacteria and the plaques are grown on agar plates. Duplicate filter membranes, which contain plaques bound to them are lifted from the plates and treated with different solutions to ensure that the phage particles are destroyed and denatured DNA is left bound to the membrane. The filter is then prehybridised in a solution containing a blocking agent, for example salmon sperm DNA or any blocking protein (such as BSA or skim milk powder). This blocking agent saturates any non-specific binding sites on the membrane. The first lifts are then hybridised with the one probe while the replicas are hybridised with the other probe. The clones of interest can be detected since they will bind differently to the two labelled oligonucleotide probes. It is very important to make sure that the probes are specific for the required DNA sequences (they should not contain any reiterated or vector sequence) to ensure that any clone selected will contain the sequence of interest. The oligonucleotide probe can be in the

---

oligonucleotide probe can be in the form of a plasmid which has the insert of interest, ds DNA, RNA or ss cDNA obtained from reverse transcribing mRNA (Huynh *et al.*, 1988). The membrane is then washed to remove any excess probe, incorrectly matched sequences and non-specific interactions, which would otherwise result in a high background. The membranes are aligned after radiography and any signal, which is present only on the one membrane is matched to the plate and the positive clone isolated and sequenced.

The construction and differential screening of a cDNA library made using RNA extracted from dry leaves of *M. flabellifolius* is described in this chapter. The Stratagene UniZap cDNA Synthesis kit (La Jolla, USA) was chosen for this study as this system combines the high efficiency of  $\lambda$ -library construction and the convenience of a plasmid system with the blue/white colour selection. The vector also accommodates inserts which are up to 10 kb long and allows for *in vivo* excision of the pBluescript phagemid. The RecA<sup>-</sup> *E. coli* host strain XL1-Blue MRF<sup>'</sup> allows the amplified library to grow efficiently. The library constructed was amplified and the titre calculated to be  $10^{10}$  pfu/ml, which is indicative of a representative library (King, 1997). Several clones were found to be present only in the dry and not the hydrated leaves of *M. flabellifolius*. These clones were, however, unsequencable for reasons discussed later.

---

## 4.2 MATERIALS AND METHODS

### 4.2.1 Pedigree of bacterial strains and phage

- **XL-Blue MRF's strain**  $\Delta(mcrA)183\Delta(mcrCB-hsdSMR-mrr)173$  *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *pro AB lac I $\phi$  Z $\Delta$ M15 Tn 10 (Tet<sup>r</sup>)*].
- **SOLR<sup>TM</sup> strain** e14' (McrA<sup>-</sup>)  $\Delta(mcrCB-hsdSMR-mrr)171$  *sbcC recB recJ uvrC umuC::Tn5 (Kan<sup>r</sup>) lac gyrA96 relA1 thi-1 endA1  $\lambda^R$  [F' *proAB lacI<sup>r</sup>Z $\Delta$ M15] Su* (nonsuppressing).*
- **ExAssist<sup>TM</sup>** interference-resistant helper phage ( $1 \times 10^{10}$  pfu/ml), supercoiled single-stranded DNA which migrates at 5 kb (Stratagene).
- **VCS M13** interference-resistant helper phage ( $1 \times 10^{11}$  pfu/ml), supercoiled single-stranded DNA which migrates at 6 kb (Stratagene).

### 4.2.2 Isolation of RNA

RNA from desiccated and hydrated leaves of *M. flabellifolius* was extracted using the protocol detailed in section 2.2.12. An aliquot of the RNA extracted was run on a 1% formaldehyde/agarose gel (section 3.2.4) to check for the integrity of the RNA isolated.

### 4.2.3 Selection of poly A<sup>+</sup> mRNA

Oligo(dT)-cellulose (25 mg) was swollen in 1 ml of 0.1 M NaOH. A 1ml column was poured in a silanized pasteur pipette. The column was washed first with 10 ml RNase-free water and then with 30 ml of 1 x column loading buffer (0.5 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% (w/v) SDS) until the pH of the column effluent was less than 8. Total RNA (2 mg) extracted from dry leaves of *M. flabellifolius* was resuspended

---

in DEPC-water and heated to 65°C for 5 min. The denaturation step was crucial as it makes the poly A<sup>+</sup> tracts more available for base pairing to the matrix (Farrell, 1993). An equal volume of 2 x loading buffer was added, the sample was cooled to room temperature and applied to the column. The flow-through was collected, heated again to 65°C, cooled and reapplied to the column. The eluate was reapplied five times in the same manner. The column was washed with 10 column volumes of wash buffer (0.15 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% (w/v) SDS). The poly-A<sup>+</sup> RNA was eluted with 4 column volumes of elution buffer (2 mM EDTA) preheated to 65°C. Fractions (1 ml) were collected and the A<sub>260</sub> of each fraction was determined. The poly A<sup>+</sup> RNA was precipitated at -20°C by the addition of 10% (v/v) 3 M Na-acetate (pH 5.2) and 2.5 volumes of absolute ethanol. The pellet was rinsed in 70% ethanol, and resuspended in DEPC water.

#### 4.2.4 First strand synthesis

The following reagents were added consecutively in an RNase free eppendorf: 5 µl of 10 x first strand buffer (250 mM Tris-HCl pH 8.3, 376 mM KCl, 15 mM MgCl<sub>2</sub>, 50 mM DTT), 3 µl of first strand methyl nucleotide mixture (10 mM dATP, dGTP and dTTP and 5 mM 5'-methyl dCTP), 2 µl of linker-primer (1.4 µg/µl), DEPC water to a final volume of 47.5 µl and 1 µl of RNase block ribonuclease inhibitor (40 U/µl). The reagents were mixed by vortexing the tube gently. 5 µg of poly (A<sup>+</sup>) RNA was added and finally 2.5 µl of StrataScript RNase H<sup>-</sup> reverse transcriptase (100U/µl) was added and the volume adjusted to 50 µl. The template and the primer were then allowed to anneal at room temperature for 10 min. 0.5 µl of [ $\alpha$ -<sup>32</sup>P]dATP (800 Ci/mmol) was aliquoted into a separate tube which would also be used for first strand synthesis. 5 µl of the sample was transferred to the control tube containing the labelled [ $\alpha$ -<sup>32</sup>P]dATP. The reactions were incubated at 37°C for 1 hr. After incubation, the tube containing the radioactivity was kept at -20°C and was later analysed on an alkaline agarose gel as detailed in section 3.2.7.

#### 4.2.5 Second strand synthesis

To the 45  $\mu\text{l}$  of first strand synthesis reaction, the following components were added consecutively: 40  $\mu\text{l}$  of 10 x second strand buffer, 6  $\mu\text{l}$  of second strand nucleotide mixture (10 mM dATP, dGTP, and dTTP, 26 mM dCTP), 88.9  $\mu\text{l}$  of sterile distilled water and 2  $\mu\text{l}$  of [ $\alpha$ - $^{32}\text{P}$ ]dATP (800 Ci/mmol). The mixture was vortexed and spun briefly in a microfuge before the addition of 2  $\mu\text{l}$  RNase H (1.5U/ $\mu\text{l}$ ) and 11.1  $\mu\text{l}$  of DNA polymerase I (9U/ $\mu\text{l}$ ). The mixture was then incubated at 16°C for 2.5 hours. The water bath was checked regularly to make sure that the temperature did not go beyond 16°C as a rise in temperature may cause the formation of hairpin secondary structures which cannot be cloned and interfere with the efficiency of insertion of the newly synthesised cDNA into the vector.

#### 4.2.6 Blunting cDNA termini

23  $\mu\text{l}$  of blunting dNTP mix (2.5 mM dATP, dGTP, dTTP and dCTP) and 2  $\mu\text{l}$  of cloned *Pfu* DNA polymerase were added to the second strand cDNA mixture which was then vortexed and centrifuged at 10 000 g briefly. The reaction was incubated for 30 min at 72°C. The cDNA was purified using a phenol/chloroform [1:1 (v/v)] extraction and precipitated for 18 h at -20°C by the addition of 20  $\mu\text{l}$  of 3M Na-acetate (pH 5.2) and 400  $\mu\text{l}$  of absolute ethanol. The cDNA pellet was resuspended in 9  $\mu\text{l}$  of *EcoRI* adaptors (0.4  $\mu\text{g}/\mu\text{l}$ ) and incubated for 30 min at 4°C. 1  $\mu\text{l}$  of this sample was run eventually together with the control from the first strand reaction on an alkaline agarose gel to check the quality and quantity of the first and second strand synthesis.

#### 4.2.7 Ligating *EcoRI* adaptors

The following components were added to the pellet which was resuspended in 3.6  $\mu\text{g}$  of *EcoRI* adaptors: 1  $\mu\text{l}$  of ligase buffer (500 mM Tris/HCl pH 7.5, 70 mM  $\text{MgCl}_2$ ), 1  $\mu\text{l}$  of 10 mM rATP and 1  $\mu\text{l}$  of  $T_4$  DNA ligase (4 WeissU/ $\mu\text{l}$ ). Ligation was allowed to proceed

---

at 8°C for 18 h after which the enzyme was heat inactivated by incubating the mixture at 70°C for 30 min.

#### 4.2.8 Kinasing *EcoRI* ends

The adaptor ends were then kinased by the addition of 1  $\mu$ l of 10 x ligase buffer, 2  $\mu$ l of 10 mM rATP, 6  $\mu$ l of sterile water and 1  $\mu$ l of T<sub>4</sub>PNK (polynucleotide kinase) (10 U/ $\mu$ l). After incubation for 30 min at 37°C, the kinase enzyme was heat inactivated (70°C for 30 min.).

#### 4.2.9 *XhoI* digestion

28  $\mu$ l of *XhoI* buffer supplement followed by 3  $\mu$ l of *XhoI* enzyme (40 U/ $\mu$ l) were added to the kinased reaction mixture which was incubated for 1.5 h at 37°C. The reaction was cooled to room temperature and 5  $\mu$ l of 10 x STE buffer (1 M NaCl, 200 mM Tris-HCl pH 7.5, 100 mM EDTA) was added. The sample was then run through a Sephacryl S-500 spin column.

#### 4.2.10 Preparation of Sephacryl S-500 spin column

The spin column was made using a 1 ml sterile syringe. The plunger was removed and sterile glass wool was used as the plug of the column. Sephacryl S-500 was added up to the top of the syringe. The syringe was transferred to a Falcon 2059 polypropylene tube and centrifuged at 400 g for 2 min. The spinning procedure at 400 g was repeated until the syringe was packed with beads up to 1 cm from the top. The spin column was loaded with the cDNA and fractionation carried out.

---

#### **4.2.11 Size fractionation**

The cDNA was pipetted onto the prepared spin column and the column centrifuged for 2 minutes. The first fraction was collected. 60  $\mu$ l of 1 x STE buffer was added, the column centrifuged for an additional 2 min and the second fraction collected. This procedure was repeated until four fractions had been collected. 5  $\mu$ l of each fraction was run on a 5% (w/v) non-denaturing acrylamide gel as described in section 4.2.12. The DNA was recovered by ethanol precipitation following a phenol:chloroform (1:1 v/v) extraction. The DNA pellet was air-dried before finally being resuspended in 6  $\mu$ l of sterile water.

#### **4.2.12 5% Non-denaturing polyacrylamide gel electrophoresis**

The 5% (w/v) non-denaturing gel (4.7 g acrylamide (Merck), 0.3 g bis-acrylamide (BDH Biorad), 10 ml 10 x TBE, 40 ml water, 650  $\mu$ l of 10% (w/v) AMPS and 65% (v/v) TEMED) was pre-electrophoresed for 1 h at 90 W. 3.5  $\mu$ l of samples were mixed with 2  $\mu$ l of formamide loading buffer (95% (v/v) formamide, 0.09% (w/v) bromophenol blue, 0.09% (w/v) xylene cyanol FF) and electrophoresed at 90 W until the slower migrating bromophenol blue front reached the bottom of the gel. The gel was then vacuum dried onto Whatman 3MM blotting paper before being exposed to X-ray Hyperfilm (Amersham).

#### **4.2.13 Quantitation of cDNA**

The concentration of cDNA was determined by aliquotting 0.5  $\mu$ l of the unknown cDNA in the centre of an ethidium bromide agar plate (10mg/ml) using 0.5  $\mu$ l of different known concentrations of ds DNA as standards (200, 150, 100, 75, 50, 25 and 10 ng/ $\mu$ l) which were spotted around the edges of the plate. The plate was viewed under UV light and the intensities of the samples and standards compared.

---

#### 4.2.14 Ligating cDNA into Unizap XR vector arms

100 ng of the resuspended cDNA was added to 0.5  $\mu$ l of 10 x ligase buffer, 0.5  $\mu$ l of 10 mM rATP (pH 7.5), 1  $\mu$ l of the Unizap XR vector (1  $\mu$ g/ $\mu$ l) and water to give a final volume of 4.5  $\mu$ l. 0.5  $\mu$ l of the enzyme T<sub>4</sub> DNA ligase (4 Weiss U/ $\mu$ l) was added. The sample was incubated at 12°C for 18 h.

#### 4.2.15 Packaging

##### 4.2.15.1 *Preparation of host bacteria*

XL-1 Blue MRF' (refer to section 4.2.1) cells from the glycerol stock were streaked onto an LB plate containing 50  $\mu$ g/ml tetracycline, and incubated overnight at 37°C. A single colony was picked off the plate and cultured in 50 ml of 2 x YT broth (10 g NaCl, 10 g yeast extract, 16 g bacto-tryptone, per litre) supplemented with 10 mM MgSO<sub>4</sub> and 0.2% (w/v) maltose. The culture was grown at 37°C with gentle shaking for 18 h. The cells were pelleted at 500 g (2000 rpm) for 10 min and resuspended in 10 mM MgSO<sub>4</sub> to an A<sub>600</sub> of 0.5.

##### 4.2.15.2 *Packaging protocol*

Packaging was carried out as per the manufacturer's recommendations using the Unizap kit (Stratagene).

#### 4.2.16 Plating and titering

A serial dilution of the packaging reaction was made and 1  $\mu$ l of the different dilutions of the phage was added to 200  $\mu$ l of the XL-1Blue MRF' cells (in 10 mM MgSO<sub>4</sub> at an A<sub>600</sub> of 0.5). The phage/cell mixture was incubated with shaking for 15 min at 37°C. This step allows the phage to adhere to the cells. 3 ml of NZY top agar (5 g NaCl, 2 g

---

MgSO<sub>4</sub>·7H<sub>2</sub>O, 7 g yeast extract, 10 g NZ amine pH 7.5 and 0.7% (w/v) agarose per litre of broth at 48°C, 15 µl of 0.5 M IPTG (in water) and 50 µl of X-Gal (250 mg/ml in DMF) were added to a sterile tube. The phage/cell mixture was added to the tube, which was shaken before the mixture was poured onto a NZY plate. The top agar was allowed to solidify for 10 min after which the plate was incubated at 37°C. The plaques were grown for 6-8 hours and then counted. Background plaques were blue while the recombinant plaques were white.

#### **4.2.17 Amplification of UniZap XR library**

The host cells were prepared as described in section 4.2.15.1. 50 000 plaque-forming bacteriophage (pfu) were mixed with 600 µl of cells and incubated at 37°C for 15 min with gentle shaking. After incubation, the phage/cell mixture was added to 6.5 ml of NZY top agar and spread onto a 150 mm NZY plate. The plaques were allowed to grow for 6-8 h. Once the plaques were formed, the plates were overlaid with 8 ml of SM buffer and stored at 4°C for 18 h with gentle rocking to allow the phage to diffuse into the buffer. The phage suspension was then recovered from each plate and pooled. The plates were rinsed with another 2 ml SM buffer which was added to the first wash. The volume of the total suspension pooled was noted and chloroform added to a final concentration of 5% (v/v). The phage suspension was incubated for 15 min at room temperature with shaking. Cell debris was pelleted by centrifuging the suspension for 10 min at 500 g. The supernatant was aliquoted into 2 ml sterile cryotubes to which DMSO was added to a final concentration of 7%. The aliquots were stored at -70°C. The titre of the amplified library was checked as described in section 4.2.16.

#### **4.2.18 Labelling of β-actin probe using Amersham Megaprime Labelling Kit**

25 ng of β-actin (kind gift from Mr A. Shoko/Professor N. Illing, Department of Biochemistry, University of Cape Town, South Africa) was combined with 2 µl random primers (Amersham) and the mixture denatured at 95°C for 5 minutes. The reaction

---

cocktail, including nucleotides (1  $\mu$ l of each dGTP, dTTP and dATP (10 mM)), 10 x reaction buffer (2  $\mu$ l), [ $\alpha$ - $^{32}$ P] dCTP (5  $\mu$ l) and 1  $\mu$ l of Klenow enzyme (2U/ $\mu$ l), was added to the denatured DNA at room temperature. The volume was adjusted to 20  $\mu$ l with water. The reaction was incubated at 37°C for 30 minutes and terminated by heat inactivation (95°C for 5 minutes). The volume was adjusted to 100  $\mu$ l with TE and the labelled DNA was purified from the unincorporated nucleotides by chromatography on a G-50 spin column. Specific activity of the labelled DNA was typically  $\sim 1 \times 10^9$  dpm/ $\mu$ g. The labelled DNA was subjected to the same conditions of prehybridisation, hybridisation and washes described in sections 4.2.22 - 4.2.23.

#### 4.2.19 Reverse prime cDNA labelling

Total RNA was labelled by reverse priming with [ $\alpha$ - $^{32}$ P] dCTP using a modified protocol described in the Reverse Prime<sup>TM</sup> cDNA labelling kit (GenHunter Corporation). Essentially 30  $\mu$ g of total leaf RNA extracted from the hydrated and dehydrated leaves of *M. flabellifolius* (section 3.2.2) was used for differential screening of the library. The reaction mixture was as follows: 3  $\mu$ l of dGTP, dATP and dTTP (500  $\mu$ M each), 5 x 1<sup>st</sup> strand buffer (125 mM Tris-HCl, pH 8.3, 188 mM KCl, 7.5 mM MgCl<sub>2</sub>, 25 mM DTT), 7.5  $\mu$ l [ $\alpha$ - $^{32}$ P] dCTP (3000Ci/mmol), a 1:1:1 cocktail of 3 anchored oligo dT primers (10  $\mu$ M each) with sequence 5' AAGCTTTTTTTTTTTN (where N= C, A or G) 3' and 6  $\mu$ l of 10% (w/v) PVP to bind any polyphenolic contaminant (Koonjul *et al.*, 1998; 1999b) in a total volume of 60  $\mu$ l. The mixture was incubated in a thermocycler programmed as follows: 65°C for 5 min, 37°C for 60 min, 75°C for 5 min. After the mixture had incubated for 10 minutes at 37°C, 400 U of MMLV reverse transcriptase (Amersham) was added and reaction allowed to proceed.

#### 4.2.20 Sephadex G-50 chromatography

The labelled cDNA was passed over a 1 ml spin column containing Sephadex G-50 in 1 x STE buffer (0.1 M NaCl, 20 mM Tris-HCl pH 7.5, 10 mM EDTA) to remove

---

unincorporated nucleotides. The efficiency of labelling was measured by Cerenkov counting. The specific activity of the labelled probe was typically  $1.5 \times 10^6$  cpm/ $\mu$ g RNA.

#### 4.2.21 Preparation of nitrocellulose filter replicas

The screening method was carried out according to a protocol described by Maniatis *et al.* (1982). The *E. coli* host strain XL-1 Blue MRF<sup>'</sup> (Stratagene) was grown for 18 h in LB medium containing 0.2% (w/v) maltose and 10 mM MgSO<sub>4</sub> at 37°C. The cells were pelleted at 500 g for 10 min and resuspended in 10 mM MgSO<sub>4</sub> to an A<sub>600</sub> of 0.5. 600  $\mu$ l aliquots of the culture were infected with  $5 \times 10^4$  pfu of the  $\lambda$ ZAP cDNA library. NZY top agarose (8 ml), equilibrated to 47 °C, was added to each aliquot of infected cells. The mixture was inverted twice and spread on prewarmed NZY plates (150 mm). The NZY plates were incubated for 6-8 hours at 37°C until tiny plaques were visible. The plates were then stored at 4°C for at least 2 h before the plate was overlaid with a nylon Hybond N filter (Amersham). The membrane was placed on the chilled plate for 2 min while transfer occurred. The position of the filter was marked with black permanent ink. A duplicate membrane was transferred for 4 min. The membranes were denatured in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 min followed by neutralisation for 5 min by submersion in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8). Finally the membranes were rinsed in 0.2 M Tris-HCl (pH 7.5) and 2 x SSC buffer for 30 s. The membranes were baked at 80°C then UV cross-linked (using a UV-cross illuminator).

#### 4.2.22 Prehybridisation and hybridisation procedures

Two batches of filters (one batch containing the first lifts and the other batch containing the duplicates) were placed in separate pyrex dishes containing prehybridisation buffer (60  $\mu$ l/cm<sup>2</sup> membrane). The prehybridisation buffer consisted of 0.5 M PB buffer (67 g Na<sub>2</sub>HPO<sub>4</sub> and 2 ml 85% (v/v) H<sub>3</sub>PO<sub>4</sub> per 500 ml), 1 mM EDTA, 7% (w/v) SDS and 0.5%

(w/v) skimmed milk powder (Church & Gilbert, 1984). Prehybridisation was carried out at 50°C for 2 hours with gentle agitation before the solution was removed and replaced with hybridisation buffer (prehybridisation buffer without skimmed milk powder). The probes (standardised to  $10^6$  cpm) were boiled for 10 min before addition to the hybridisation buffer. Hybridisation was carried out for 18 h at 50°C with gentle shaking.

#### **4.2.23 Washes and autoradiography**

The membranes were removed from the pyrex dishes and washed as follows: twice in 1 x SSC/ 0.1% (w/v) SDS for 15 min at room temperature. If the membranes had high levels of radioactivity remaining, they were washed once in 0.25 x SSC/0.1% (w/v) SDS for 15 min at 60°C. After the washes, the filters were air-dried on Whatman 3MM blotting paper before being exposed to Hyper-film (Amersham). Autoradiography was performed for 2-3 days at -70°C with an intensifying screen.

#### **4.2.24 Identification and purification of specific clones**

The putative negative plaques were identified by aligning autoradiographs of duplicate filters and identifying overlapping signals. These would correspond to clones present in both the dry and the hydrated leaves of the plant. Agarose plugs corresponding to clones which were only present in the dry plant were cored out of the plates with a pasteur pipette. The plaque was transferred to a sterile eppendorf tube containing 500  $\mu$ l of SM buffer and 20  $\mu$ l of chloroform. The eppendorf was vortexed to release the phage particles into the SM buffer. The tube was then incubated at room temperature for 1-2 h or for 18 h at 4°C. This secondary phage was then subjected to a second round of screening and plaque lifts prehybridised and hybridised as before. These secondary phage stocks were stored at 4°C.

---

#### 4.2.25 *In vivo* single clone automatic excision process

The pBluescript phagemid (containing the cDNAs of interest) was excised from the secondary phage stocks according to the following protocol. The two *E. coli* host strains, SOLR™ and XL1-Blue MRF' were streaked onto LB plates containing kanamycin (20 µg/µl) and tetracycline (12.5 µg/µl) respectively, and incubated overnight at 37°C. Single colonies were inoculated into 100 ml LB and in the case of the XL1-Blue MRF' cells, the medium was supplemented with 0.2% (w/v) maltose and 10 mM MgSO<sub>4</sub>. Cultures were grown for 18 h at 30°C. The cells were pelleted at 1000 g for 10 min and resuspended in 10 mM MgSO<sub>4</sub> to an A<sub>600</sub> of 1.0. The following components were combined in a Falcon 2059 polypropylene tube: 200 µl XL1-Blue MRF' cells (A<sub>600</sub> = 1), 250 µl of isolated phage stock and 1 µl ExAssist (Stratagene) helper phage (> 10<sup>6</sup> pfu/ml). The mixture was incubated for 15 min at 37°C, 3 ml of LB broth was added and the mixture incubated for 2.5 - 3 h at 37°C with shaking. The tubes were then heated at 65°C - 70°C for 20 min and centrifuged for 15 min at 1000 g at 4°C. The supernatant, containing the plasmid packaged as a filamentous phage particle, was decanted into a sterile tube and stored at 4°C for up to 2 months. The excised phagemid was plated by adding 200 µl of freshly grown SOLR cells (A<sub>600</sub> = 1) to three separate eppendorfs and adding 100 µl, 10 µl or 1 µl of the phage stock to each tube. The tubes were incubated at 37°C for 15 min before 200 µl and 100 µl of cells from each phage mixture were plated on LB/ampicillin plates (50 µg/ml) and incubated at 37°C for 18 h. To maintain the pBluescript phagemid, colonies were restreaked onto new LB/ampicillin plates. Glycerol stocks for longterm storage were kept at -70°C.

#### 4.2.26 DNA isolation using High Pure Plasmid Isolation Kit (Boehringer Mannheim)

Single bacterial colonies containing plasmid DNA were picked from agar plates and inoculated into 4 ml LB containing ampicillin (50 µg/ml). Cells were grown overnight at 37°C and pelleted for 15 min at 4°C at 10 000 g. The cells were resuspended in 250 µl cell suspension solution (50 mM Tris-HCl, pH 8, 10 mM EDTA and 100 µg/ml RNase

A), and lysis was achieved by addition of 250  $\mu$ l of cell lysis solution (0.2 M NaOH, 1% (w/v) SDS). Plasmid was released from the lysed cells by gentle mixing and the mixture supplemented with 350  $\mu$ l neutralising and binding solution (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2). Cell debris and chromosomal material was precipitated for 10 min by centrifugation at 12 000 g. The supernatant was retained and passed directly over a High Pure filter column. The column was centrifuged for 60 s after which it was washed with 500  $\mu$ l of wash buffer I (5 M guanidine hydrochloride, 20 mM Tris-HCl, pH 6.6) and 700  $\mu$ l of wash buffer II (20 mM NaCl, 2 mM Tris-HCl, pH 7.5). The DNA was finally eluted using 100  $\mu$ l sterile water and the concentration determined spectrophotometrically at 260 nm.

#### **4.2.27 PCR using T3/T7 and M13 reverse and forward primers**

PCR using plasmid DNA (section 4.2.26) was performed in a final reaction volume of 50  $\mu$ l. The reaction mix was prepared by combining template DNA (100-500 ng), 5  $\mu$ l 10 x PCR buffer, 0.2 mM each dNTP, 0.4  $\mu$ l Taq (5U/ $\mu$ l), 2  $\mu$ l of each primer (10  $\mu$ M) and adjusting the final MgCl<sub>2</sub> concentration to 1.5 mM. The primers were M13F (5' TGTAACGACGGCCAGT), M13R (5' AACAGCTATGACCATG 3'), T3 (5' ATTAACCCTCACTAAAG 3') and T7 (5' AATACGACTCACTATAG 3') and were used in pairs (M13F and M13R used together and similarly T3 and T7 were used together). The cycling programme was set for 25 cycles with each cycle constituting incubation at 94°C for 30 s, followed by 55°C for 30 s, and 72°C for 40 s. A final incubation was performed for 5 min at 72°C. 15  $\mu$ l of the PCR product was checked on a 1% (w/v) agarose gel.

#### **4.2.28 Sequence analysis**

DNA sequencing was performed in a Perkin Elmer ABI Prism 377 automated DNA sequencer by Carel Van Heeren (Central Sequencing Facility, University of Stellenbosch, South Africa) using M13 reverse and forward primers as well as T3 and T7 primers.

---

Analysis of the DNA sequences of the different clones was carried out using the BLAST network services at the National Centre for Biotechnology Information (NCBI). BLASTN (Altschul *et al.*, 1990) and BLASTP (Gish & States, 1993) searches were performed which both make comparisons with the public sequence databases. The BLASTN program is optimised to find nearly identical nucleotide sequences, whereas BLASTP is used for database similarity searches of protein coding regions.

#### 4.2.29 Recovery of single stranded DNA from pBluescript

Single stranded DNA was isolated according to the Stratagene instruction manual for pBluescript Exo/Mung DNA Sequencing system. Single colonies were picked from the ampicillin plates (section 4.2.25) and grown in 5 ml of 2 x YT containing 50 µg/ml ampicillin and VCM 13 helper phage at  $10^7$ - $10^8$  pfu/ml (multiplicity of infection ~ 10). After a 2 h incubation at 37°C with vigorous aeration, kanamycin was added to a final concentration of 70 µg/ml and the culture was allowed to grow for another 16 h at 37°C. The cells were pelleted at 12 000 g for 5 min at 4°C and the phage-containing supernatant mixed with 0.2 volumes 20% (w/v) PEG 8000/2.5 M NaCl. The mixture was vortexed gently and incubated at room temperature for 15 min. The phage particles were pelleted at 12 000 g for 5 min at 4°C and the supernatant carefully removed. The pellet was resuspended in 400 µl of 0.3 M NaOAc, pH 6, and 1 mM EDTA by vortexing vigorously. Phage were extracted in 1 volume of phenol/chloroform and DNA recovered by ethanol precipitation. The DNA pellet was evaporated to dryness and resuspended in 20 µl water.

---

## 4.3 RESULTS

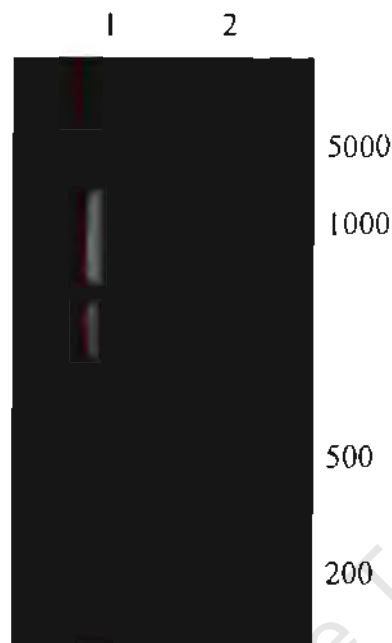
### 4.3.1 Construction of the cDNA library

The primer used in the first strand synthesis was a 50-mer oligonucleotide containing an 18-mer poly dT sequence and a (GAGA)<sub>5</sub> sequence as well as a *XhoI* restriction site (shown in bold). The sequence was as follows:

5'TTTTTTTTTTTTTTTTTTTT**GAGCTCTGATCAAGAGAGAGAGAGAGAGAGA** 3'

The *XhoI* recognition site allows the cDNA to be inserted into the UniZap XR vector (cut with *EcoRI* and *XhoI*) in a sense orientation with respect to the *lacZ* promoter. The GAGA sequence protects the *XhoI* restriction site and the poly dT sequence as well as producing more efficient cutting of the *XhoI* site by the enzyme. The poly dT region binds to the poly A tail of the template mRNA and acts as the primer for the first strand synthesis. First strand synthesis utilises dATP, dGTP, dTTP and 5-methyl dCTP instead of the usual dCTP. 5-methyl cytosine protects the newly synthesised cDNA from digestion by particular nucleases such as *XhoI*. RNase H, used in the second strand synthesis mixture nicks any RNA still bound to the first strand cDNA and gives rise to a range of fragments which in turn become the primers for DNA polymerase I enzyme. The quality of first and second strand synthesis was checked by running an aliquot of the reactions spiked with radioactive nucleotides on an alkaline agarose gel. This result is shown in fig. 4.2.

---



**Fig. 4.2:** Alkaline agarose gel showing the quality of first (lane 1) and second strand synthesis (lane 2). The migration of fragments of bacteriophage lambda digested with *Pst*I is depicted on the right of the gel.

As can be seen from fig. 4.2, the intensity of the labelling of the two strands was different because of the relative ratio of [ $\alpha$ - $^{32}$ P]dATP ATP to the amount of dNTPs used in the first- or second-strand reaction. Typically, the second strand should be only 1/10 to 1/20<sup>th</sup> of the intensity of the first strand band. The bright smear of bands corresponds to abundant mRNAs and the distribution of molecular weights (0.5 - 3 kb) implies that the cDNA is pure and intact. The ratio of the intensity of the first strand to the second strand was about 10:1 (which falls within the desired range).

*Eco*RI adaptors (sequences shown below) were added to the blunt ends of the ds cDNA:

5'-AATTCGGCACGAG-3' (13-mer)

3'- GCCGTGCTC-5' (9-mer)

The 9-mer adaptor was kinased so that it would ligate to the blunt ends of the ds cDNA and other adaptors while the 13-mer adaptor was not phosphorylated so as to prevent it from ligating to other cohesive ends. Once the ligation of the adaptors was completed, the *EcoRI* ends were kinased to allow it to ligate to the dephosphorylated vector arms. *XhoI* digestion was then performed in order to release the *EcoRI* adaptor and the residual linker-primer from the 3' end of the cDNA leaving a 3' *XhoI* sticky end. The fragments after separation on a Sephacryl S-400 spin column are shown in Fig. 4.3.



**Fig. 4.3:** 5% non-denaturing acrylamide gel used to check the sizes of cDNA fragments eluted from the spin column. Lane 1 (eluate 1) contains the desired large fragments, lanes 2-4 (eluates 2-4) contain smaller fragments.

Fraction 1 contained the largest fragments of cDNA which were then ligated to the UniZap XR vector arms and packaged. Ligation was carried out only because the cDNA concentration was more than 50ng/ $\mu$ l. This concentration favours the formation of concatamers. Optimal efficiency of packaging is obtained when the  $\lambda$  DNAs are concatameric (Maniatis *et al.*, 1982) hence the ligated cDNA-vector samples were kept at 4°C to prevent multiple freeze-thawing which can break concatameric strands.

The bacterial cells used in the titering step were an *E. coli* strain called XL1-Blue MRF'. This strain is *mcrA*<sup>-</sup> and *mcrB*<sup>-</sup> which prevents digestion of the hemimethylated DNA by the *mcrA* and the *mcrB* restriction systems. XL1-Blue MRF' contains an F' episome which fulfills several functions. The main advantage is that the episome contains the  $\Delta$ M15 mutation in the *lacZ* gene which is required for  $\alpha$ -complementation with the amino terminus of the *lacZ* gene in the vector. Both of these partial genes must be present to generate a functional  $\beta$ -galactosidase ( $\beta$ -gal) protein from the *lac* operon. If present  $\beta$ -gal cleaves the substrate X-gal, which is added to the plates resulting in blue plaques. If a cDNA clone is inserted into the polylinker region upstream of the *lacZ* gene in the vector, it will disrupt the production of the  $\beta$ -gal and no cleavage of the X-gal will occur resulting in recombinant white plaques. The F' episome also contains the *lac Z* repressor which blocks the transcription of  $\beta$ -gal from the *lacZ* promoter unless the inducer IPTG is present. Finally the F' episome contains genes for expression of the bacterial F' pili which is necessary for phage infection. The cells were grown on LB plates supplemented with tetracycline to maintain the F' episome since the *Tn10* tetracycline resistance gene is found on the episome. When the cells were cultured, one colony was picked from the plate and transferred to 2 x YT broth supplemented with magnesium sulphate and maltose which both induce the production of a maltose receptor on the cell surface to which the phage attaches.

A primary library, such as this, tends to be unstable and hence was amplified to produce a large, stable quantity of a high stock (10<sup>4</sup> pfu/ml) of the initial library. Once the library had been amplified, chloroform was added to a final concentration of 5% (v/v) to prevent

---

contamination from filamentous helper phages which convert the UniZap XR phage to pBluescript phagemids. The amplified library was titered and the results shown in Table 6.

The titre of the cDNA library was calculated using the following formula:

Titre = number of plaques x dilution factor x total volume

**Table 6:** Table showing number of plaques, from which the titre of the library can be calculated.

Dilution	No. of white plaques	No. of blue plaques
$10^{-2}$	TNTC	TNTC
$10^{-3}$	TNTC	215
$10^{-4}$	2400	21
$10^{-5}$	244	2
$10^{-6}$	17	0

TNTC: too numerous to count

From the table, the titre was calculated and found to be:

$$\begin{aligned} ((2400 \times 10^4) + (244 \times 10^5) + (17 \times 10^6))/3 &= 2.18 \times 10^7 \text{ pfu}/\mu\text{l} \\ &= 2.18 \times 10^{10} \text{ pfu/ml} \end{aligned}$$

The ratio of blue:white plaques was about 1:100 which gave a preliminary indication of a good library in that most plaques contained a cDNA insert. The titre of the amplified library was  $10^{10}$  pfu/ml typical of a good library (King, 1997). Furthermore, when the library was screened with  $\beta$ -actin, at least 5% of the plaques gave a positive signal which would indicate that the cDNA library constructed was representative since  $\beta$ -actin is the most abundant transcript in the eukaryotic cell and represents 5-7% of the total genome

(Alberts *et al.*, 1989). This means that the probability of isolating a rare clone is quite high. One out of every 800 clones screened should represent a rare transcript (Alberts *et al.*, 1989).

### 4.3.2 Differential screening of the cDNA library

Since the cDNA library appears to be a representative one, recombinant clones involved in desiccation tolerance from *M. flabellifolius* were identified. Differential screening of the library using labelled RNA from desiccated and rehydrated leaves resulted in several putative clones present only during desiccation. These initial positive clones were subjected to a second round of screening.

A total of  $1 \times 10^6$  pfu was screened at a density of  $1 \times 10^3$  pfu/plate. Duplicate filters were lifted from each plate and all filters were subsequently incubated with radiolabelled RNA probes from dry and green leaves of *M. flabellifolius*. The two probes were standardised and  $10^6$  cpm per ml prehybridisation/hybridisation buffer was used in each case to ensure that the same level of radioactivity was used on each membrane. This allows comparisons between clones expressed during hydration and desiccation conditions since the amount of probe used in both cases was the same.

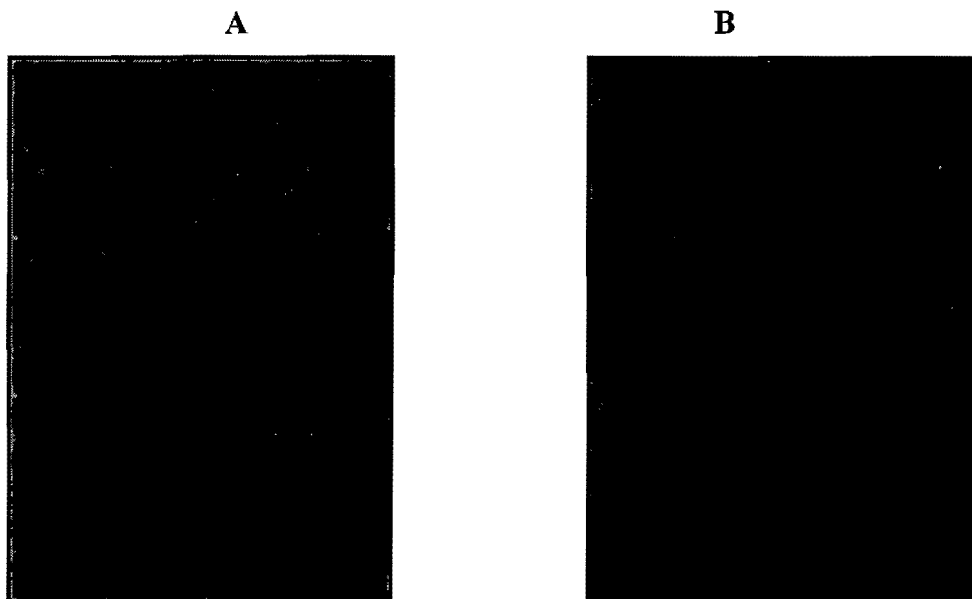
After a second round of screening 44 plaques were found to be still positive, i.e. gave a signal with the probe from the dry RNA and not with the probe from the hydrated leaves. Excised phagemids were titred and plated on LB-ampicillin plates containing X-gal and IPTG. 90% of the colonies were white indicating the phagemids contained an insert.

### 4.3.3 Expression analysis of clones isolated from the library

Plasmid DNA was isolated from all 44 positive phagemids and slot blot analysis was performed using labelled RNA from hydrated and dry leaves (as per section 4.2.19.1). This analysis demonstrated that 27 of the 44 DNAs were expressed only in dry leaves

---

(Fig. 4.4). No RNA corresponding to these 27 clones could be detected in hydrated leaves, even after overexposure of the slot blot. This analysis was repeated three times and the same results obtained. Again, the probes were standardised to ensure equal binding between template and probe. This result is in accordance to those obtained from the secondary screening of phage whereby these clones were found to be only present in RNA from dry leaves and not hydrated leaves. The fact these 27 clones were only expressed in dry leaves implies that they might play an important role in conferring desiccation tolerance to this plant.



**Fig. 4.4:** Expression analysis of clones isolated from differential screening of cDNA library. 1  $\mu$ g of plasmid DNA from each clone was used. Results of slot blot analysis (A) when RNA extracted from dry leaves and (B) RNA from green leaves was used as the probe.

#### **4.3.4 PCR screening of clones expressed during desiccation of *M. flabellifolius***

To determine the size of the insert, PCR using the T3/T7 and M13 reverse and forward primer pairs was carried out using plasmid DNA from these clones as a template. This

---

PCR screening technique using primers specific to the vector will amplify across the MCS (multiple cloning site) and any DNA inserted into the plasmid. All 27 clones produced a product of approximately 250-300 bp during amplification with both the T3/T7 or M13 primer pairs. The size of the amplified product was smaller than expected. Expected sizes were between 1-3 kb since results from the reverse transcription (Fig. 4.2) and the size fractionation using a Sephacryl column (Fig. 4.3) indicated that the sizes of the transcripts were indeed larger than 250-300 bp. However, since these 250-300 bp inserts gave strong signals when hybridised to RNA from dry leaves, these clones were subjected to sequence analysis.

#### **4.3.5 Sequence analysis of clones expressed during desiccation of *M. flabellifolius***

23 of the clones could not be sequenced either using T3/T7 or the M13 primer pairs. Clones 5, 15, 16 and 22 were sequenced, however, the entire sequence was identical to the MCS of pBluescript plasmid. Since no sequence could be obtained from the ds template, ss DNA was isolated from these same colonies and sequenced. Once again, no sequence could be obtained.

It is important to remember that these clones gave positive signals with RNA from only dry leaves during primary and secondary screening of the library, as well as during slot blot analysis. In the presence of IPTG/X-gal these clones gave white colonies indicating the presence of an insert. Furthermore, bacteria containing no insert in their plasmid should not grow on the ampicillin plate. All these results indicate that an insert was indeed present in the MCS. The most likely explanation for the lack of informative sequence appears to be that the vector was fragmented during the *in vivo* excision step and rearrangement of the MCS and insert occurred. This would lead to complexity of the template which could therefore not be sequenced or produced a sequence corresponding to the MCS.

---

#### 4.4 DISCUSSION

A cDNA library was constructed using RNA isolated from desiccated leaves of *M. flabellifolius*. The amplified cDNA library had a titre of  $2 \times 10^{10}$  pfu/ml, indicative of a representative library (Alberts *et al.*, 1989). The ratio of recombinant to non-recombinant plaques of 1:100 was another indication of a good library. Finally screening of the library with  $\beta$ -actin confirmed that the cDNA library was indeed representative. Secondary screening of the library resulted in 27 clones which only gave positive signals when hybridised with RNA isolated from dry leaves from this species. The fact that these clones are differentially expressed in dry tissues indicates that they could be potential candidates involved in protecting *M. flabellifolius* during dehydration.

However, clones purified and excised from the library were either unsequencable or contained no insert within the MCS of the vector. This would suggest that the subcloning step (*in vivo* excision) was inefficient since all the reactions prior to this step worked successfully. The *in vivo* excision depends totally on the f1 bacteriophage-derived helper proteins which are contained within the UniZap XR vector. These proteins recognise a region of DNA usually serving as the "origin of replication" for positive strand synthesis. The origin of replication, in turn, contains a site of initiation as well as a site of termination for DNA synthesis. The helper proteins recognise the site of initiation, nick one of the two DNA strands starting DNA synthesis and duplication of any DNA fragment found downstream (3' end) of the nicking site. DNA synthesis continues through the cloned insert DNA until the site of termination is reached. The ss DNA molecule thus formed is circularized by the gene II product from the f1 proteins, producing a circular DNA molecule containing pBluescript SK (-) phagemid as well as the insert DNA. The *in vivo* excision involves ExAssist helper phage and SOLR strain. The helper phage contains an amber mutation that prevents replication of the phage genome in the nonsuppressing SOLR strain and hence only excised phagemids containing an insert can replicate in the host. Although this subcloning step might seem obvious, it actually is a complicated procedure whereby any slight damage to the helper

---

proteins (through freeze-thawing or possible degradation) could result in inefficient DNA synthesis and hence no insert within the initiator and terminator sites. If the vector was fragmented during the procedure for example by freeze-thawing, pieces of the vector could easily be inserted during the circularisation step hence giving results obtained in this study. The other possible problem associated with *in vivo* excision could be low viability of the helper phage and/or the host cells which would both result in inefficient excision. It has been shown previously that insert DNAs can get deleted during the circularization step of the subcloning (King, 1997; Maniatis *et al.*, 1982).

The other possibilities for the failure of sequencing the clones obtained from this cDNA library could be that they contained complex sequences (possibly GC rich) and/or secondary structures which would prevent sequencing (Thomas *et al.*, 1997). Furthermore, a current problem with sequencing clones from cDNA libraries is the difficulty in reading through poly (A) tail sequences that are present at the 3' end of most cDNA sequences. This could be due to random insertions and deletions caused by polymerase slippage occurring at high frequency within homopolymeric tracts (Thomas *et al.*, 1997).

To conclude, although no sequence data was obtained several clones were found to be only expressed in dry and not hydrated leaves of *M. flabellifolius* and could therefore play a role in desiccation tolerance in this plant. Further investigation is required to identify the nature of these clones. The library could be rescreened and a different strain of helper phage used during the *in vivo* excision step. Another possibility would be to screen the library with antibodies to proteins known to confer desiccation tolerance, however, this will not isolate novel proteins involved in desiccation tolerance. Ultimately the same clones can be sequenced again with primers going from both sides of the MCS. However, in this thesis, a second strategy, that of differential display, was employed to isolate differentially expressed genes (see chapter 5).

---

## CHAPTER 5

### ANALYSIS OF ALTERED GENE EXPRESSION DURING DEHYDRATION IN *M. flabellifolius* BY DIFFERENTIAL DISPLAY

#### 5.1 INTRODUCTION

The complexity of the genome of a particular organism and the relative abundance of a particular mRNA within a cell type affects the ease with which one can isolate a gene or cDNA clone of interest. Prior to the advent of PCR, the principal techniques to isolate mRNAs in comparative studies were differential or subtractive hybridisation of a library. These methods work very well for abundant messages (representing  $\geq 10\%$  of total mRNA) whose representation is sufficient to give positive signals during the hybridisation technique. However, rare messages (as low as 1 in  $10^6$  mRNAs) are very difficult to detect and even if a million plaques are screened, the chance of picking up a rare message is very low (Travis *et al.*, 1987). Furthermore, the construction and screening of a library poses several intrinsic problems (see chapter 4). Hence, in addition to the cDNA library construction and screening, the technique of differential display (Liang & Pardee, 1992; Liang *et al.*, 1993) was performed concurrently as a second strategy for obtaining genes expressed during dehydration in *M. flabellifolius*. This technique has been widely used in obtaining genes which are differentially expressed in plants (Benito *et al.*, 1996; Bertioli *et al.*, 1995; Truesdell & Dickamn, 1997; Visioli *et al.*, 1997).

The differential display technique is basically a combination of three techniques: (i) reverse transcription from anchored primers (ii) amplification of cDNAs by PCR and (iii) resolving the cDNAs on a sequencing gel. This technique reveals differential gene expression immediately and each set of primers chosen so as to display between 50 and 100 cDNAs, as this is optimal for visualisation on a sequencing gel (Liang & Pardee,



Reverse transcription essentially converts all mRNAs to cDNAs. The primers chosen are anchored oligo dT primers consisting of a stretch of Ts plus one or more 3' bases, which take advantage of the polyadenylate tail present on most mRNAs. It has been shown that anchored oligo dT primers reduce the redundancy and the number of reverse transcription reactions required whilst avoiding the use of degenerate primers which tend to under-represent certain populations of mRNAs. Furthermore, these anchored primers select only a subpopulation of mRNAs which allows optimal visualisation. For example, a primer such as 5' dT<sub>n</sub>G would allow anchored annealing to mRNAs containing C immediately upstream of the poly A tail. By systematically changing the anchored base(s) at the 3' end maximum coverage of mRNAs can be obtained. Oligo dT primers without anchors result in complete smearing on the gel since the primers would bind to all mRNAs in different places on the poly A tail (Liang *et al.*, 1993). A restriction site can be introduced at the 5' end of the primers so that amplified cDNAs can be easily cloned and retrieved (Liang & Pardee, 1995).

After cDNA synthesis of a mRNA subpopulation, a short random primer (10-15 bp) is used in a PCR. Although it could be argued that such short primers may result in non-specific DNA amplification by PCR (a standard PCR uses primers of 20 or more nucleotides), it has been shown that a short primer together with an anchored dT primer amplify about 100 mRNAs which is the optimal number of bands for display on a sequencing gel (Liang & Pardee, 1995). Since the primers are short, the annealing temperature is dropped to 40°C to allow priming (Liang & Pardee, 1995). It has been shown that an annealing temperature of between 40 and 42°C is optimal and many successful applications using plant tissues have been achieved (for example, Benito *et al.*, 1996; Vielle-Calzada *et al.*, 1996).

The final step in differential display is the resolution of the amplified bands in a polyacrylamide gel. A denaturing gel enables up to 100 cDNAs to be separated with high resolution although these molecules must be isotopically labelled during amplification. It has been shown that, if non-denaturing gels are used to separate the DD-PCR products,

---

incompletely annealed ssDNA and heteroduplexes of the same gene were usually resolved as different bands (Bauer *et al.*, 1993). Since agarose gels with ethidium bromide staining are not sensitive enough to depict all the bands, radioisotope detection of the cDNAs on a denaturing gel appears to be the most appropriate method for DD-PCR. Since  $^{35}\text{S}$  can evaporate and contaminate PCR machines,  $^{33}\text{P}$  (which is as sensitive as  $^{35}\text{S}$ ) is the preferred isotope (Trentmann *et al.*, 1995). After display on a sequencing gel, bands can be cut out and DNA eluted. These DNAs (with or without cloning) can be used for further analysis.

In this chapter, the strategy of differential display was used to isolate genes which are expressed during desiccation in *M. flabellifolius*. DD-PCR products from control (hydrated) and desiccated plants were compared and 8 transcripts were identified which were present only in the dry and not the hydrated plant. Expression analysis performed on these clones confirmed their differential expression. These products were isolated and cloned before being subjected to sequence analysis which suggested that most of the clones contained partial ORFs (open reading frames). These partial clones contained very short stretches of nucleotides (nt) and amino acids (aa) identical to sequences in the public databases.

---

## 5.2 MATERIALS AND METHODS

### 5.2.1 RNA isolation and DNase treatment

RNA was extracted from young dry and hydrated leaves of *M. flabellifolius* using the methodology described in section 3.2.2. Any contaminating chromosomal DNA was removed from the RNA using the protocol outlined in the Messageclean™ Kit (Genhunter Corporation). 50 µg of total cellular RNA was incubated for 30 min at 37°C with 10 units of DNase I (Boehringer Mannheim) in 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 1% (w/v) PVP. After extraction with phenol/chloroform (3:1), the RNA was recovered by ethanol precipitation in the presence of 3M NaOAc.

### 5.2.2 Primers

The primers used in this section were synthesised by P. Ma (Department of Biochemistry, University of Cape Town, South Africa) using a Beckman oligo 1000M DNA synthesiser and purified by established procedures (Ausubel *et al.*, 1987). Concentrations were determined spectrophotometrically. The molar extinction coefficient for each oligonucleotide was estimated from the extinction coefficients of the individual bases (Ausubel *et al.*, 1987). The primers used were HT<sub>11</sub>M (5' AAGCTTTTTTTTTTTM where M = G, C or T) and HAP<sub>1</sub> (5' AAGCTTGATTGCC).

### 5.2.3 Reverse transcription of mRNA

0.2 µg of total RNA was used for reverse transcription in a 20 µl reaction volume as described in section 3.2.6. The samples were done in duplicate.

---

### **5.2.4 DNA polyacrylamide gel**

6% (w/v) polyacrylamide denaturing gels were prepared and run as described in section 3.2.8.

### **5.2.5 Amplification of cDNAs**

PCR was performed in 20  $\mu$ l final volume reactions as described in section 3.2.6.

### **5.2.6 Recovery and reamplification of cDNA bands**

The DNA polyacrylamide gel was blotted onto a piece of Whatman 3MM paper and dried without methanol/acetic acid fixing. The autoradiogram and dried gel were orientated using radioactive ink. After developing the film, cDNA bands corresponding to transcripts which were differentially expressed, were cut out from the dried gel. The gel slice along with the 3MM paper was incubated in 100  $\mu$ l sterile H<sub>2</sub>O for 10 min. After rehydration of the polyacrylamide gel, the cDNA was recovered by boiling the gel slice for 15 min in a tightly capped eppendorf (the piece of paper was left at the bottom of the tube). cDNA was recovered by ethanol precipitation in the presence of 3M NaOAc and 5  $\mu$ l of glycogen (10mg/ml) as a carrier. The eluted cDNA was redissolved in 10  $\mu$ l H<sub>2</sub>O. 4  $\mu$ l of the cDNA was reamplified in a 40  $\mu$ l reaction volume using the same primer set and PCR conditions as used in the mRNA display except that the dNTP concentrations were 20  $\mu$ M instead of 2  $\mu$ M and no isotope was added. 30  $\mu$ l of the PCR samples were run on a 1% (w/v) agarose/TAE gel and stained with ethidium bromide (as per section 3.2.4). The remaining samples were stored at -20° C.

---

### 5.2.7 Purification of PCR product from gel

The amplified product from above was purified from the gel using the Agarose Gel DNA Extraction Kit (Boehringer Mannheim) as per the manufacturer's instructions. The cDNA fragments were then used for reverse Northern slot blot analysis and cloning.

### 5.2.8 Reverse Northern blot analysis

The reamplified cDNA fragments were used in slot blot reverse Northern analysis. 1  $\mu$ g of each reamplified band was slot blotted as described in section 2.2.13. Total RNA from the dry and the hydrated plant was labelled and used as probes for the slot blot analysis (see section 4.2.19). Prehybridisation and hybridisation were performed according to sections 4.2.22 - 4.2.23.

### 5.2.9 Cloning of cDNA fragments

Reamplified cDNA fragments, which were purified from the agarose gel, were cloned into pBluescript SK(+) vector at the *EcoRV* sites. The vector was cut with *EcoRV* restriction enzyme and run on a 1% (w/v) agarose gel. The cut/linearised vector was purified using the procedure described in section 5.2.7.  $MgCl_2$  was added at a final concentration of 5 mM to the vector mixture. The protruding termini were blunted by adding  $T_4$  DNA polymerase and 1  $\mu$ l of dNTPs (each at a final concentration of 2 mM) and the mixture incubated at 11°C for 20 min after which the enzyme was heat inactivated by further incubation at 75°C for 10 min. The vector was then dephosphorylated for 60 min at 37°C by addition of 3 units of CIP enzyme and 1 x CIP buffer (Boehringer Mannheim) after which the reaction was terminated by the addition of EGTA (to a final concentration of 20 mM). The CIP enzyme was heat denatured by incubation for 10 min at 65°C. The cDNA insert (amplified and purified cDNA) was then ligated to the vector in a total ligation volume of 10  $\mu$ l. The ligation mixture contained 5  $\mu$ l insert DNA, 1  $\mu$ l vector DNA, 1  $\mu$ l 10 x ligation buffer (500 mM Tris-HCl, pH 7.6, 50

mM MgCl<sub>2</sub>, 50 mM DTT, 10 mM ATP) and 2 units T<sub>4</sub> DNA ligase. Ligation was performed at 16°C for 18 hours.

### 5.2.10 Transformation of competent cells

JM 109 High Efficiency Cells (kind gift of Di James, Department of Microbiology, University of Cape Town, South Africa) were transformed with the ligation reactions. A 50 µl aliquot of competent cells was combined with 5 µl of the ligation reaction mixture and incubated on ice for 20 min. The cells were heat shocked for 60 s in a water bath at 37°C, and placed on ice for 2 min. The transformed cells were supplemented with 800 µl LB (room temperature) and incubated for 50 min at 37°C with shaking. The cells were then centrifuged for 15 s in a microfuge, the supernatant discarded and the cells resuspended in 100 µl fresh LB. Transformants were selected by plating 100 µl of a serial dilution of the cells (from 10<sup>0</sup> to 10<sup>-6</sup> dilution) onto LB plates containing ampicillin (50 µg/µl) and incubated at 37°C overnight.

### 5.2.11 DNA isolation

Plasmid DNA was isolated as described in section 4.2.26.

### 5.2.12 Sequence analysis

DNA sequencing was performed as described in section 4.2.28. The algorithms 'DNASIS' and 'ORF finder' at NCBI were used to check for ORFs.

### 5.2.13 End labelling of DD-PCR products

End labelling reactions were performed in 25 µl final volume reactions containing 100 ng of the DD-PCR product, 1 x T<sub>4</sub> PNK buffer, 5 units of T<sub>4</sub> PNK enzyme and 100 µCi [ $\gamma$ -<sup>32</sup>P] ATP. The reaction mixture was incubated at 37°C for 30 min before the enzyme was

---

denatured at 65°C for 5 min. 25 µl of 4M ammonium acetate followed by 250 µl were added and mixture vortexed. The labelled DNA was allowed to precipitate at -20°C for 18 h and was recovered by centrifugation at 12 000 g for 15 min at 4°C. The DNA pellet was resuspended in 25 µl of sterile water. This labelled DNA was then used as a probe to screen a cDNA library, constructed using RNA from dry leaves of *M. flabellifolius* (chapter 4).

#### **5.2.14 Screening of library**

The end labelled DD-PCR products were used as probes to screen a cDNA library as described in sections 4.2.21. The prehybridisation/hybridisation and washes were done as per sections 4.2.22-4.2.23. The putative positive clones were subjected to a second round of screening and purified as described in section 4.2.24. These clones were excised and sequenced (sections 4.2.25 & 4.2.28).

---

## 5.3 RESULTS

### 5.3.1 Optimization of DD-PCR

It was necessary to optimise RNA isolation since various published protocols that were tested did not yield any RNA (see chapter 3). Addition of PVP to the RNA sample prior to checking the absorbance readings at a final concentration of 1% (w/v), resulted in an increased  $A_{260} : A_{280}$  ratio (from 1.6-1.7 to 1.9-2.0), characteristic of good quality RNA (Lessard *et al.*, 1997; Wilkins & Smart, 1996). However, it was found that the RNA isolated from *M. flabellifolius* still contained traces of polyphenolics which were inhibitory to the DD-PCR resulting in no cDNA fragments observed on the sequencing gel (not shown).

To determine whether the reverse transcription step of the DD-PCR was successful, 50 ng RNA was reverse transcribed in the presence of [ $\alpha$ - $^{32}$ P]dATP. A range of different length fragments was obtained suggesting that failure of the DD-PCR was due to inhibition of the PCR step. This may be due to the larger amount of enzyme used for reverse transcription (200 U) than for PCR (1 U) (see chapter 3). This result is in agreement with those of Singh *et al.* (1998) who demonstrated that PCR was inhibited by the presence of 1.2 - 1.8 mg chlorogenic acid whereas reverse transcription was only inhibited by much higher concentrations (6-7.5 mg).

Different sets of PCR (including optimisation of  $Mg^{2+}$ , annealing temperature and final concentration of anchored primer) were carried out to determine the optimum conditions for analysis of gene expression patterns between dry and hydrated leaves of *M. flabellifolius*. The best results, in terms of reproducibility and optimal number of bands, were achieved when reverse transcription and PCR were performed in the presence of soluble PVP (Koonjul *et al.*, 1999b), using 4.5  $\mu$ M  $Mg^{2+}$  (usually 1.5  $\mu$ M), 10  $\mu$ M final concentration of anchored primer (usually 2  $\mu$ M), and an annealing temperature of 55°C for 1 minute (usually 40-42°C for 2 min) (Liang & Pardee, 1992). In all experiments,

---

duplicate reactions were included in order to reduce the number of false positives.

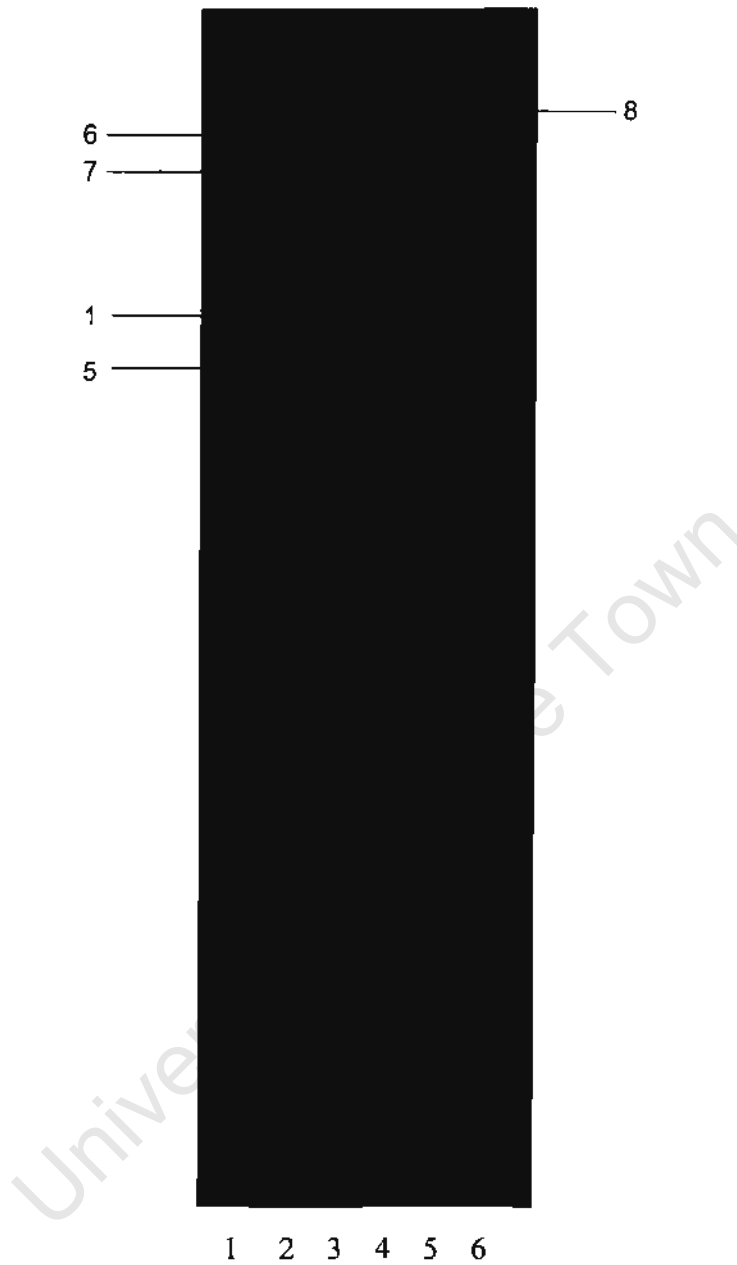
### 5.3.2 Analysis of banding pattern on sequencing gel

Figure 5.2 shows a representative example of results obtained. In addition to bands representing constitutively expressed mRNAs from both the dry and the hydrated leaves of *M. flabellifolius*, a few novel bands were detected only in the dry or the hydrated samples. A total of 3 primer combinations were tested (3 anchored primers and 1 random 13-mer). Although differences in the number of bands were observed between the different primer combinations, an average of 70-80 bands was detected in the different samples. Several bands were apparently specific to the dry plant, of which 8 were chosen and recovered from the gel (arrowed in Fig. 5.2). All 8 bands were successfully amplified in the first round of PCR (Fig 5.3). These fragments were in the range of 170-315 bp. Fragments 1, 5, 6, 7 and 8 appeared to be homogeneous, while fragments 2, 3 and 4 were mixtures of at least two different DNA species. Several workers have found that the likelihood of obtaining a homogeneous fragment after cutting out the band from the sequencing gel and reamplifying is very low (Benito *et al.*, 1996). Fragments 1, 5, 6, 7 and 8 were used for further analysis.

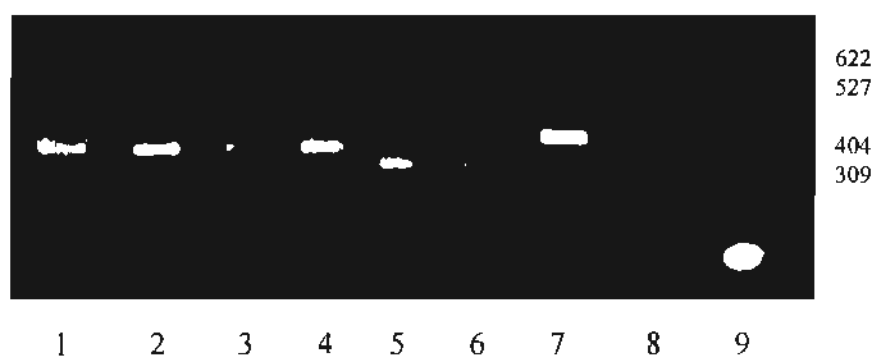
### 5.3.3 Reverse Northern analysis

1  $\mu$ g of amplified DNA from clones 1, 5, 6, 7 and 8 was slot blotted onto duplicate blots. These blots were hybridised with RNA from the dry and green plants which was labelled as described in section 4.2.19. All five fragments corresponded to mRNAs only expressed in the dry plant and not in hydrated leaves (Fig. 5.4). The blot hybridised with RNA from hydrated leaves was overexposed to check for very low levels of expression and still no signal was obtained. These differentially expressed fragments were therefore cloned and sequenced.

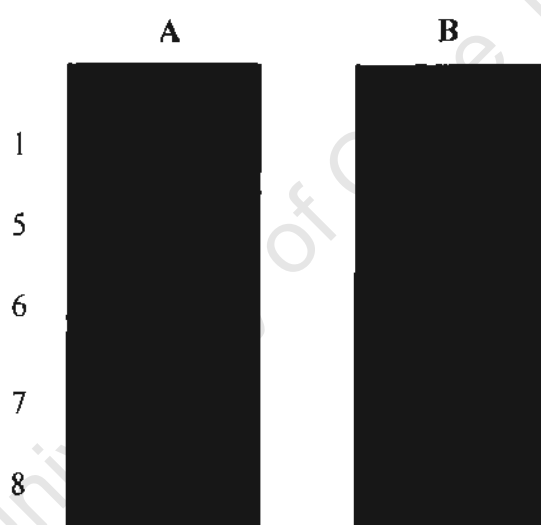
---



**Fig 5.2:** Comparison of mRNA subpopulations in the dry and hydrated leaves of *M. flabellifolius*. RNA from dry (lanes 1, 3, 5) and hydrated leaves (lanes 2, 4, 6) were used as templates in reverse transcription in the presence of primers HT<sub>11</sub>C (lanes 1, 2), HT<sub>11</sub>A (lanes 3, 4) or HT<sub>11</sub>G (lanes 5, 6). The same primers were then used in combination with an arbitrary primer HAP<sub>1</sub> during PCR and the amplification products were separated on a denaturing 6% polyacrylamide gel. Arrowheads indicate the bands which were cut out of the gel and purified.



**Fig. 5.3:** Reamplification of fragments 1-8 cut out from sequencing gel. Lanes 1-8 represent fragments 1-8 respectively while lane 9 is the molecular size marker (*Hpa*II digest of pBR322).



**Fig. 5.4:** Reverse Northern analysis of fragments 1, 5, 6, 7 and 8 to check their expression in the dry (blot A) and hydrated (blot B) leaves of *M. flabellifolius*.

#### 5.3.4 Sequence analysis of cloned fragments

DD-PCR has resulted in mainly the amplification of 3' untranslated sequences (for example, Benito *et al.*, 1996; Truesdell & Dickman, 1997; Vielle-Calzada *et al.*, 1996), although sequences corresponding to the 3' end of the coding regions of genes have also

been isolated (O'Mahogany & Oliver, 1999). To determine the nature of the sequences obtained in this study, they were analysed for putative ORFs using the 'DNASIS' and 'NCBI ORF finder' algorithms. Two different algorithms for finding ORFs were used to ensure that similar ORF results were obtained. Finding a putative ORF in the partial sequence gives an indication as to whether the sequence formed part of the coding or the non-coding region of a gene. Although the probability that the anchored oligo dT primer binds to the poly A tail of a transcript is higher than the probability of binding to an internal stretch of A s, the latter is still possible. Should the dT primer bind only to the poly A tail, then only positive frames would be considered but if the dT primer bound to an internal stretch of A s, then the three negative frames would also have to be considered. All six frames of the ORFs were therefore considered.

Figure 5.5 shows the nucleotide sequence of cloned fragment 1 (dd-1). The 180 bp fragment contained both primers used for the initial differential display analysis. ORF analysis using both algorithms mentioned earlier revealed 3 putative ORFs. The first ORF was in frame -1 (nt 1-171). This ORF encoded for a polypeptide of 56 aa, did not have a start or a stop codon and could therefore represent an internal fragment of a gene (Fig 5.6a). The second ORF was in frame +1 (from nt 1-120) and although no start codon was found, it did have a stop codon and coded for a polypeptide of 39 aa (Fig 5.6b). Since there was no start codon, this clone could represent the 3' end of the coding region of a gene. The last ORF in frame +2 was from nt 65-179 (Fig 5.6c) and encoded a polypeptide of 38 aa. This ORF did not have a stop or a start codon and could hence be part of the coding region of a gene. Nucleotide searches of fragment dd-1 revealed very high homologies (100% identity over a 18-19 bp overlap) to several extracellular matrix genes isolated from different species, including humans (nt 44-62 from sequence is identical to nt 1011-1029 in human) and mouse (nt 57-74 from sequence corresponds to nt 6787-6804 from mouse) (AF029694). Both these homologous regions fall within the second ORF described (Fig. 5.6b). At the protein database homology search (BLASTP), it was found that clone dd-1 scored homology to several glycoprotein precursors including those from *Mus musculus* and *Bos taurus* (A39808) (33% identity over a

stretch of 54 aa; aa 19-180 from sequence and aa 823-877 from *B. taurus*). This stretch of aa falls within the second ORF as well. These results would indicate that the second ORF (nt 1-120) would be the most probable ORF since the homology searches at the nucleotide and the protein levels produce results that fall within this region of the gene.

```

1   AAGCTTGATT GCCGTAGAAA GCCCGTTTCC ATAGAAAGCC AGTTTCCCTT
51  TAGAAAGCCA GTTTCTGAGA AAGCCAGTTT CCGTAGAAAG CCAGTTTCCC
101 AGAGAGAGCC AGTTCCGTAG AAAGCTAGTT TCCCCTAGAA AGCTAGTTTC
151 AGTCAAAAGC CAGTTTTCCA AAAAAAAAAA

```

**Fig. 5.5:** DNA sequence analysis of fragment dd-1. Fragment dd-1 (180 bp) was sequenced with M13 forward primer from pBluescript plasmid. The primers used for the differential display reaction are shown in red (HAP<sub>1</sub>) and green (HT<sub>11</sub>M).

**a: ORF 1 (56 aa in frame -1)**

```

171  ttggaaaactggcttttgactgaaactagctttctaggggaaact
      L E N W L L T E T S F L G E T
126  agctttctacggaaactggctctctctgggaaactggctttctacg
      S F L R N W L S L G N W L S T
81   gaaactggctttctcagaaactggctttctaaagggaaactccgt
      E T G F L R N W L S K G K L A
36   ttctatggaaacgggctttctacggcaatcaagctt l
      F Y G N G L S T A I K L

```

**b: ORF 2 (39 aa in frame +1)**

```

1   aagcttgattgccgtagaaagcccgtttccatagaaagccagttt
    K L D C R R K P V S I E S Q F
46  cccttagaaagccagtttctgagaaagccagtttccgtagaaag
    P F R K P V S E K A S F R R K
91  ccagtttcccagagagagccagttccgtag 120
    P V S Q R E P V P *

```

**c: ORF 3 (38 aa in frame +2)**

```

65  ctgagaaagccagtttccgtagaaagccagtttcccagagagagc
    L R K P V S V E S Q F P R E S
110 cagttccgtagaaagctagtttcccctagaaagctagtttcagtc
    Q F R R K L V S P R K L V S V
155 aaaagccagtttccaaaaaaaaaaaaa 180
    K S Q F S K K K

```

**Fig 5.6:** Analysis of the putative open reading frames for clone dd-1 was performed using the 'NCBI ORF finder' and 'DNASIS' algorithms. Stop codon is shown in bold.

The insert size of fragment dd-5 is 177 bp (Fig 5.7). This clone contained both primers used for the initial differential display reaction. The 'DNASIS' and NCBI 'ORF finder' algorithms were used to analyse the clone for ORFs. One putative ORF was found in frame -3 and encoded a polypeptide of 41 aa. This ORF started with ATG but did not have a stop codon (Fig. 5.8). Protein database homology searches revealed a 32% identity (aa 80 to aa 172 from sequence and aa 257-287 from query) to a fructokinase from *Rhizobium trifolii* (AAB52373) while at the nucleotide level searches, an 18 nt overlap of 100% identity to a hexophosphate transport protein (spanning nt 99-116 from query and nt 9143-9160 from subject) from *Chlamydomophila pneumoniae* (AAD18804) was found. The results from the nucleotide and the protein searches show that both regions of homologies fall within the same part of the gene.

```

1   AAGCTTGATT GCGGAGTAAA AACTGTTTAG GTAACCTAAC ATTAACCGTT
51  GGGGTGGACC TTTGGTTTAA ACCTTGTCCTA TCCGAGATCA GCTTACAAAG
101 ACTGGCTACA GAGAAATGTA ACATGTAATG ACATTACTTA AAGTTCCAAG
151 GTGGAAAGTT CATACCAAAA AAAAAA

```

**Fig. 5.7:** DNA sequence analysis of fragment dd-5. Fragment dd-5 (177 bp) was sequenced with M13 forward primer from Bluescript plasmid. The primers used for the differential display reaction are shown in red (HAP<sub>1</sub>) and green (HT<sub>1(M)</sub>).

#### ORF 1 (41 aa in frame -3)

```

124 atgttacatttctctgtagccagtcctttgtaagctgactcggct
      M L H F S V A S L C K L I S D
79  ggacaagggttaaaccaaaggtccaccccaacggctaatgtagg
      G Q G L N Q R S T P T V N V R
34  ttacctaaacagtttttactcggcaatcaagctt 1
      L P K Q F L L G N Q A

```

**Fig 5.8:** Analysis of the putative open reading frames for clone dd-5 was performed using the 'NCBI ORF finder' and 'DNASIS' algorithms. Start codon is shown in bold.

Clone dd-6 contains an insert of 286 bp (Fig. 5.9) and three putative ORFs encoding for 37 aa (nt 129 to nt 242 in frame -3), 36 aa (nt 19 to nt 129 in frame -2) and 33 aa respectively (nt 1 to nt 101 in frame -3). The first two ORFs both have a start codon with a stop codon (Fig 5.10a, b). Both ORFs also contained other methionine residues within their sequences. It is possible that these clones could be full length (although the polypeptides encoded are short) but are more likely to be part of the 3' coding region of a gene since they contain a stop codon. ORF 3, on the other hand, did not have a start or a stop codon (Fig 5.10c). Nucleotide database homology searches revealed that there was

100% identity (between nt 144 to nt 161 in frame +1) to part of *A. thaliana* chromosome 1 BAC T7A14 (from nt 41222-41239) (AC005322). This BAC sequence has homology to an integral membrane protein from *Beta vulgaris* (AAC98002) and a member of the sugar transport family (AAC98002). At the protein level, this clone showed 34% homology (aa 31-177) to part of an unknown ORF (aa 33-82) from *Lactococcus lactis* (U44843).

```

1   AAGCTTGATT GCCCTAATCT AGCTCTGCAT ATCCAATGAG TCGTCTGTGT
51  CGTAGTGGCA GCTGACTTCA CTCTCGGTGT GAGTTTCGCA TGGAAACGCA
101 GAGTTAGAAC TGTTTAGGTA ACCTAACATT AACCGYTTGT GGCTGGACCA
151 TTTGPTTTAT ACCTTGTCCT TCCGAGACTC AGCATTACAT AAGACTGGAC
201 ATACAGCAGT ATAATGTCAC CACTGTCACT GACAATTAAC ATTAAACGTT
251 AACCAAGGTG GAAACGTTCA TACAAAAAAAAAAAAAAAA

```

**Fig. 5.9:** DNA sequence analysis of fragment dd-6. Fragment dd-6 (286 bp) was sequenced with M13 forward primer from Bluescript plasmid. The primers used for the differential display reaction are shown in red (HAP<sub>1</sub>) and green (HT<sub>11</sub>M).

**a: ORF 1 (37 aa in frame -3)**

```

242 atgttaagggtcagtgacagtggtgacattatactgctgtatgtc
    M L I V S D S G D I I L L Y V
197 cagtcttatgtaatgctgagtcctcggatggacaaggtataaaaca
    Q S Y V M L S L G W T R Y K T
152 aatggteccagccacaarcggttaa 129
    N G P A T X G *

```

**b: ORF 2 (36 aa in frame -2)**

129 **atg**ttaggtttaoctaaacagtttctaactctgcggtttccatgcgaa  
 M L G Y L N S S N S A F P C E  
 84 actcacaccgagagtggaagtcagctgccactacgacacagacgac  
 T H T E S E V S C H Y D T D D  
 39 tcattggatatgcagagct**tag**  
 S L D M Q S \*

**c: ORF 3 (33 aa in frame -3)**

101 ctgcggtttccatgcgaaactcacaccgagagtggaagtcagctgcc  
 L R F H A K L T P R V K S A A  
 56 actacgacacagacgactcattggatatgcagagctagattaggg  
 T T T Q T T H W I C R A R L G  
 11 caatcaagcctt l  
 Q S S

**Fig 5.10:** Analysis of the putative open reading frames for clone dd-6 was performed using the 'NCBI ORF finder' and 'DNASIS' algorithms. Start and stop codons are shown in bold. Any methionine (other than the start codon) is shown in pink.

A total of 249 nt were sequenced for clone dd-7 (Fig. 5.11). As with the previous sequences, this clone contained both primers used in the initial differential display analysis.

```

1  AAGCTTGATT GCCCTACGTC TATTCTCTAC TAAGTCATAG TAGCTAGTGG
51 CGCTTACATA ATCTCGATAG TCAGTTTGCA GTAAAGAGAG TAAACTGTTA
101 GGTACCTAAC ATTAACCGTT GGGGTGGACC TTTGGTTTAA ACCTTGTTCA
151 TCCGAGATCA GCTTACTAAA GACTGGCTAC AGAGAAATGT AACATGTAAT
201 GACATTACTT AAAGTTCCAA GGTGGAAAGT TCATACCAAAA

```

**Fig. 5.11:** DNA sequence analysis of fragment dd-7. Fragment dd-7 (249 bp) was sequenced with M13 forward primer from Bluescript plasmid. The primers used for the differential display reaction are shown in red (HAP<sub>I</sub>) and green (HT<sub>I</sub>M).

Using the 'DNASIS' and 'NCBI ORF finder' algorithms, no ORF could be found since there were several stop codons within all six frames. A typical frame (frame +1) is shown in figure 5.12. This clone could therefore represent the 3' non-coding region of a gene and this result correlates with what most workers have found after using the strategy of DD-PCR to isolate clones (for example Benito *et al.*, 1996). Nucleotide searches depicted 100% homology to a short stretch of 18 nt (nt 83-100) to a mitogen-activated protein kinase from *A. thaliana* (nt 90121-90138) (AF076275). However, no homology was found at the protein level.

```

1  agcttgattgccctacgtctattctctactaagtcatagtagctagtggcgcttacataa
   S L I A L R L F S T K S * * L V A L T *
62  tctcgatagtgagtttgcagtaaagagagtaaactgttaggtacctaacattaaccgttg
   S R * * V C S K E S K L L G T * H * P L
122 gggtggacctttggttaaaccttgtccatccgagatcagcttactaaagactggctaca
   G W T F G L N L V H P R S A Y * R L A T
182 gagaaatgtaacatgtaatgacattacttaaagttccaaggtggaaagttcataccaaaa
   E K C N M * * H Y L K F Q G G K F I P K
242 aaaaaaa
   K K

```

**Fig 5.12:** Analysis of the putative open reading frames for clone dd-7 was performed using the 'NCBI ORF finder' and 'DNASIS' algorithms. Stop codons are shown in bold. Methionine residue is shown in pink.

Clone dd-8 represents an insert of 315 bp and contains both primers used in the initial differential display PCR (fig 5.13). The 'DNASIS' and 'NCBI ORF finder' algorithms were used to analyse the clone for ORFs. One putative full length ORF was found in frame -1 and encoded a polypeptide of 39 aa (nt 61-180). Nucleotide database homology searches revealed no substantial scores, whereas the highest score for the protein searches showed a 45% identity (aa 151-261 from sequence) to a putative transposase (aa 70-107) from *Yersinia pestis* (AAC69769).

```

1   AAGCTTGATT GCCCTAGTCA CACAAGCAAC TATCCAACAA CATAAGCTGT
51  TGGCTCCGCC TTAAGCATCA AAGCGCATGA ATGCTTTTTT GCCCGTTAAC
101 GCAGAAGTTA AAAACTGTTT TAGGTTATCC TGAACATTAA CCATATGGGG
151 TTGGACCTTT GGTTTTAAAC CTTGGTCCAT CCGAGATCGC TTACAGAGAA
201 CTGGGCTACA GAGTATAATG TCCCGTGTCC CTGCACATTT ACTTTAAACG
251 TTCCAACGGT GTAAACGCTT CATACCAAAA AAAACGCTAG AGCTTATGCT
300 TGAGAAAAAAAAAAAA

```

**Fig. 5.13:** DNA sequence analysis of fragment dd-8. Fragment dd-8 (315 bp) was sequenced with M13 forward primer from Bluescript plasmid. The primers used for the differential display reaction are shown in red (HAP<sub>1</sub>) and green (HT<sub>11</sub>M).

#### ORF 1 (39 aa in frame -1)

```

180 atggaccaaggttttaaaaaccaaaggtccaaccccatatggttaat
      M D Q G L K P K V Q P H M V N
135 gttcaggataacctaaaacagtttttaacttctgcgttaacgggc
      V Q D N L K Q F L T S A L T G
90  aaaaaagcattcatgcgctttgatgcttaa 61
      K K A F M R F D A *

```

**Fig 5.14:** Analysis of the putative open reading frames for clone dd-8 was performed using the 'NCBI ORF finder' and 'DNASIS' algorithms. Stop and start codons are shown in bold. Any other methionine is shown in pink.

**Table 7:** Results of nucleotide homology searches with the database and fragments expressed only in desiccated leaves of *M. flabellifolius* as revealed during DD-PCR.

Clone	Length (nt)	Results of nucleotide homology search with the database
dd-1	180	100% identity over an 18 nt stretch to extracellular matrix genes (AF029694)
dd-5	177	100% identity over an 18 nt stretch to hexosephosphate transport protein (AAD18804)
dd-6	286	100% identity over an 18 nt stretch to a sugar transporter (AAC98002)
dd-7	249	100% identity over an 18 nt stretch to a mitogen activated protein kinase (AF066275)
dd-8	315	No score

### 5.3.5 Screening of cDNA library with DD-PCR products

In order to obtain the full length clones from the partial sequences, one of the strategies that can be used is screening of a cDNA library using these partial clones as probes. The DD-PCR products were therefore end-labelled with phosphonucleotide kinase in the presence of  $\gamma$ -<sup>32</sup>P[ATP] and screening carried out as described earlier (section 4.2.21). All five probes resulted in putative positive clones which were subjected to a second round of screening. From the secondary screening 10 clones corresponding to each probe were plaque purified, the phagemid excised (see section 4.2.25) and plasmid DNA isolated. These DNAs were then sequenced and it was found once again (as in chapter 4) that these clones could not be sequenced. As explained earlier, it would seem that the *in vivo* excision step was unsuccessful resulting in sequences which were impure and unsequencable.

## 5.4 DISCUSSION

The isolation using DD-PCR of several cDNA fragments representing genes potentially involved in desiccation tolerance in *M. flabellifolius* is reported. This procedure has been used successfully in several different organisms, including plants, for the analysis of differential gene expression (Benito *et al.*, 1996; O'Mahogany & Oliver, 1999; Sharma & Davis, 1995; van der Knaap & Kende, 1995). Amongst the resurrection plant species more attention has been devoted to *Craterostigma spp* (Bartels *et al.*, 1990; 1992; 1993; Piatkowski *et al.*, 1990) from which several cDNAs of transcripts expressed during desiccation have been isolated and characterised. However, this is the first report focussing on differential gene expression in the resurrection plant *M. flabellifolius*.

It was necessary to optimise several parameters of the DD-PCR technique to be able to analyse gene expression pattern between dry and hydrated leaves of *M. flabellifolius*. During desiccation *M. flabellifolius* accumulates polyphenolics (see sections 2.3.1 & 2.3.7), which are known to be inhibitory in molecular manipulations. Therefore, RNA extraction had to be optimised to yield RNA that could be reverse transcribed and used as a template during PCR. Although the modified protocol for RNA extraction (see section 3.2.2) yielded good quality RNA (as per integrity on agarose gel and an  $A_{260}:A_{280}$  of 1.9-2.0), it could be used for reverse transcription but not for the PCR. However, addition of PVP into the PCR mixture attenuated the inhibitory effects (Koonjul *et al.*, 1999b). It was found that changing the final concentration of the anchored dT primer from 2  $\mu\text{M}$  to 10  $\mu\text{M}$  as well as increasing the final  $\text{Mg}^{2+}$  concentration to 4.5  $\mu\text{M}$  (as opposed to 1.5  $\mu\text{M}$  from the original method, Liang & Pardee, 1992) gave a better banding pattern on the sequencing gel. The optimal annealing conditions in the PCR step were found to be 55°C for 1 minute compared to the lower temperatures (40-42°C for 2 minutes) used by several workers (for example Liang & Pardee, 1992).

The technique of DD-PCR has resulted mainly in isolation of fragments corresponding to untranslated regions of genes (Benito *et al.*, 1996; Truesdell & Dickman, 1997; Vielle-

---

Calzada *et al.*, 1996) although some partial clones corresponding to the 3' end of coding regions have also been found (for example O'Mahogany & Oliver, 1999). Homology searches with the database have therefore resulted in very low score identity since it is well documented that 3' untranslated regions are less conserved than the coding regions between homologous genes in different organisms (Benito *et al.*, 1996). In the study carried out in this chapter, it was found that all fragments isolated and purified were found to contain both the primers used in the initial differential display reaction despite the report that some clones isolated using this procedure only contain the shorter primer at both extremes (Benito *et al.*, 1996). According to the principles of differential display, the anchored primer should anneal to the poly (A) tail of the mRNA and, therefore, amplified fragments should represent the extreme 3' end of the message (Liang & Pardee, 1992). Sequence analysis showed that clone dd-7 did not contain any putative ORF and could therefore represent the untranslated region of a differentially expressed gene. The other clones all contained putative ORFs suggesting that they may encode the 3' end of coding regions. The 100% identity found between short stretches of nucleotides in these clones and genes in the database could represent conserved motifs of proteins. The absence of longer stretches of homology suggests that besides having the same conserved regions, genes from *M. flabellifolius* have quite different sequences to their paralogues. Alternatively, these transcripts could represent novel genes hence showing very insignificant homology with existing genes. It must be emphasised that no genes have been cloned from *M. flabellifolius* and hence the results obtained here cannot really be compared to other genes isolated from this species.

Clones dd-5 and dd-6 are especially interesting since they showed homology to a hexophosphate transport protein and a sugar transporter respectively. Although it should be noted that the homology extended only over a short stretch of nucleotides and might not be significant, it is tempting to speculate about the roles of sugar transporters during desiccation. It was shown earlier (section 2.3.7) that during desiccation, *M. flabellifolius* accumulates hexoses in its chloroplasts and these were postulated to confer protection to the membranes. Sugar transporters are likely to play a role in the transport of these

---

hexoses from the cytoplasm into the chloroplasts. It has been reported that during drying of *C. plantagineum*, transcripts showing homology to sucrose synthetase and sucrose phosphate synthetase are induced (Ingram & Bartels, 1996). Similarly, betaine aldehyde dehydrogenase (Ishitani *et al.*, 1995) and aldose reductase (Mundree *et al.*, unpublished results) have been shown to be over expressed in barley and *X. viscosa* respectively. All these sugar metabolic enzymes encode for metabolites which have been demonstrated to afford cellular protection and the putative sugar transporters identified from *M. flabellifolius* could be postulated to play similar roles.

To confirm whether these clones represent novel genes or form part of the 3' coding/non-coding regions of existing genes, full length clones must be isolated. The strategy of obtaining the full length clones using the partial sequences as probes to screen a cDNA library was unsuccessful. As a second strategy to obtain the full length clones, 5'RACE was performed and will be discussed in chapter 6.

In conclusion, the use of DD-PCR to isolate differentially expressed genes was successful. Although the clones isolated represented only partial sequences of genes showing homology to short stretches of nt or aa from the public database, these clones are potentially involved in conferring desiccation tolerance to *M. flabellifolius*. Expression analysis confirmed that all the fragments isolated and purified were only present in the dry and not in the hydrated plant.

---

---

## CHAPTER 6

### RAPID AMPLIFICATION OF cDNA ENDS

#### 6.1 INTRODUCTION

DD-PCR methodology mainly identifies the downstream sequence of a gene since the oligo dT primer binds to the 3' end of the mRNA. Homology searches would therefore reveal no positive score as 3' untranslated regions of genes are known to be less conserved than coding regions (Benito *et al.*, 1996). Despite this, DD-PCR has resulted in the isolation of both known and novel genes. In chapter 5, it was shown that the five partial clones obtained using DD-PCR technique from dry leaves of *M. flabellifolius* did not show any conclusive homology to sequences found in the database. These clones could either represent partial sequences of novel genes or form part of the non-coding region of a known gene. In order to differentiate between these alternatives, the 5' end of the gene has to be isolated and the homology search repeated. Various techniques enable the 5' end of a gene to be isolated. These include screening a cDNA library using the 3' end fragment as a probe, or using several applications of PCR such as inverse PCR (Huang, 1997), step-out PCR (Wesley & Wesley, 1997), or rapid amplification of the 5' end (RACE). Screening of a cDNA library proved unsuccessful in this study (chapter 5) and therefore the strategy of 5' RACE was chosen in order to obtain the 5' end of the partial genes isolated earlier.

5' RACE or "anchored PCR" is a technique that allows the isolation and characterization of 5' ends from low copy number messages (Frohman *et al.*, 1988; Ohara *et al.*, 1989). A schematic representation of 5' RACE is shown in figure 6.1. Essentially total RNA is converted to cDNA using either a gene specific primer or an oligo dT primer. In either case, a subpopulation of the mRNAs will be selected and extended to the 5' end of the message. Following cDNA synthesis, the first strand product is separated from unincorporated dNTPs and primer. Terminal deoxynucleotidyl transferase (TdT) is then used to add homopolymeric tails to the 3'

---

end of the cDNA. The tailed cDNA is eventually amplified by PCR using a mixture of a gene specific primer and an abridged anchor primer (AAP) which allow for amplification for the homopolymeric tail. The AAP contains 3' sequence complementary to the homopolymeric tail and additional 5' sequence that encodes an adapter region containing restriction sites to facilitate cloning and characterization of the 5' RACE products. Since homopolymeric sequences alter the annealing temperatures, an AAP containing deoxyinosine residues in the poly dG portion was designed. Deoxyinosine has the capacity to base pair with a variable affinity with all four bases. The order of stabilities for the different combinations are I:C > I:A > I:T > I:G (Martin *et al.*, 1985). The inclusion of deoxyinosine bases in the 3' end of the sequence helps in maintaining a  $T_m$  comparable to a typical 20-mer primer despite the increased length (Rychlik & Rhodes, 1989). This results in the maximisation of specific primer binding to the oligo dC tail and minimisation of binding to internal C-rich regions of the template, factors required for efficient PCR.

This chapter describes the use of 5' RACE in order to identify the 5' end of the DD-PCR products obtained in chapter 5. The methodology was a combination of that described by Bertioli, 1997 and the 5' RACE system for rapid amplification of cDNA ends (Life Technologies). This method differed from that of Frohman *et al.*, 1998 in several ways including: (1) The use of dC instead of dA for the homopolymer tailing reaction and (2) the use of total RNA as the initial substrate for reverse transcription (3) the use of an oligo dT primer during the reverse transcription step. The reason for using dC tailing was that it enabled the use of a higher annealing temperature and hence prevented non-specific amplification of cDNA that would occur when the "non-specific" primer is complementary to both ends of the cDNA. Total RNA was preferred to mRNA since the structural RNAs are known to act as carriers for the much less abundant mRNA and cDNAs (Bertioli, 1997). Finally an oligo dT primer was chosen instead of a gene specific primer as the oligo dT primer would select for all mRNAs as opposed to a specific subpopulation of messages.

This methodology proved successful in the isolation of the 5' end of three of the five partial genes investigated. Sequence analysis of the products showed that all

---

contained either a partial or a full ORF. All the clones scored high homology when homology searches with the public database were performed. One of the fragment was particularly interesting as it showed high homology to fructokinase. It is interesting that the fragment obtained using DD-PCR corresponding to this same clone already showed homology to a protein involved in hexosephosphate metabolism although the homology extended only over a few nucleotides. This suggests that RACE has indeed amplified the 5' end of the partial fragments obtained during DD-PCR.

---

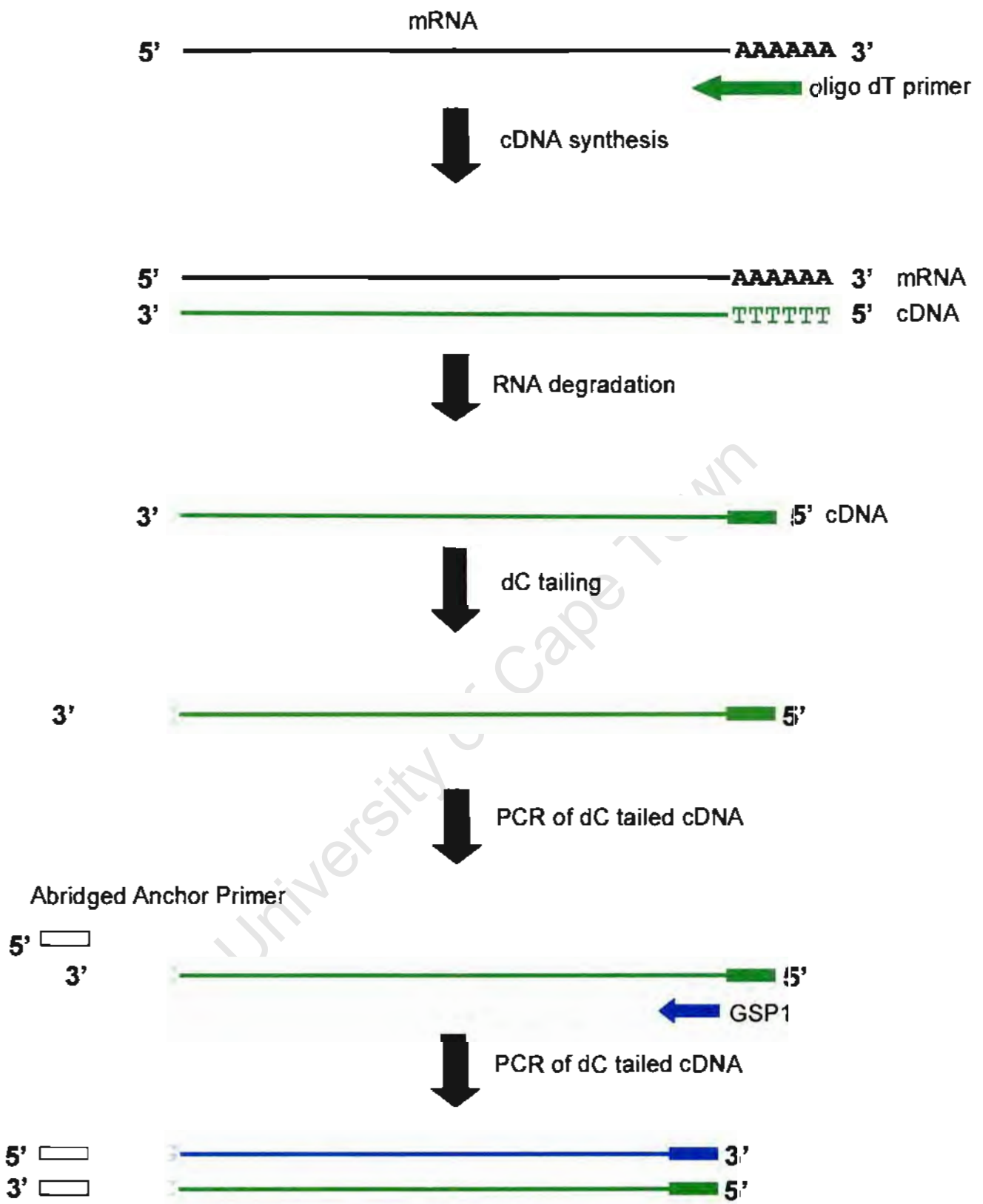


Fig. 6.1: Schematic diagram showing the steps involved during 5' RACE

## 6.2 MATERIALS AND METHODS

### 6.2.1 Primers used

Gene specific primers DD<sub>1</sub> GSP<sub>1</sub> (5' CTAGCTTTCTAGGGGAAA 3'), DD<sub>5</sub> GSP<sub>1</sub> (5' GTAAGCTGATCTCGGATGGAC 3'), DD<sub>6</sub>GSP<sub>1</sub> (5' CCACCTTGGTTAACGT-TTAATG 3'), DD<sub>7</sub>GSP<sub>1</sub> (5' CATTACATGTTACATTTCTC 3'), DD<sub>8</sub> GSP<sub>1</sub> (5' GC-AGGGACACGGGACATTATAC 3'), a 20-mer oligo primer (5' TTTTTTTTTT-TTTTTTTTTTA 3') and the 5' RACE Abridged Anchor Primer AAP (5' GGCCA-CGCGTCGACTAGTACGGGIIGGGIIGGGIIG 3') were synthesized as described before (section 5.2.2). Control GSP<sub>1</sub> (5' TTGTAATTCATTAAGCATTCTGCC 3'), GSP<sub>2</sub> (5' GACATGGAAGCCATCACAGAC 3') and GSP<sub>3</sub> (5' CGACCGTTCAG-CTGGATATTA 3') used to amplify the control RNA (see section 6.2.2) were purchased from Life Technologies. The T<sub>m</sub> of all the primers were determined using DNAMAN Lynnon Biosoft, version 4.13 software.

### 6.2.2 Isolation of RNA

Total RNA was isolated from dry leaves of *M. flabellifolius* as described in section 3.2.2. Control RNA (an *in vitro* transcribed RNA from the chloramphenicol acetyltransferase (CAT) gene) was purchased from Life Technologies.

### 6.2.3 First strand synthesis

Refer to Fig. 6.2.

20 µg of total RNA isolated from *M. flabellifolius* (tube 1) and 100 ng of control RNA (tube 2) were reverse transcribed in the presence of 400 nM oligo dT primer and 200 nM GSP<sub>1</sub> primer respectively. The RNA was denatured at 70°C for 10 min. The final composition of the reaction mixture consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 mM DTT and 400 µM of each dNTP (dATP, dCTP, dGTP and dTTP). PVP was added at a final concentration of 1% (w/v) to the *M.*

---

*flabellifolius* RNA sample (tube 1) to prevent any polyphenolic inhibition (Koonjul *et al.*, 1999b). 200 units of Superscript™ II reverse transcriptase (Life Technologies) were added to each reaction which was incubated for 50 min at 42°C before enzymatic denaturation by incubation for 15 min at 72°C. The tubes were then incubated at 37°C in the presence of the RNase mix (RNase H and RNase T1); samples were incubated for 30 min.

To check for the efficiency of first strand synthesis, an aliquot of the cDNA from the control RNA (tube 2) was amplified in the presence of GSP<sub>2</sub> and GSP<sub>3</sub> (tube A<sub>1</sub>). This PCR should result in a product of 500 bp if the PCR goes to completion. To check whether the sample of RNA from *M. flabellifolius* contained any inhibitors of the first strand reaction, an aliquot of the control RNA together with sample RNA (tube 3) were reverse transcribed and subjected to PCR with primers GSP<sub>2</sub> and GSP<sub>3</sub> (tube A<sub>2</sub>).

#### 6.2.4 Purification of cDNA

cDNA synthesized from sample RNA (tube 1), control RNA (tube 2) and mixture of sample together with control RNA (tube 3) were purified by GLASSMAX DNA isolation spin cartridges (Life Technologies). 120 µl of binding buffer (6 M sodium iodide) was added to each sample and purification performed as per the manufacturer's protocol. The cDNA was eluted with 50 µl of sterile water. The eluates were labelled E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub> and corresponded to cDNA from sample RNA, control RNA and mixture of control together with sample RNA respectively. 5 µl of the reverse transcribed control RNA (sample E<sub>2</sub>) was transferred into a new tube (tube B) and retained for PCR.

#### 6.2.5 dC Tailing of cDNA

Purified cDNA (tubes E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub>) from section 6.2.3 was used in the terminal transferase tailing reaction. The reaction mixture consisted of 1 % final volume PVP (w/v), 10 mM Tris-HCl pH 8.4, 25 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dCTP and 20

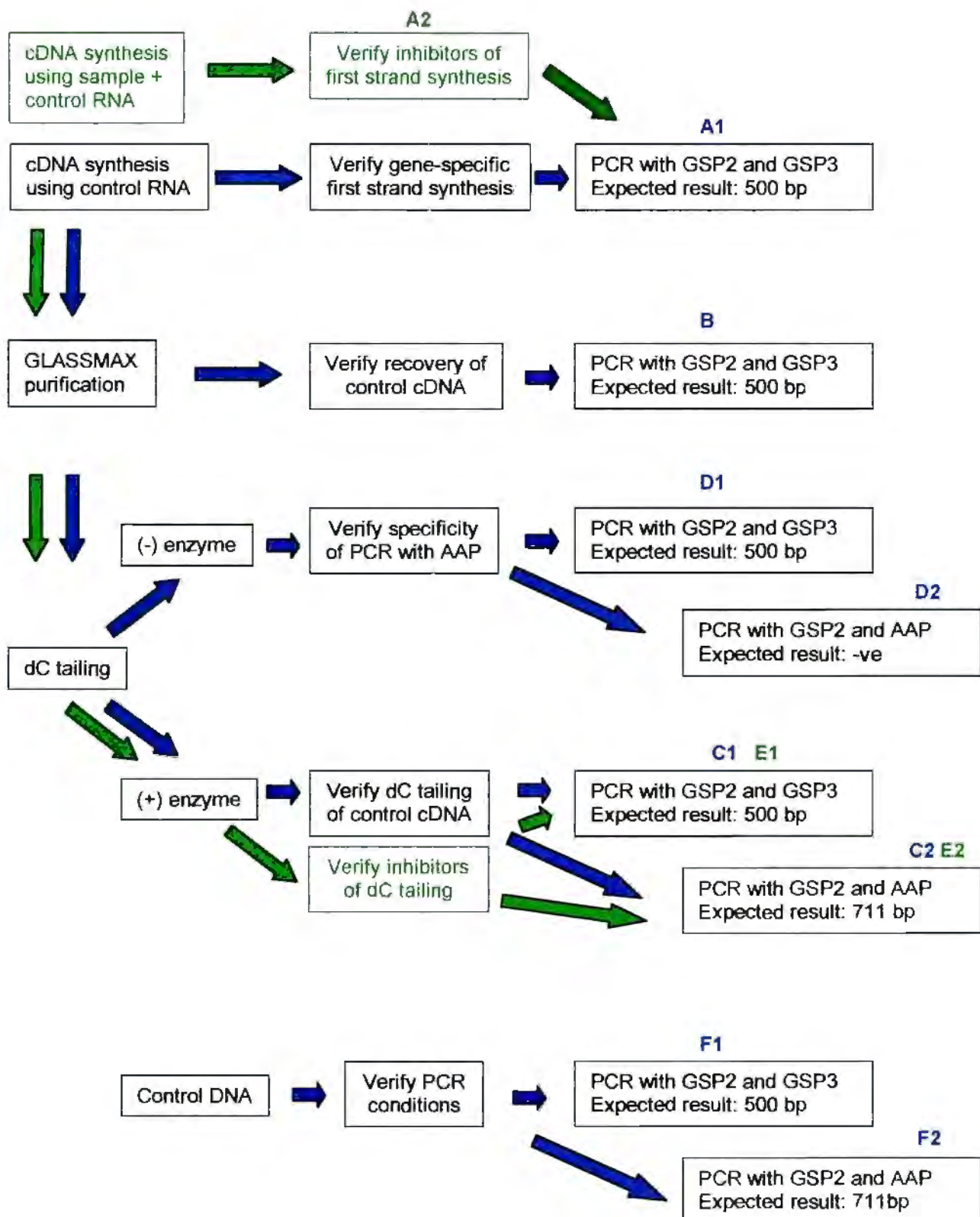
---

$\mu\text{l}$  of sample cDNA (tube T), or 20  $\mu\text{l}$  of control cDNA (diluted to 1:10) (tubes C & D) or 20  $\mu\text{l}$  of RNA mix (tube E). All tubes were incubated for 3 min. at 94°C and chilled on ice. Terminal deoxynucleotidyl transferase enzyme (10 units) were added to tubes T, C and E which were incubated at 37°C for 15 min. The tailing reaction was terminated by incubating the tubes for another 10 min. at 65°C. An aliquot of tubes C, D and E were used in PCR.

### 6.2.6 PCR of dC-tailed cDNA

5  $\mu\text{l}$  of sample cDNA was used during PCR in a total reaction volume of 50  $\mu\text{l}$ . The PCR mix consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 400 nM AAP, 400 nM of either DD<sub>1</sub> GSP<sub>1</sub>, DD<sub>5</sub> GSP<sub>1</sub>, DD<sub>6</sub> GSP<sub>1</sub>, DD<sub>7</sub> GSP<sub>1</sub> and DD<sub>8</sub> GSP<sub>1</sub>, 200  $\mu\text{M}$  of each dNTP, 1% PVP (w/v) and 2.5 units of *Taq* DNA polymerase (Life Technologies). The cycling parameters were 35 cycles of 94°C for 1 min., 55°C for 1 min and 72°C for 2 min followed by a final extension of 10 min at 72°C.

2  $\mu\text{l}$  of samples from tube A<sub>1</sub>, A<sub>2</sub>, B, C, D, E and F were used to check for the various steps involved in the procedure. The PCR mix (mix 1) consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 200 nM of GSP<sub>2</sub> and 200 nM GSP<sub>3</sub> while mix 2 was similar to mix 1 except the primers were 400 nM GSP<sub>2</sub> and 400 nM AAP. In each reaction mix, 5 units of *Taq* DNA polymerase were added. The cycling conditions was set for 35 cycles with each cycle constituting of 94°C for 1 min., 63°C for 30 s and 72°C for 2 min. A final extension of 72°C for 10 min. was performed. A flow diagram showing the various controls taken during the RACE procedure is shown in figure 6.2.



**Fig. 6.2:** Flow diagram showing controls taken during 5' RACE using control RNA and DNA purchased from Life Technologies.

### **6.2.7 Agarose gel electrophoresis**

20  $\mu$ l of each sample was analysed by gel electrophoresis as described in section 3.2.3.

### **6.2.8 Purification of bands from agarose gel**

Bands of interest were cut out from the agarose gel and purified as per section 5.2.7.

### **6.2.9 Sequencing and homology searches**

Sequencing and homology searches were done as described in section 4.2.28.

---

## 6.3 RESULTS

### 6.3.1 Design of primers

Primer design is critical for efficient and specific PCR, but especially for 5' RACE where only one gene specific primer is used. The software DNAMAN was used to check the sequences of the designed primers. The primers DD<sub>1</sub> GSP<sub>1</sub>, DD<sub>5</sub> GSP<sub>1</sub>, DD<sub>6</sub> GSP<sub>1</sub>, DD<sub>7</sub> GSP<sub>1</sub> and DD<sub>8</sub> GSP<sub>1</sub> were designed from the sequences of the partial fragments obtained from chapter 5. These primers, designed so that they were free of secondary structures (for example, hairpin loops and dimers) represented part of the 3' end of fragments dd-1, dd-5, dd-6, dd-7 and dd-8. In order to maintain high specificity with the template DNA, the primers were designed so that at least two of the last five 3' bases were G or C residues (Rychlik & Rhodes, 1989). 3' terminal complementarity was also minimised since primer-dimer artifacts may significantly reduce PCR efficiency.

The next important factor considered in the design of the gene specific primers was the  $T_m$  of the primer. The  $T_m$  of a primer is defined as the temperature at which 50% of the primer and its complementary sequence are present in a duplex DNA molecule. The  $T_m$  is essential for the establishment of the annealing temperature used during the PCR cycling. The annealing temperature should be low enough to guarantee efficient annealing of the primer to the template but high enough to minimize non-specific binding. The  $T_m$  of all the gene specific primers were found to be between 55°C and 60°C. Hence an annealing temperature of 55°C was chosen.

### 6.3.2 First strand cDNA synthesis

RNA isolated from dry leaves of *M. flabellifolius* was reverse transcribed as detailed in section 6.3.3. The primer used during the reverse transcription (RT) reaction was an oligo dT primer as opposed to a gene specific primer. The oligo dT primer was chosen so that all poly A RNA would be transcribed instead of selecting for a specific sub-population of the messages. The enzyme Superscript™ II (Life Technologies)

---

was chosen as it is a derivative of MMLV reverse transcriptase lacking RNase H activity therefore allowing for longer cDNAs to be produced.

The efficiency of first strand synthesis was monitored by reverse transcribing a control RNA sample (an 891 bp *in vitro* transcribed RNA from the chloramphenicol acetyltransferase (CAT) gene that has been engineered to contain a 3' poly A tail) in the presence of a gene specific primer (GSP<sub>1</sub>). Conversion of first strand cDNA from the control RNA sample were assayed by a CAT cDNA-specific PCR using the control GSP<sub>3</sub> and GSP<sub>2</sub> resulting in a product of 500 bp (product A<sub>1</sub> in Fig. 6.2). This strategy is depicted schematically in figure 6.2 and the result shown in figure 6.3 (lane 1).

To check whether the RNA sample isolated from desiccated leaves of *M. flabellifolius* contained any inhibitors of the reverse transcription step, an aliquot of the sample was mixed with an aliquot of the control RNA. After reverse transcription, the cDNA was used in a PCR containing the control GSP<sub>2</sub> and GSP<sub>3</sub> primers. Should any inhibitors (such as polyphenolics) be present, they would bind to either the enzyme or the control RNA sample (see chapter 3) and prevent formation of the product. Figure 6.3 shows that product formation occurred (product A<sub>2</sub> in Fig. 6.2) (lane 2) demonstrating that there no inhibitors were present in the RNA isolated from dry leaves of *M. flabellifolius*. This is most probably due to the presence of PVP which was included in the reverse transcription reaction mixture (Koonjul *et al.*, 1999b).

After cDNA synthesis, RNase mix (RNase H and RNase T1) was used to degrade the RNA. The digestion was performed following the thermal inactivation of the MMLV RT enzyme so as to reduce any potential formation of hairpin-primed second strand synthesis catalysed by MMLV RT. This might obscure the accessibility of the cDNA ends to the terminal transferase enzyme. Template RNA in the cDNA:RNA hybrid was degraded by RNase H and ss RNAs were degraded by RNase T1 preventing possible renaturation of template RNA to cDNA. Furthermore, any carry over RNA may inhibit the tailing reaction and subsequent PCR (Pikaart & Villeponteau, 1993)

---

as terminal transferase exhibits a marked preference for RNA (Lobban & Kaiser, 1973; Nelson & Brutlag, 1979).

### **6.3.3 GLASSMAX cDNA isolation spin cartridge purification of cDNA**

The cDNA was passed onto a GLASSMAX spin cartridge (Life Technologies) to separate it from any residual primer and dNTPS (from the RT reaction) and contaminating proteins. A stringent purification procedure was required as residual primer can be tailed by terminal transferase and compete with the abridged anchor primer during PCR (Frohman, 1990). The GLASSMAX spin column binds any cDNAs > 200 bp in the presence of the chaotropic agent, sodium iodide, whilst removing any dNTPs, proteins and excess primers. This purified cDNA can be used directly during the tailing reaction.

The efficiency of the purification was assayed by using an aliquot of the control eluate cDNA as template in a PCR reaction containing the CAT specific primers. It can be clearly seen in figure 6.3 (lane 3 corresponding to product B in Fig. 6.2) that the purification was successful as there was product formation (500 bp).

### **6.3.4 dC tailing of cDNA**

Tailing creates the abridged anchor primer binding site on the 3' end of the cDNA (see figure 6.1). The choice of the nucleotide for homopolymeric tailing is still debatable. Each nucleotide has its advantages and disadvantages. In this case, dC tailing was chosen as it complements the abridged anchor primer used and also because it enables the use of a higher annealing temperature. This would prevent non-specific amplification of total cDNA that could arise when the non-specific oligo dT primer (in the case of the sample cDNA) is complementary to both ends of the cDNA. Furthermore, since G:C base pairs are more stable than A:T base pairs, shorter stretches of dCs are required compared with dAs or dTs.

Efficient tailing is important to ensure that there is enough tailed cDNA molecules for PCR. Besides, homopolymeric tails of sufficient length are needed for efficient

---

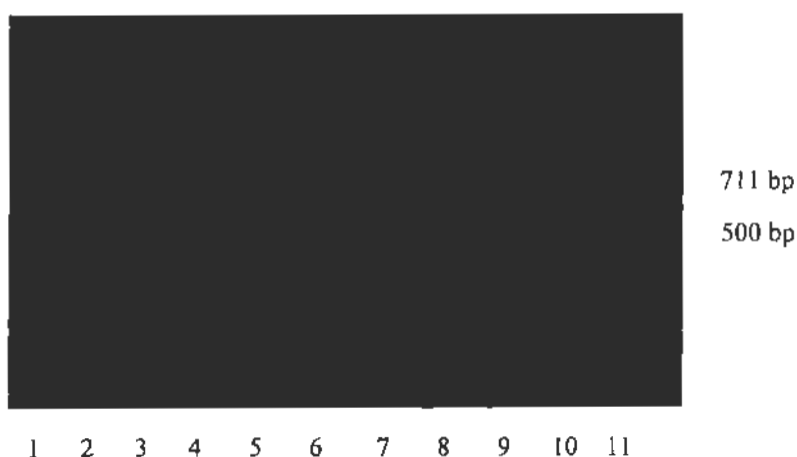
binding of primer to template. A brief denaturation procedure prior to the tailing reaction was included to ensure disruption of any secondary structures that might be present in the cDNA. Tailing efficiency was assayed by using an aliquot of the tailed control eluted cDNA as template in a PCR using CAT specific primers. As can be seen in figure 6.3, in the presence of GSP<sub>2</sub> and GSP<sub>3</sub> primer combination (C1), a product of 500 bp was formed (lane 8) while in the presence of the AAP and GSP<sub>2</sub> (C2), a major product of size 711 bp was formed (lane 9). In the absence of the terminal transferase enzyme (D1 & D2), a product of 500 bp was obtained with primer combination GSP<sub>2</sub> and GSP<sub>3</sub> while with the primer combination of GSP<sub>2</sub> and AAP, no product was observed (lanes 4 & 5). These results show that the tailing reaction was indeed successful and was not dependent on any contaminating DNA.

To check whether the tailed sample cDNA from *M. flabellifolius* contained any inhibitors despite the use of PVP during the tailing reaction, an aliquot of this sample was mixed with an aliquot of the control tailed cDNA and the mix used as template during PCR. Figure 6.3 (lanes 6 & 7) show that the expected product of size 500 and 711 bp respectively was still formed with the primer combinations GSP<sub>2</sub>/GSP<sub>3</sub> and GSP<sub>2</sub>/AAP respectively. This indicated that the PVP included in the tailing reaction was reversing any inhibitory effects of any contaminants which could be present in the sample cDNA.

### 6.3.5 Amplification of target cDNA

Successful 5' RACE is extremely dependent on the efficiency and specificity of the PCR. Although conditions for amplification are dependent on the nature of each particular primer and target sequence used, in this study a control DNA (Life technologies) was used to optimize the PCR conditions. The control DNA was constructed by cloning the 5'RACE product from the control RNA sample (coding for the CAT gene) into pAMP1. The 4.8 kb pAMP1 5'RACE recombinant contains the oligo dC tail and can thus be used as a template to check the performance of the GSP<sub>3</sub> or AAP primers in conjunction with the control GSP<sub>2</sub> producing a band of size 500 bp or 711 bp respectively (Figure 6.3, lanes 10 & 11 respectively).

---



**Fig. 6.3:** PCR of controls taken to check various steps involved during the RACE procedure. Lanes 1-11 represent samples A<sub>1</sub>, A<sub>2</sub>, B, D<sub>1</sub>, D<sub>2</sub>, E<sub>1</sub>, E<sub>2</sub>, C<sub>1</sub>, C<sub>2</sub>, F<sub>1</sub> and F<sub>2</sub> respectively (see figure 6.2 for details).

Once the PCR parameters were optimized, the sample cDNA was used as template in the presence of DD<sub>1</sub> GSP<sub>1</sub>, DD<sub>5</sub> GSP<sub>1</sub>, DD<sub>6</sub> GSP<sub>1</sub>, DD<sub>7</sub> GSP<sub>1</sub> or DD<sub>8</sub> GSP<sub>1</sub> gene specific primer and AAP. The results of the amplification products are shown in figure 6.4. Lanes 2, 3 and 6 represent the products formed when the primers DD<sub>1</sub> GSP<sub>1</sub>, DD<sub>5</sub> GSP<sub>1</sub> and DD<sub>8</sub> GSP<sub>1</sub> were used. In the case of DD<sub>8</sub> GSP<sub>1</sub>, only one product was formed while in the case of DD<sub>1</sub> GSP<sub>1</sub> and DD<sub>5</sub> GSP<sub>1</sub>, a range of products was formed. In the case of DD<sub>6</sub> GSP<sub>1</sub> and DD<sub>7</sub> GSP<sub>1</sub> (lanes 4 & 5), no product was observed. The samples in lanes 2, 3 & 4 were diluted and used as the template for another PCR using AAP and a nested primer. No product was formed from samples corresponding to lanes 4 & 5. However, similar results to those in Fig. 6.4 were obtained for samples 2 & 3. These results are very similar to those obtained by Bertoli, 1997, who showed that only in very rare cases does one amplify just one product. A range of products is usually obtained. Bands containing DNA of interest (shown by arrows in Fig. 6.4) were excised from the gel and the DNA purified and sequenced.



**Fig. 6.4:** RACE products separated by agarose gel electrophoresis. Lane 1,  $\lambda$ -DNA digested with *Pst*I; lanes 2-6 represent RACE products obtained from RNA isolated from dry leaves of *M. flabellifolius* using gene specific primers DD<sub>1</sub> GSP<sub>1</sub>, DD<sub>5</sub> GSP<sub>1</sub>, DD<sub>6</sub> GSP<sub>1</sub>, DD<sub>7</sub> GSP<sub>1</sub> or DD<sub>8</sub> GSP<sub>1</sub> respectively. Arrows indicate DNA excised from gel.

### 6.3.6 Sequencing of 5' RACE products

The products obtained after gel purification were sequenced using the appropriate gene specific primer. As before (section 5.3.4), the two algorithms 'DNASIS' and 'NCBI ORF finder' were used to analyse the open reading frames of the sequences obtained and all six frames of the ORFs were considered.

Figure 6.5 shows the nucleotide sequence of RACE product obtained when using the DD<sub>1</sub>GSP<sub>1</sub> primer (arrow a in figure 6.4). The 281 bp fragment contained part of the sequence corresponding to the same clone obtained by DD-PCR (shown in bold). ORF analysis using both algorithms revealed 2 putative ORFs. The first ORF was in frame +2 (nt 53 - nt 154) and encoded for a polypeptide of 33 aa. This ORF had both a start and a stop (Figure 6.6a). Since the polypeptide encoded by this ORF is small, it

is unlikely that the methionine residue is the start codon and instead is part of the internal sequence of the gene. The second ORF was in frame +3 (nt 120 - nt 280) and coded for a polypeptide of 53 aa. This second ORF had two methionine residues and contained no stop codon (Figure 6.6b). Once again, it is unlikely that these methionine residues are start codons. Nucleotide homology searches with the public database revealed 100 % identity over a stretch of 19 nucleotides to the P1 clone isolated from *A. thalania* (AP000419). Nucleotides 105-123 from query were identical to nt 33135-33153 from subject and since the full length clone of the P1 clone from *A. thalania* is 81875 nt, it would therefore imply that the sequence obtained from DD<sub>1</sub>GSP<sub>1</sub> is likely to be part of the coding region of the full length gene. Therefore it would seem that although RACE has resulted in extending the sequence data in the 5' direction of this fragment, the full length gene for this clone is still to be obtained.

```

1 GGGTCCGAGA GGGCTCATTG CATGGCTTTT TACGGGATTG AATGTGGCAT
51 CCATGCTGAT AAATGACGTT CCTGTTGTTG GGGGTGAGGA AGCTATTCAT
101 CATCTCTGA TAATGAGTAA TGATGCATGC TTCGGGGAGT TTTCTTCAAC
151 GTAGAAAGCC CGTTTCCATA GAAAGCCAGT TTCCCTTTAG AAAGCCAGTT
201 TCTGAGAAAG CCAGTTTCCG TAGAAAAGCC AGTTTGCCCA GAGAGAGTTC
251 CGTAGAAACG CTAGATTCCC CTAGAAAGC

```

**Fig. 6.5:** DNA sequence analysis of fragment DD<sub>1</sub>GSP<sub>1</sub>. The nucleotides shown in bold represent those that were present in the DD-PCR fragment dd-1.

**a: ORF 1 (33 aa in frame +2)**

```

53  atgctgataaatgacgttcctgttggtgggggtgaggaagctatt
    M L I N D V P V V G G E E A I
98  catcattctctgataatgagtaatgatgcatgcttcggggagttt
    H H S L I M S N D A C F G E F
143 tcttcaacgtag 154
    S S T *

```

**b: ORF 2 (53 aa in frame +3)**

```

120 atgatgcatgcttcggggagttttcttcaacgtagaaagcccggtt
    M M H A S G S F L Q R R K P V
165 tccatagaaagccagtttcccttagaaagccagtttctgagaaa
    S I E S Q F P F R K P V S E K
210 gccagtttccgtagaaaagccagtttgcccagagagagttccgta
    A S F R R K A S L P R E S S V
255 gaaacgctagattcccctagaaagcta 281
    E T L D S P R K L

```

**Fig. 6.6:** Analysis of the putative open reading frames for fragment DD<sub>1</sub>GSP<sub>1</sub> using the 'NCBI ORF finder' and 'DNASIS' algorithms. Methionine residues and stop codons are shown in bold.

Fragment DD<sub>5a</sub>GSP<sub>1</sub> was 323 nt and contained some of the nucleotides already obtained by DD-PCR using another set of primers; these nucleotides are shown in bold (Fig. 6.7). The 'DNASIS' and 'NCBI ORF finder' algorithms were used to analyse the clone for ORFs. Two putative reading frames were obtained (Fig. 6.8). The first ORF (nt 58 – nt 192) had both a stop and a start codon. Since the polypeptide encoded by this ORF has only 44 aa, it is unlikely that the methionine residue represents the start codon. The second ORF (nt 2 – nt 184) encoded a polypeptide of 60 aa and included a stop codon. Homology searches revealed 90% identity over 41 nt between nt 161 and nt 201 from query and nt 9189 – nt 9149 from

subject) to a gene encoding a hexosephosphate transport protein in *A. thaliana* (ATAC0110796). No homology was however found at the protein level. This fragment obtained using the 5' RACE methodology was significantly longer than that obtained using DD-PCR with an additional 232 nt present at the 5' end. The length of the fragment suggests that it forms part of the coding region of a gene, full length of which still needs to be determined. It is interesting to note that the homology to the hexophosphate protein was already observed with the much shorter 3' DD-PCR fragment corresponding to this RACE product.

```

1  GTTTAAGGTT  GTTCTGAAGT  ATTGGCCAGG  CTTCAGAATC  GGAGGTCACG
51  ACTGTACATG  CAACCCATTA  AATGTCTCAG  GGATCCATTG  CTGCTCGTCC
101 AACTCTCGAG  GTTCGTCAGT  AAATACGTTT  CTGTACCGGG  TTTTGTTCGG
151 TCAAACGCCC  CTCAACAAAA  AATCCGAAAC  TTAACATATT  GAAGCAAACA
201 ATAACCCAGC  AGATTCAAAT  TATCACTTTT  CCATTATTCC  GGAGTAAAAA
251 CTGTTAGGTA  ACCTAACATT  AACCGTTGGG  GTGGACCTTT  GGTTTAAACC
301 TTGTCCATCC  GAGATCAGCT  TAC

```

**Fig. 6.7:** DNA sequence analysis of fragment DD<sub>5</sub>GSP<sub>1</sub>. The nucleotides shown in bold represent those that were present in the DD-PCR fragment dd-5.

**a: ORF 1 (44 aa in frame +1)**

```

58  atgcaaccattaaatgtctcagggatccattgctgctcgtccaa
    M  Q  P  I  K  C  L  R  D  P  L  L  L  V  Q
103  ctctcgagggttcgctcagtaaatacgtttctgtaccggggttttgtt
    L  S  R  F  V  S  K  Y  V  S  V  P  G  F  V
148  ccgtcaaacgcccctcaacaaaaaatccgaaacttaacatattga 192
    P  S  N  A  P  Q  Q  K  I  R  N  L  T  Y  *

```

**b: ORF 2 (60 aa in frame +2)**

```

2  tttaaggttggttctgaagtattggccaggcttcagaatcggaggt
   F K V V L K Y W P G F R I G G
47  cacgactgtacatgcaaccatttaaagtgtctcagggatccattgc
   H D C T C N P L N V S G I H C
92  tgctcgtccaactctcgaggttcgtcagtaaatacgtttctgtac
   C S S N S R G S S V N T F L Y
137 cgggttttggtccgtcaaacgccctcaacaaaaaatccgaaact
   R V L F R Q T P L N K K S E T
182 taa 184
   *
```

**Fig. 6.8:** Analysis of the putative open reading frames for fragment DD<sub>5b</sub>GSP<sub>1</sub> using the 'NCBI ORF finder' and 'DNASIS' algorithms. Methionine residues and stop codons are shown in bold.

Fragment DD<sub>5b</sub>GSP<sub>1</sub> was 223 nt (Fig. 6.9) and did not include any nucleotides found earlier during sequencing of the dd-5 fragment obtained using DD-PCR (see chapter 5). This fragment contained two putative ORFs in frames +1 and +2 respectively. The first ORF had both a stop and a start codon (Fig. 6.10a). This ORF encoded for a polypeptide of 60 aa while the second ORF did not have a stop or a start codon although several methionine residues were found within the sequence (Fig. 6.10b) and corresponded to a polypeptide of 64 aa. Homology searches at the nucleotide level showed 64% identity between nt 51- nt 273 and nt 226 - nt 448 of the cDNA clone cLEC33M20 from *Lycopersicon esculentum* (EST 277604). This clone is similar to fructokinase isolated from the same species (AW34109). The EST from *L. esculentum* is 575 bp long indicating that fragment DD<sub>5b</sub>GSP<sub>1</sub> is part of the coding region of the full length gene. Although ORF 1 contained both a start and a stop codon, the polypeptide encoded by that portion of the gene is too short to represent the full length clone.

```

51 GGGTTTGTCTG GCCACGATGG GACCGACCAC TTGCGCGGCG GAGTTGGATA
   |||   |||  || |||||  /  ||  |||  |||  ||  ||  |  ||  |
226 GGGAAACTCG GCGACGATGA GTTCGGTCAC ATGCTCGCCG GGATTCTGAA

101 CCGGAACCGC ACCCATCAAG AGATCGTTAA CCTGCGGAAG GATATCAGGA
   ||||  ||  ||  |  |  ||||  |  |||  |  ||||
276 AACGAACGGC GTACAAGCCG AAGGAATTAA TTTTGACAAG GGCGCCAGGA

151 CGGCTAGGGA GTGCGCGCGG AGCAGAGCCG ACGCGGTTCTG TGAICTGATG
   ||||  ||  ||  ||  |  |  |  ||||  ||||  |  ||  |||  |  |||
326 CGGCTTTGGC GTTCGTGACG CTACGCGCCG ACGGAGAGCG TGAGTTTATG

201 TATCGCACAA ATCCCAGTGC CGATTTTTGG CCTATGTCCG CGGCGTTGAA
   |  |  ||  ||  |||||  ||||  |||  |  |  |||  |  |  |||||
376 TTTTACAGAA ATCCCAGTGC CGATATGTTG CTCACGCCCG CTGAGTTGAA

251 TCGGGAGCTT TTGGTCTATA GTA
   ||  ||  |||  |  |  ||  ||
426 TCTTGATCTT ATTAGATCTG CTA

```

**Fig. 6.9:** DNA sequence alignment of fragment DD<sub>5b</sub>GSP<sub>1</sub> (shown in black) and fructokinase EST isolated from *L. esculentum* (shown in blue).

**a: ORF 1 (60 aa in frame +1)**

```

17  atgggaccgaccacttgcgcgggaggagttggataccggaaccgca
   M G P T T C A A E L D T G T A
62  cccatcaagagatcgттаacctgcggaaggatatcaggacggcta
   P I K R S L T C G R I S G R L
107 gggagtgcgcgcgagcagagccgacgcggttcgtgactcgatgt
   G S A R G A E P T R F V T R C
152 atcgcacaaatcccagtgccgatttttggcctatgtccgcgcgct
   I A Q I P V P I F G L C P R R
197 tga 199
   *

```

**b: ORF 2 (64 aa in frame +2)**

```

31  ttgcgcggcggagttggataccggaaccgcacccatcaagagatc
    L R G G V G Y R N R T H Q E I
76  gttaacctgcggaaggatatcaggacggctagggagtgcgcgcgg
    V N L R K D I R T A R E C A R
121 agcagagccgacgcggttcgtgactcgatgtatcgcaaaatccc
    S R A D A V R D S M Y R T N P
166 agtgccgatttttggcctatgtccgcggcggttgaatcggaggctt
    S A D F W P M S A A L N R R L
211 ttggtctatagt 222
    L V Y S

```

**Fig. 6.10:** Analysis of the putative open reading frames for fragment DD<sub>5b</sub>GSP<sub>1</sub> using the 'NCBI ORF finder' and 'DNASIS' algorithms. Methionine residues are shown in bold.

Fragment DD<sub>8</sub>GSP<sub>1</sub> was 409 nucleotides long (Fig. 6.11) and contained several nucleotides already identified during DD-PCR. These are shown in bold in figure 6.11. Sequence analysis (Fig. 6.12) showed that there were two putative ORFs although they were both in the negative frame. The sequence obtained contained a few bases which could not be identified. These might have contributed to no ORF being found in any of the three possible positive frames. Both ORF 1 & ORF 2 coding polypeptides of 42 aa and 39 aa respectively contained a start as well as a stop codon. Homology searches at the nucleotide level showed high homology (100% identity) to several genes coding for iodothyronine deiodinase protein in *Homo sapiens* (AL031427) although this homology only extended over a short stretch of 18 nucleotide (between nt 95 – nt 113 from query and nt 101831 – nt 101849 from subject). Homology was not detected at the protein level.

```

1  CTCCCGGTTT TAAATTGATC AACTAGATTA AAATCGTGAA TCGGGTAATA
51  TTCGAACAAA TCCCTTTTAG TAAGGGGAGC TTTTGTATTG GCAGAATATA
101 GGAAACAATT TTAGTAACTC TCCTCTCTGC ACTATATACA AATAGGGGCA
151 TTTTYNAYM ACTMTCCCGC CCCCTTATAC CCCATGAATT CTAGTCACAC
201 AAGCAACTAT CCAACAACAT AAGCTGTTGG CTCCGCCTTA AGCATCAAAG
251 CGCATGAATG CTTTTTTGCC CGTTAACGCA GAAGTTAAAA ACTGTTTTAG
301 GTTATCCTGA ACATTAACCA TATGGGGTTG GACCTTTGGT TTTAAACCTT
351 GGTCCATCCG ATCGCTTACA GAGAACTGGG CTACAGAGTA TAATGTCCCG
401 TGTCCCTGC

```

**Fig. 6.11:** DNA sequence analysis of fragment DD<sub>8</sub>GSP<sub>1</sub>. The nucleotides shown in bold represent those that were present in the DD-PCR fragment dd-8.

**a: ORF 1 (42 aa in frame -1)**

```

151 atgccccctatttgtatatagtgagagaggagagttactaaaatt
    M P L F V Y S A E R R V T K I
106 gtttcctatattctgccaatcaaaaagctccccttactaaaaggg
    V S Y I L P I K K L P L L K G
61  atttggttegaatattaccgcattcagatTTtaatctag 23
    I C S N I T A F T I L I *

```

**b: ORF 2 (39 aa in frame -2)**

```

357 atggaccaaggttttaaaccacaaaggtccaaccccatatggttaat
    M D Q G L K P K V Q P H M V N
312 gttcaggataacctaataaacagtttttaacttctgcttaacgggg
    V Q D N L K Q F L T S A L T G
267 aaaaaagcattcatgcgctttgatgcttaa 238
    K K A F M R F D A *

```

**Fig. 6.12:** Analysis of the putative open reading frames for fragment DD<sub>8</sub>GSP<sub>1</sub> using the 'NCBI ORF finder' and 'DNASIS' algorithms. Methionine residues and stop codons are shown in bold.

**Table 8:** Results of nucleotide homology searches with the database and fragments expressed only in desiccated leaves of *M. flabellifolius* as revealed during 5' RACE PCR.

Clone	Length (nt)	Results of nucleotide homology search with the database
DD <sub>1</sub> GSP <sub>1</sub>	281	100% identity over a 19 nt stretch to P1 clone from <i>A. thaliana</i> (AP000419)
DD <sub>1a</sub> GSP <sub>1</sub>	323	90% identity over a 41 nt stretch to a gene encoding a hexosephosphate transport protein (ATAC0110796)
DD <sub>5b</sub> GSP <sub>1</sub>	223	64% identity over a 222 nt stretch to a fructokinase EST (AW34109)
DD <sub>8</sub> GSP <sub>1</sub>	409	100% identity over an 18 nt stretch to a gene encoding an iodothyronine deiodinase protein (AL031427)

## 6.4 DISCUSSION

The 5' end of three genes expressed in dry *M. flabellifolius* plant but not in the hydrated plant are given. In chapter 5, the partial fragments corresponding to the 3' end of mRNAs were obtained using the strategy of differential display. Since no conclusive homology searches were found for those fragments, sequence towards the 5' end of the message was obtained using 5'RACE. The different steps during the RACE procedure had to be optimised since it was found that traces of polyphenolics present in the RNA isolated from dry leaves of *M. flabellifolius* inhibited all the molecular reactions involved (results not shown). Thus it was necessary to include PVP during all reactions performed in order to reverse the inhibitory effects of polyphenolics (Koonjul *et al.*, 1998; 1999b). Appropriate controls were included to check the efficiency of all steps involved during this procedure.

No 5' RACE product was of sufficient length to represent the full length clone, even though some of the ORFs contained both start and stop codons. However, 5' RACE products were significantly longer than the original fragments identified by DD-PCR. Homology comparisons with the public database showed that two of the RACE products had sequences homologous to enzymes involved in sugar metabolism. Fragment DD<sub>5a</sub>GSP<sub>1</sub> had an overlap of 41 nt to a hexophosphate protein from *A. thaliana* while fragment DD<sub>5b</sub>GSP<sub>1</sub> shared 64% identity over a stretch of 243 nt to a fructokinase isolated from *L. esculentum*. It was interesting to note that the DD-PCR products corresponding to these two 5' RACE products both showed homology to hexosephosphate although the homology was only over a stretch of 18 nt. These results indicated that sugar transporters and enzymes involved in sugar metabolism are very important in protecting *M. flabellifolius* during desiccation.

Similar transcripts have been reported to be induced during water loss in several desiccation sensitive as well as desiccation tolerant species (Ingram & Bartels, 1996; Mundree *et al.*, unpublished results). These transcripts have been shown to confer tolerance in these species. It can therefore be postulated that the sugar transporters and the enzymes involved in sugar metabolism identified in *M. flabellifolius* could play a similar role in this species. Furthermore this plant was shown to accumulate

---

hexoses in its chloroplasts during desiccation, which further indicate the putative roles of these sugar transporters during water limiting conditions.

---

---

## CHAPTER 7

### GENERAL DISCUSSION AND CONCLUSION

This thesis has demonstrated that *M. flabellifolius* uses a variety of strategies to confer tolerance against desiccation and/or light stress. Resurrection plants must tolerate light stress as they grow in rocky outcrops with little shade (Sherwin & Farrant, 1996). The presence of light in conditions of limiting water can be detrimental to the plant due to the formation of free radicals. These, if unquenched, can result in denaturation of proteins, pigment loss, photoinhibition and lipid oxidation (McKersie, 1991; Smirnoff, 1993).

In the first part of the thesis, the physical and biochemical properties of chloroplasts from *M. flabellifolius* were investigated. The chloroplast is an important organelle that needs to be protected for the survival of the plant. Since it is known that free radical formation is common in chloroplasts (Navari-Izzo *et al.*, 1996), resurrection plants need to devise protective mechanisms to maintain the integrity of their chloroplasts. It was found that chloroplasts from *M. flabellifolius* had a higher buoyant density compared to the control resurrection plant *C. wilmsii* and the desiccation sensitive plant *P. sativum*. This higher buoyant density was ascribed to the unusual staircase arrangement of thylakoids in *M. flabellifolius* (Koonjul *et al.*, 1999a). Intact chloroplasts from this plant could only be isolated in the presence of trehalose and not sucrose. Despite the apparent fragility of these chloroplasts, biochemical studies have revealed several means by which they might achieve protection against the stresses associated with water loss in the presence of light. Thus chloroplasts from *M. flabellifolius* were found to accumulate sugars, polyphenolics (including anthocyanins) and antioxidants, all of which are implicated in protection during water and/or light stress (Koonjul *et al.*, 1999a). Although chloroplasts isolated from *M. flabellifolius* had a very different protein complement to that of chloroplasts isolated from *P. sativum* and *C. wilmsii*, a protein recognised by a LEA group III antibody was present in *M. flabellifolius*. LEA proteins are known to play a role in water stress (Asghar *et al.*, 1994; Bray, 1993; Dure, 1993; Goday *et al.*, 1993). There were also a number of small proteins (10-15 kDa) which were unique to

---

chloroplasts isolated from *M. flabellifolius*. It is possible that these may help protect the sub-cellular organelle structure during desiccation.

Ultrastructural studies carried out on chloroplasts of *M. flabellifolius* showed that separation of thylakoid membranes occurred in desiccated leaves. This mechanism has been postulated to prevent photo-oxidation. It has been suggested that the discontinuity between adjacent thylakoid membranes is eased by the staircase arrangement of thylakoid membranes and may more easily accommodate their regular separation and reaggregation during drying and rehydration (Koonjul *et al.*, 1999a).

The polyphenolic content in leaf tissues of *M. flabellifolius* was found to be significantly higher than in other desiccation sensitive or desiccation tolerant plants and was found to further increase on dehydration. Polyphenolics are known to protect the plant in several ways. They are known to have antioxidant properties and thus act as free-radical scavengers (Larson, 1988; Rice-Evans, 1995; Rice-Evans *et al.*, 1996; Sherwin & Farrant, 1996; Yu *et al.*, 1997) and act as growth regulators (Dixon & Paiva, 1995). Polyphenolics are also known for their antagonising action on plant hormones (Ray *et al.*, 1980; Tamagnone *et al.*, 1998) and their role in the defence mechanisms of plants (Frey *et al.*, 1997). Furthermore, polyphenolics help strengthen the cell wall of the plant (Wallace & Fry, 1994). Their function during desiccation tolerance relies mainly on the fact that they are able to maintain the fluidity of membranes by partitioning from the aqueous cytoplasm into the lipid phase during desiccation (Golovina *et al.*, 1998; Hoesktra *et al.*, 1997). The presence of polyphenolics, however, was problematic in all molecular biological reactions carried out throughout this study. Polyphenolics are known to bind to proteins and nucleic acids (Loomis, 1974; Wilkins & Smart, 1996) and so it was not surprising that even traces of these molecules proved inhibitory during molecular biological manipulation. All molecular biological reactions thus required to be optimised and included the presence of PVP in the reaction mixture in order to reverse the inhibitory effects of the polyphenolics (Koonjul *et al.*, 1999b).

Two strategies were used to identify genes expressed during desiccation in *M. flabellifolius*. These strategies included the construction and differential screening of a cDNA library and the use of differential display polymerase chain reaction. The cDNA library constructed was found to have a titre of  $10^{10}$  pfu/ml after amplification.

---

---

**REFERENCES**

- Adam Z.** 1996. Protein stability and degradation in chloroplasts. *Plant Mol. Biol.* **32**, 773-783.
- Adams CA, Rinne RW.** 1980. Moisture content as a controlling factor in seed development and germination. *International Review of Cytology* **68**, 1-8.
- Ainsworth C.** 1994. Isolation of RNA from floral tissue of *Rumex acetosa* (sorrel). *Plant Mol. Biol. Rep.* **12**, 198-203
- Alamillo JM, Bartels D.** 1996. Light and stage of development influence the expression of desiccation-induced genes in the resurrection plant *Craterostigma plantagineum*. *Plant, Cell and Environment* **19**, 300-310.
- Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD.** 1989. Molecular biology of the cell, 2<sup>nd</sup> ed. Garland Publishing. New York and London.
- Altschul M, Gish W, Miller W, Meyers EW, Lipman DJ.** 1990. Basic local alignment search. *J. Mol. Biol.* **215**, 403-410.
- Amuti KS, Pollard CJ.** 1977. Soluble carbohydrates of dry and developing seeds. *Phytochemistry* **16**, 529-532.
- Anderson JM.** 1968. Extraction of enzymes and subcellular organelles from plant tissues. *Phytochemistry* **7**, 1973-1988.
- Anderson JM, Aro EM.** 1994. Grana stacking and protection of Photosystem II in thylakoid membranes of higher plant leaves under sustained high irradiance: An hypothesis. *Photosyn. Res.* **41**, 315-326.
- Anderson JM, Chow WS, Park YI.** 1995. The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. *Photosyn. Res.* **46**, 129-139.
- Asada K.** 1994. Mechanisms for scavenging reactive molecules generated in chloroplasts under light stress. In *Photoinhibition of photosynthesis* pp 131-145. Baker NR and Bower JR eds. Bios Scientific Publishers, Oxford.
- Asada K.** 1996. Radical production and scavenging in the chloroplasts. In *Photosynthesis and the Environment* pp 123-150. Baker NR ed. Kluwer, Dordrecht, Netherlands.
- Asghar R, Fenton RD, DeMason DA, Close TJ.** 1994. Nuclear and cytoplasmic localization of maize embryo and aleurone dehydrin. *Protoplasma* **177**, 87-94.
- Aspinall D, Paleg LG.** 1981. Proline accumulation. In *The Physiology and Biochemistry of Drought Resistance in Plants* pp 206-242. Paleg LG and Aspinall D. eds. Academic Press, Sydney.
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seideman JG, Smith JA, Struhl K.** 1987. In *Current Protocols in Molecular Biology*. John Wiley and Sons ed., New York.
- Bachem CWB, Van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RGF.** 1996. Visualization of differential gene expression using a novel
-

method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *The Plant Journal* **9** (5), 745-753.

**Bahloul M, Burkard G. 1993.** An improved method for the isolation of total RNA from spruce tissues. *Plant Mol. Biol. Rep* **11**, 212-215.

**Baker JC, Steele C, Dure III L. 1988.** Sequence and characterisation of 6 Lea proteins and their genes from cotton. *Plant Mol. Biol.* **11**, 277-291.

**Baker SS, Rugh CL, Kamalay JC. 1990.** RNA and DNA isolation from recalcitrant plant tissues. *Biotechniques* **9**(3), 268-272.

**Bartels D, Schneider K, Terstappen G, Piatkowski D, 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, Hanke C, Schneider K, Michel D, Salamini F. 1992.** A desiccation-related *Elip*-like gene from the resurrection plant *Craterostigma plantagineum* is regulated by light and ABA. *EMBO J.* **11**(8), 2771-2778.

**Bartels DR, Alexander K, Schneider R, Elster R, Velasco J, Alamillo G, Bianchi G, Nelson D, Salamini F. 1993.** Desiccation-related gene products analyzed in a resurrection plant and in barley embryos. In *Plant responses to cellular dehydration during environmental stress: Current topics in plant physiology*. Close TJ, Bray EA (eds). Am. Soc. Plant Physiol. Series Vol. **10** pp 119-127. .

**Bate-Smith EC. 1981.** Astringent tannins of the leaves of *Geranium* species. *Phytochemistry* **20**, 211-216.

**Bathurst NO. 1954.** The amino-acids of grass pollen. *J. Exp. Bot.* **5**, 253-256.

**Bauer D, Muller H, Reich J, Ahrenkiel V, Warthoe P, Strauss M. 1993.** Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucl. Acids. Res.* **21** (18) 4272-4280.

**Baxter NJ, Lilley TH, Haslam E, Williamson MP. 1997.** Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **36**, 5566-5577.

**Beeson RC. 1992.** Restricting overhead irrigation to dawn limits growth in container-grown woody ornamentals. *Hort. Science* **27**, 996-999.

**Benito EP, Prins T, Van Kan JAL. 1996.** Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction. *Plant Mol. Biol.* **32**, 947-957.

**Bergmeyer H, Bernt E. 1974.** Sucrose. In *Methods of enzymatic analysis* **2** (3) pp 1176-1179. Verlag Chemie Weinheim Academic Press, New York.

**Bernacchia G, Schwall G, Lottspeich F, Salamini F, Bartels D. 1995.** The transketolase gene family of the resurrection plant *Craterostigma plantagineum* plant: differential expression during the rehydration phase. *EMBO J.* **14** (3), 610-618.

**Bernardini PM, Cersosimo A. 1979.** Histochemical and biophysical study of cuticle sclerotization in *Pycnoscelus surinamensis* L. (Blattaria). *Basic Appl. Histochem.* **23** (3), 203-210.

- Bertioli D.** 1997. Rapid amplification of cDNA ends. In: *PCR Cloning Protocols* pp 233-238. White BA (ed.). Humana Press, Totowa, New Jersey.
- Bertioli DJ, Schlichter UHA, Adams MJ, Burrows PR, Steinbiss H-H, Antoniw JF.** 1995. An analysis of differential display shows a strong bias towards high copy number mRNAs. *Nucl. Acids. Res.* **23**, 4520-4523.
- Bewley JD.** 1979. Physiological aspects of desiccation tolerance. *Annual Review of Plant Physiol.* **30**, 195-238.
- Bewley JD, Krochko JE.** 1981. Desiccation-tolerance. In *Encyclopedia of plant physiology: Physiological plant ecology II. Water and carbon assimilation Vol 12A*, 325-378. Lange OL, Nobel PS, Osmond CB, Ziegler H eds. Springer Verlag, Berlin.
- Bewley JD, Larrsen KM, Papp JET.** 1983. Water-stressed induced changes in the pattern of protein synthesis in maize seedlings mesocotyls: A comparison with the effects of heat shock. *J. of Exp. Bot.* **34**, 1126-1133.
- Bewley JD, Oliver 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. In *Water and Life: a comparative analysis of water relationships at the organismic, cellular and molecular levels* pp 141-160. Osmond CB., Somero G. and bolis C.L. eds. Springer-Verlag, Berlin.
- Bianchi G, Gamba A, Murelli C, Salamini F, Bartels D.** 1991. Novel carbohydrate metabolism in the resurrection plant *Craterostigma plantagineum*. *Plant J.* **1**, 355-359.
- Bianchi G, Gamba A, Limiroli R, Pozzi N, Elster R, Salamini F, Bartels D.** 1993. The unusual sugar composition in leaves of the resurrection plant *Myrothamnus flabellifolius*. *Physiologia Plantarum* **87**, 223-226.
- Blomstedt CK, Gianello RD, Hamill JD, Neale AD, Gaff DF.** 1998. Drought-stimulated genes correlated with desiccation tolerance of the resurrection grass *Sporobolus stapfianus*. *Plant Growth Reg.* **24**, 153-161.
- Borle F, Seelig J.** 1983. Hydration of *Escherichia coli* lipids: deuterium T<sub>1</sub> relaxation time studies of phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta* **735**, 131-136.
- Boubriak I, Kargiolaki H, Lyne L, Osborne DJ.** 1997. The requirement for DNA repair in desiccation tolerance of germinating embryos. *Seed Sci. Res.* **7**, 97-105.
- Bowler C, Van Montagu M, Inzé D.** 1992. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 83-116.
- Bradford KJ, Chandler PM.** 1992. Expression of "dehydrin-like" proteins in embryos and seedlings of *Zizania palustris* and *Oryza sativa* during dehydration. *Plant Physiology* **99**, 488-494.
- Bray EA.** 1993. Molecular responses in water deficit. *Plant Physiol.* **103**, 1035-1040.
- Brenac P, Horbowicz M, Downer SM, Dickerman AM, Smith ME, Obendorf RL.** 1997. Raffinose accumulation related to desiccation tolerance during maize (*Zea mays* L.) seed development and maturation. *J. Plant Physiol.* **150**, 481-488.
- Bruce RJ, West CA.** 1989. Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiol.* **91**,

889-897.

**Buitink J, Walters-Vertucci C, Hoekstra FA, Leprince O. 1996.** Calorimetric properties of dehydrating pollen: Analysis of a desiccation tolerant and an intolerant species. *Plant Physiol.* **111**, 235-242.

**Burke MJ. 1986.** The glassy state and survival of anhydrous biological systems. In *Membranes, metabolism and dry organisms* pp 358-363. Leopold AC ed. Cornell university Press, Ithaca, NY.

**Büssis D, Heineke D. 1998.** Acclimation of potato plants to polyethylene glycol-induced water deficit. II. Contents and subcellular distribution of organic solutes. *J. Exp. Bot.* **49**, 1361-1370.

**Caffrey M, Fonseca V, Leopold AC. 1988.** Lipid-sugar interactions. *Plant Physiol.* **86**, 754-758.

**Cai Q, Moore GA, Guy CL. 1995.** An unusual group 2 lea gene family in citrus responsive to low temperature. *Plant Mol. Biol.* **29**(1), 11-23.

**Casper T, Huber SC, Somerville CR. 1986.** Alterations in growth, photosynthesis and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) Heynh. deficient in chloroplast phosphoglucomutase activity. *Plant Physiol.* **79**, 1-7.

**Ceccardi TL, Meyer NC, Close TJ. 1994.** Purification of a maize dehydrin. *Protein Expression and Purification* **5**, 266-269.

**Cerovic ZG, Plesnicar M. 1984.** An improved procedure for the isolation of intact chloroplasts of high photosynthetic capacity. *Biochem. J.* **223**, 543-545.

**Chalker-Scott L. 1999.** Environmental significance of anthocyanins in plant stress responses. *Phytochem. Photobiol.* **70** (1), 1-9.

**Chandler PM, Robertson M. 1994.** Gene expression regulated by abscisic acid and its relation to stress tolerance. *Ann. Rev. of Plant Physiol. And Plant Mol. Biol.* **45**, 113-141.

**Chandrasekhar I, Gaber BP. 1988.** Stabilization of the biomembrane by small molecules: Interaction of trehalose with the phospholipid bilayer. *Journal of Biomolecular structure & Dynamics* **5**(6), 1163-1171.

**Cherath L, Tkachenko A, D'Armiento J, Welsh J, McClelland M, Chada K. 1996.** In *A Laboratory guide to RNA: Isolation, Analysis, and Synthesis*. RNA fingerprinting by arbitrarily primed PCR pp 251-271. Krieg PA ed., Wiley-Liss, Inc.

**Child GF. 1960.** Brief notes on the ecology of the resurrection plant (*Myrothamnus flabellifolia*) with mention of its water-absorbing abilities. *J. of South African Botany* **26**, 1-8.

**Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. 1979.** Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**(24), 5294-5299.

**Choinski JS, Johnson JM. 1993.** Changes in photosynthesis and water status of developing leaves of *Brachystegia spiciformis* Benth. *Tree Physiol.* **13**, 17-27.

**Chomeczynski P, Sacchi N. 1987.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

---

- Chow WS, Miller C, Anderson JM. 1991.** Surface charges, the heterogeneous lateral distribution of the two photosystems and thylakoid stacking. *Biochim. Biophys. Acta* **1057**, 69-77.
- Church GM, Gilbert W. 1984.** Genomic Sequencing. *Proc. Natl. Acad. Sci. USA.* **81** (7), 1991-1995.
- Clegg JS. 1986.** The physical properties and metabolic status of *Artemia* cysts at low water contents: the 'Water Replacement Hypothesis.' In *Membranes, metabolism and dry organisms*, pp 169-187. Leopold, AC. ed. Cornell University Press, Ithaca. NY.
- Close TJ, Kortt AA, Chandler PM. 1989.** A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Mol. Biol.* **13**, 95-108.
- Close TJ, Fenton RD, Moonan F. 1993.** A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Molecular Biology* **23**, 279-286.
- Cohen A, Bray EA. 1990.** Characterisation of three mRNA that accumulate in wilted tomato leaves in response to elevated levels of endogenous abscisic acid. *Planta* **182**, 27-33.
- Colaco C, Sen S, Thangavelu M, Pinder S, Roser B. 1992.** Extraordinary stability of enzymes dried in trehalose; simplified molecular biology. *Bio/Technology* **10**, 1007-1011.
- Coley PD, Aide TM. 1989.** Red coloration of tropical young leaves: a possible antifungal defence? *J. Trop. Ecol.* **5**, 293-300.
- Collins RA, Ng TB, Fong WP, Wan CC, Yeung HW. 1998.** Removal of polyphenolic compounds from aqueous plant extracts using polyamide minicolumns. *Biochemistry and Molecular Biology International* **45** (4), 791-796.
- Colpan M, Schumacher J, Brüggemann W, Sanger HL, Riesner D. 1983.** Large-scale purification of viroid RNA using  $\text{Cs}_2\text{SO}_4$  gradient centrifugation and high-performance liquid chromatography. *Anal. Biochem.* **131**, 257-265.
- Cox RA. 1968.** The use of guanidium chloride in the isolation of nucleic acids: *Methods in Enzymology* **12** (B), 120-129.
- Crowe JH. 1971.** Anhydrobiosis: An unsolved problem. *The American Naturalist* **105**, 563-573.
- Crowe JH, Crowe LM, Jackson SA. 1983.** Preservation of structural and functional activity in lyophilized sarcoplasmic reticulum. *Arch. Biochem. Biophys.* **220**, 477-484.
- Crowe JH, Crowe LM, Chapman D. 1984.** Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **223**, 701.
- Crowe LM, Crowe JH, Rudolph A, Womersley C, Appel L. 1985.** Preservation of freeze-dried liposomes by trehalose. *Archives of Biochemistry and Biophysics* **242** (1), 240-247.
- Crowe LM, Womersley C, Crowe JH, Reid D, Appel L, Rudolph A. 1986.** Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. *Biochim. Biophys. Acta.* **861**, 131-140.
-

- Crowe JH, Crowe LM, Carpenter JF, Wistrom CA. 1987.** Stabilisation of dry phospholipid bilayers and proteins by sugars. *Biochem J.* **242**, 1-10.
- Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ, Anchordoguy TJ. 1988.** Interactions of sugars with membranes. *Biochimica et Biophysica Acta* **947**, 367-384.
- Crowe JH, Hoekstra FA, Crowe LM. 1992.** Anhydrobiosis. *Annu. Rev. Physiol.* **54**, 570-599.
- Crowe JH, Hoekstra FA, Nguyen KHN, Crowe LM. 1996.** Is vitrification involved in depression of the phase transition temperature in dry phospholipids? *Biochim. Biophys. Acta.* **1280**, 187-196.
- Crowe JH, Crowe LM, Carpenter JF, Prestrelski SJ, Hoekstra FA. 1997a.** Anhydrobiosis: cellular adaptations to extreme dehydration. In *Handbook of Physiology section 14, Comparative Physiology, Vol 11* pp 1445-1477. Dantzler WH ed. Oxford University Press, Oxford.
- Crowe JH, Oliver AE, Hoekstra FA, Crowe LM. 1997b.** Stabilization of dry membranes by mixtures of hydroxyethyl starch and glucose: The role of vitrification. *Cryobiology* **35**, 20-30.
- Dabo SM, Mitchell Jr, ED, Melcher U. 1993.** A method for the isolation of nuclear DNA from cotton (*Gossypium*) leaves. *Anal. Biochem.* **210**, 34-38.
- Dace H, Sherwin HW, Illing N, Farrant JM. 1998.** Use of metabolic inhibitors to elucidate mechanisms of recovery from desiccation stress in the resurrection plant *Xerophyta humilis*. *Plant Growth Regulation* **24**, 219-228.
- Dalla Vecchia F, El Asmar T, Calamassi R, Rascio N, Vazzana C. 1998.** Morphological and ultrastructural aspects of dehydration and rehydration in leaves of *Sporobolus stapfianus*. *Plant Growth Reg.* **24**, 219-228.
- Dalton DA, Russell SA, Hanus FJ, Pascoe GA, Evans HJ. 1986.** Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc. Natl. Acad. Sci. USA.* **83**, 3811-3815.
- Deisenhofer J, Michel H. 1991.** High-resolution structures of photosynthetic reaction centers. *Annu. Rev. Biophys. Chem.* **20**, 247-266.
- Demmig-Adams, Adams WW III. 1992.** Photoprotection and other responses of plants to high light stress. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 599 - 626.
- Dhindsa RS, Matowe W. 1981.** Drought tolerance in two mosses: Correlated with enzymatic defence against lipid peroxidation. *J. of Exp. Bot.* **32**, 79-91.
- Dixon RA, Paiva NL. 1995.** Stress-induced phenylpropanoid metabolism *Plant Cell* **7**, 1085-1097.
- Dizdaroglu M. 1991.** Chemical determination of free radical-induced damage to DNA. *Free Radical Biology and Medicine* **10**, 225-242.
- Donaldson K, Beswick DH, Gilmour PS. 1996.** Free radical activity associated with the surface of particles: a unifying factor in determining biological activity? *Toxicol. Lett.* **88** (1-3), 293-298.
-

- Drennan PM, Smith MT, Goldsworthy DA, Van Staden J. 1993.** The occurrence of trehalose in leaves of the desiccation tolerant angiosperm *Myrothamnus flabellifolius* Welw. *Journal of Plant Physiology* **142**, 493-496.
- Drumm-Herrel H, Mohr H. 1982.** Effects of blue/UV light on anthocyanin synthesis in tomato seedlings in the absence of bulk carotenoids. *Phytochem. Photobiol.* **36**, 229-233.
- Dry IB, Robinson SP. 1994.** Molecular cloning and characterisation of grape berry polyphenol oxidase. *Plant Mol. Biol.* **26**, 495-502.
- Dure III L. 1993.** A repeating 11-mer amino acid motif and plant desiccation. *Plant J.* **3**, 363-369.
- Dure III L, Crouch M, Harada J, Ho THD, Mundy J, Quatrano R, Sung ZR. 1989.** Common amino acid sequence domains along the LEA proteins of higher plants. *Plant Mol. Biol.* **12**, 475-486.
- Dutt SK, Bal AR, Bandyopadhyay AK. 1991.** Salinity induced chemical changes in *Casuarina equisetifolia* Forst. *Egypt. J. Soil Sci.* **31**, 57-63.
- Dyer RA. 1975.** *The Genera of Southern African Flowering Plants*. Vol 1. Department of Agriculture. Pretoria.
- Ellis RH, Roberts EH. 1980.** Improved equations for the prediction of seed longevity. *Ann. Bot.* **45**, 13-30.
- Ellis RH, Hong TD, Roberts EH, Tao KL. 1990.** Low moisture content limits to relations between seed longevity and moisture. *Ann. Bot.* **65**, 493-504.
- Elsner EF, Heupel A. 1976.** Inhibition of nitrite formation from hydroxyl ammonium chloride: a simple assay for superoxide dismutase. *Anal. Biochem.* **70**, 616-620.
- Espelund M, Saeboe-Larssen S, Hughes DW, Galau GA, Larsen F, Jakobsen KS. 1992.** Late embryogenesis-abundant genes encoding proteins with different numbers of hydrophilic repeats are regulated differentially by abscisic acid and osmotic stress. *Plant J.* **2**, 241-252.
- Farrant JM. 2000.** Comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plants. *Plant Ecology* (In press)
- Farrant JM, Sherwin HW. 1998.** Mechanisms of desiccation tolerance in seeds and resurrection plants. In: *Progress in Seed Science Research: Communication Services of the New York State Agricultural Experimental Station*, pp 109-120. Taylor NY Ag Huang X-L eds. Geneva.
- Farrant JM, Cooper K, Kruger LA, Sherwin HW. 1999.** The effect of drying rate on the survival of three desiccation-tolerant angiosperm species. *Annals of Botany* (In press).
- Farrell Jr RE. 1993.** RNA Methodologies: A laboratory guide for isolation and characterisation. Academic Press, Harcourt Brace Jovannovich Publishers, New York.
- Fava F, Di Gioia D, Romagnoli C, Marchetti L, Mares D. 1993.** Biosynthesis and cytoplasmic accumulation of a chlorinated catechol pigment during 3-chlorobenzoate aerobic co-metabolism in *Pseudomonas fluorescens*. *Arch. Microbiol.* **160**, 350-357.
-

- Finch-Savage WE, Pramanik SK, Bewley JD. 1994.** The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. *Planta* **193**, 478-485.
- Frank HA, McLean MB, Sauer K. 1979.** Triplet states in photosystem I of spinach chloroplasts and subchloroplast particles. *Proc. Natl. Acad. Sci. USA* **76** (10), 5124-5128.
- Frank W, Phillips J, Salamini F, Bartels D. 1998.** Two dehydration-inducible transcripts from the resurrection plant *Craterostigma plantagineum* encode interacting homeodomain-leucine zipper proteins. *Plant J.* **15** (3), 413-421.
- Franks F, Hatley RHM, Mathias SF. 1991.** Materials science and production of shelf stable biologicals. *Bio-Pharm* **4**, 38-42.
- Frey M, Chomet P, Glawischnig E, Stettner C, Grun S, Winklmaier A, Eisenreich W, Bacher A, Meeley RB, Sriggs SP, Simuox K, Gierl A. 1997.** Analysis of a chemical plant defence mechanism in grasses. *Science* **227**, 696-699.
- Frohman MA, Dush MK, Martin GR. 1988.** Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA.* **85**, 8998-9002.
- Furini A, Koncz C, Salamini F, Bartels D. 1997.** High level transcription of a member of a repeated gene family confers dehydration tolerance to callus tissue of *Craterostigma plantagineum*. *EMBO J.* **16** (12), 3599-3608.
- Gabrielska J, Oszmianski J, Komorowska M. 1999.** Anthocyanin extracts with antioxidant and radical scavenging effect. *Z. Naturforsch.* **54c**, 319-324.
- Gadd GM, Chalmers K, Reed RH. 1987.** The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. *FEMS Microbiology Letters* **48**, 249-254.
- Gaff DF. 1971.** Desiccation tolerant flowering plants in Southern Africa. *Science* **174**, 1033-1034.
- Gaff DF. 1977.** Desiccation-tolerant vascular plants of Southern Africa. *Oecologia* **31**, 95-109.
- Gaff DF. 1989.** Responses of desiccation tolerant "resurrection" plants to water stress. In: *Structural and functional responses to environmental stresses: water shortages*. K.H. Kreeb, Richter, H., Hinckley, T.M. eds. The Hague Netherlands: SPB Academic Publishing, 264-311.
- Gaff DF, Ellis RP. 1974.** Southern African grasses with foliage that revives after dehydration. *Bothalia* **11**, 305-308.
- Galau GA, Hughes DW, Dure III L. 1986.** Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. *Plant Mol. Biol.* **7**, 155-170.
- Galau GA, Close TJ. 1992.** Sequences of the cotton Group 2 LEA/RAB/dehydrin proteins encoded by LEA 3 cDNA's. *Plant Physiology* **98**, 1523-1525.
- Genckel PA, Pronina ND. 1969.** Anabioz pri vysykhaniy u tsvetkovogo poikilokserofitnogo rasteniya *Myrothamnus flabellifolia* (Anabiosis during desiccation of the flowering poikiloxerophytic plant *M. flabellifolia*). *Fiziologiya Rastenii.* **16** (5), 896-901 (Biol. Abstracts).
-

- Ghasempour HR, Gaff DF, Williams RPW, Gianello RD. 1998.** Contents of sugars in leaves of drying desiccation tolerant flowering plants, particularly grasses. *Plant Growth Reg.* **24**, 185-191.
- Gish W, States DJ. 1993.** Identification of protein regions by database similarity search. *Nat. Genet.* **3**, 266-272.
- Glisin V, Crkvenjakov, Byus C. 1974.** Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13** (12), 2633-2637.
- Goday A, Albà MM, Torrent M, Pagès M. 1993.** Immunolocalization of RAB-17 (LEA D-11 Family) in maize embryo cells. In *Responses of plants to cellular dehydration during environmental stress*. Close TJ, Bray EA eds. pp 267. American Society of Plant Physiologists. Rockville MD.
- Goldsworthy DA, Drennan PM. 1991.** Anhydrous fixation of desiccated leaves of *Myrothamnus flabellifolia* Welw. *Electron Micro. Soc. of S.A.* **21**, 105-106.
- Goldsworthy DA. 1992.** Desiccation tolerance in *Myrothamnus flabellifolius* Welw. MSc Thesis, University of Natal, Pietermaritzburg, South Africa (1992).
- Golovina EA, Hoekstra FA, Hemminga MA. 1998.** Drying increases intracellular partitioning of amphiphilic substances into lipid phase. *Plant Phys.* **118**, 975-992.
- Grimm B, Kloppstech K. 1987.** The early light-inducible proteins of barley. *Eur. J. Biochem.* **167**, 493-499.
- Grimm B, Kruse E, Kloppstech K. 1989.** Transiently expressed early light-inducible thylakoid proteins share transmembrane domains with light-harvesting chlorophyll binding proteins. *Plant Mol. Biol.* **13**, 583-593.
- Gussin AES. 1972.** Does trehalose occur in Angiospermae? *Phytochemistry* **11**, 1827-1828.
- Hall TC, Ma Y, Buchbinder BU, Pyne JW, Sun SM, Bliss FA. 1978.** Messenger RNA for G1 protein of French bean seeds. *Proc. Natl. Acad. Sci. (USA)* **75**, 3196-3200.
- Hallam ND, Luff SE. 1980.** Fine structural changes in the mesophyll tissue of the leaves of *Xerophyta villosa* during desiccation. *Bot. Gaz.* **141** (2), 173-179.
- Hambler DJ. 1961.** A poikilohydrous, poikilochlorophyllous angiosperm from Africa. *Nature* **191**, 1615 - 1416.
- Hanafusa N. 1969.** Denaturation of enzyme protein by freeze-thawing and freeze-drying. In: *Freezing and freeze-drying of microorganisms* pp 117-129. Tokio N ed. University Park Press, Baltimore.
- Harlow E, Lane D. 1988.** Antibodies - A Laboratory Manual. Cold Spring Harbour Laboratory Press, New York.
- Haslam E. 1974.** Polyphenol-protein interactions. *Biochem. J.* **139**, 285-288.
- Havaux M, Niyogi KK. 1999.** The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. *Proc. Natl. Acad. Sci. USA.* **96** (15), 8762-8767.
- Heber U, Santarius KA. 1964.** Loss of adenosine triphosphate synthesis caused by
-

- freezing and its relationship to frost hardiness problem. *Plant Physiol.* **39**, 712-719 (1964).
- Hegarty TW. 1978.** The physiology of seed hydration and dehydration and the relation between water stress and the control of germination: A review. *Plant Cell and Environment* **1**, 101-119.
- Helm KW, LaFayette PR, Nagao RT, Vierling E. 1993.** Localisation of small heat shock proteins to the higher plant endomembrane system. *Mol. and Cell. Biol.* **13**, 238-247.
- Hetherington SE, Hallam ND, Smillie RM. 1982.** Ultrastructure and compositional changes in chloroplast thylakoids of leaves of *Borya nitida* during humidity-sensitive degreening. *Aust. J. Plant Physiology* **9**, 601-609.
- Hoekstra FA, Crowe JH, Crowe LM. 1991.** Effect of sucrose on phase behaviour of membranes in intact pollen of *Typha latifolia* L., as measured with Fourier transform infrared spectroscopy. *Plant Physiol.* **97**, 1073-1079.
- Hoekstra FA, Crowe JH, Crowe LM. 1992.** Germination and ion leakage are linked with phase transitions of membrane lipids during imbibition of *Typha latifolia* pollen. *Physiologia Plantarum* **84**, 29-34.
- Hoekstra FA., Wolkers WF, Buitink J, Golovina EA, Crowe JH, Crowe LM. 1997.** Membrane stabilization in the dry state. *Comp. Biochem. Physiol.* **117**, 335-341.
- Hoffman P. 1968.** Pigmentgehalt und gaswechsel von Myrothamnus-Blättern nach austrocknung und wiederaufsattigung. *Photosynthetica* **2** (4), 245-252.
- Hong TD, Linington S, Ellis RH. 1996.** Compendium of information on seed storage behaviour. International Plant Genetic Resources Institute, Rome.
- Hopkins WG. 1992.** Introduction to plant physiology pp 125-140. Wiley John & Sons Inc. eds. New York.
- Horbowicz M, Obendorf RL. 1992.** Changes in sterols and fatty acids of buchwheat endosperm and embryo during seed development. *Journal of Agricultural Food Chemistry* **40**, 745-750.
- Horbowicz M, Obendorf RL. 1994.** Seed desiccation tolerance and storability: dependence on flatulence-producing oligosaccharides and cyclitols: review and survey. *Seed Sci. Res.* **4**, 385-406.
- Huang SH. 1997.** Inverse PCR: An efficient approach to cloning cDNA ends. In: *PCR Cloning Protocols*. pp 287-294. White BA (ed.) Humana Press, Totowa, New Jersey.
- Huh NW, Porter NA, McIntosh TJ, Simon SA. 1996.** The interaction of polyphenols with bilayers: conditions for increasing bilayer adhesion. *Biophys. J.* **71** (6), 3261-3277.
- Huynh TV, Young RA, Davis RW. 1988.** Constructing and screening cDNA libraries in  $\lambda$ gt10 and  $\lambda$ gt11. In: *DNA cloning: A Practical Approach*. Glover DM (ed.), IRL, Oxford, UK.
-

- Ijtin WS. 1957.** Drought resistance in plants and physiological processes. *Ann. Rev. Plant. Physiol.* **3**, 341-363.
- Imai R, Chang L, Ohta A, Bray EA, Takagi M. 1996.** A lea-class gene of tomato confers salt and freezing tolerance when expressed in *Saccharomyces cerevisiae*. *Gene* **170(2)**, 243-248.
- Ingram J, Bartels D. 1996.** The molecular basis of dehydration tolerance in plants. *Ann. Rev. of Plant Physiol and Mol. Biol.* **47**, 377-403.
- Ingram J, Chandler JW, Gallagher L, Salamini F, 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 Physiol.* **115**, 113-121.
- Ishitani M, Nakamura T, Han Sy, Takabe T. 1995.** Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Mol. Biol.* **27 (2)**, 307-315.
- Ismail AM, Hall AE, Close TJ. 1999.** Purification and partial characterization of a dehydrin involved in chilling tolerance during seedling emergence of cowpea. *Plant Physiol.* **120(1)**, 237-244.
- Iturragia G, Leyns L, Villegas A, Gharaibeh R, Salamini F, Bartels D. 1996.** A family of novel Myb-related genes from the resurrection plant *Craterostigma plantagineum* are specifically expressed in callus and roots in response to ABA or desiccation. *Plant Mol. Biol.* **32 (4)**, 707-716.
- John ME. 1992.** An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acids Res.* **20 (9)**, 2381.
- Johnson CM, Carswell GK, Shillito RD. 1989.** Direct gene transfer via polyethylene glycol. *Journal of Tissue Culture Methods* **12 (4)**, 127-132.
- Kaliemoorthy S, Rao AS. 1994.** Effect of salinity on anthocyanin accumulation in the root of maize. *Ind. J. Plant Physiol.* **37**, 169-170.
- Kander O., Senser M. 1965.** Vorkommen von trehalose in *Botrychium lunaria*. *Z. Pflanzenphysiol.* **53**, 157-161.
- Kander O. 1967.** Biosynthesis of poly- and oligosaccharides during photosynthesis in green plants. In *Harvesting the sun: Photosynthesis in plant life* pp 134-151. San Pietro F, Greer FA, Army TJ eds. Academic Press New York, NY and London.
- Kashiwada Y, Nonaka GI, Nishioka I, Bori I, Fukushima Y, Bastow KF, Lee KH. 1993.** Tannins as potent inhibitors of DNA topoisomerase II in vitro. *J. Pharm Sci.* **82 (5)**, 487-492.
- Katterman FRH, Shattuck VI. 1983.** An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. *Prep. Biochem.* **13**, 347-359.
- Keegstra K, Yousif AE. 1986.** Isolation and characterisation of chloroplast envelope membranes. *Methods in Enzymology* **118**, 316-325.
- Kermode AR. 1990.** Regulatory mechanisms involved in the transition from seed
-

- development to germination. *CRC Critical Reviews in Plant Science* **9**, 155-195.
- Kieselbach T, Hagman A, Andersson B, Schröder W.P.** 1998. The thylakoid lumen of chloroplasts. *The Journal of Biological Chemistry* **273** (12), 6710-6716.
- Kim CS, Lee CH, Shin JS, Chung YS, Hyung NI.** 1997. A simple and rapid method for isolation of high quality genomic DNA from fruit trees and conifers using PVP. *Nucleic Acids Res.* **25** (5), 1085-1086.
- King MW.** 1997. Rapid and nonradioactive screening of recombinant libraries by PCR. In: *PCR Cloning Protocols: From Molecular Cloning to Genetic Engineering*. pp 331-338. White BA (ed.). Humana Press Inc. Totowa, New Jersey.
- Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K.** 1994. Characterization of two cDNAs (ERD10 and ERD14) corresponding to genes that respond rapidly to dehydration stress in *Arabidopsis thaliana*. *Plant Cell Physiol.* **35**(2), 225-231.
- Koonjul PK.** 1999. Isolation of RNA from the polyphenolic rich resurrection plant *Myrothamnus flabellifolius* welw. (Qiagen Newsletter, In press).
- Koonjul PK, Brandt WF, Lindsey GG, Farrant JM.** 1998. Inhibitory effect of polyphenolics during PCR. In: *Polyphenols Communications*. Vol. II. pp 441-442. Charbonnier F, Delacotte JM, Rolando C. (Eds.). Lille, France.
- Koonjul PK, Brandt WF, Lindsey GG, Farrant JM.** 1999a. Isolation and characterisation of chloroplasts from the resurrection plant *Myrothamnus flabellifolius* welw. *J. Plant Physiol.* (In press)
- Koonjul PK, Brandt WF, Farrant JM, Lindsey GG.** 1999b. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nucleic Acids Res.* **27** (3), 915-916.
- Koonjul PK, Kruger LA, Farrant JM.** 1999c. The antioxidant systems used by the resurrection plant *Myrothamnus flabellifolius* welw. during desiccation (In preparation).
- Koster KL, Leopold AC.** 1988. Sugars and desiccation tolerance in seeds. *Plant Physiol.* **88**, 829-832.
- Kruger LA.** 1998. Towards an understanding of the mechanisms of desiccation tolerance in *Myrothamnus flabellifolius* (Welw.). *MSC thesis*, Department of Botany, University of Cape Town, South Africa.
- Kuang J, Gaff DF, Gianello RD, Blomstedt CK, Neale AD, Hamill JD.** 1995. Changes in *in vivo* protein complements in drying leaves of the desiccation-tolerant grass *Sporobolus stapfianus* and the desiccation-sensitive grass *Sporobolus pyramidalis*. *Australian J. of Plant Physiol.* **22**, 1027-1034.
- Kühlbrandt W, Wang DN, Fujiyoshi Y.** 1994. Atomic model of plant light-harvesting complex by electron crystallography. *Nature* **367** (6464), 614-621.
- Kuntz ID, Kauzmann W.** 1974. Hydration of proteins and polypeptides. *Adv. Protein. Chem.* **28**, 239345.
- Kut SA, Flick CF.** 1986. A minipreparation system for the analysis of chloroplast DNA restriction enzyme digest fragments in *Nicotiana*. *Plant Molecular Biology Reporter* **4**(1), 48-55.
-

- Labrude P, Chaillot B, Vigneron C. 1987.** Problems of freezing haemoglobin freeze drying: evidence that water removal is the key to iron oxidation. *Journal of Pharmaceutics and Pharmacology* **39**, 344-348.
- Laemmli UK. 1970.** Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* **227**, 680-685.
- Larson RA. 1988.** The antioxidants of higher plants. *Phytochemistry* **27** (4), 969-978.
- Lee GJ, Pokala N, Vierling E. 1995.** Structure and in vitro molecular chaperone activity of cytosolic small heat shock protein from pea. *J. Biol. Chem.* **279**, 10432-10438.
- Lenne C, Douce R. 1994.** A low molecular mass heat-shock protein is localised to higher plant mitochondria. *Plant Physiol.* **195**, 1255-1261.
- Leopold AC, Vertucci CW. 1987.** In: *Membranes, macromolecules, and stability in the dry state*. Leopold C. ed. pp 22-34, Cornell University Press, Ithaca.
- Leopold AC, Vertucci CW. 1989.** Moisture as a regulator of physiological reaction in seeds. In *Seed Moisture* pp 51-67. Stanwood PC. and McDonald MB. eds. Crop Science society America Special Publ # 14. Madison, WI.
- Leopold AC, Sun WQ, Bernal-Lugo I. 1994.** The glassy state in seeds: analysis and function. *Seed Sci. Res.* **4**, 267-274.
- Leslie SB, Teter S, Crowe LM, Crowe JH. 1994.** Trehalose lowers membrane phase transitions in dry yeast cells. *Biochimica et Biophysica Acta* **1192**, 7-13.
- Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM. 1995.** Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology* **61** (10), 3592-3597.
- Lessard P, Decroocq V, Thomas M. 1997.** In *Plant Molecular Biology, A laboratory manual: Extraction of RNA, cloning and subtractive hybridisation* pp 154-220. Krieg PA eds. Springer-Verlag Berlin Heidelberg.
- Levine H, Slade L. 1992.** In *Physical chemistry of foods* pp 83-221. Schwartzberg H, Hatal RW eds. Marcel Dekker, New York.
- Levitt J. 1980a.** Responses of plants to experimental stresses. Vol 1: chilling, Freezing and high temperature stresses. Academic Press, New York and London.
- Levitt J. 1980b.** Responses of plants to experimental stresses. Vol 2: water, radiation, salt and other stresses. Academic Press, New York and London.
- Liang P, Pardee AB. 1992.** Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.
- Liang P, Averboukh L, Pardee AB. 1993.** Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Research* **21** (14), 3269-3275.
- Liang P, Pardee AB. 1995.** Recent advances in differential display. *Curr. Op. in Immunol.* **7**, 274-280.
- Lichtenthaler HK. 1987.** Chlorophylls and carotenoids: pigments of photosynthetic
-

- biomembranes. *Methods in Enzymology* **148**, 350-383.
- Lichtenthaler HK, Miehe JA. 1997.** Fluorescence as a diagnostic tool for plant stress. *Trends in Plant Science* **2** (8), 316-320.
- Litts JC, Colwell GW, Charekian RL, Quatranno RS. 1991.** Sequence analysis of a functional member of the Em gene family from wheat. *J. of Sequencing and Mapping* **1**, 263-274.
- Loban PE, Kaiser AD. 1973.** Enzymatic end-to end joining of DNA molecules. *J Mol Biol.* **78**(3), 453-471.
- Logemann J, Schell J, Willmitzer L. 1987.** Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16-20.
- Loomis WD. 1974.** Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods in enzymology* **31**, 528-545.
- Luzzati V, Husson F. 1962.** The structure of the liquid-crystalline phase of lipid-water systems. *J. Cell Biol.* **12**, 207-219.
- MacDonald RJ, Swift GH, Przybyla AE, Chirgwin JM. 1987.** Isolation of RNA using guanidium salts. *Methods in Enzymology* **152**, 219-227.
- Madin KAC, Crowe JH. 1975.** Anhydrobiosis in nematodes: carbohydrate and lipid metabolism during dehydration. *J. of Exp. Zoology* **193**, 335-342.
- Malan HL, Farrant JM. 1998.** Effects of the metal pollutants cadmium and nickel on soybean seed development. *Seed Sci. Res.* **8**, 445-453.
- Malan C, Greyling MM, Gressel J. 1990.** Correlation between CuZn superoxide dismutase and glutathione reductase, and environmental and xenobiotic stress tolerance in maize inbreds. *Plant Science* **69**, 157-166.
- Maniatis T, Fritsch EF, Sambrook J. 1982.** Molecular cloning. In *A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
- Manning K. 1991.** Isolation of nucleic acids from plants by differential solvent precipitation. *Anal. Biochem.* **195**, 45-50 (1991).
- Mariaux JB, Bockel C, Salamini F, Bartels D. 1998.** Desiccation- and abscisic acid-response genes encoding major intrinsic proteins (MIPs) from the resurrection plant *Craterostigma plantagineum*. *Plant Mol. Biol.* **38** (6), 1089-1099.
- Martin W, Nock S, Meyer-Gauen G, Hager KP, Jensen U, Cerff R. 1993.** A method for isolation of cDNA-quality mRNA from immature seeds of a gymnosperm rich in polyphenolics. *Plant Mol. Biol.* **22**, 555-556.
- Mauve AA. 1966.** Resurrection plants. *Fauna Flora* **17**, 26-31.
- McIntyre GI. 1987.** The role of water in the regulation of plant development. *Canadian Journal of Botany* **65**, 1287-1298.
- McKersie BD, Stinson RH. 1980.** Effect of dehydration on leakage and membrane structure in *Lotus corniculatus* L. seeds. *Plant Physiol.* **66**, 316-320.
-

- McKersie B, 1991.** The role of oxygen free radicals in mediating freezing and desiccation stress in plants. In *Current Topics in Plant Physiology Am. Soc. Plant Physiol.: Active oxygen and oxidative stress and plant metabolism*. Vol. 8 pp 107-118. Pell E. and Staffen K eds.
- McKord JM, Fridovich I. 1969.** Superoxide dismutase, and enzymatic function for erythrocyte hemocuprein (hemocuprein). *J. Biol. Chem.* **244**, 6049-6055.
- McKown R, Kuroki G, Warren G. 1996.** Cold responses of *Arabidopsis* mutants impaired in freezing tolerance. *J. Exp. Bot.* **47**, 1919-1925.
- Mendes EJ. 1978.** *Flora Zambesiaca* **4**, 68-71.
- Metcalf CR, Chalk L. 1950.** Leaves, stem, and wood in relation to taxonomy with notes on economic uses. In: *Anatomy of the dicotyledons* Vol 1.
- Milner RJ, Sutcliffe JG. 1983.** Gene expression in rat brain. *Nucl. Acids Res.* **11** (16), 5497-5520.
- Mittelheuser CJ, van Steveninck. 1969.** Stomatal closure and inhibition of transpiration by RS-abscisic acid. *Nature* **221**, 281-282.
- Mozer TJ. 1980.** Partial purification and characterization of the mRNA from  $\alpha$ -amylase from barley aleurone layers. *Plant Physiol.* **65**, 834-837 (1980).
- Mtwisha L, Brandt W, McReady S, Lindsey GG. 1998.** HSP 12 is a LEA-like protein in *Saccharomyces cerevisiae*. *Plant Mol. Biol.* **37**, 513-521.
- Mundree SG. 1996.** Genetic determinants of salinity tolerance in tobacco. PhD dissertation. Auburn University, Auburn USA.
- Mundree SG, Farrant JM. 1999.** Some physiological and molecular insights into the mechanisms of desiccation tolerance in the resurrection plant *Xerophyta viscosa* Baker. In: *Plant tolerance to abiotic stresses in agriculture: role of genetic engineering*. Cherry J (ed.) (In Press).
- Mundy J, Chua N.H. 1988.** Abscisic acid and water-stress induce the expression of a novel rice gene. *The EMBO Journal* **7**, 2279-2286.
- Nakane H, Fukushima M, Ono K. 1990.** Differential inhibition of reverse transcriptase and various DNA polymerases by digallic acid and its derivatives. *J. Nat. Prod.* **53** (5), 1234-1240.
- Navari-Izzo F, Ricci F, Vanazza C, Quartacci MF. 1995.** Unusual composition of thylakoid membranes of the resurrection plant *Boea hydroscopica*: Changes in lipids upon dehydration and rehydration. *Physiologia Plantarum* **94**, 135-142.
- Navari-Izzo F, Quartacci MF, Sgherri CM. 1996.** Superoxide generation in relation to dehydration and rehydration. *Biochem. Soc. Trans.* **24** (2), 447-451.
- Nelson T, Brutlag D. 1979.** Addition of homopolymers to the 3'-ends of duplex DNA with terminal transferase. 1979. *Methods. Enzymol.* **68**, 41-50.
- Nishizawa M, Yamagishi T, Dutschman GE, Parker WB, Bodner AJ, Kilkuskie RE, Cheng YC,**
- Lee KH. 1989.** Anti-AIDS agents, 1. Isolation and characterization of four new
-

tetragalloylquinic acids as a new class of HIV reverse transcriptase inhibitors from tannic acid. *J. Nat. Prod.* **52** (4), 762-768.

**Ohara O, Dorit RL, Gilbert W. 1989.** One-sided polymerase chain reaction: the amplification of cDNA. *Proc. Natl. Acad. Sci. USA.* **86**, 5673-5677.

**Oliver MJ, Bewley JD. 1997.** Desiccation tolerance of plant tissues: a mechanistic overview. *Horticultural Science* **18**, 171-213.

**Oliver MJ, Wood AJ, O'Mahony P. 1998.** "To dryness and beyond"- preparations for the dried state and rehydration in vegetative desiccation-tolerant plants. *Plant Growth Regulation* **24**, 193-201.

**O'Mahony PJ, Oliver MJ. 1999.** Characterization of a desiccation-responsive small GTP-binding protein (Rab2) from the desiccation-tolerant grass *Sporobolus stapianus*. *Plant Mol. Biol.* **39**, 809-821.

**Ooms JJJ, Wilmer JA, Karssen CM. 1994.** Carbohydrates are not the sole factor determining desiccation tolerance in seeds of *Arabidopsis thaliana*. *Physiologia Plantarum* **90**, 431-436.

**Pammenter NW, Berjak P. 1999.** A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Sci. Res.* **9**, 13-37.

**Parker J. 1962.** Relationships among cold hardiness, water-soluble protein, anthocyanins, and free sugars in *Hedera helix* L. *Plant Physiol.* **37**, 809-813.

**Pellegrini L. 1980.** Cytological studies on physodes in the vegetative cells of *Cystoseira stricta* Sauvageau (Phaeophyta, Fucales). *J. Cell Sci.* **41**, 209-231.

**Peterson DG, Boehm KS, Stack SM. 1997.** Isolation of milligram quantities of nuclear DNA from tomato (*Lycopersicon esculentum*), a plant containing high levels of polyphenolic compounds. *Plant Mol. Biol. Rep.* **15** (2), 148-153.

**Pfündel E, Bilger W. 1994.** Regulation and possible function of the violaxanthin cycle. *Photosynth. Res.* **42**, 89-109.

**Piatkowski D, Schneider K, Salamini F, Bartels D. 1990.** Characterisation of five abscisic acid-responsive cDNA clones isolated from the desiccation-tolerant plant *Craterostigma plantagineum* and their relationship to other water-stress genes. *Plant Physiol.* **94**, 1682-1688.

**Pich U, Schubert I. 1993.** Midiprep method for isolation of DNA from plants with a high content of polyphenolics. *Nucleic Acids Res.* **21** (14), 3328.

**Pikaart MJ, Villeponteau B. 1993.** Suppression of PCR amplification by high levels of RNA. *Biotechniques* **14**(1), 24-25.

**Polle A. 1996.** Mehler reaction: Friend or foe in photosynthesis? *Bot. Acta* **109**, 84-89.

**Prestrelski SJ, Tedeschi N, Arakawa T, Carpenter JF. 1993.** Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys. J.* **65**, 661-671.

**Priestley DA, DeKruiff B. 1982.** Phospholipid motional characteristics in a dry biological system. *Plant Physiol.* **70**, 1075-1078.

---

- Quartacci MF, Navari-Izzo F. 1997.** Water stress and free radical mediated changes in sunflower seedlings. *J. Plant. Physiol.* **139**, 621-625.
- Quartacci MF, Forli M, Rascio N, Dalla Vecchia F, Bochocchio A, Navari-Izzo F. 1997.** Desiccation-tolerant *Sporobolus stafianus*: lipid composition and cellular ultrastructure during dehydration and rehydration. *J. Experimental Botany* **48** (311), 1269-1279.
- Ramanathan V. 1988.** The greenhouse theory of climate change: A test by an inadvertant global experiment. *Science* **240**, 293-299.
- Ray SD, Guruprasad KN, Laloraya MM. 1980.** Antagonistic action of phenolic compounds on abscisic acid-induced inhibition of hypocotyl growth. *J. of Exp. Bot.* **31** (125), 1651-1656.
- Reynolds ES. 1963.** The use of lead citrate at high pH as an electron opaque stain for electron microscopy. *Journal of Cell Biology* **17**, 208-212.
- Rice-Evans C. 1995.** Plant polyphenols: free radical scavengers or chain-breaking antioxidants? *Biochem. Soc. Symp.* **61**, 103-116.
- Rice-Evans CA, Miller NJ, Paganga G. 1996.** Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad. Biol. Med.* **20** (7), 933-956.
- Rice-Evans C, Miller N. 1997.** Measurement of the antioxidant status of dietary constituents, low density lipoproteins and plasma. *Prostaglandins Leukot Essent. Fatty Acids* **57** (4-5), 499-505.
- Roberts EH. 1973.** Predicting the storage life of seeds. *Seed Sci. Technol.* **1**, 499-514.
- Roberts EH, King MW, Ellis RH. 1984.** Recalcitrant seeds: their recognition and storage. In: *Crops Genetic Resources* pp 38-52. Holden JHW and Williams JT eds. Allen and Unwin, London.
- Ross JH. 1972.** *The Flora of Natal*. Government Printer, Pretoria.
- Russouw PS, Farrant J, Brant W, Maeder D, Lindsey GG. 1995.** Isolation and characterisation of a heat-soluble protein from pea (*Pisum sativum*) embryos. *Seed Science Research* **5**, 137-144.
- Russouw PS, Farrant J, Brandt W, Lindsey GG. 1997.** The most prevalent protein in a heat-treated extract of pea (*Pisum sativum*) embryos is not an LEA group 1 protein; its conformation is not affected by exposure to high temperature. *Seed Science Research* **7**, 117-123.
- Rychlik W, Rhodes RE. 1989.** A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. *Nucl. Acids Res.* **17**(21), 8543-51.
- Sales K, Brandt W, Rumbak E, Lindsey G.** The Lea-like protein HSP12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation. *Biochem. Biophys. Acta* (In Press).
- Salunkhe DK, Jadhav SJ, Kadam SS, Chavan JK. 1982.** Chemical, biochemical, and biological significance of polyphenols in cereals and legumes. *Crit. Rev. Food Sci. Nutr.* **17** (3), 277-305 (1982).
-

- Sanches R, WLB Melo, Colombo MF. 1986.** Effects of hydration in the oxygenation and autooxidation of carbon-monoxihaemoglobin powder. *Biochim. Biophys. Acta.* **874**, 19-22.
- Saradhi A, Saradhi PP. 1991.** Proline accumulation under heavy metal stress. *J. Plant Physiol.* **138**, 554-558.
- Sargent T. 1987.** Isolation of differentially expressed genes. *Meth. Enzymol.* **152**, 423-432.
- Sastry GM, Agmon N. 1997.** Trehalose prevents myoglobin collapse and preserves its internal mobility. *Biochemistry* **36**, 7097-7108.
- Schneider K, Wells B, Schmelzer E, Salamini F, 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.
- Schneiderbauer A, Sandermann Jr. H, Ernst D. 1991.** Isolation of functional RNA from plant tissues rich in phenolic compounds. *Anal. Biochem.* **197**, 91-95.
- Schwab KB, Heber U. 1984.** Thylakoid membrane stability in drought-tolerant and drought-sensitive plants. *Planta* **161**, 37-45.
- Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H. 1999.** LeProT1, a transporter for proline, glycine betaine, and  $\gamma$ - amino butyric acid in tomato pollen. *The Plant Cell* **11**, 377-391.
- Seel W, Hendry G, Atherton N, Lee J. 1991.** Radical formation and accumulation in vivo in desiccation tolerant and intolerant mosses. *Free Radic. Res. Commun.* **15** (3), 133-141.
- Seel WE, Hendry GA, Lee JA. 1992.** Effects of desiccation on some activated oxygen processing enzymes and antioxidant in mosses. *J. Exp. Bot.* **43** (253), 1031-1037.
- Senaratna T, McKersie BD. 1983.** Dehydration injury in germinating soybean (*Glycine max L. Merr.*) seeds. *Plant Physiol.* **72**, 620-624.
- Senaratna T, McKersie BD, Stinson RH. 1985.** Simulation of dehydration injury to membranes from soybean axes by free radicals. *Plant Physiol.* **77**, 472-474.
- Sgherri CLM, Pinzino C, Navari-Izzo, F. 1993.** Chemical changes and  $O_2^-$  production in thylakoid membranes under water stress. *Plant Physiol.* **87**, 211-216.
- Sgherri CLM, Loggini B, Bochicchio A, Navari-Izzo F. 1994a.** Antioxidant system in *Boea hydroscopica*: changes in response to desiccation and rehydration. *Phytochemistry* **35** (3), 377-381.
- Sgherri CLM, Loggini B, Puliga S, Navari-Izzo F. 1994b.** Antioxidant system in *Sporobolus stapfianus*: changes in response to desiccation and rehydration. *Phytochemistry* **35** (3), 561-565.
- Sharma YK, Davis KR. 1995.** Isolation of a novel Arabidopsis ozone-induced cDNA by differential display. *Plant Mol. Biol.* **29** (1), 91-98.
- Sherwin HW. 1995.** Desiccation tolerance and sensitivity of vegetative plant tissue. PhD thesis, University of Natal, Durban, South Africa.
-

- Sherwin HW, Farrant JM. 1996.** Differences in rehydration of three desiccation-tolerant angiosperm species. *Annals of Botany* **78**, 703-710.
- Sherwin HW, Farrant JM. 1998.** Protection mechanisms against excess light in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*. *Plant Growth Regulation* **24**, 203-210.
- Sherwin HW, Pammenter NW, February E, Van Der Willigen C, Farrant JM. 1998.** Xylem hydraulic characteristics, water relations and wood anatomy of the resurrection plant *Myrothamnus flabellifolius* Welw. *Annals of Bot.* **81**, 567-575.
- Simon EW. 1974.** Phospholipids and plant membrane permeability. *New Phytologist* **73**, 377-420.
- Singh J, Beckwell BA, Miller RW, Bewley JD. 1984.** Membrane organisation in the desiccation-tolerant moss *Tortula ruralis* in dehydrated states. *Plant Physiol.* **75**, 1075-1079.
- Singh RP, Singh M, King RR. 1998.** Use of citric acid for the neutralizing polymerase chain reaction inhibition by chlorogenic acid in potato extracts. *J. Virol. Methods* **74** (2), 231-235.
- Smirnoff N. 1993.** The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytology* **125**, 27-58.
- Soni R, Murray JAH. 1994.** Isolation of intact DNA and RNA from plant tissues. *Anal. Biochem.* **218**, 474-476.
- Spurr AR. 1969.** A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructural Research* **26**, 31-43.
- Staub U, Polivka H, Gross HJ. 1995.** Two rapid microscale procedures for isolation of total RNA from leaves rich in polyphenols and polysaccharides: application for sensitive detection of grapevine viroids. *J. of Virological Methods* **52**, 209-218.
- Stefanov K, Markoska YK, Kimenov GP, Popov SS. 1992.** Lipid and sterol changes in leaves of *Haberlea rhodopensis* and *Ramonda* species at transition from biosis into anabiosis and vice versa caused by water stress. *Phytochemistry* **30**, 461-466.
- Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF. 1998.** Mode of action of the COR 15a gene on the freezing tolerance of *Arabidopsis thaliana*. *PNAS* **95** (24), 14570-14575.
- Stevanovic B, Pham Thi AT, Monteiro de Paula F, Vieira da Silva J. 1992.** Effects of dehydration and rehydration on the polar lipid and fatty acid composition of *Ramonda* species. *Canadian Journal of Botany* **70**, 107-113.
- Stewart RC, Bewley JD. 1982.** Stability and synthesis of phospholipids during desiccation and rehydration of a desiccation-tolerant and a desiccation-intolerant moss. *Plant Physiol.* **69**, 724-727.
- Su X, Gibor A. 1988.** A method for RNA isolation from marine macro-algae. *Anal. Biochem.* **174**, 650-657.
- Suau R, Cuevas A, Valpuesta V, Reid MS. 1991.** Arbutin and sucrose in the leaves of the resurrection plant *Myrothamnus flabellifolius*. *Phytochemistry* **30** (8), 2555-2556.
-

- Swain T.** 1965. Nature and properties of flavonoids. In *Chemistry and Biochemistry of plant pigments*. T.W Goodwin (ed). Academic Press. London and New York.
- Tamagnone L, Merida A, Stacey N, Plaskitt K, Parr A, Chang C, Lynn D, Dow JM, Roberts K, Martin C.** 1998. Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. *The Plant Cell* **10** (11), 1801-1816.
- Tan GT, Pezzuto JA, Kinghorn AD, Hughes SH.** 1991. Evaluation of natural products as inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. *J. Nat. Prod.* **54** (1), 143-154.
- Telfer A, Dhami S, Bishop SM, Phillips D, Barber J.** 1994.  $\beta$ -Carotene quenches singlet oxygen formed by isolated photosystem II reaction centers. *Biochem.* **33**, 14469-14474.
- Terao J, Piskula M, Yao Q.** 1994. Protective effect of epicatechin, epicatechin gallate and quercetin on lipid peroxidation in phospholipid bilayers. *Arch. of Biochem. and Biophys.* **308**, 278-284.
- Tetteroo FAA, de Brujn AY, Henselmans RNM, Wolkers WF, van Aelst Ac, Hoekstra FA.** 1996. Characterization of membrane properties in desiccation-tolerant and intolerant carrot somatic embryos. *Plant Physiol.* **111**, 403-412.
- Thomas MG, Hesse SA, Foss YJ, Farzaneh F.** 1997. The use of PCR for differential screening of cDNA libraries. In: *PCR Cloning Protocols*. Vol 67, pp 405-418. White BA (ed.). Humana Press Inc. Totowa, New Jersey.
- Travis GH, Naus GG, Morrison JH, Bloom FE, Sutcliffe JG.** 1987. Subtractive cDNA cloning and analysis of primate neocortex mRNAs with regionally-heterogeneous distributions. *Neuro-pharmacology* **26**, 845-854.
- Trentmann SM, Knaap E, Liang P, Pardee AB.** 1995. Alternatives to  $^{35}\text{S}$  as a label for the differential display of eukaryotic messenger RNA. *Science* **267**, 1186-1187.
- Truesdell GM, Dickman MB.** 1997. Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display. *Plant Mol. Biol.* **33**, 737-743.
- Tsuda T, Watanabe M, Ohshima K, Norinobu S, Choi SW, Kawakishi S, Osawa T.** 1994. Antioxidative activity of the anthocyanin pigments cyanidin 3-O-beta-D-glucoside and cyanidin. *J. Agric. Food Chem.* **42**, 2407-2410.
- Tsuda T, Shiga K, Ohshima K, Kawakishi S, Osawa T.** 1996. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Biochem. Pharmacol.* **52**, 1033-1039.
- Tuba Z, Lichtenthaler HK, Maroti I, Csintalan Z.** 1993. Resynthesis of thylakoids and functional chloroplasts in the desiccated leaves of the poikilochlorophyllous plant *Xerophyta scabrida* upon rehydration. *Journal of Plant Physiology* **142**, 742-748.
- Tuba Z, Lichtenthaler HK, Csintalan Z, Nagy Z, Sente K.** 1994. Reconstitution of chlorophylls and photosynthetic  $\text{CO}_2$  assimilation upon rehydration of the desiccated poikilochlorophyllous plant *Xerophyta scabrida* (Pax) Th. Dur. et Schinz. *Planta* **192**, 414-420.
- Tymms MJ, Gaff DF.** 1979. Proline accumulation during water stress in resurrection

- plants. *Journal of Experimental Botany* **30**, 165-168.
- Van Camp W, Capiou K, Van Montagu M, Inze D, Slooten L. 1996.** Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. *Plant Physiol.* **112**, 1703-1714.
- van der Knaap E, Kende H. 1995.** Identification of a gibberellin-induced gene in deepwater rice using differential display of mRNA. *Plant Mol. Biol.* **28** (3), 589-592.
- Velasco R, Salamini F, Bartels DR. 1994.** Dehydration and ABA increase mRNA levels and enzyme activity of cytosolic GAAPDH in the resurrection plant *Craterostigma plantagineum*. *Plant Mol. Biol.* **26** (1), 541-546.
- Velasco R, Salamini F, 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.
- Veluthambi K, Mahadevan S, Maheshivari R. 1981.** Trehalose toxicity in *Cuscuta reflexa*. *Plant Physiol.* **68**, 1369-1374.
- Vertucci CW, Farrant JM. 1995.** Acquisition and loss of desiccation tolerance. In: *Seed development and Germination*. Kigel, J., Galilli, G. eds. New York: Marcel Dekker, Inc., 237-271.
- Verwoerd TC, Dekker BMM, Hoekema A. 1989.** A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* **17**, 2362.
- Vicre M, Sherwin HW, Driouich A, Jaffer MA, Farrant JM. 1999.** Cell wall characteristics and structure of hydrated and dry leaves of the resurrection plant *Craterostigma wilmsii*, a microscopical study. *J. of Plant Physiol.* (In press).
- Vielle-Calzada JP, Nuccio ML, Budiman MA, Thomas TL, Burson BL, Hussey MA, Wing RA. 1996.** Comparative gene expression in sexual and apomictic ovaries of *Pennisetum ciliare* (L.) Link. *Plant Mol. Biol.* **32** (6), 1085-1092.
- Vierling E. 1991.** The role of heat shock proteins in plants. *Annu. Rev. Plant Physiol Plant Mol. Biol.* **42**, 579-620.
- Visioli G, Maestri E, Marmiroli N. 1997.** Differential display-mediated isolation of a genomic sequence for a putative mitochondrial LMW HSP specifically expressed in condition of induced thermotolerance in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* **34**, 517-527.
- Von Holt G, Brandt WF, Greyling HJ, Lindsey GG, Retief JD, Rodrigues JA, Schwager S, Sewell BT. 1989.** Isolation and characterization of histones. *Methods in Enzymology* **170**, 428-492.
- Wallace DM. 1987.** Large- and small-scale phenol extractions. *Methods in Enzymology* **12(A/B)**, 33-49.
- Wallace G, Fry SC. 1994.** Phenolic components of the plant cell wall. *Int. Rev Cytol* **151**, 229-267.
- Wan CY, Wilkins TA. 1994.** A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* **223**, 7-12.
- Wang SY, Jiao HJ, Faust M. 1991.** Changes in ascorbate, glutathione, and related
-

enzyme activities during thidiazuron-induced bud break of apple. *Physiol. Plant* **82**, 231-236.

**Waters ER, Lee GJ, Vierling E.** 1996. Evolution, structure and function of the small heat shock proteins in plants. *J Exp. Bot.* **47** (296), 325-338.

**Watson JB, Battenberg EF, Wong KK, Bloom FE, Sutcliffe JG.** 1990. Subtractive complementary DNA cloning of RC3, a rodent-cortex-enriched mRNA encoding a novel 78 residue protein. *J. Neurosci. Res.* **26**, 397-408.

**Wellburn FAM, Wellburn AR.** 1976. Novel chloroplasts and unusual cellular ultrastructure in the "resurrection" plant *Myrothamnus flabellifolius* Welw. (Myrothamnaceae). *Botanical Journal of the Linnean Society* **72**, 51-54.

**Wesley UV, Wesley CS.** 1997. Rapid directional walk within DNA clones by step-out PCR. In: *PCR Cloning Protocols* pp 279-286. White BA (ed.), Humana Press, Totowa, New Jersey.

**Wettstein-Westersheim W, Minelli H.** 1962. Breeding intersectional *Populus* hybrids. *Allg. Forstztg.* **73**, 2.

**Whitsitt M.S, Collins RG, Mullet JE.** 1997. Modulation of dehydration tolerance in soybean seedlings : Dehydrin Mat I is induced by dehydration but not abscisic acid. *Plant Physiol.* **114**, 917-925.

**Wilkins TA, Smart LB.** 1996. Isolation of RNA from plant tissue. In *A Laboratory guide to RNA isolation, analysis, and synthesis* pp 21-41. Krieg PA (eds). Wiley-Liss Inc, NY.

**Williams RJ, Leopold AC.** 1989. The glassy state in corn embryos. *Plant Physiol.* **89**, 977-981.

**Wood AJ, Duff RJ, Oliver MJ.** 1999. Expressed sequence tags (ESTs) from desiccated *Tortula ruralis* identify a large number of novel plant genes. *Plant Cell Physiol.* **40** (4), 361-368.

**Wolff S, Garner A, Dean RT.** 1986. Free radicals, lipids and protein degradation. *Trends in Biochemical Science* **11**, 27-31.

**Wolkers WF, Alberda M, Koornneef M, Léon-Kloosterziel KM, Hoekstra FA.** 1998a. Properties of proteins and the glassy matrix in maturation-defective mutant seeds of *Arabidopsis thaliana*. *Plant J.* **16** (2), 133-143.

**Wolkers WF, Oldenhof H, Alberda M, Hoekstra FA.** 1998b. A Fourier transform infrared microspectroscopy study of sugar glasses: Application to anhydrobiotic higher plant cells. *Biochim. Biophys. Acta.* **1379**, 83-96.

**Wolkers WF, Brandt W, Lindsey G.** Isolation of a D-7 LEA like protein from *Typha latifolia* pollen (unpublished results).

**Womersley C.** 1981. Biochemical and physiological aspects of anhydrobiosis. *Comparative Biochemistry and Physiology* **70B**, 669-678.

**Wray W, Boulikas T, Wray VP, Hancock R.** 1981. Silver staining of proteins in polyacrylamide gels. *Analytical Biochemistry* **118**, 197-203

**Xu D, Duan X, Wang B, Hong B, Ho TD, Wu R.** 1996. Expression of late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water

deficit and salt stress in transgenic rice. *Plant Physiol.* **110**, 249-257.

**Yoshida Y, Kiyosue T, Nakashima K, Yamaguchi-Shinosaki K, Shinosaki K. 1997.** Regulations of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.* **38** (10), 1095-1102.

**Young AJ. 1991.** The photoprotective role of carotenoids in higher plants. *Physiol. Plantarum* **83**, 702-708.

**Yu R, Jiao JJ, Duh JL, Krishnamurthy G, Tan TH, Kong ANT. 1997.** Activation of mitogen-activated protein kinases by green tea polyphenols: potential signaling pathways in the regulation of antioxidant-responsive element-mediated Phase II enzyme gene expression. *Carcinogenesis* **18** (2), 451-456.

**Yung MH, Schaffer R, Putterill J. 1999.** Identification of genes expressed during early Arabidopsis carpel development by mRNA differential display: characterisation of ATCEL2, a novel endo-1,4-beta-D-glucanase gene. *Plant J.* **17** (2), 203-208.

**Zhang H, Croes AF, Linskens HF. 1984.** Protein synthesis in germinating pollen of *Petunia*: Role of proline. *Planta* **154**, 199-203.

---