GENE EXPRESSION ASSOCIATED WITH DROUGHT TOLERANCE IN *Xerophyta viscosa* BAKER

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Dedication

In loving memory of my late father Zweli, sister Mandlakazi and nephew Xhanti to whom I would like to dedicate this dissertation. I felt your presence even though you were miles away and sincere thanks to you for everything.

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ABBREVIATIONS

ABA	abscisic acid
ABRE	abscisic acid response elememnt
Bzip	a family of transcription factors with basic region and Leu-zipper motif
C	Celsius
cDNA	copy deoxyribonucleic acid
CDPK	calcium-dependent protein kinase
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DRE	drought response element
DRE/C	DRE coupling element
DREBP	DRE binding protein
DS	differential screening
EDTA	ethylenediamenetetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
Fig.	figure
g	gram(s)
G-box	ubiquitous regulatory elements
Gly	glycine
h	hour
kb	kilobase(s)
kDa	kilodalton(s)
λ	lambda
l	litre(s)
LB	Luria-Bertani broth
LEA	late embryogenesis abundant
Lys	lysine
M	molar concentration
MAP	mitogen-activated protein
MAPK	mitogen-actvated protein kinase
MAPKKK	MAP KINASE KINASE KINASE
min	minute(s)
mg	milligram(s)
ml	millilitre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
MTD	mannitol dehydrogenase
MYB	a family of transcription factors with Trp cluster motif
MYC	a family of transcription factors with basic-helix loop-helix (bHLHL) and Leu-zipper motif
N	normal concentration
NaCl	sodium chloride
NaOH	sodium hydroxide

nm	nannometre(s)
O_2	oxygen
ORF	open reading frame
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PR	pathogenesis related proteins
PRK	phosphoribulokinase
RAB	abscisic acid response element
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RSK	ribosomal S6 protein kinase
RT-PCR	reverse transcriptase PCR
RT	room temperature
RWC	relative water content
SDS	sodium dodecyl sulphate
SSC	sodium chloride tri-sodium citrate
TE	tris-EDTA buffer
Tris	tris (hydrxymethyl) aminomethane
Tween 20	sorbitan monolaurate
µg	microgram(s)
UV	ultraviolet
w/v	weight per volume

ABSTRACT

Xerophyta viscosa (Baker) is a monocotyledonous resurrection plant that can tolerate extremes of desiccation. Upon rewatering, it rehydrates completely and assumes its full physiological activities. Studies on changes in gene expression associated with dehydration stress tolerance were conducted. A cDNA library constructed from mRNA isolated from dehydrated (85%, 37% and 5% relative water content) *X. viscosa* leaves, was differentially screened. Of the 192 randomly selected cDNAs screened, 30 showed higher expression levels when *X. viscosa* was dehydrated while 20 showed lower expression. *XVLEA*, *XVDH* and *XVLEC* represent three cDNAs that were upregulated during dehydration stress. *XVLEA* showed the highest identity at the amino acid level with a late embryogenesis abundant protein, LEA29G, from *Gossipium hirsutum* (30%) and a LEA D-29 from cotton (50%). *XVDH* exhibited significant identity to dehydrin proteins from *Arabidopsis thaliana* (45%) and *Pisum sativum* (43%) at the amino acid level. It encodes a glycine-rich protein (27 kDa) which is largely hydrophilic and contains a hydrophobic segment at the C-terminus. *XVLEC* showed 28% identity and 50% similarity to a lectin-like protein from *Arabidopsis thaliana*. Southern blot analysis confirmed the presence of the three cDNAs in the *X. viscosa* genome. Both *XVLEA* and *XVDH* transcripts were highly expressed during dehydration- (37% RWC) and rehydration (4%, 32%, 72% RWC) treatment of the plant ≈ 1.0kb was observed. However, with *XVDH* a transcript of ≈1.0 kb and 1.09kb were observed. *XVDH* transcripts accumulated in *X. viscosa* plants in response to low temperature, heat and dehydration stresses, as well as to exogenous supply of abscisic acid, ethylene and methyl jasmonate. Localization studies of the XVDH encoded protein showed that XVDH is located in the plasma membrane-cell wall region.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Water deficit

Water deficit varies from plant to plant. What is constituted as water stress from one species may not be conceived as a stress to another at all. The survival of a plant depends on its ability to respond to everchanging environments (Hwang & Goodman 1995). The availability of water is a critical environmental determinant of plant growth and distribution. In nature as well as in agriculture, plants are constantly exposed to highly variable water conditions (flooding through to desiccation) and other adverse environmental conditions such as low and high temperatures and high salt concentrations (Soderman *et al.* 1996; Conley *et al.* 1997; Shinozaki & Shinozaki-Yamaguchi 1997; Liu *et al.* 1998). Water plays a very important role in the survival of plants. It not only acts as a solvent for biochemical reactions in organisms, but as a stabiliser of the structure of macromolecules and organelles within cells. Membrane structure, in particular, depends on complex cellular interactions and is often regarded as the primary site of desiccation damage (Vertucci & Farrant 1995; Oliver & Bewely 1997; Oliver *et al.* 1998; Berjak & Pammenter 1999).

1.1.1 Plants responses to water deficit

Under water-stressed conditions plant cells lose water, leading to decreases in turgor pressure. The amount of water loss as well as the rate and duration of the stressed condition may result in cell damage, thus limiting plant growth and productivity from inhibition of photosynthesis and other diverse changes in cell metabolism (Koster 1991; Mitchell *et al.* 1994; Shinozaki & Shinozaki-Yamaguchi 1996; Cellier *et al.* 1998). Plants respond to water deficit by modifying their morphological, physiological and metabolic processes occurring in all organs (Bray 1994; Gosti *et al.* 1995; Cellier *et al.* 1998). Depending on the efficiency of the response, plant species can withstand longer or shorter periods of water deficits. Some responses may result from cell damage while others may correspond to adaptive processes. Plant responses to water deficit are very complex and depend on several factors such as developmental stage and the physiological condition of the affected plants. Water deficit responses elicited by sensitive plants and drought tolerant plants are perceived differently. Among desiccation-tolerant species, responses to water deficit vary. Some plants lose chlorophyll on drying (poikilochlorophyllous) and thylakoid membranes are dismantled, but upon rewatering they resynthesise their chlorophyll and

thyllakoids. Whereas others retain their chlorophylls (homoiochlorophyllous). The plant hormone, abscisic acid, is thought to regulate these changes.

Short-term effects of water deficit induce a rapid physiological response whereby stomata are closed in the leaves to prevent further loss of water (Chou *et al.* 1991). Long-term changes in response to abiotic stresses (drought, low temperature, high salinity) which may all disturb the intracellular water balance, result in the synthesis and accumulation of osmotically active, low molecular weight compounds. Among these compounds are sugar alcohols, proline and glycinebetaine which are collectively referred to as osmolytes, osmoprotectants or compatible solutes (Fig.1.1).

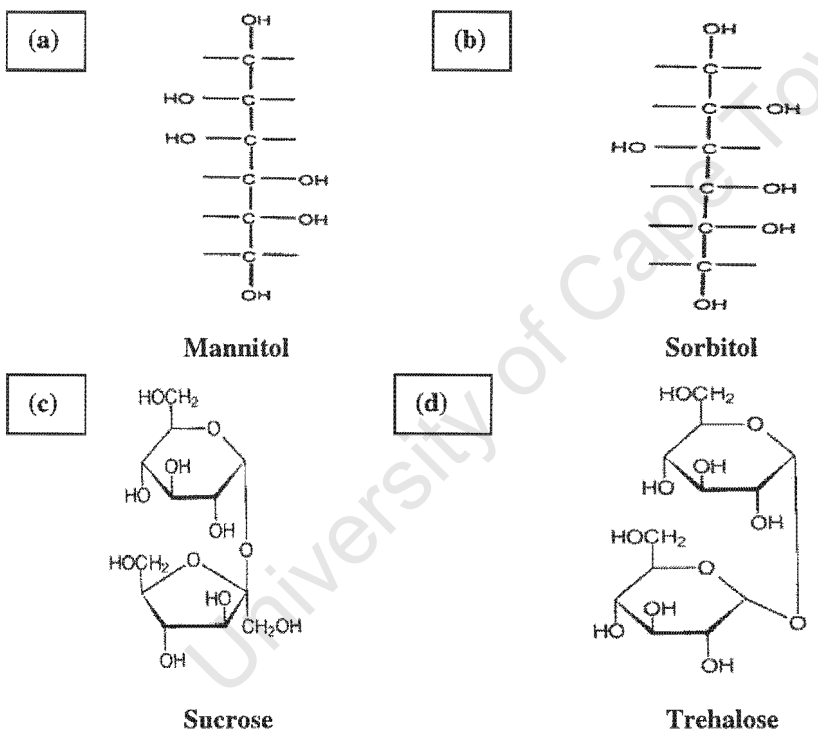


Figure 1.1 Chemical structures of some common six-carbon sugar alcohols, and energy storage compounds: a) Mannitol; b) Sorbitol or glucitol; c) Sucrose; d) Trehalose

Although their exact function in plants is unknown, these osmolytes have been suggested to protect the plant from abiotic stress (Chou *et al.* 1991). For example, osmolytes accumulate in plant cells in response to water or salinity stress and are subsequently degraded or lost after stress relief. Other studies indicate a macromolecular protective effect: *in vitro* incubation of osmolytes with protein extracts from plants often alleviates the adverse effects of electrolytes and temperature stress on enzymatic activity (Steward 1990). Additionally, accumulation of

these compounds may be the result of a stress-induced metabolic impairement (Jones & Gorham 1983).

Plant survival in most environments requires an ability to withstand or avoid extremes of stress caused by drought, salinity and temperature. Long-term changes in photosynthetic chemistry are also used by certain plants to reduce evaporative water loss (Bohnert *et al.*1988). Water stress, on the other hand, involves subtle changes in cellular chemistry. It appears to be the result of the accumulation of compatible solutes and of specific proteins that can be rapidly induced by osmotic stress (Rhodes 1987). Cellular responses to water deficit occur as a result of increasing solute concentrations, changes in cell volume and membrane shape, loss of turgor, disruption of membrane integrity and protein denaturation. Cells respond to these signals by employing mechanisms to avoid water deficit and the consequent damage that may result. An example of this avoidance is maintenance of turgor pressure by osmotic adjustment (Bray 1997).

1.2 Desiccation-tolerant plants

For plants to be truly desiccation-tolerant, they need to be able to reduce mechanical damage caused by loss of turgor, maintain macromolecular and membrane integrity, minimise toxin accumulation as well as free radical damage from impaired metabolism; and following rehydration, they need to repair the damage caused by desiccation (Bewely 1979, Vertucci & Farrant 1995; Oliver & Bewely 1997,Oliver *et al.* 1998). These plants provide excellent models to study water stress tolerance at the genetic and biochemical level. They occur in a niche where water frequently becomes the limiting factor but can withstand severe water loss and recover quickly from the stress upon rehydration (Sherwin & Farrant 1996). For example most resurrection plants occur on shallow soil on rocky outcrops. Consequently they experience frequent soil drying, especially during the non-rainy (usually winter) season (Gaff 1977; Farrant *et al.*1999; Farrant 2000). These modifications require alterations in gene expression within the cells even though the plants are multicellular (Shinozaki & Shinozaki-Yamaguchi 1997).

***Xerophyta viscosa* Baker**

The desiccation tolerant plant *Xerophyta viscosa* Baker (Family Velloziaceae) belongs to a small group of angiosperms, referred to as "resurrection plants", that are capable of tolerating extremes of desiccation (Gaff 1971; Bewley & Oliver 1992; Vertucci & Farrant 1995; Ingram & Bartels 1996). *X. viscosa* can be dehydrated down to 5 % relative water content (RWC) (Fig. 1.2a) and upon rewatering, the desiccated plant rehydrates completely within 80 h (Fig. 1.2b) with resumsion of full physiological activities (Sherwin & Farrant 1996). This unique ability to tolerate severe water loss is often shared with certain algae and bryophytes (Oliver & Bewley 1997), a few ferns (Reynolds & Bewley 1993), specialised structures of higher plants such as seeds and pollen grains (Kermode *et al.* 1986) and some 60 other angiosperms (Sherwin & Farrant 1996).

A variety of mechanisms have been identified which enable these extremophiles to survive severe water loss. It has been hypothesised that the mechanisms essential for desiccation tolerance in algae and bryophytes are constitutively present in cells (Oliver 1991; Oliver & Bewley 1997) and encompass repair processes during rehydration.

Resurrection plants on the other hand appear to activate the synthesis of specific transcripts and proteins during dehydration (Ingrams & Bartels 1996), although it has been proposed that different plants rely on these processes of protection and repair to different degrees (Oliver & Bewley 1997). It is not clear how resurrection plants tolerate desiccation but studies on the species involved have revealed the occurrence of cellular processes during desiccation (Ingram & Bartels 1996; Oliver & Bewely 1997; Dace *et al.*1998; Farrant *et al.* 1999; Farrant 2000) It has been suggested that accumulation of protectants such as proteins, sugars and compatible solutes may protect the membrane against desiccation and play a role in the osmotic adjustment (Ingram & Bartels 1996; Oliver & Bewely 1997).

Figure 1.2a *Xerophyta viscosa* Baker plant in a dehydrated state of about 5 % RWC.

Figure 1.2b Typical *Xerophyta viscosa* Baker in its fully hydrated condition.

1.3 Mechanisms of desiccation tolerance and other abiotic stresses

1.3.1 Osmoprotectants

Organisms and cellular systems with adaptation to stresses such as high temperature, desiccation and high urea environments often respond by accumulating particular organic solutes of lower molecular weight such as sugars polyhydric alcohols, free amino acids and their derivatives

(glycine & glycinebetaine) and methylamines. Collectively, these compounds have been referred to as osmolytes, osmoprotectants or compatible solutes (Hellebust 1976; Yancey 1979, 1982). Osmolytes accumulate in plant cells in response to water or salinity stress and are subsequently degraded or lost after stress relief (Hellebust 1976). The presence of these molecules has been shown *in vitro* to provide stability to enzymes without substantive alteration in their catalytic action (Borowitz & Brown 1974; Bowlus & Somero 1979; Pollard & Wyne Jones 1972; Yancey 1985). Glycinebetaine specifically stabilises proteins, not by interacting with them directly, but by altering the solvent properties of the surrounding water and therefore the macromolecule-solvent interactions. This results in a shift in the unfolded-folded equilibrium to favour the folded species (Yancey 1982; Timashef 1992, 1993). Their effect seems to be general to all proteins and has no inhibitory or enhancing effects on biological activity (Foord *et al.* 1998).

Several osmoregulatory mechanisms used by plants have been intensively studied in other organisms. These include pathways controlling the biosynthesis and transport of compatible solutes such as the polyamines and sugar alcohols, and osmoprotectants such as glycine-betaine and proline (Flores *et al.* 1985; Ostrem *et al.* 1987).

It has been shown by Yoshiba and his co-workers (1991) in a pilot study that many plants, including halophytes , accumulate proline to high levels in response to osmotic stresses such as salinity and water deficit (cited in Chou *et al.* 1991). It has been suggested that proline (similar to glycinebetaine) is a compatible osmolyte that plays a role in counteracting the effects of osmotic stress (Fig. 1.3). Recently, it has been demonstrated that proline acts as an osmoprotectant and that overproduction of proline results in increased tolerance to osmotic stress of transgenic tobacco plants (Yoshiba *et al.* 1997, Nanjo *et al.* 1999). Genetically engineered crop plants that overproduce proline might, thus, acquire osmotolerance, namely, the ability to tolerate environmental stresses such as drought and high salinity. These results indicate that proline is not just an osmoregulator in stressed plants but has a unique function involved in osmotolerance as well as in morphogenesis as a major constituent of cell wall structural proteins in plants (Yoshiba *et al.*1997; Nanjo *et al.* 1999)

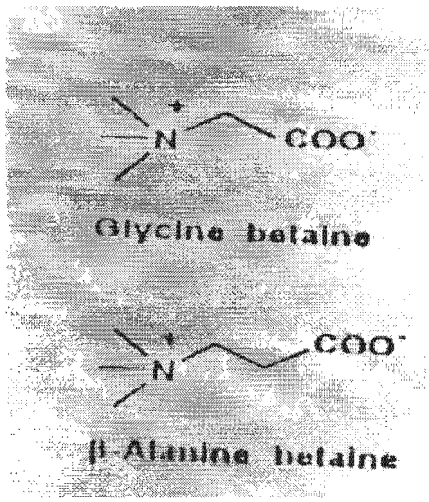


Figure 1. 3 Glycinebetaine similar to proline

On the other hand glycinebetaine protects cells from salt stress by maintaining an osmotic balance with the environment and by stabilizing the quaternary structure of complex proteins. Furthermore, it stabilises the oxygen-evolving photosystem Π complex and ribulose biphosphate carboxylase of the photosynthetic systems (Bernard *et al.* 1988)

The water replacement hypothesis suggests that polyhydroxy compounds can substitute for water in stabilising membrane structure in the dry state. In the case of membranes, the hydroxy groups can hydrogen-bond to polar head groups providing the hydrophilic interactions necessary for membrane structure and stability. This was demonstrated by the glucose dimer, trehalose, which occurs in many desiccation-tolerant organisms (Berjark & Pammenter 1999) (Fig.1.1c).

Mannitol

Mannitol is a six-carbon sugar alcohol that functions as a 'compatible solute' in very few families in higher plants (Fig. 1.1a). It has been shown to accumulate in the cytosol during osmotic stress preventing inactivation of osmotic processes (Stoop *et al.* 1996; Shen *et al.* 1997). Because of its structure which contains a water-like hydroxyl group, mannitol can enhance cell integrity thus maintaining cellular hydration around macromolecules. Secondly, it can act as sink of activated O_2 species to prevent peroxidation of fats which could result in cell damage (Smirnoff & Cumbes 1989). In response to high salinity e.g. celery, the dry weight of the plant can remain the same as those grown under normal conditions (Stoop *et al.*1996). The water content of the plant decreased with increased salinity, but in the presence of mannitol salt accumulation within the cell is prevented simply because mannitol acts as a 'salt excluder'.

On the other hand mannitol has been shown to function as a hydroxyl radical scavenger (Smirnoof & Cumbes 1989; Shen *et al.* 1997). It has been shown that in the presence of mannitol in the chloroplasts, plants can protect themselves against oxidative damage by hydroxyl radicals. This function is well documented in transgenic tobacco and *Arabidopsis* which were transformed targeting mannitol biosynthesis to chloroplasts. These plants were resistant to salinity for several generations (Shen *et al.* 1997). The mechanism of protection by mannitol is still unclear. It is thought that polyols react chemically, thus removing the hydroxyl radicals that would affect phosphoribulokinase (PRK), and the resulting products of the reaction between mannitol and hydroxyl radicals in the chloroplast may be highly variable (Shen *et al.* 1997).

Lastly, it has been documented that mannitol and its mannitol-utilizing enzyme mannitol dehydrogenase (MTD) are also involved in plant responses to pathogen attack. Two classes of proteins showing homology to MTD have been identified using protein databases. They showed close resemblance to the EL13 pathogenesis related proteins (PR proteins). For example, EL13 proteins in *Arabidopsis* are thought to be resistant to *Pseudomonas* (Kiedrowski 1992). That MTD is related to PR proteins and can confer resistance to pathogen attack, stems from the fact that mannitol is a potent antioxidant (Smirnoff & Cumbes 1989).

Sorbitol

The reduction of glucose to its sugar alcohol, catalysed by aldose reductase (AR), results in the formation of sorbitol (Fig.1.1b). Sorbitol is found in the seeds of a variety of plant species, and in *Rosaceae* species (Bartels & Nelson 1994). It is also expressed in vegetative tissues in the halotoerant *Plantago maritima*. Its function in vegetative tissues is mainly regulatory both in animal and plant systems whilst in seeds it contributes to the desiccation tolerance of the mature embryo (Bartels & Nelson 1994). In animal tissues during hyperglycemia associated with diabetes, the flux through AR increases and sorbitol accumulates inside the cell because it is not readily converted to fructose and because the plasma membrane is not readily permeable to sorbitol. The increase of sorbitol on its own has been proposed to result in diabetic complications (Bhatnagar & Srivastava 1992). AR also functions as an osmoregulator in the renal medulla by modulating sorbitol to maintain osmotic balance and cellular volume (Roncarati *et al.* 1995). The levels of expression have been shown to increase in both animal and

plant systems during water stress. The induction of sorbitol accumulation has been proposed to be the result of a response to ABA (Skriver & Mundy 1990).

Trehalose

Trehalose (Fig. 1.1d) occurs rarely in plants but its occurrence in resurrection plants is universal although in low concentrations. This has led to the idea that trehalose contributes significantly to desiccation tolerance in resurrection plants. It is the most effective osmoprotectant sugar in terms of minimum concentration required and it can effectively replace water in artificial membrane systems (Crowe *et al.*1992). Among other possible roles of trehalose in microorganisms and higher plants are sugar sensing and osmotic stress protection (Zentella *et al.* 1999) although the mechanism of action is still unknown. On the other hand, trehalose has been proposed to stabilize membranes. It is also well known as the most potent sugar in the protection of enzyme activity during dry storage with regard to duration of storage. It has been implicated as the most effective of the non-reducing sugars that replace water according to the 'Water Replacement Hypothesis' (Crowe *et al.* 1992). This hypothesis suggests that specific sugars replace the water normally associated with membrane surfaces, thereby maintaining the correct lipid head-group spacing and so preventing liquid crystalline to gel phase transition in the lipid bilayer (Hoekstra *et al.* 1997). It has been shown by Crowe *et al.* (1992) that *in vitro* drying and rehydration of the model-membrane sarcoplasmic reticulum usually results in the fusion of vesicles and loss of their ability to transport calcium. However, when trehalose was present at concentrations equivalent to those found in tolerant organisms, functional vesicles were preserved. These sugars are thought to protect membranes *in vitro*. Hence it has been suggested that sugars alter physical properties of dry membranes so that they resemble those of fully hydrated biomolecules (Crowe *et al.* 1992).

Sucrose

Sucrose (Fig. 1.1.c) occurs in high abundance in resurrection plants studied to date (Ghasempour *et al.* 1998; Mundree & Farrant 2000). The accumulation of non-reducing sugars, especially sucrose and rafinose, is well documented with regard to desiccation, during dehydration and/or following rehydration (Oliver & Bewely 1997; Kleins *et al.* 1999). Accumulation of sucrose in dehydrating leaves is thought to originate from different sources such as complete utilisation of carbohydrate storage reserves in source tissues whereas it is mobilised and reserved in sink

tissues (Ghasempour *et al.* 1998; Hartung *et al.* 1998; Norwood *et al.* 1999; Scott 2000, Whittaker *et al.* 2000). Reports on the concomitant increase of sucrose together with fructose and glucose in leaf tissue during the initial stage of drought stress in *Sporobolus stapfianus* is well documented (Ghasempour *et al.* 1998; Whittaker *et al.* 2000). Sucrose accumulation is known to increase with the decrease of both glucose and fructose during the final stages of dehydration (Ghasempour *et al.* 1998; Whittaker *et al.* 2000).

Late embryogenesis abundant (LEA) proteins

Apart from the osmoprotectants described above, the production of new proteins such as LEA-like and heat shock proteins, which protect the macromolecular functioning have been shown to occur during the dehydration state (Shonozaki & Shinozaki-Yamaguchi 1997). LEA-like proteins are extremely hydrophylic and have therefore been predicted to play various roles during dehydration; amongst their roles, which include sequestration of ions and binding of water, they may operate as molecular chaperones (Bray 1997). These fold proteins to protect them against the damage occurring during water stress. The production of most proteins is regulated mainly by abscisic acid (ABA) (Ingrams & Bartels 1996; Bray, 1997).

LEA proteins are a subset of abscisic acid (ABA) responsive proteins, expression of which has been observed to occur during embryo maturation in most angiosperms (Galau *et al.* 1986). Characteristically they are small hydrophilic proteins that remain soluble at high temperatures (Baker *et al.* 1986). Six groups of LEA proteins have been identified based on common amino acid sequence domains (Dure *et al.* 1989; Bray 1993) (Table 1.1). At least five groups have been proposed to contribute towards desiccation tolerance in the embryo. Although the actual biological mechanism of action is unclear, roles in ion sequestration and the maintenance of shell hydration during desiccation have been proposed (Dure *et. al* 1989; Xu *et. al* 1996; Whitsitt *et al.* 1997). Although the *lea* mRNAS and LEA proteins are rapidly degraded upon germination, accumulation of these proteins has been reported in several plants in response to water deficit or ABA ((Bewely *et al.* 1983; Russouw *et al.* 1995; Close *et al.* 1989; Cohen *et al.* 1991; Mundy & Chua 1988). It has been proposed that transcription of LEA proteins increases in drought stressed plants (Baker *et al.* 1986; Robertson & Chandler 1994) and that these proteins play a protective role against desiccation-induced cellular damage. Evidence supporting this hypothesis is that expression of the barley group 3 HVA 1 LEA protein in rice resulted in increased tolerance to water stress (Xu *et al.* 1996). Moreover recombinant yeast expressing the wheat group 1 LEA Em protein have recently

been reported to be less succeptible to inhibition of growth media of high osmolarity (Swire-Clark & Marcotte 1999).

Table 1.1 Six groups of LEA proteins, five of which are thought to be regulated by drought stress in vegetative tissues have been identified based on their common acid domains (Bray 1992).

	Gene	Species	Stress Induction	ABA regulated	References
Group 1	D19	cotton	?	?	Baker *et al.*, 1988
	Em	wheat	D	+	Marcotte *et al.*1988
	B19.1	barley	DSL-†	+†	Espelund *et al.*, 1992
	B19.3	barley	DS-L-†	+†	Espelund *et al.*, 1992
	B19.4	barley	DS-L-†	+†	Espelund *et al.*, 1992
	Ha ds10	sunflower	DH‡	+‡	Almoguera & Jordano, 1992
Group 2	D11	cotton	?	?	Baker *et al.*, 1988
	*rab*16A-D	rice	DS	+	Mundy & Chua, 1988 Yamaguchi-Shinozaki *et al.*, 1989
	*dhn*1-4	barley	D‡	+‡	Close *et al.*, 1989
	*rab*17	maize	D	+*	Pla *et al.*, 1989
	pcC27-04	res. plant	D	+	Piatkowski *et al.*, 1990
	pcC6-19	res. plant	D	+	Piatkowski *et al.*, 1990
	TAS14	tomato	DSL-	+	Godoy *et al.*, 1990
	*le*4	tomato	DSL-H-	+*	Cohen *et al.*, 1991
	*cor*47	*Arabidopsis*	L	+	Gilmour *et al.*, 1992
	*rab*15	wheat	D‡	?	King *et al.*, 1992a
	D147b	cotton	D†	+†	Galau & Close, 1992
	D71	cotton	D†	+†	Galau & Close, 1992
	D131	cotton	D†	+†	Galau & Close, 1992
	*rab*25	rice	DL-§	+§	Kusano *et al.*, 1992
Group 3	D7	cotton	?	?	Baker *et al.*, 1988
	HVA1	barley	D‡	+	Hong *et al.*, 1988, 1992
	pLEA 76	rape	D‡	+†	Harada *et al.*, 1989
	pMA20005	wheat	D‡	?	Curry *et al.*, 1991
Group 4	D113	cotton	?	?	Baker *et al.*, 1988
	*le*25	tomato	DSL-H-	+*	Cohen *et al.*1991
	Ha ds11	sunflower	HD‡	+‡	Almoguera & Jordano, 1992
Group 5	D29	cotton	?	?	Baker *et al.*, 1988
	pcC3-06	res. plant	D	+	Iturriaga *et al.*, 1992
Group 6	D34	cotton	?	?	Baker *et al.*, 1988
	*rab*28	maize	D	+*	Pla *et al.*, 1991

* regulated by endogenous ABA.
† studies only completed on embryos.
‡ studies completed on seedlings.
§ studies completed on callus.

LEA proteins have also been reported to be induced by osmotic stress in vegetative tissues (Roberts *et al.* 1993). They are thought to be localized in all embryos during growth. Moreover, tissue-specific and time-dependent expression of *Rab28* has been shown to be restricted to the meristem and vascular elements of the plumule. So far there is less information about the localization of LEA proteins in the vegetative tissues of plants subjected to water stress (Niogret *et al.* 1996), but they are predominantly localized in the cytosol in many cell types in high concentrations (Dure 1993). Apart from the preservation of membrane and protein structure and function, LEA proteins are thought to bind to water (Goday *et al.* 1993). The major problem faced by plants during dehydration is that the loss of water results in crystallization of cellular

components, which consequently damages the cellular structures. LEA proteins counteract this problem by acting as compatible solutes, which supports the likely role of sugars in maintaining the structure of cellular constituents in the absence of water (Bartels & Ingram 1996).

Dehydrins

Dehydrins belong to a distinct family of proteins known as LEA D11 or dehydrin class, which is a subset of LEA proteins. Their expression has been shown to occur in most angiosperms and gymnosperms (Close *et. al* 1993; Dure III 1993; Close 1996). Characteristically dehydrins remain soluble even at high temperatures and have low hydropathy scores, hence they have been proposed to be hydrophilic in nature. Based on their common amino acid sequence domains these proteins have shown no similarity with any enzymes or proteins. They are composed of different amounts of the K segment (lysine-rich repeat), the S segment (Tract of Ser residues), and the Y segment (conserved N-terminal sequence). On the basis of their composition dehydrins have been divided into five subtypes (Close 1996).

Characteristically dehydrins have been shown to have a consensus of 15 amino acid sequence residues EKKGIMDKIKEKLPG (Lys rich) that is always present near the C-terminus. It has been well documented that accumulation of dehydrins in plants coincides with adaptation to dehydration stress (Close 1996). Other features include a run of Ser residues, repeating GTs, a DEYGNP consensus upstream of the N-terminal-most Lys-rich blocks, and Gly-rich tandem repeats on the amino side of the DEYGNP consensus. These are not always present in all dehydrins by the feature of strict conservation of amino acids in the Lys-rich mortif is the key to dehydrin function Also dehydrin accumulation and stress tolerance have been correlated in physiological studies (Close 1992). Dehydrins are thought to be localised mostly in the cytoplasm. The induction of this group of LEA proteins has been shown in independent studies on drought stress, cold acclimation, salinity stress, embryo development and responses to ABA (Close 1996).

Their role is still unclear but recently it has been proposed that being a family of LEA proteins, they do not confer desiccation tolerance but interact with other protectants such as oligosaccharides (Blackman *et. al* 1992). However, not much is known about their physiological regulation. The accumulation of dehydrins is thought to be stimulated by detachment of a grain irrespective of desiccation tolerance.

Lectin-like proteins

Lectins are any group of specific agglutinins and other antibody-like proteins of non-immune origin, defined by the Oxford dictionary as sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and /or precipitate glyco-conjugates (Herve *et al.* 1996). Lectins are also known as cell surface receptors of the protein kinase family which are thought to play a role in signal transduction. Characteristically these putative receptors have been grouped into three major classes based on their extracellular domains (Stein *et al.* 1991; Goring & Rothstein 1992; Herve *et al.* 1996).

They are widely distributed in nature, found mainly in seeds, but also in other parts of certain plants, and in many organisms from bacteria to mammals. Lectins bear at least two sugar-binding sites: they bind specific sugars and thereby precipitate certain polysaccharides, glycoproteins and glycolipids, and/or agglutinate animal and plant cells. They are widely used experimentally, especially concanavalin A as tools in carbohydrate biochemistry for studying cell surfaces and for inducing transformation lymphocytes. Plant lectins are also referred to as phyto-agglutinins.

Among other roles of this protein in plants in connection with intercellular communication is the role of foreign and endogenous oligosaccharides. A putative function in plant intercellular communication is the transduction of foreign and endogenous oligosaccharide signals. The possible role of this new plant receptor class in transduction pathways was discussed in relation to carbohydrate and plant hormone signals (Herve *et al.* 1996, 1999). The manner in which they interact is through the hydrophobic cavity of the lectins where they bind with hydrophobic molecules such as auxins and cytokinins (Edelman *et al.* 1978; Roberts & Goldstein 1983). It is widely accepted that lectins occur in storage organs in special cell organelles called protein bodies (van Driessche 1988). Some plants are known to produce large amounts of exudates on wounding, an example being that of the rubber tree (*Hevea brasiliensis)* in which a hevein lectin occurs (van Parijs *et al.* 1991; Gidrol *et al.* 1994). Another lectin-like protein has been shown to appear in *Dolichos biflorus* on wounding preceding heat shock (Etzler 1996).

Hypothetically, biological functions of plant lectins can be divided into extrinsic roles and intrinsic roles. The extrinsic roles include the protection of plants against herbivory and

predation by microbial and insect invaders through the production of toxic substances. Intrinsic roles of lectins include functioning as storage proteins (Hasselback *et. al*1990; Peumans & van Damme 1994b; Calderon *et. al* 1997).

Lectins, also interact with membranes. It has been shown that the thylakoid membranes from spinach (*Spinacia oleracea* Chenopodiaceae) chloroplasts are stabilised against freezing damage when bound to galactose-specific lectins from members of the Leguminosae, Moraceae or Euphorbiaceae (Peumans & van Damme 1994c). They also function as cryoprotectants as observed in lectin content in mistletoe which undergoes seasonal changes and those that occur in hybernating plant organs. The correlation between lectin content/specificity and cold adaptation has been shown from experiments with wheat plants (Komarova *et. al* 1997). The biological function for these proteins has been debated for decades and has opened an important area to investigate adaptive and developmental processes in plants (Herve 1996; Rudiger 1998).

Osmotic adjustment

During mild drought, total water potential in plants can be maintained by osmotic adjustment which involves the utilisation of sugars and other compatible solutes (Bohnert *et al.* 1995) (Fig. 1.4). Changes in intracellular water potential can occur as a result of accumulation of osmolytes in the cytoplasm. This results in a lower water potential inside the cell than outside the cell with the result that the cell shrinks. Both ion and water channels are thought to be important in regulating water flux. Evidence of this has been the isolation of channel proteins which are expressed during periods of water deficit. Channel proteins that accumulate in the tonoplast during stress result in increased movement of water and solutes from the vacuole to the cytoplasm. This alters either the water content or the osmotic potential of the cytoplasm. Besides these channel proteins, osmotin and other non-specific proteins such as proteases, ubiquitin and chaperones, protect the plant cells against pathogen infection. The expression of osmotin is induced in the cell by salt stress, while the induction of non-specific proteins in the cells occur as a response to water deficit (Bray 1997).

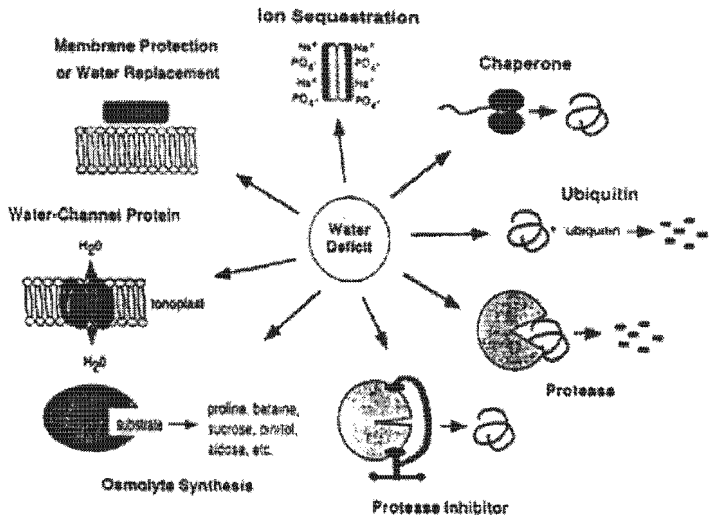


Figure 1. 4. Osmotic adjustment to protect cellular processes and structures (Bray 1993).

1.3.2 The role of abscisic acid (ABA)

ABA is a naturally occurring phytohormone that was discovered in the 1960s affecting leaf abscission and bud dormancy (Addicitt & Carns 1983). Water deficit in plants occurs when the rate of transpiration exceeds the rate of water uptake or when water itself becomes the limiting factor in the environment. Water deficit occurs not only during drought, but also during cold conditions (Shinozaki & Yamaguchi-Shinozaki 1996). Morphological, physiological and metabolic changes within all plant organs allow the whole plant to respond and to avoid water stress. To date, this hormone has been suggested to be involved in this type of regulation. Its accumulation within plant tissues increases as a result of water stress, and is involved in plant tolerance to drought and high salinity and other cold stresses (Henson 1984; Mohapatra 1988).

Under drought conditions leaf ABA concentrations can increase up to 40 times and is very effective in stomatal closure (Raschke 1987). Its accumulation in stressed levels plays an important role in the reduction of water loss by transpiration under water stressed conditions. It enhances water uptake by plants. Its application to root tissue stimulates water flow and ion flux, suggesting that it regulates turgor pressure not only by decreasing transpiration but also by increasing water influx into roots (Glinka & Reinhold 1971). The antagonistic effects of ABA on roots and leaves cause a reduction in leaf area and an increase in the water absorbing area of roots, which helps the plant cope with drought conditions.

1.4 Cellular events leading to water induced gene expression

The molecular mechanisms by which plants detect water deficit, transduce the signal to intracellular machinery and regulate adaptive responses is not well understood and needs further investigation (Conley *et al.* 1997). These processes have been studied at the cellular level despite the fact that plants are multicellular, and it is crucial for plants to integrate and regulate all cellular responses in order to survive water deficit.

1.4.1 Cellular perception of the genes

The first step in the regulation of water response is the recognition of the stress. Loss of water from the cells is perceived, triggering a cellular signal transduction pathway, converting a physical response into a biochemical response. There are already clues to the recognition mechanism in bacteria and in yeast, where mutants with altered cellular perception of osmotic stress have been isolated and genes responsible described (Bray 1997). Even in these model systems the component of cellular stress that triggers the signal transduction pathway is not understood. It has been predicted that there are multiple receptors involved in the mechanism whereby yeast cells sense osmotic stress. Not much is known of how a cell perceives water stress, but possible sources of information such as decrease in turgor pressure, change in cell volume, loss of membrane flexibility, change in solute concentrations may signal water deficit to the cell (Bray 1997). It has been suggested that a change in osmotic pressure is a major trigger of the water stress response at the molecular level (Yamaguchi &Yamaguchi-Shinozaki 1997).

1.4.2 Signal transduction events

Once the stress is perceived by the cell, a signal is transduced to induce the expression of specific genes. Not all stress-induced genes are induced under the same conditions or in the same cell types, implying that there are different signalling mechanisms (Bray 1997). Most of the drought inducible genes that have been studied respond to high salinity. Likewise not all the genes responding to cold stress also respond to high salinity indicating that various pathways are involved in the stimulation of drought inducible genes. Hence it is not clear whether perception of a signal directly induces gene expression or whether it leads to the accumulation of other molecules to induce gene expression indirectly (Shinozaki & Shinozaki-Yamaguchi 1997).

Endogenous ABA levels are thought to increase in plant tissues in response to water stress (Henson 1984; Mohapatra *et al.* 1988). This plant hormone has been proposed to be an essential mediator between these environmental stimuli and plant responses in general through alterations in gene expression. Molecular studies done to date have focused on identifying genes that are regulated positively or negatively by ABA and/or water stress. Most of the genes that respond to drought, salt and cold stresses require endogenous ABA, and some of genes respond to exogenous application of ABA. This evidence suggests the existence of both ABA-dependent and ABA-independent pathways involved in drought and cold conditions (Shinozaki & Yamaguchi-Shinozaki 1997).

1.4.3 DNA elements controlling gene expression during water deficit

Reports on the mechanism of regulation of gene expression in response to water deficit come from the investigation of DNA elements and sequence-specific DNA binding proteins. So far, two classes of DNA elements have been identified, namely, the ABA-response element (ABRE); and the drought-responsive element (DRE). The ABRE has been shown to be sufficient for ABA-regulated gene expression, but in some genes it must be associated with a coupling element (Bray 1997).

ABA response element

The consensus for the ABRE is RYACGTGGYR, where R denotes a nucleotide with a purine base and Y is a nucleotide with a pyrimidine base. Their core element ACGT was first reported in the Em gene from wheat and is bound by the bZIP protein EmBP-1 (Bray 1997; Shinozaki & Yamaguchi-Shinozaki 1997). This core has been reported to be responsible for the regulation of gene expression in maize, barley, rice, tobacco and *Arabidopsis*, and also in other DNA elements containing the G box which is believed to regulate expression of genes by light, auxin, jasmonic acid and salicylic acid. Although the ABRE is sufficient for most ABA-regulated genes, in some it requires an association with a coupling element (CE 1). For example CE 3 (ACGCGTGTCCTC) is an ABA-induced gene element of barley group 3 HVA-1 LEA (Shen *et al.* 1996).

Drought response element

DRE has been shown to be contained in some drought inducible genes. The DNA sequence,

TACCGACAT, of the DRE from rd29A gene from *Arabidopsis* which regulates the expression of this gene by ABA-independent pathway. The gene rd29A is differentially induced under conditions of dehydration, low temperature and high salt (Thomashow 1994; Shinozaki & Shinozaki-Yamaguchi 1997). Based on this it can be seen that some of the genes that are induced during dehydration, cold and high salinity, do not require ABA for their expression under cold or drought conditions but do respond to ABA. The DRE in this instance has been shown to act as a cis-acting element responsible for the induction of rd29A gene expression in response to low temperature stress or cold.

So far, a number of genes that respond to both drought and cold stresses have been shown to activate DRE-dependent transcription of rd29A although the signals are transmitted separately. As a result of this, transacting factors regulating DRE-dependent gene expression have been identified (Fig. 1.5). Two binding proteins DREB1A and DREB 2A, have been shown to interact with the DRE sequence in the promoter region of rd29A during dehydration and cold conditions. Based on their deduced amino acid sequences, no significant sequence identity has been shown except in the conserved DNA binding domain. This idea has lead to the conclusion that they function as transcriptional activators in plants in two signal transduction pathways (ABA-dependent and ABA-independent pathways) in response to cold, drought and high salinity stresses (Shinozaki & Shinozaki-Yamaguchi 1994; Liu *et al.* 1998).

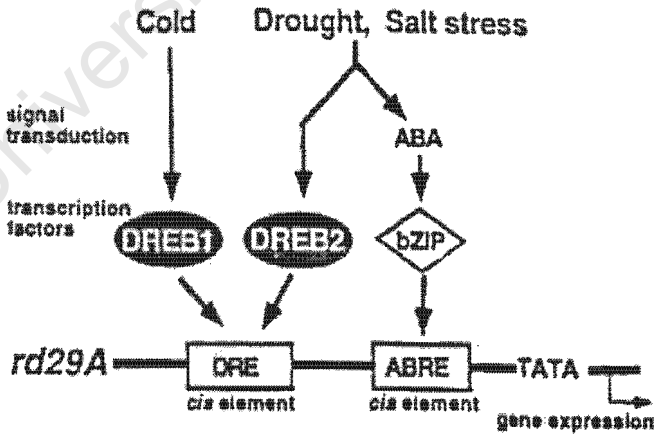


Figure 1.5 A model for the induction of rd29A gene expression under dehydration, high salinity and low temperature conditions (Liu *et al.* 1998)

1.4.4 ABA-dependent pathway

Endogenous ABA levels increase under drought and salt stresses in many plants. The ABA

dependent pathway includes pathways I and II (Fig.1.6). Pathway I synthesises protein factors for the expression of water stress-inducible genes such as *rd22* from *Arabidopsis* (Fig.1.6). The *rd22* promoter contains two conserved motifs of DNA-binding proteins, namely MYC and MYB, but does not have the ABREs. Many bZIP transcription factors in rice, maize and *Arabidopsis* plants respond to cold, dehydration and exogenous ABA treatment (Shinozaki & Yamaguchi-Shinozaki 1997). The bZIP proteins bind to G box-like sequences and this indicate that they are also involved in one of the ABA-dependent pathways (Fig. 1.6)

Pathway Π, which is an ABA dependent pathway, does not require protein biosynthesis in order to be expressed. These dehydration inducible genes contain ABREs (PyACGTGGC) in their promoter regions. The ABRE in turn has a basic region adjacent to the Leu-zipper motif called bZIP specificity binding. The issue of how ABA activates the bZIP proteins to bind to ABRE and initiate transcription of ABA-inducible genes needs to be studied as not much is known (Shinozaki & Yamaguchi-Shinozaki 1997).

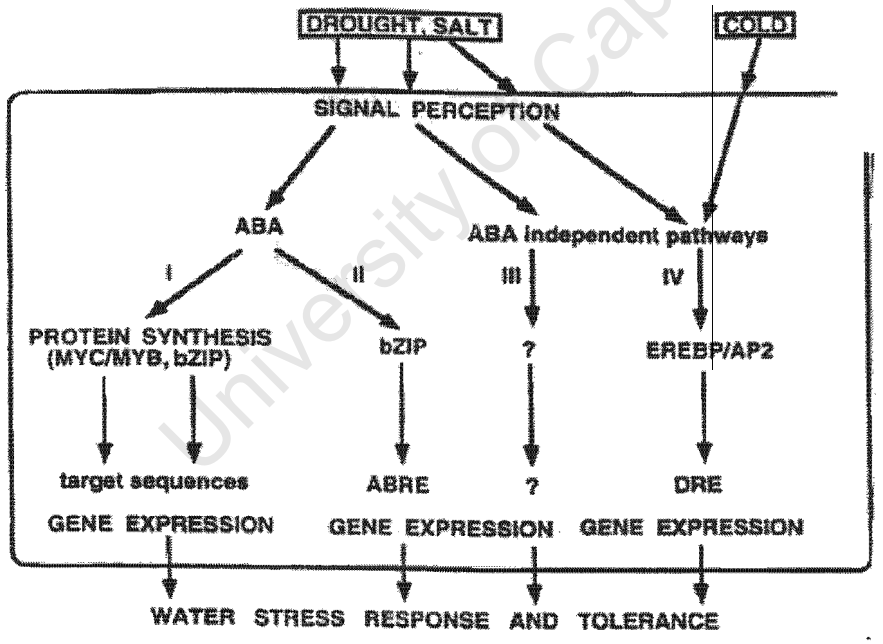


Figure 1.6 Signal transduction pathways between perception of stress and gene expression (Shinozaki & Yamaguchi-Shinozaki 1997).

1.4.5 ABA-independent pathway

Most of the genes that respond to drought, cold or salt stresses do so in ABA-deficient mutants do

not require ABA to be expressed under these stresses. However they do respond to exogenous ABA (Shinozaki & Yamaguchi-Shinozaki 1997). These genes are found in pathways III and IV. The genes in pathway IV contain DRE-related motifs which are found in most cold- and drought-inducible genes but DRE does not function as an ABRE. This has led to the idea that DRE-related mortifs included a C-repeat containing a core mortif CCGAC. DRE/C-repeating-binding proteins contain a conserved DNA-binding mortifs, EREBP and AP2, involved in ethylene-responsive genes and floral morphogenesis respectively.

There are several drought inducible genes that do respond to drought, cold, high salinity and/or ABA treatment, suggesting that a fourth pathway in the dehydration stress response, i.e. pathway IV, is present. Genes involved in this pathway have been found to contain DREs. Although it has been shown that some of the genes in this pathway do respond to exogenous ABA. The induction of gene expression and integration of the above pathways result in a water stress response and subsequent tolerance (Shinozaki & Shinozaki-Yamaguchi 1997). This is an example of how the whole plant integrates cellular responses; the plant produces ABA which then regulates gene expression in individual cells to aid protection against water deficit. But the ABA independent pathways do not require synthesis of proteins for the expression of genes, the mechanism so far is still unclear.

Pathway III includes drought inducible genes that do not respond to cold or ABA. Not much is known about this pathway. It has been hypothesised that the promoter region may reveal more about the regulation of the pathway.

1.4.6 Perception and signal transduction of ABA

Signal transduction pathways must be multilayered . Just as there are recognition mechanisms for environmental stimuli, there must also be a mechanism for the recognition of ABA in the cell (Fig. 1.7). Currently, the location, number and type of receptors for ABA is not known, although there is evidence that ABA can be recognised both inside and outside the cell (Allan *et al.* 1994; Schwartz *et al.* 1994). The ABA signal transduction pathway probably comprises a protein kinase/phosphatase cascade interacting with Ca^{2+}. However, not all of the components have been identified, and a clear picture of the interactions has not been established (Bray 1997).

Current models suggest that osmotic stress is first perceived by cells as plasmalemma perturbations. This is caused by loss of turgor pressure, followed by an increase in cystolic and apoplastic ABA due to *de novo* synthesis and/or release of the sequestered ABA in organelles (Zeevaart & Creelman 1988). Current evidence links changes in ABA levels and the expression of *rab* genes with increased osmotic stress tolerance. For example, glycophyte plants and cells respond to high osmoticum by changes in the composition of cell wall polysaccharides and proteins (Iraki *et al.*1989; Singh *et al.* 1987) and osmoprotectants such as proline (Rhodes *et al.* 1986). However, it is unclear what role ABA plays in regulating the levels of osmoprotectant compounds such as proline (Steward & Voetberg 1987).

Identification of potential members of the signal transduction pathways by DNA sequence homology

Many components of signal transduction pathways operational in other organisms have also been identified in plants, and many of these genes are induced during the periods of water deficit: phospholipase C; calcium-dependent protein kinase and a MAP kinase (MAPK) cascade; MAP kinase kinase kinase (MAPKKK) and ribosomal S6 kinase are upstream and downstream components cascade respectively of MAPK (Mizoguchi *et al.*1996). The expression patterns of these genes indicate that the plants have similar signalling pathways to animals in response to environmental stimuli, although the exact pathways have yet to be determined (Bray 1997).

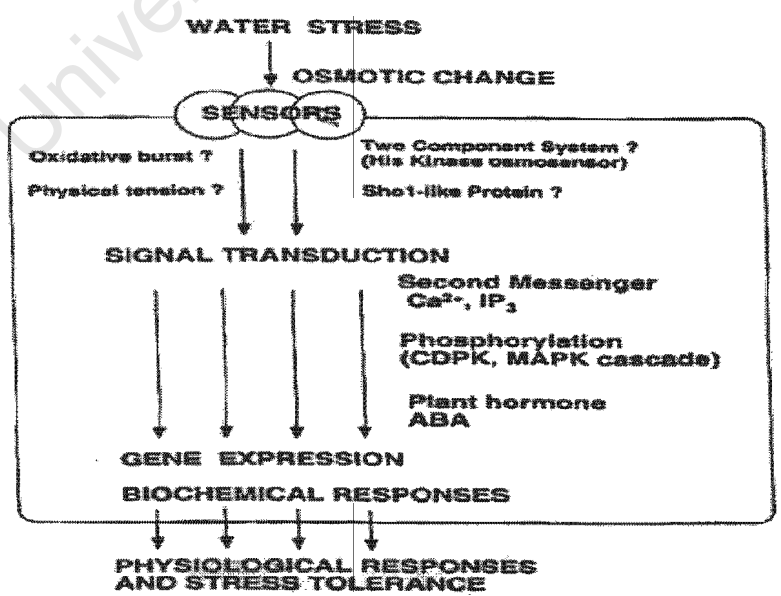


Figure 1.7 Second messengers and factors involved in the signal perception and transduction in water stress response. (Shinozaki & Shinozaki-Yamaguchi 1997).

Functions of ABA- and osmotic stress-responsive genes

Molecular studies have characterised the rapid induction of *rab* (responsive to abscisic acid) genes by ABA. These novel genes have been isolated by differential screening of cDNA libraries synthesised from mRNAs of hormone-treated tissues. Therefore, they probably encode abundant mRNAs and proteins. They are also expressed during the desiccation phase of embryogenesis and may, therefore be referred to as the *lea* genes (Mundy 1989). Other conserved, positively charged domains found in RAB proteins initially suggested that they may bind to nucleic acids (Mundy & Chua 1988). Other studies have shown that these proteins in fact bind single-stranded DNA, poly rU, and poly rG, and remain bound to the latter at high salt concentrations. Some data indicate that RAB and LEA proteins are ubiquitous in plants, and it is possible that homologous proteins are expressed during osmotic stress in other organisms (Shrer & Potts 1989).

Signal transduction mechanisms connecting osmosensing with changes in gene expression have been shown, and indicate that ion channels and active transport are involved in osmoregulation and signalling in plant cells. Stress osmosensors have been connected to gene activation via protein phosphorylation in a pathway involving Ca^{2+} and phosphoinositol second messengers conditions (Shinozaki &Shinozaki-Yamaguchi 1997). These messengers do not require gene activation but may induce physiological changes in specific cells. For example, increased cytosolic Ca^{2+} in guard cells by the ABA-dependent pathway stimulate closure of stomata through cation and anion effluxes (Skriver & Mundy1990). Hence inositol 3-phosphate and Ca^{2+} are suggested to act as secondary messengers in the signal transduction pathways under drought and cold conditions (Shinozaki &Shinozaki-Yamaguchi 1997).

1.5 Water stress-inducible genes

Many species have genes which respond to water stress. Such genes do not only protect cells through expression of metabolic proteins, but also regulate genes involved in transduction pathway during water deficit (Shinozaki & Yamaguchi-Shinozaki 1997) (Fig.1.8). Hence these genes are grouped into two; those that encode proteins that act during stress tolerance which includes water channel proteins to alter cellular water potential, enzymes to synthesise various osmoprotectants and protective proteins such as *lea* proteins, proteinases detoxifying enzymes and chaperones. The second group of genes encode products that act as regulatory proteins during stress response. These

include transcription factors (MYB, MYC, bZIP) and protein kinases (MAPK, MAPKKK, S6R, CDPK, SNF) (Shinozaki & Shinozaki-Yamaguchi 1997).

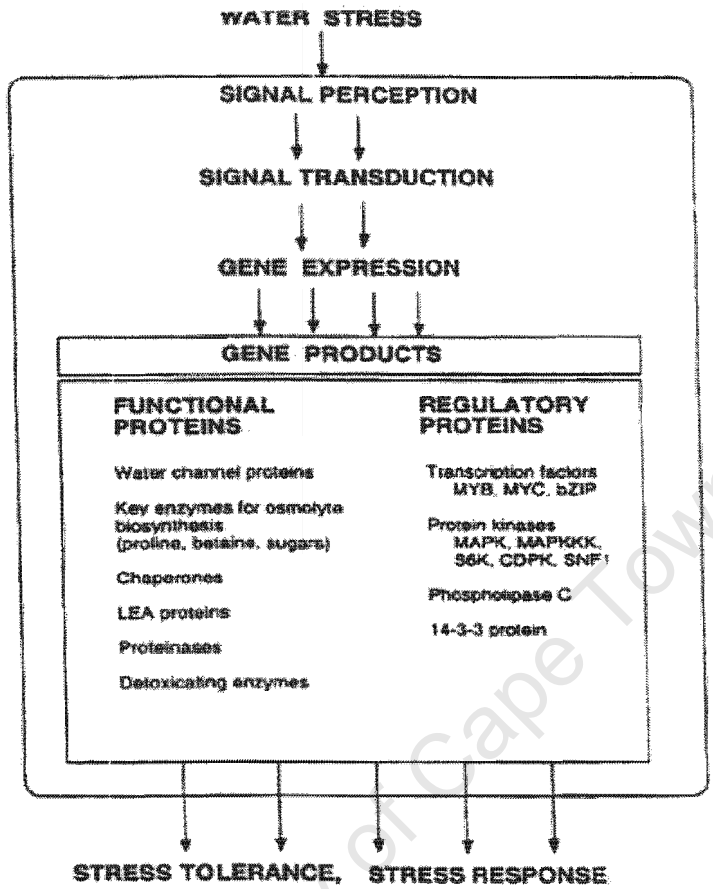


Figure 1. 8 Function of water-stress-inducible gene products in stress tolerance and stress response. (Shinozaki & Shinozaki-Yamaguchi 1997).

1.6 Concluding remarks and objectives of the study

A number of genes that are induced in plants subjected to water stress have been identified. Using a number of molecular biological strategies, new insights have been provided to study plant responses to the environment with respect to the functioning of these changes, and the regulation of these responses among different stresses within a species. To investigate the basis of extremes of water deficit tolerance, a resurrection plant *Xerophyta viscosa* was used to characterise genes that were induced during water deficit. As already mentioned, proteins act as protectants which may stabilise macromolecular structures during dehydration and/or repair processes.

In this study, a cDNA library was constructed from dehydrated *X. viscosa* leaves [85 %, 37 % and 5 5 relative water content (RWC)] was used (Ndima *et al.* 2000). The main objective was to differentially screen the library and to identify cDNAs that are differentially expressed and characterise them. Using this strategy it is possible to isolate clones with unknown functions and identities. Randomly selected clones were sequenced and analysed using the BLAST network service, to reveal identities and homologies with known proteins. Expression patterns of the clones were determined when *X. viscosa* was exposed to dehydration-rehydration treatment, cold, heat shock, high salinity stress, ABA, methyl jasmonate and ethylene treatments. Finally, the immunogold and localisation studies of the protein were performed.

CHAPTER 2

2.0 CONSTRUCTION AND DIFFERENTIAL SCREENING OF A cDNA LIBRARY FROM *Xerophyta viscosa* Baker

2.1 Introduction

A number of molecular techniques have been employed extensively in the past decade for the cloning of specific genes. Standard techniques for screening libraries with heterologous DNA probes and expression libraries with specific antibodies have been successful in isolating many genes. This led to the identification of many genes that were over- and under-expressed in plants under stressed conditions. Using differential screening cDNA libraries have been screened successfully and stress-induced plant genes were successfully isolated (Roncarati *et al.* 1995; Furini *et al.* 1996; Ingram & Bartels 1996; Hajela *et al.* 1997; Mariaux *et al.* 1998). A number of genes associated with salinity tolerance (Redkar 1995) have been identified using the subtractive hybridization strategy. Genes of low abundance mRNAs have been identified successfully using a PCR-

based differential display method (Oh *et al.* 1995; Song *et al.* 1995). O'Mahony & Oliver (1999) employed differential display to detect altered mRNA levels in response to desiccation and rehydration in the leaves of the desiccation-tolerant grass *Sporobolus stapfianus*. Other PCR-based methods including reverse transcription PCR (RT-PCR) (Chang *et al.* 1993) and Inverse PCR (Wang *et al.* 1991) have also been successfully used to clone genes. The complementation of *E. coli* mutants using expression libraries has been used to clone eukaryotic genes associated with specific functions (Hu *et al.*1992; Ilag *et al.*1994; Ravanel *et al.*1996).

Subtractive hybridisation

An effective molecular cloning technique called subtractive hybridisation has been developed for identifying and isolating cDNAs of differentially expressed genes. It is based primarily on a suppression PCR technique and it combines normalisation and subtraction steps on a single procedure. The former step deals with the exclusion of the common sequences between 'tracer and driver' populations. Numerous cDNA subtraction methods have been reported which involve hybridisation of cDNA from one population (driver) to excess mRNA (cDNA) from another population (tracer), and then separation of the un-hybridised fraction (tracer) from hybridised common sequences (Diatchenko *et al.* 1996; Sagerstrom *et al.* 1997). The subtractive method results in the production of a library of specific DNA sequence tags present in only one strain (tracer) but absent from the other (driver) (Schmdt *et al.* 1998).

Differential display

Another powerful technique that is widely used is differential display. It uses mRNA in eukaryotes and also combines PCR (Zhang *et al.* 1996; Fislage 1998). It is the most useful tool to identify genes with altered transcription rates in response to exogenous or endogenous stimuli. The mRNA differential display RT-PCR method provides a sensitive and flexible approach to identify novel individual differentially transcribed genes in

different sets of eukaryotic cells. Primed RT-PCR techniques concentrate on screening the bacterial transcripts that respond to external stimuli. Using arrayed cDNA fragments, differential display can identify transcriptionally regulated gene in respect to its position in the cDNA. This technique can be used on its own or in combination with other techniques.

Complementation by functional sufficiency

Complementation by functional sufficiency is a novel strategy to isolate plant cDNA clones which can independently confer tolerance to high NaCl and other abiotic stresses upon *E. coli* cells (Mundree & Farrant 2000). It was used to isolate nine genes from the resurrection plant *Xerophyta viscosa* Baker which conferred tolerance to sorbitol stress.

Differential screening (DS)

Differential screening (DS) is the most frequently used technique. It is also the most powerful technique for detecting biological differences between two characteristics, e.g. stress-responsive and stress-unresponsive genes at the molecular level (Kuang 1998). With this technique duplicate replicas of the cDNA libraries are screened by hybridization to the source from which the library was constructed, such as cDNA synthesized from RNA isolated from stressed organs. cDNAs that hybridize to the source of the library but not to the non-stressed comparison are candidates for stress-responsive genes. Two approaches can be used to isolate novel genes effectively viz. a random or differential approach. In the former cDNA clones are isolated at random from the cDNA library. With the differential approach only cDNA clones with rare sequences are analyzed further (Hoog 1991). DS is capable of isolating differentially expressed genes of high abundance. However, genes which are differentially expressed at low levels cannot be isolated with this technique resulting in the necessity for employment of other techniques which can be used synergistically with DS to identify these genes (Maser 1995;

Diatchenko *et al.* 1996). The possible function of the products of genes isolated by DS are unknown (Bray 1992) and further characterization is required.

For the purpose of this study DS was used to screen a cDNA library that was constructed by Mundree *et al.* (unpublished). This strategy was used to screen 192 randomly selected clones, and identify genes that are differentially expressed during dehydration stress.

2.2 Materials and methods

2.2.1 Plant material

X. viscosa Baker plants were collected from Buffelskloof Nature Reserve (Mpumalanga Province, South Africa) and were grown in a mixture of river sand, peat and potting soilin the Greenhouse under 30% shadecloth (Sherwin & Farrant 1996; Walford 1998). Plant drying and determination of water content (on a dry mass basis) and RWC was measured and calculated suing the standard formula: RWC = water content/ water content at full turgor expressed as a percentage (Sherwin and Farrant 1996; Farrant 2000).

2.2.2 Dehydration stress

X. viscosa was dried down to 85%, 37% and 5% RWCs by withholding water. The relative RWC of the plant were measured and the samples were harvested at the correct percentages. Four large leaves were harvested, flash frozen in liquid nitrogen and stored at -70^{0}C. After the treatment the plant was re-watered and allowed to recover and maintained under the usual glasshouse conditions. The percentages of RWC were selected based on the dehydration curve against the relative times or durations for the leaves to be harvested (Fig. 2.1).

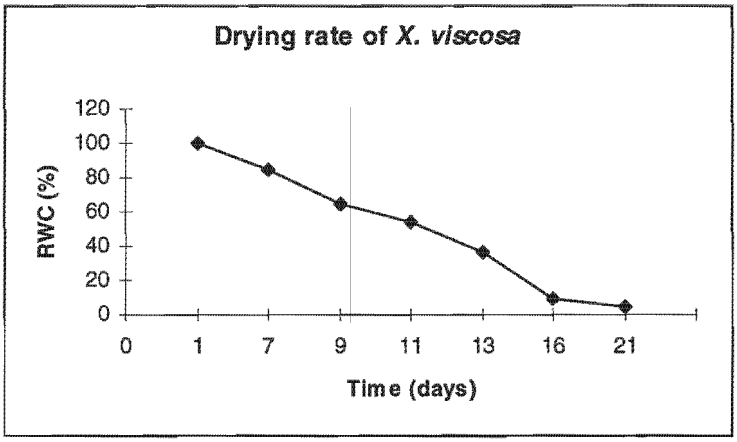


Figure 2.1 *X. viscosa* leaves at various relative water contents plotted against the number of days during the dehydration process.

RNA isolation and extraction

Total RNA was extracted from leaf explants of *X. viscosa* according to the protocol of Chomczynski (1987) (Appendix B.4). Three to four leaves were frozen in liquid nitrogen and ground with a mortar and pestle. Aliqouts (1 g) were transferred to microcentrifuge tubes and tissue samples were homogenized in 1 ml Trizol Reagent (Life Technologies, GIBCOBRL). The mixture was vortexed for 1 min, and stored at room temperature (RT) for 5 min to permit complete dissociation of the nucleoprotein complex. Chloroform (200 µl) was added and the contents mixed gently by inverting the tube for 15 seconds. The resulting mixture was stored at RT for 5 min, following which it was centrifuged at 12000 x g for 15 min at 4 ^{0}C. The aqueous phase was then transferred to a new micro-centrifuge tube and 500 ml of isopropanol was added to precipitate the RNA. Samples were incubated at RT for 15 min followed by centrifugation at 12000 x g for 8-10 min at 4 ^{0}C. The supernatant was discarded, the resulting pellet was washed with 75% ethanol and the pellet air dried. The pellet was resuspended in 50 µl of DEPC (diethyl pyrocarbonate, Appendix A.3.3.2) treated water. The samples were quantitated spectrophotometrically (Sambrook *et al.* 1989) and stored at –70 ^{0}C.

Preparation of probes

Total RNA (5 μg) isolated from hydrated (100% RWC) and dehydrated (37% RWC) *X. viscosa* leaves was reverse transcribed, incorporating [^{32} P] dCTP (Amersham, England), and used to probe the membranes. First strand cDNAs from hydrated and dehydrated samples were radiolabelled. Following this, slot blots were hybridized with radiolabelled cDNAs from the two treatments. Differentially expressed genes were identified following autoradiography.

2.3 Differential screening of the cDNA clones

A cDNA library constructed from dehydrated *X. viscosa* (85%-5% RWC) was differentially screened. Two hundred pBluescript phagemids were randomly selected and restriction analysis carried out to verify existence of cDNA inserts. Plasmid preparations from 192 cDNAs were quantitated spectrophotometrically and 1 μg was slot blotted in duplicate onto nylon membranes (MSI, 0.45 μ) in a slot apparatus (Hoefer Scientific, San Fransisco). mRNA from hydrated and dehydrated (37% RWC) plants were isolated and the first strand cDNAs were radiolabelled. Slot blots were hybridized with radiolabelled cDNAs from two treatments. Filters were pre-hybridized at 68^{0}C for 4 hours and hybridized for 18 hours at 68 ^{0}C in 6x SSC (1x SSC in 150 mM NaCl, 17 mM sodium citrate), 5x Denhardt's solution, 0.1% SDS, and 100 μg/ml herring sperm DNA. Filters were washed at room temperature in 2x SSC, 0.1% SDS for 3x 5 min, and finally at 68 ^{0}C in 0.1x SSC, 0.1% SDS for 2x 1 hour. The membranes were exposed to X-ray film at –70 ^{0}C for appropriate durations. Differentially expressed genes were identified following auto-radiography.

2.4 Results

A restriction digestion (*EcoR*1 and *Xho*1) was carried out on each plasmid to verify the presence of cDNA insert (Figure 2.2). The cDNA library represented genes that were expressed in *X. viscosa* leaves during dehydration (85%, 37% and 5% RWC) (Fig. 2.1). DS was used to screen 192 randomly selected cDNA clones from the *X. viscosa* cDNA library (Figs. 2.3, 2.4 and 2.5).

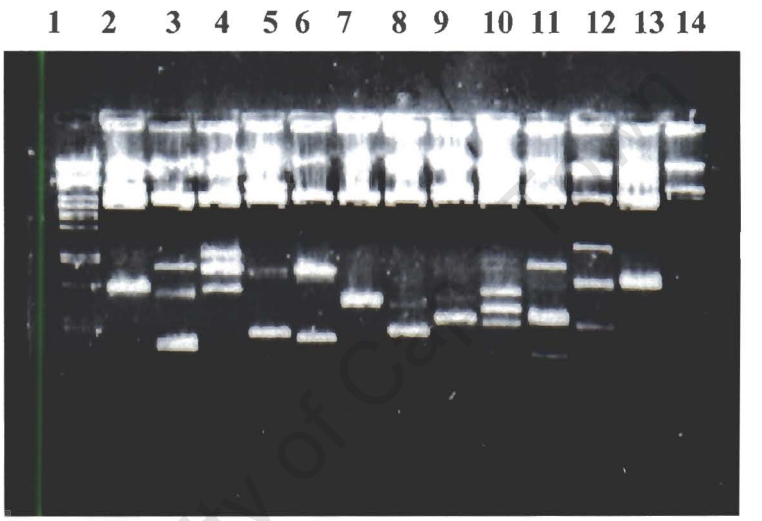


Figure 2.2 EtBr stained 1% agarose gel electrophoresis of restriction digestion of the cDNA inserts of the randomly selected clones.

Total RNA isolated from hydrated (100% RWC) and dehydrated (37% RWC) *X. viscosa* leaves were reverse transcribed and each used to probe one replicate of the clones, respectively. Of the 192 cDNAs screened, 30 showed higher expression levels when the plant was dehydrated (Fig. 2.3 B, 2.4 B and 2.5 B), while 20 exhibited higher levels when the plant was hydrated. Three of the former group (clones marked in Fig. 2.3: 8A, 1B and 3B) were chosen for further study.

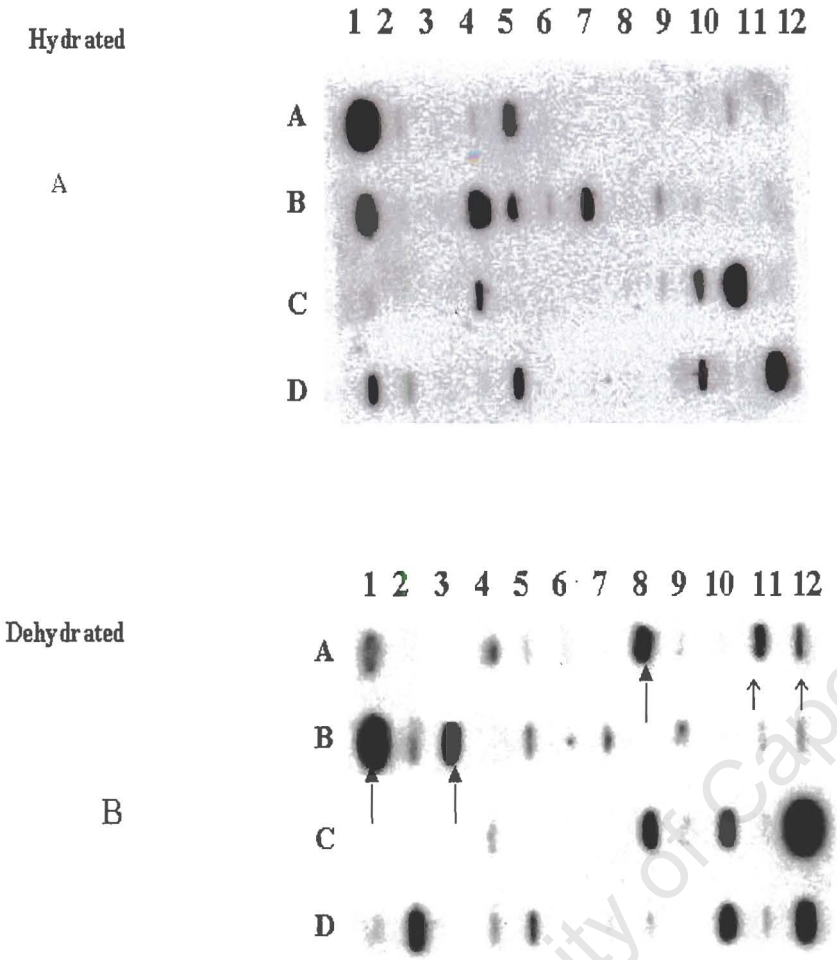


Figure 2.3 Slot blot showing differential screening of 192 cDNAs probed with ^{32}P-labeled cDNA isolated from A) hydrated and B) dehydrated *X. viscosa* leaves. Arrows indicate the cDNAs that were up-regulated during dehydration. Thick-headed arrows indicate the cDNAs chosen for the study, while the thin-headed arrows indicate the rest of the 30 cDNAs. Numbers and alphabetical letters in a slot blot apparatus represents the location of cDNAS from 1A to 12D.

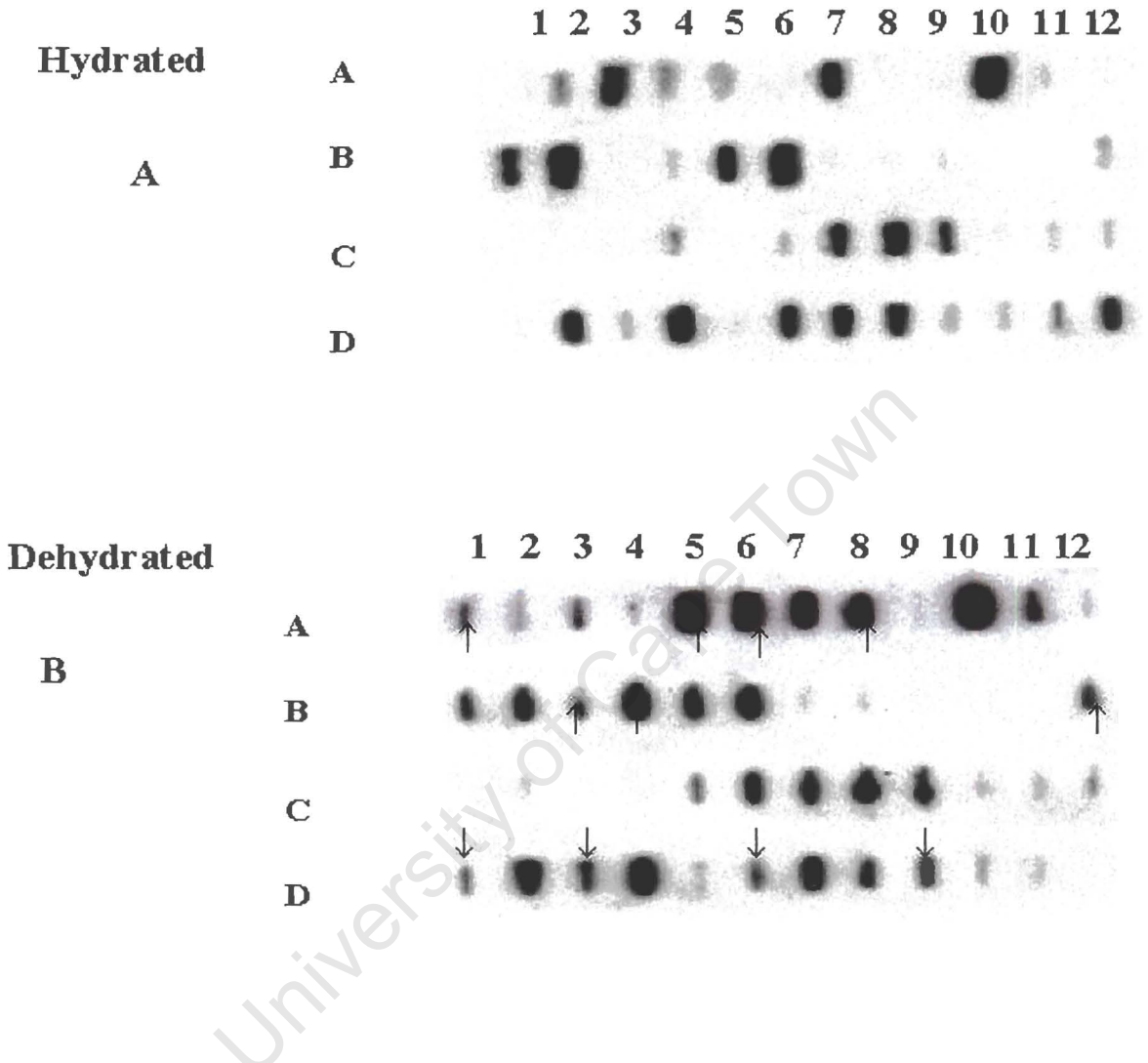


Figure 2.4 Slot blot showing differential screening of 192 cDNAs (second duplicate) probed with ^{32}P-labelled cDNA isolated from A) hydrated and B) dehydrated *X. viscosa* leaves. Arrows indicate the differentially expressed cDNAs. Numbers and alphabetical letters in a slot blot apparatus represents the location of cDNAS from 1A to 12D.

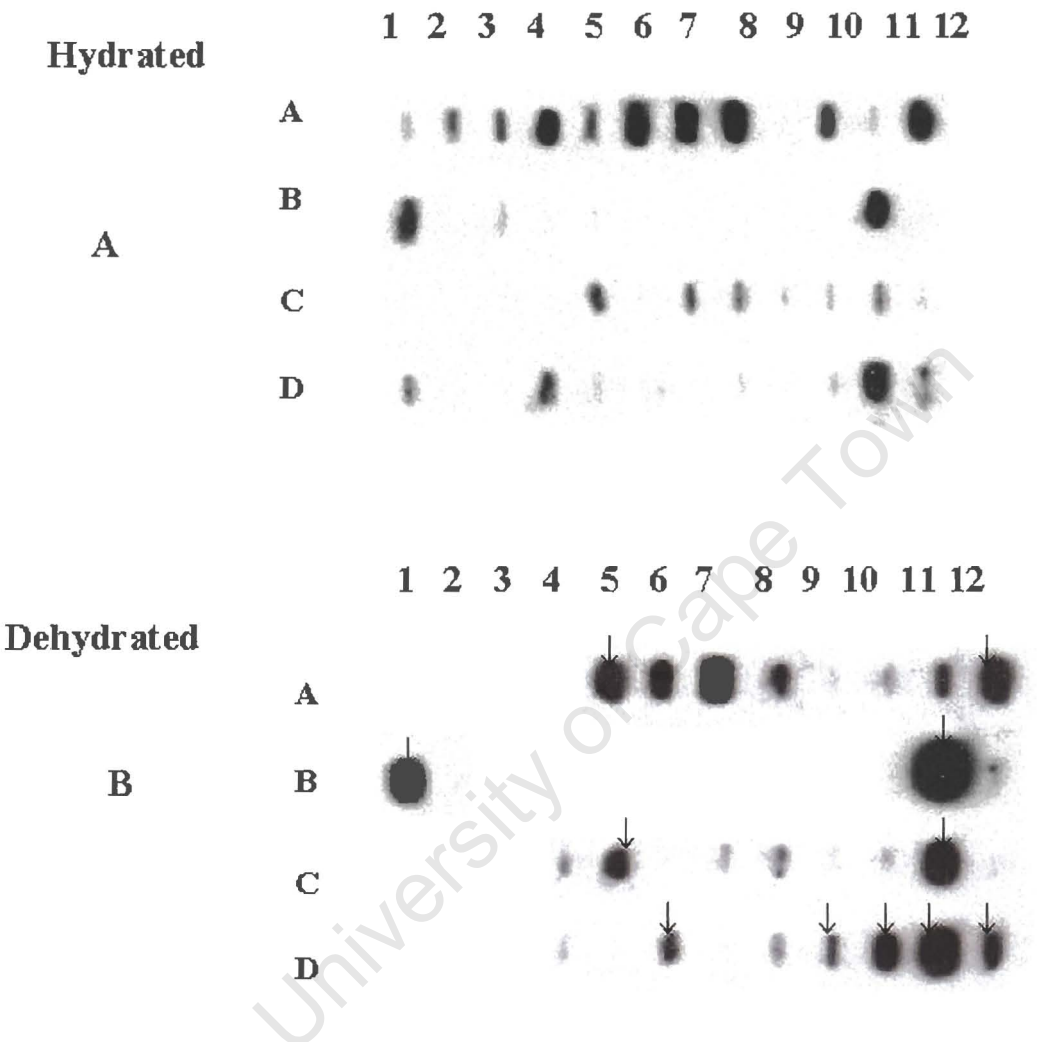


Figure 2.5 Third duplicate of slot blot showing differential screening of 192 cDNAs probed with ^{32}P-labelled cDNA isolated from A) hydrated and B) dehydrated *X. viscosa*. Arrows indicate the differentially expressed cDNAs. Numbers and alphabetical letters in a slot blot apparatus represents the location of cDNAS from 1A to 12D.

2.5 Discussion

The strategy of differential screening was used for the isolation of cDNA clones from *X. viscosa*. The cDNA library represented genes that were expressed in the leaves of this plant during dehydration to 85%, 37% and 5% RWC. The λ ZAPIII vector allowed for the directional cloning of cDNAs and the efficient rescue of phagemids from this vector (Short *et al.* 1988). DS was used to screen 192 randomly selected cDNA clones from the *X. viscosa* cDNA library. Total RNA isolated from hydrated (100% RWC) and dehydrated (37% RWC) *X. viscosa* leaves were reverse transcribed and used to probe one replicate of the 192 cDNAs, respectively. Of the 192, 30 were up-regulated during dehydration stress and 20 that were down-regulated. 8A, 1B and 3B (Fig. 2.3) were the representatives of the 30 cDNAs, for further characterisation in this study.

However, this is not a directed method and the resulting cDNAs do not represent genes with known functions. (Cohen & Bray 1990). Other cloning strategies *viz* differential display, subtractive hybridization, differential display and complementation by functional sufficiency are more direct and can be used to isolate a desired cDNA when more information is known about the gene product to be studied. The simplicity of DS is advantageous, however, it is limited by its ability to isolate only those genes that are abundantly expressed. In addition, its inability to equalize differing levels of mRNA contributes to repeated isolation of abundant genes (Kuang *et al.* 1998). Based on the results, it could be seen that changes in gene expression during dehydration have been observed mostly in desiccation tolerant plants studied so far through induction of specific genes and repression of others (Bray 1992). Their mRNAs have been shown to accumulate increasingly during stress and decrease once the stress subsides.

CHAPTER 3

3.0 SEQUENCING AND SOUTHERN BLOT ANALYSIS OF *XVLEA*, *XVDH* & *XVLEC*

3.1 Introduction

Vegetative tissues from resurrection plants survive dehydration (Oliver *et al.* 1998). It has been suggested from previous studies that plants tend to employ multiple signal transduction pathways to sense changes in water status thereby activating specific changes in gene expression (Bostock & Quatrano 1992; Buttler & Cumming 1993; Espelund *et al.* 1995). These genes are induced positively or negatively by ABA depending on the developmental stage of the plant as well as on the species concerned. As a result new proteins are synthesised which allow the plant to tolerate and respond to stressful situations. To determine the proteins encoded by the differentially expressed cDNAs, their sequences were determined, translated and compared for homology with other sequences available in the databases. Furthermore, the copy number of the genes

was determined using Southern blot analysis and also used to confirm that the genes corresponding to the three cDNAs were indeed present in the *X. viscosa* genome.

3.2 Materials and methods

3.2.1 Sequencing and sequence analysis

The nucleotide sequence of the cDNA clones were determined on both strands using the ALFexpress™ automated DNA Sequencer AMV3.0 (Pharmacia Biotech AB, Uppsala, Sweden). The sequencing reactions were carried out using the Thermo Sequenase Fluorescent Labeled Primer cycle sequencing kit (Amersham International, Buckinghamshire, England). The inferred amino acid sequences were used to search for identities in protein sequence databases using the BLAST network service (Altschul *et al.* 1990). Amino acid comparisons were done with the CLUSTAL program of DNAMAN (Version 3.0, 1997). The design of primers and nucleotide restriction analysis were carried out using DNAMAN.

3.2.2 Isolation of genomic DNA

Genomic DNA was isolated from the leaves of *X. viscosa* according to a modification of the protocol of Dellaporta *et al.* (1983) (Appendix B.9). Four to five young leaves from fully hydrated plants were frozen in liquid nitrogen, ground into a fine powder using a mortar and pestle and 0.5 to 1g transferred into an SS34 tube. Extraction buffer (15 ml of 100 mM, Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol) was added to the mixture and the contents vortexed vigorously for 1 min. One ml of 20% SDS was added and the tubes were shaken vigorously for 1 min. The mixture was then incubated at 65 ^{0}C for 10 min. Following this, 5 ml of 5 M potassium acetate was added and the tubes were shaken gently by inverting. They were incubated on ice for 20 min and subsequently centrifuged at 12,000 rpm for 20 min. The supernatant was transferred into

fresh SS34 tubes containing 10 ml of isopropanol. Tubes were shaken gently and incubated at –20 ^{0}C for 2 hours. The tubes were centrifuged at 10,000 rpm for 15 min. Following the centrifugation, the supernatant was discarded and the pellet air dried. The pellet was resuspended in 1 ml TE buffer. RNase A (10 µl of 10 mg/ml) was added and the tube incubated at 37 ^{0}C for 30 min. A phenol, phenol-chloroform and chloroform extraction was carried out followed by precipitation of the DNA with sodium acetate and absolute ethanol (Sambrook *et al.* 1989). The DNA pellet was resuspended in 50 µl TE buffer and quantitated spectrophotometrically. Genomic DNA (20 µg) was restricted with enzymes *EcoR*1, *EcoR*V and *Hind* III. To determine the copy number, enzymes that cut only once in the insert or not at all, and enzymes that cut in flanking regions, were chosen.

3.2.3 Southern blot analysis

The endonuclease restricted genomic DNA was electrophoresed on an 0.8% agarose gel at 30V overnight. DNA fragment sizes were estimated using the λ DNA *PstI* marker. The gel was treated by soaking in 0.2 N HCl for 5 min with gentle shaking, and subsequently rinsed twice with dH_2O. The gel was then placed in a denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 30 min with gentle shaking. This was followed by placing the gel in a neutralization solution (1.5 M NaCl and 0.5 M Tris-HCl) with gentle shaking for 30 min. Four sheets of Whatman 3MM filter paper and 1 nylon membrane (MSI, 0.45 µm) were cut to the size of each gel. The gel was then inverted in a gel tray and placed over the gel tank. A presoaked nylon membrane and 4 Whatman filter papers were placed over the gel, and a 15 cm stack of dry absorbent paper towels was placed over the Whatman filter paper. The transfer occurred for 18 hours by capillary action. Following this, the membrane was cross-linked in a short UV lamp to fix DNA onto the membrane.

Preparation of labeled probes

The probes were prepared using the cDNA inserts of the three differentially expressed clones. The plasmids were restricted with both *EcoR*1 and *Xho*1 to release the inserts. The digested DNA was subjected to electrophoresis on a 1% agarose gel to separate the vector from the insert. The insert DNA was cut out of the gel electroeluted and labeled with ^{32}P [dCTP] using a Random Primed labeling kit (Boehringer Mannheim) as described by the distributor. To separate the unincorporated nucleotides, the radiolabeled probe was passed through a spin-column according to the protocol.

Southern blot hybridization

The UV crosslinked membranes were hybridized with the radioactively labeled probe according to the modified protocol by Church and Gilbert (1984). The membrane was placed in a plastic container with 100 ml of hybridization buffer (10 ml of 10% BSA stock, 200 ml of 0.5 M EDTA pH 8.0, 50 ml NaH_2PO_4, 35 ml of 20% SDS, 4.8 ml distilled water). It was then pre-hybridized at 65 ^{0}C for 2 hours with shaking. The probe was denatured by heating at 95 ^{0}C and added directly to the buffer with the membrane. The membrane was then incubated at 65 ^{0}C for 18 hours with agitation. It was washed twice for 15 min, first in high stringency buffer (2X SSC, 0.5% SDS) and then for 10 min in low stringency buffer (0.5X SSC, 0.1 % SDS). The membrane was sealed in a plastic bag with no air bubbles entrapped and exposed to X-ray film at –70 ^{0}C for 5 days and thereafter developed.

3.3 Results

After DNA sequencing, uncertain bases were corrected to produce error free data (Figures 3.1, 3.5 and 3.6).

XVLEA

The partial sequence data of the 770-bp insert of the cDNA clone *XVLEA* revealed a putative open reading frame (ORF) of 732 bp and a calculated *Mr* of 26,972. The deduced amino acid sequence showed a high level of identity with the LEA group 5 or LEA D29 family (Appendix C.1.2). Based on their deduced common amino acid sequences, the protein has been hypothesized to have a highly conserved 11-amino acid motif (T/A A/T Q/E A/T A/T K/R Q/ED K/R A/T X ED/Q) (Dure 1993). Cys and Trp are not found in the proteins of this group. The sequence shows highest identity of 50% to LEA D29G from *Gorsipium hisurtum* (302 amino acids) and 30% identity to LEA D29C from cotton (337 amino acids) (Fig. 3.1). These LEA proteins are small hydrophyllic proteins that are induced by environmental stresses (Close 1996). LEA proteins remain soluble even at high temperatures. Hydropathy analysis of the protein revealed that they are hydrophyllic in nature (Fig. 3.2).

```
XVLEA          IRHEVHG----------TTV---------------------------------------------------- 10
LEA D29G       MASSWFFIVFLVLTVASVRCTTVDHMPSTDEDARDYSKLKTKTEEATDEH 50
LEA D29C       MASSWFFIVFLVLTVASVRCTTVDHMPSTDEDARDYSKLKTKTEEATDEH 50
                                  ***

XVLEA          --------DETKERVNAAIPTM----DETKEKVSPAMQAAKEKKEAWSEW 48
LEA D29G       HSRTQQAKDELKSKADHAANEVKSNTQQAKDRASEVGKEAKEYTESWTEW 100
LEA D29C       HSRTQQAKDELKSKADHAANEVKSNTQQAKDRASEVGKEAKEYTESWTEW 100
                       ** * . . *          . *.. *    . ***  *.*.**

XVLEA          VEDKL-EGLGLKMENK--AAADDTTDKAAEVAMKAKDS----ASGAAETS 92
LEA D29G       AKEKISEGLGFKQDDDPKGSVEKAFDSVADTATKTKDKLQDMASGAGEYS 150
LEA D29C       AKEKISEGLGFKQDDDPKGSVEKAFDSVADTATKTKDKLQDMASGAGEYS 150
                ..*. **** * ..     . .    *  *. * * **     **** * *

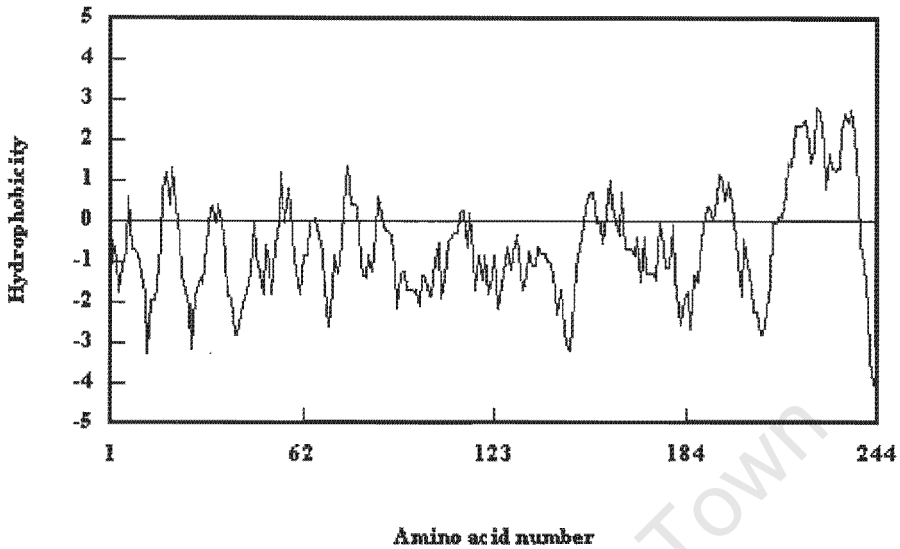
XVLEA          SEKAGESKNTVKDKAAKSADCMSGKAEDVKGKTTETAGKASEDAGGSTRA 142
LEA D29G       AGKAKDMKDTAYKKTDDVKNAAKGKSSEMRQATTEKARELADSAKENANT 200
LEA D29C       AGKAKDMKDTAYKKTDDVKNAAKGKSSEMRQATTEKARELADSAKENANT 200
               . ** . * *          .   **. ...  *** * . .. *

XVLEA          PSRRWKKPTLLPG--TSWLRILRQAMKLRRIRCPRRLVPRRAKGKSEELY 190
LEA D29G       AYIAAKEKVRDMADRTSEMTNEAQERGARKAEEAKEVVAEKAEGAAEETK 250
LEA D29C       AYIAAKEKVRDMADRTSEMTNEAQERAARKAEEAKEVAAEKAKDAAEETK 250
                              **      *     **       *   * *  **

XVLEA          ------------------------------------------- TISQA 195
LEA D29G       KKNEERGESLKWAKEKAKQGYD---------------AAKSKAEETIESA 285
LEA D29C       KKNEETGESLKWAKEKAKQGYDATTEKAKETARQGYDAAKSKAGETVESA 300
                                                          *.  *
```

Figure 3.1 Multiple sequence alignments of the amino acid sequence of *XVLEA* with related proteins. Conserved and similar residues are indicated with dot (.) while an asterix (*) indicates identity in all three proteins. Highly conserved 11-amino acid motif is shown in bold and shading.

A



B

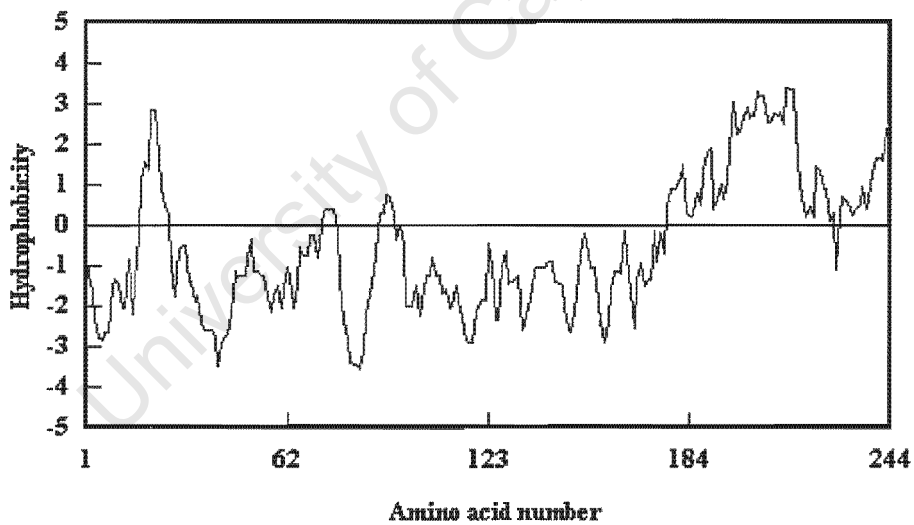


Figure 3.2 Hydropathic index of (a) *XVLEA* and (b) *XVDH* from amino acid 1 to 244. computed using an interval of 11 amino acids (Gravy = -5.75)

Southern blot hybridization patterns indicated that there is only one copy number of *XVLEA* present in the *X. viscosa* genome (Figure 3.4(a)). Only one band was observed in lane 1, *EcoR*1 in the Southern blot of the *EcoR*1 digest This enzyme cuts once in the insert. No bands were observed with *EcoR*V and *Hind* III that cut outside the insert.

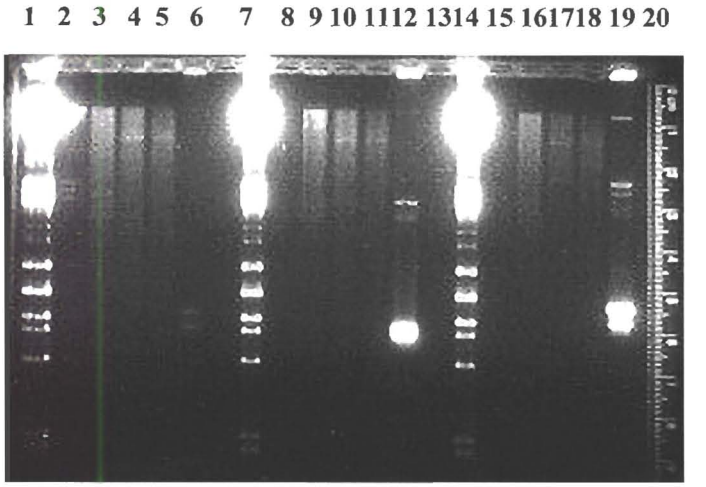


Figure 3.3 EtBr stained 0.8 % gel electrophoresis of restriction digestion of *X.viscosa* genomic DNA with different enzymes. Lanes: 1, λ*Pst*1, 2, positive control (*XVLEA)*, 3,4,5, *EcoR*1, *EcoR*V and *Hind*III respectively for *XVLEA*; 6, blank; 7, λ*Pst*1, 8, blank, 9,10,11, *EcoR*1, *EcoR*V, *Hind*III respectively for *XVDH*; 12, positive control *XVDH*; 13, blank; 14, λ*Pst*1, 15, blank; 16,17,18, (*EcoR*1, *EcoR*V, *Hind*III, respectively); 19, positive control *XVLEC*; 20, blank.

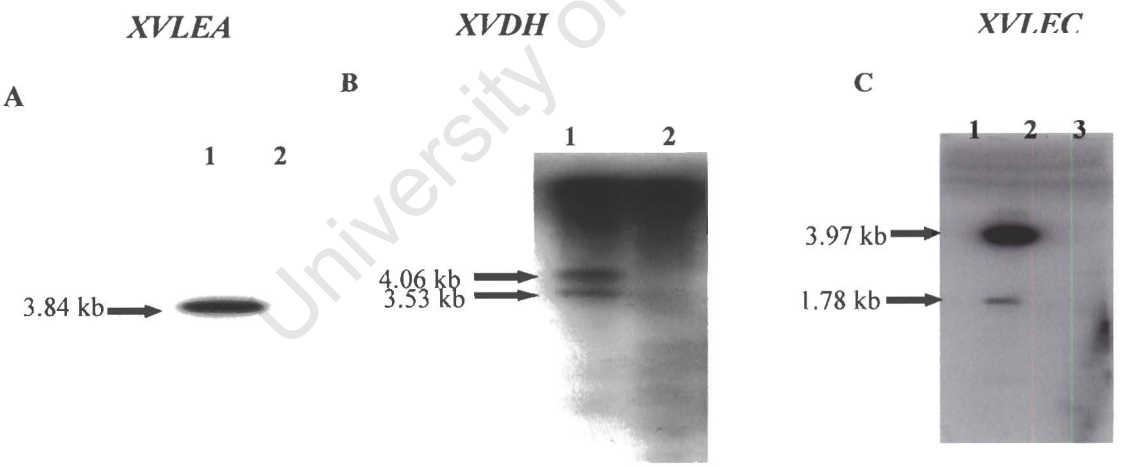


Figure 3.4 Southerrn blot analysis of the three cDNAs present in *X. viscosa* genome. (a) *XVLEA*: Lane 1, *EcoR*1; lane 2, *EcoR*V, (b) *XVDH*: lane 1, *EcoR*1; lane 2, *EcoR*V and lastly (c) *XVLEC* with same enzymes in each lane.

XVDH

The *XVDH* cDNA insert is 770-bp long, with an ORF of 732-bp (Appendix C.2.1). The predicted protein contains 244 amino acids, a calculated *Mr* of 27,000 and a pI of 9.5. The protein contains two highly conserved motifs, SSSSSSESDGEGGRRKK and KIKEKIPG, which are characteristic signature motifs of dehydrins (Appendix C.2.1). Hydropathy analysis (Kyte & Doolittle, 1982) predicted a largely hydrophilic protein, while a segment at the C-terminus was hydrophobic (Figure 3.2). A computer search for homologies revealed that *XVDH* has significant identity with several dehydrins. The highest identities were with ATDEHYDRIN and PSDEHYDRIN, dehydrins from *A. thaliana* and *P. sativum*, in the order of 45% and 43 % respectively (Fig. 3.5).

Southern blot analysis of genomic DNA isolated from *X. viscosa* and probed with the *XVDH* insert produced two bands (Fig 3.4).

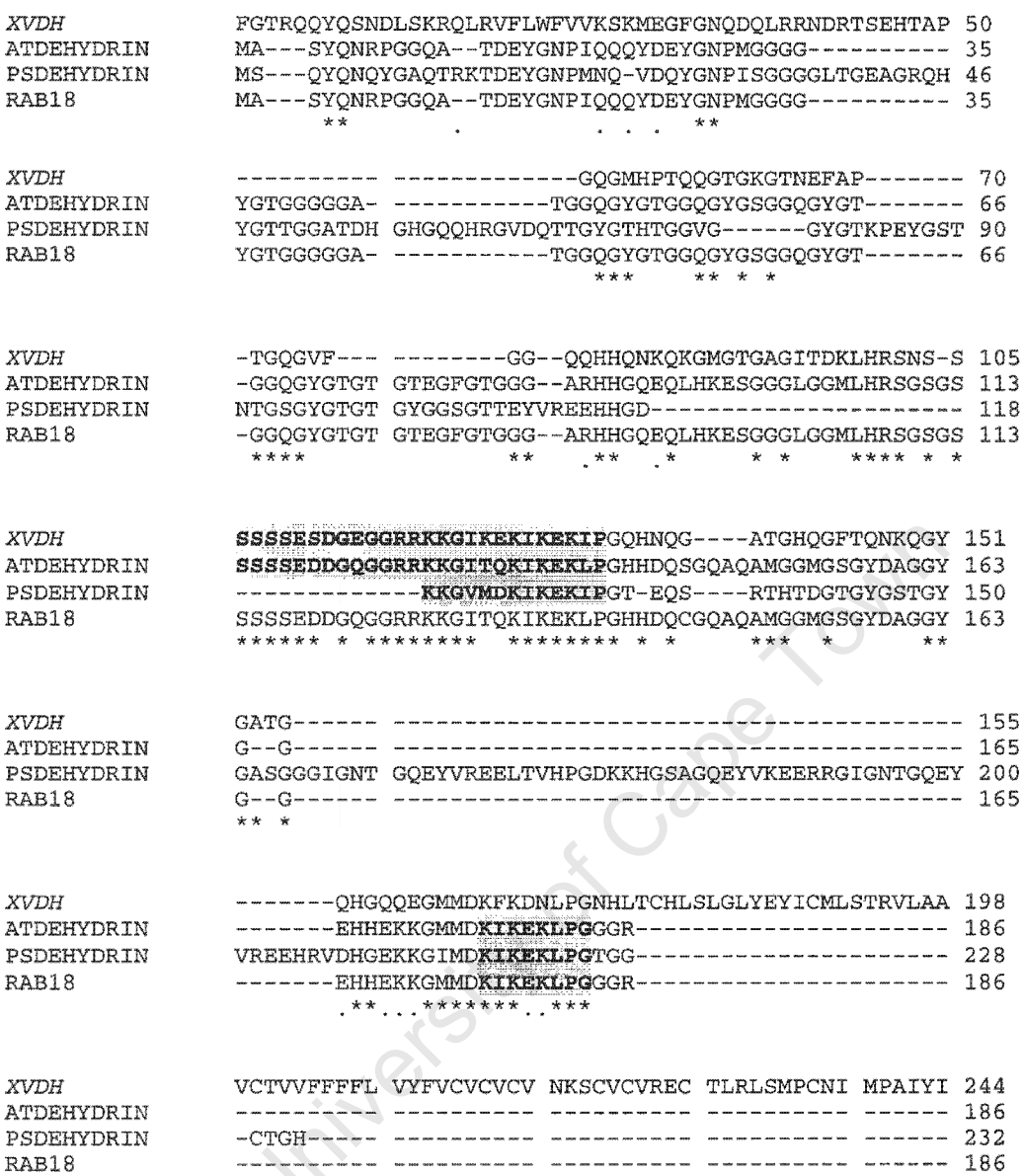


Figure 3.5 Amino acid comparison of *XVDH* with related proteins. An asterix (*) indicates identity with the *XVDH* sequence while a dot (.) indicates similarity. The sequences are: ATDEHYDRIN, a dehydrin protein from *A. thaliana* (Lang and Palva., 1992); PSDEHYDRIN, a dehydrin protein from *P. sativum* (U91969). Gaps (-) were introduced to optimise sequence alignment. Two highly conserved residues are indicated by bold and shading sequences.

XVLEC

The *XVLEC* has a nucleotide sequence of 652 bp and an ORF of 432 bp which codes for 144 amino acids (Appendix 3.2). The sequence shows the highest identity of 28% and 50% similarity with a lectin-like protein from *A. thaliana* (Figure 3.6). The alignment of *XVLEC* to this lectin-like protein from *A. thaliana* is shown in Figure 3.6 below. The sequence shows that about 30% of the sequence is missing from the 5' end since the alignment starts around 150 amino acid, hence *XVLEC* is not a full length clone.

```
XVLEC       VALQQIWEVG--EDPYLFLLGRPSKRLGRRTYNDGWDKV 189
            ::****::::  :*:**:*:              ::::*:
ALPROT      VAMERVWEVGKFEEPYVFVL-------------DGWDRV 122

XVLEC       FQL--TDQQLDWMGDFVVYSKLKFSMQIKG 363
            :*:  :***:*:*:*:***::**:::**::
ALPROT      FKLVLTEKELEWVGEFMIHSKIEFSMEVKG 182

 XVLEC      LVVIPK 396
            ::::::
 ALPROT     LVVIPK 193
```

Figure 3.6 Multiple sequence alignment of the amino acid sequence of incomplete *XVLEC* encoded by the ORF of ALPROT. Identity and similar residues are indicated by an asterix (.) and a dot (*) respectively.

Southern hybridization revealed that *XVLEC* was present in the *X. viscosa* genome. Two bands were observed with *EcoRV* that cuts once within *XVLEC*.

3.4 Discussion

XVLEA and *XVDH* are glycine-rich proteins of 27 kDa, largely hydrophilic and contain the highly conserved serine- and lysine-rich motifs of LEA proteins (Appendix C.2.1 and C.2.3). *XVDH* shows significant identity to ATDEHYDRIN and PSDEHYDRIN, dehydrins from *A. thaliana* and *P. sativum.* (Lang and Palva 1992). In addition, *XVDH* also has significant identity to dehydrins from *Hordeum vulgare*, *Sorghum bicolor* and

Helianthus annuus (data not shown). The properties described above together with the results from the BLAST search, supported our hypothesis that XVLEA and XVDH are likely to be a LEA protein and a dehydrin respectively. The size of LEA proteins varies from 14 to 85 kDa according to sequence data (Close *et al.* 1992). They were also found to be truncated at the 5' ends with no starting codon ATG hence they do not represent the full length sequences of the genes.

Southern blot analysis confirmed that the clones indeed came from *X. viscosa*. However, only one enzyme in each case produced hybridizing band. In each case these enzymes cut the cDNA internally. No bands were observed with the endonucleases that cut outside the insert. It could be possible that the absence of bands in the external cutters would be that the resulting band from the digest was too big and it might have not left the well when the gel was stopped and the small sized band had migrated to the bottom. Or it could be that the high stringency used washed away the bands with the result that only those were seen.

On the other hand the role of lectin-like proteins during dehydration is unclear in plants so far, although there are some clues of being more to a storage protein associated with membranes. Among other things it has been suggested to mediate cell recognition processes. Lectins have been proposed to be involved in plant-microorganisms interactions, as defense mechanisms against pathogens, or in legumes in the recognition of the plant root by the nitrogen-fixing *Rhizobium* (Cote & Hann 1994).

CHAPTER 4

4.0 ANALYSIS OF *XLEA* AND *XVDH* EXPRESSION IN *X. VISCOSA*

4.1 Introduction

Previous studies have shown that the expression of many drought inducible genes also respond to other environmental stresses such as high salinity, heat and cold or freezing stresses (Bray 1997; Shinozaki & Yamaguchi-Shinozaki 1997). Most of these genes require ABA, while others do not, but do respond to exogenous ABA. This hormone is therefore regarded as an important mediator involved in stress response resulting in the expression of genes and/or synthesis of many proteins.

To gain insight into the function the genes, *XVLEA* and *XVDH*, which encode LEA and dehydrin-like proteins respectively detailed studies to investigate the properties and expression patterns during dehydration-rehydration , cold, heat, ABA, ethylene and MeJa stresses were imposed on *X. viscosa*. Expression levels of these cDNAs were determined in *X. viscosa* plants exposed to different treatments. Estimated 1.0 kb transcripts were observed in both cDNAs and based on this they were determined to be near full length copies.

4.2 Materials and methods

4.2.1 Treatments imposed on *X. viscosa*

X. viscosa was exposed to dehydration-rehydration, cold, NaCl, heat, ABA and ethylene treatments. Prior to each treatment four expanded leaves were harvested, flash frozen in liquid nitrogen and stored at –70 ^{0}C. After each treatment the plant was allowed to recover and was maintained under the usual glasshouse conditions.

Dehydration was initiated by withholding water from plants grown in soil thus allowing plants to dry naturally according to Sherwin & Farrant (1996). Rehydration was achieved by watering the same plant after dehydration.

For cold treatment the plant was placed at 4 ^{0}C with a 16 hour light period and watered daily. Four fully expanded leaves were harvested on a daily basis for seven days, flash frozen in liquid nitrogen and stored at –70 ^{0}C.

For heat treatment the plant was placed at 42 ^{0}C for seven days. A 16 hour light period was maintained. On a daily basis, four fully expanded leaves were harvested and treated as above.

For salt treatment the plant was flooded once with 100 mM NaCl and leaf samples were removed once daily for 7 days and treated as above.

The plant was sprayed once daily with 100 µM ABA solution (Appendix A.4.1). Leaves were harvested at six hourly intervals for three consecutive days and treated as above.

For ethylene treatment a *X. viscosa* plant was put under a closed chamber with oxygen and light supplied directly to it. Ethylene was administered in the form of a gas at 6 ppm. The samples were harvested for three days at six hourly intervals and treated as above.

For methyl jasmonate treatment *X. viscosa* was kept at room temperature and sprayed once a day with 100 μM MeJA solution (Appendix A.4.1.2). Leaves were collected at six hourly intervals and treated as above.

4.3 Northern blot hybridization

The isolation of total RNA was carried out according to a modification of the protocol of Chomczynski (1987), (Appendix B.4 and Chapter 2) 10 μg of each sample of RNA isolated from the treatments imposed on *X. viscosa* leaves were electrophoresed on 1.2% formaldehyde gels in 20 mM MOPS (3-[N-morpholino] propanesulfonic acid) pH 7.0, and transferred to nylon membranes (MSI, 0.45 μ) by capillary elution. For the northern slot blot analysis (Appendix B.6) of cold, heat, NaCl, ABA and ethylene treated *X. viscosa* plants, 4 μg of total RNA was slot blotted onto nylon membranes (MSI, 0.45 μ) in a slot blot apparatus (Hoefer Scientific, San Francisco). The *β-actin* DNA of 2.1 kb was released from the pBluescript (pSK) vector after subjecting it on BamH1 restriction endonuclease. This was then electrophoresed on 1% TBE-agarose gel stained with ethidium bromide (EtBr) and the insert was electroeluted (Appendix B.1.3). The *XVLEA*, *XVDH* and *β-actin* inserts were labeled with [^{32}P] dCTP using polymerase chain reaction (PCR). PCR-labeling (Appendix B.1.7) was devised to label the slot blot and the regular Northerns and the labeling intensity was good. Following PCR the labeled probe was purified as described earlier. Membranes were hybridized as described in Chapter 3 section 3.3.4. They were exposed to x-ray film at –70 ^{0}C for appropriate durations.

4.4 Results

The effect of dehydration-rehydration on *XVLEA* and *XVDH* expression

Northern blot analysis of total RNA isolated from hydrated (100% RWC) and dehydrated (37% RWC) *X. viscosa* leaves exhibited a single transcript of 0.71 kb in dehydrated (37% RWC) leaves only when probed with the *XVLEA* insert [Fig. 4.1A]. The cDNA insert was deduced to be a near full-length copy based on the length of the RNA transcript to which

it hybridized. A transcript of 1.0 kb was observed [Fig. 4.2A] during dehydration-rehydration treatment. Total RNA isolated from hydrated (92% RWC) and rehydrated (4%, 32% and 72% RWC) *X. viscosa* leaves was probed with the same insert

The Northern blot of RNA isolated from *X. viscosa* leaves dehydrated to 37% RWC and subjected to rehydration treatment (4%, 32%, 72% and 92%) was probed with the *XVDH* insert (Figs. 4.1B and 4.2B). It exhibited a transcript of 1.0 kb.

Effect of cold and heat stresses on *XVDH* expression

Northern slot blot analysis of cold and heat treated *X. viscosa* plants revealed that the *XVDH* transcripts were detected within one day after the above treatments, (Fig. 4.3). The abundance of *XVDH* transcripts increased significantly within two and three days after exposure to 4°C [Fig. 4.3A(i)]. At three days, there appeared to be basal levels of *XVDH* transcripts once again, and further exposure to the low temperature stress resulted in a gradual increase in transcript levels. The abundance of *XVDH* transcripts following exposure to heat stress (Fig. 4.3B(i) was not as dramatic as observed for cold stress. A gradual increase in *XVDH* transcripts was observed until three days, followed by a decrease and subsequent increase in transcript levels. No *XVDH* transcripts were detected under control conditions prior to the cold and heat treatments. The blots were probed with β-actin to verify equal loading of RNA samples.

Effect of ABA, ethylene and MeJa on *XVDH* expression

Northern slot blot analysis of ABA treated plants revealed that *XVDH* expression was induced after 6 hours of ABA treatment, [Fig. 4.4A]. A gradual increase in *XVDH* transcripts was observed following ABA treatment, until a saturation level was reached at 36 hours. This level of *XVDH* transcripts was observed for the following 30 hours.

XVDH transcripts were observed 12 hours after ethylene treatment [Fig. 4.4B] and their

expression levels remained the same for the following 24 hours. A significant increase in *XVDH* transcript levels was observed 42 hours after ethylene treatment, followed by a gradual decrease. A similar pattern of *XVDH* expression was observed at 60 and 66 hours where an increase and subsequent decrease in transcript levels was observed. The blots were probed with β-actin to verify equal loading of RNA samples [Figs. 4.4A(ii) and 4.4B(ii)].

In the case of MeJa, *XVDH* transcripts had minimal expression until 12 hours after treatment. Thereafter an increase was observed until 78 hours [Fig. 4.5].

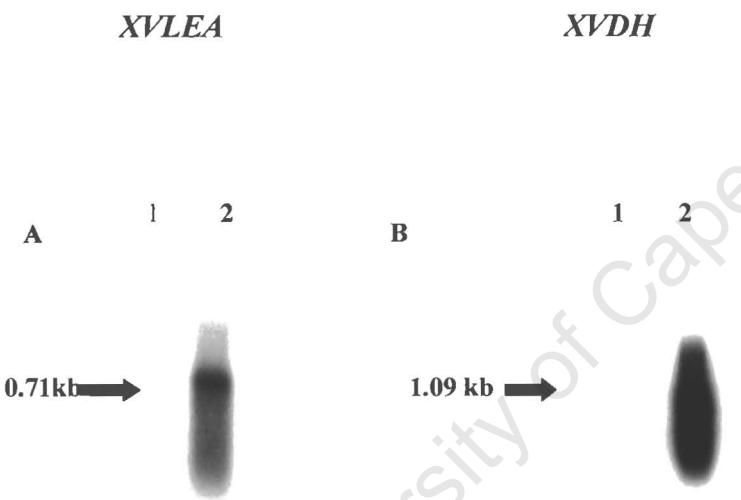


Figure 4.1. Northern blot analysis of hydrated (100% RWC) (lane 1) and dehydrated (37% RWC) (lane 2) *X. viscosa* leaves. 10 μg of total RNA was fractionated on a 1.2 % agarose gel and transferred to a nylon membrane. The blot was probed with ^{32}P-labelled *XVLEA* (A) and *XVDH* (B) inserts. Equivalent amounts of RNA were loaded as determined from Northern analysis using a β-actin probe.

XVLEA *XVDH*

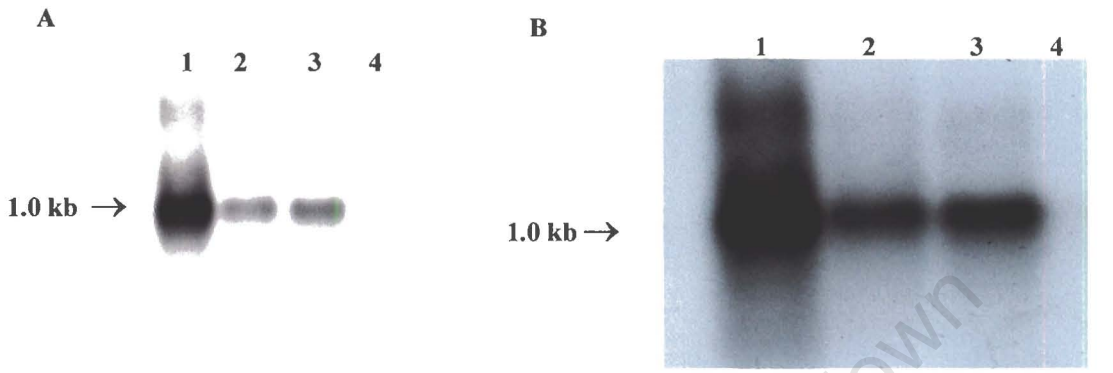


Figure 4.2 Northern blot analysis of XVLEA *XVDH* of dehydration-rehydration treatment lane1, 4%; 2, 32%; 3, 37%; 4, 92% RWC. a) *XVLEA* rehydration; b) *XVDH* rehydration. RNA was electrophoresed on 1% agarose gel containing EtBr, and transferred onto a nylon membrane, transferred to a nylon membrane and probed with *XVLEA* and *XVDH* insert.

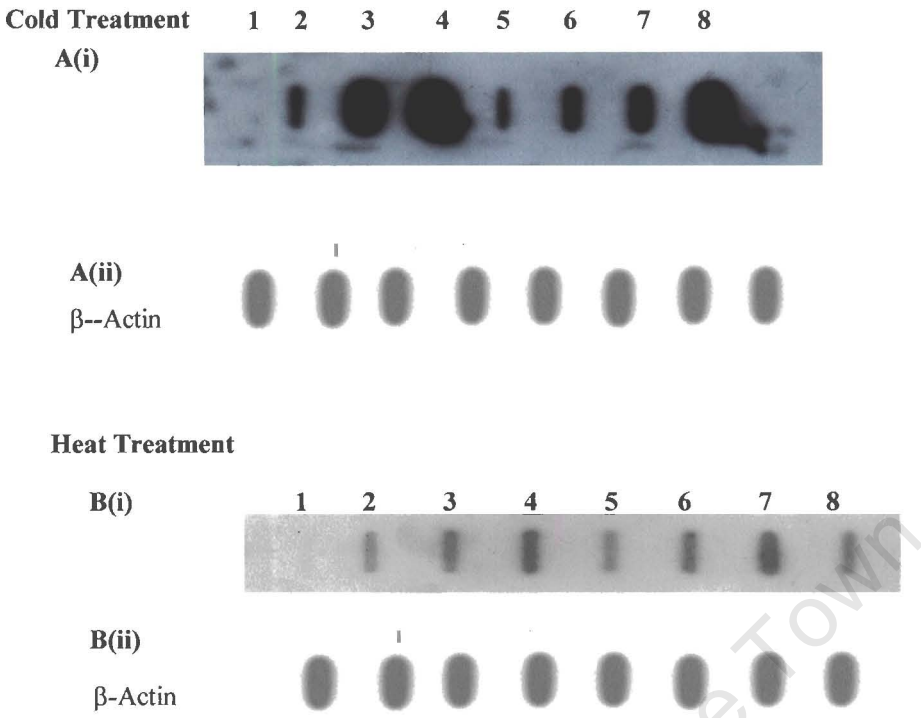


Figure 4.3 Northern slot blot analysis of low temperature (A) and heat (B) stressed *X. viscosa* plants. 4 μg of total RNA was slot blotted onto nylon membranes in a slot blot apparatus (Hoefer Scientific, San Francisco). The blot was probed with ^{32}P-labelled *XVDH* insert. Equivalent amounts of RNA were loaded as determined from using a β-actin probe (A(ii) and B(ii)). Lane1, day 0 prior to the treatment; lane 2, day 1; lane 3, day 2; lane 4, day 3; lane 5, day 4; lane 6, day 5; lane 7, day 6; lane 8, day 7.

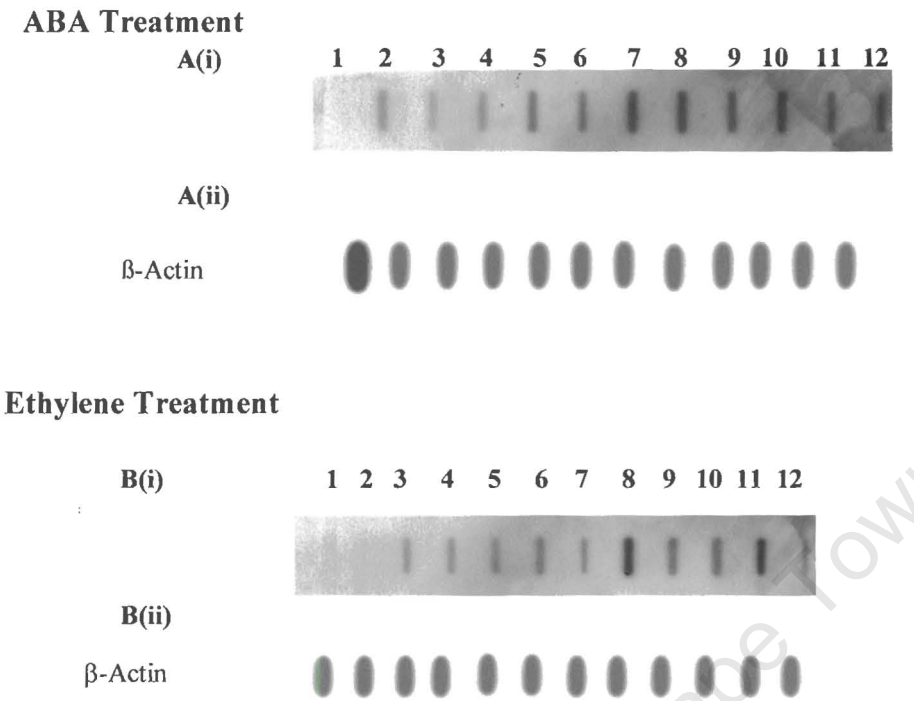


Figure 4.4 Northern slot blot analysis of ABA (a) and ethylene (b) treated *X. viscosa* plants. 4 μg of total RNA was slot blotted onto nylon membranes in a slot blot apparatus (Hoefer Scientific, San Francisco). The blot was probed with ^{32}P-labelled *XVDH* insert. Equivalent amounts of RNA were loaded as determined from using a β-actin probe (A(ii)and B(Fig. 4.3B(i) (ii)). Time (t) is at 6 hourly intervals. Lane 1, time 0 (t0); lane 2, t6; lane 3, t12; lane 4, t18; lane 5, t24; lane 6, t30; lane 7, t36; lane 8, t42; lane 9, t48; lane 10, t54; lane 11, t66; lane 12, t66.

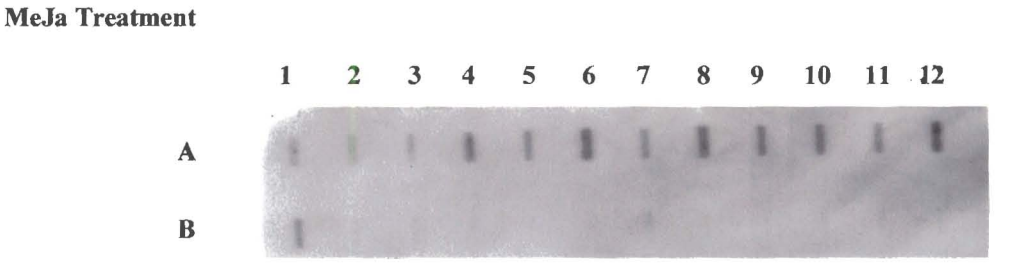


Figure 4.5 Northern slot blot analysis of MeJa treated *X. viscosa* plants. 4 µg of total RNA was slot blotted onto nylon membrane in a slot blot apparatus (Hoefer Scientific, San Francisco). The blot was probed with ^{32}P-labelled *XVDH* insert. Time (t) is at 6 hourly intervals. Lane 1, time 0 (t0); lane 2, t6; lane 3, t12; lane 4, t18; lane 5, t24; lane 6, t30; lane 7, t36; lane 8, t42; lane 9, t48; lane 10, t54; lane 11, t60; lane 12, 66; lane 13 (1B), t72. Samples were 13 in total and the slot blot accommodate 12 in a row such that the last t72 was loaded in the second row of the slot.

4.5 Discussion

It has been well documented that late embryogenesis abundant proteins and dehydrins accumulate in response to dehydration (Dure III 1993; Close 1996; Chermidae 1997; Han *et al.* 1997) and also cold and salt stress (Close 1996). Therefore, the effects of dehydration-rehydration on both cDNAs, and the effects of low temperature and heat stress, exogenous ABA and ethylene on *XVDH* expression in plant *X. viscosa* were tested. The *XVDH* transcripts accumulated in response to each of the above treatments. There was particularly abundant accumulation of the transcripts when plant was dehydrated to 37% RWC (Fig. 4.1B), suggesting that *XVDH* and *XVLEA* do indeed play a role in tolerance of water loss in this plant as has been reported for other desiccation tolerant organisms (Dure III 1993; Close 1996; Chermidae 1997; Han *et al.* 1997). The precise nature of that role is still unclear (Black *et al.* 1999)

XVDH transcripts accumulated in response to ABA, heat, cold and ethylene treatments. There is good evidence that dehydrins play important roles in response to various stresses,

including heat, salt, low temperature and dehydration. Although dehydrins are known to accumulate during the above stresses, their role is still unclear (Black *et al.* 1999).

The accumulation of *XVDH* transcripts in response to low temperature stress is consistent with results from other studies (Lang & Palva 1992; Close *et al.* 1993). Dehydrins have been found to accumulate in response to any environmental stress that has a dehydration component (Close 1996). The two-phase induction of *XVDH* expression could imply that there is a turnover of the dehydrin proteins. The accumulation of *XVDH* transcripts in response to heat stress does not have great effect. Dehydrins are not known to respond to heat stress. The adaptation of *X. viscosa* to hot and dry habitats could justify the existence of a dehydrin that accumulates during heat stress.

The transcription of dehydrin genes is widely known to be inducible by ABA-dependent and ABA-independent signals (Robertson & Chandler 1994). We have observed that *XVDH* expression was slightly induced 6 hours after exposure to exogenous ABA. This indicates that the *XVDH* promoter may respond to ABA at the whole plant level. The induction of *XVDH* expression in *X. viscosa* leaves by dehydration may be associated with dehydration-induced increases in endogenous ABA. A number of studies have revealed an increase in endogenous ethylene in plants exposed to hypoxia (He *et al.* 1996; Drew 1997). We observed that the exposure of *X. viscosa* plants to exogenous ethylene resulted in a slight accumulation of *XVDH* transcripts, with the maximum occurring after 42 hours of treatment. This suggests that ethylene could be a signal for *XVDH* expression when *X. viscosa* is exposed to hypoxic conditions. The accumulation of *XVDH* transcripts is only slightly affected by MeJa although it has been reported that MeJa treatment mimics plant responses several environmental stimuli (Creelman & Mullet 1997).

CHAPTER 5

IMMUNOLOCALIZATION STUDIES OF *XVDH* ON HYDRATED AND DEHYDRATED LEAVES OF *X. viscosa*

5.1 Introduction

Dehydrins have highly conserved lysine-rich residues (KIKEKLPG) near the C-terminus which may be present in one or more copies. This sequence appears to be characteristic and unique to dehydrins (Mundy & Chua 1988; Close *et al.* 1993). The proteins are known to accumulate abundantly during osmotic stress and have no similarity with any other enzyme and/or protein (Close & Lammers 1993; Close 1996). The number and size of dehydrins vary widely from species, and ranges from 15 to 150 kDa. Dehydrin proteins have been found to be localized in the cytosol in *Craterostigma plantagineum* (Schneider *et al.* 1993) and in nuclei of different cell types in sections of maize kernels (Colmenro-Flores *et al.* 1999). WCOR410 from wheat is a cold regulated dehydrin that has been found to be localized in the vicinity of plasma membranes (Danyluk 1998) and this functions as a cryoprotective protein. Some dehydrins contain serine tract residues which bind to nuclear localization peptides (Godoy *et al.* 1994; Giordani *et al.* 1999). In this study, the immunolocalization of XVDH dehydrin in hydrated (100% RWC) and dehydrated (37% and 5% RWC) leaves was determined. At 100% RWC, XVDH was found to be associated with the cell wall and membranes of leaf mesophyll cells. At 37% and 5%

RWC the gold particles were more concentrated at and near the plasma membrane and to a lesser extent in the cytoplasm and vacuole.

5.2 Materials and methods

5.2.1 Tissue processing

X. viscosa leaf samples were fixed in 2.5% glutaldehyde in pH 7.4 at 4 ^{0}C for 16 hours (Appendix B.10). Post fixation was in 1% PBS for 60-90 min. Following this, washes were done twice for 5 min. Ethanol dehydration was carried out and the leaf material was embedded in epoxy resin (Spur 1969). The 90 nm resin was hardened at 60 ^{0}C for 16 hours. Thin sectioning was done using a Reichert Ultracut-S (Leica, Astria) ultramicrotome and collected on nickel grids.

5.2.2 Dehydrin antibody (PLA-100) production

Dehydrin (PLA-100) is a rabbit polyclonal antibody (StressGen, Canada) that was commercially prepared by Close *et al.* (1993). It was generated against a dehydrin C-terminal consensus sequence of a highly conserved lysine-rich block (KIKEKLPG).

5.2.3 Immunogold labeling

Sections from fully hydrated and dehydrated leaves of *X. viscosa* plants were taken and mounted on Formvar-coated nickel grids. Sections were floated on a 15 µl drop of water for 5 min, followed by floating in 0.02 M glycine twice for 3 min, and finally in 1X PBS and 1% BSA for 3 min. Sections were then floated on drops of purified dehydrin antibodies (PLA-100) diluted to 1:25 and 1:50 in blotto. The incubation with the primary antibody was carried out for 16 hours at room temperature. After washing with Tween-20 in 1% BSA and 1X PBS, the grids were placed on drops of goat anti-rabbit IgG coupled with 10 nm gold particles, diluted 1:100. The sections were incubated in the secondary antibody at room temperature for 2 hours. Sections were fixed with glutaraldehyde in 1X PBS for 3 min and rinsed with water 5 times for 2 min, respectively. Staining with uranyl acetate and lead citrate was carried out

separately for 10 min each. The sections were rinsed in water between stains. The grids were viewed under a JEOL (JEOL, Japan) X 1 200 Electron microscope. Control experiments consisted of replacing the dehydrin antibody with pre-immune serum or PBS supplemented with 1% (w/v) BSA.

5.3 Results

Ultrastructure of hydrated (100%RWC) and dehydrated (37% RWC) *X. viscosa* leaf tissue

Examination of the ultrastructure under low magnification using electron microscopy was carried out on hydrated (100% RWC) and dehydrated (37% RWC) leaf sections. At 100% RWC, cell walls were thickened, and centrally arranged vacuoles, peripheral chloroplasts with abundant thylakoid and several grana, and mitochondria with well developed cristae were observed (Fig. 5.1A). The plasma membrane was pressed to the thickened cell wall. At 37% RWC, the cell walls were contracted and cells no longer exhibited a regular compartmentation (Fig 5.1B). Chloroplasts with disordered thylakoid system and numerous small, well defined and vacuoles were observed.

Subcellular localization of XVDH dehydrin

Examination of leaf sections (100% RWC) revealed an accumulation of some protein along the cell wall and very few gold particles were detected in the cytoplasm (Fig. 5.2). The control experiments in which the PLA-100 antibody was replaced with the pre-immune serum resulted in no labeling in any of the cell compartments.

Examination of dehydrated (37% and 5% RWC) leaf sections revealed that the XVDH protein accumulated mainly in the cytoplasm and in close association with the plasma membrane (Figs. 5.3 and 5.4). No labeling was observed in leaf sections that were treated with pre-immune serum as a control (Figs. 5.3D and 5.4D).

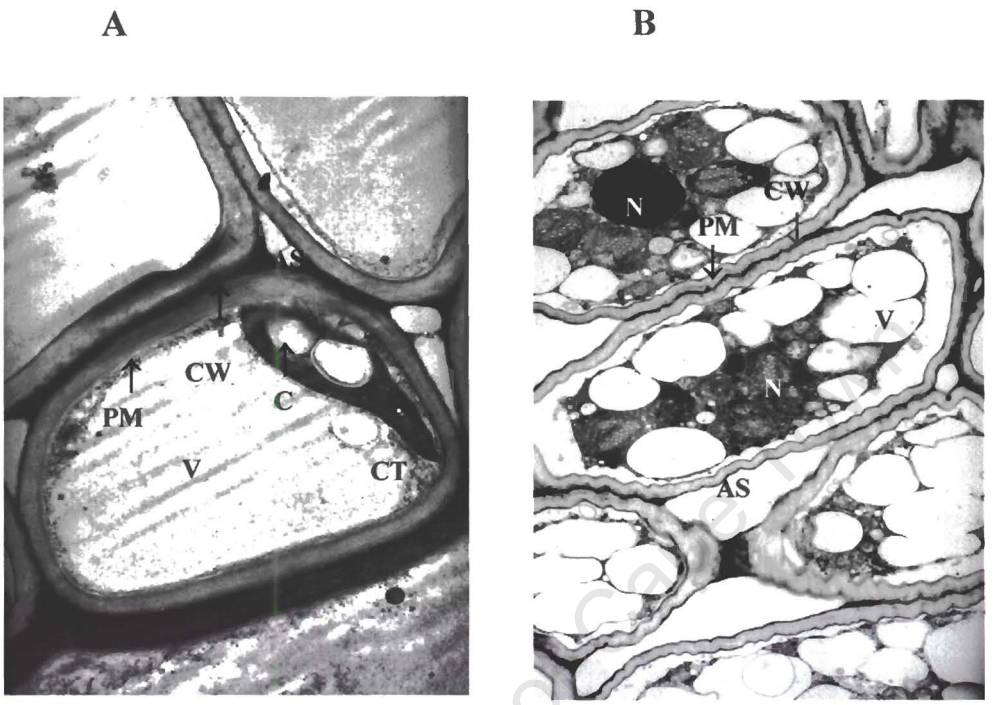


Figure 5.1 Electron micrographs showing ultrastructure of mesophyll cells of hydrated (A) and dehydrated (B) *X. viscosa* leaves. AS, intercellular air space; C, chloroplast; CT, cytoplasm; CW, cell wall; N, nucleus; PM, plasma membrane; V, vacuole. Magnification: A, 48000X; B, 30000X.

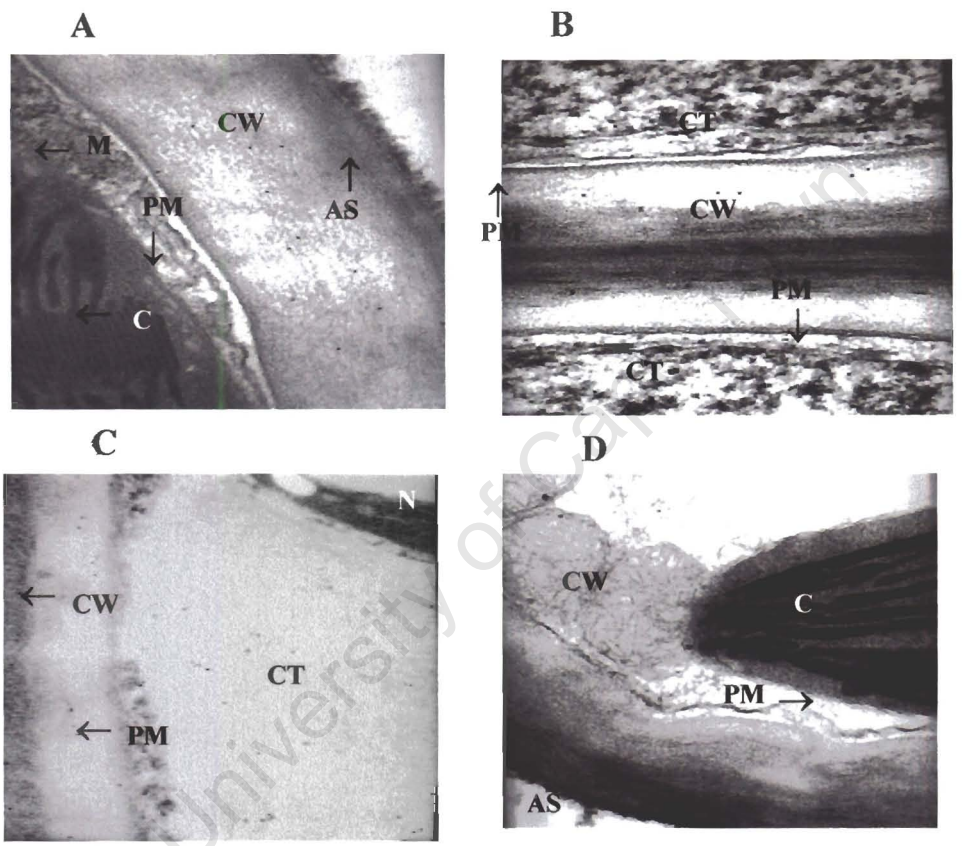


Figure 5.2 Electron micrographs of hydrated (100% RWC) leaf sections of *X viscosa* labelled with PLA-100 antibody (A, B and C) and with pre-immune serum (D). AS, intercellular air space; C, chloroplast; CT, cytoplasm; CW, cell wall; M, mitochondria; N, nucleus; PM, plasma membrane; V, vacuole. Magnification: A, B, C, 28000X; D, 23000X.

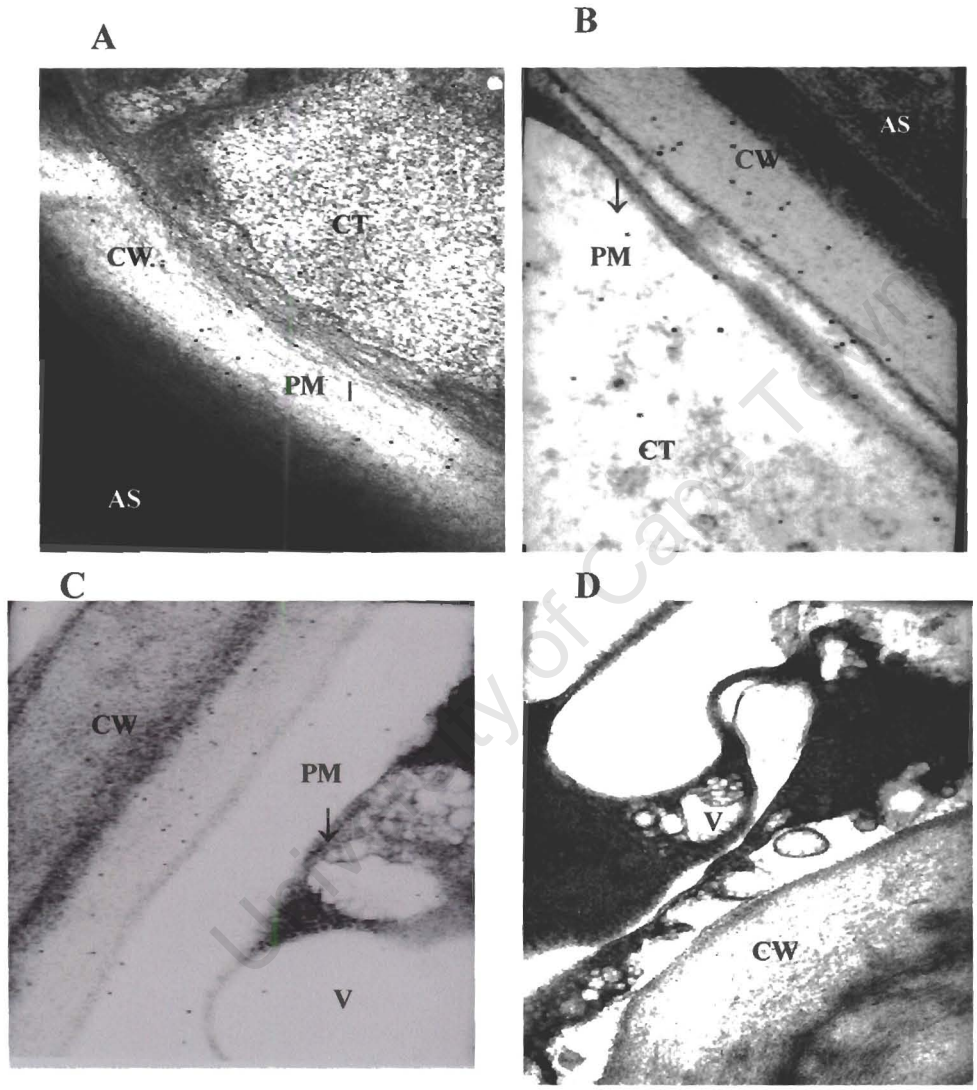


Figure 5.3 Electron micrographs of dehydrated (37% RWC) leaf sections of *X viscosa* labelled with PLA-100 antibody (A, B and C) and with pre-immune serum (D). AS, intercellular air space; C, chloroplast; CT, cytoplasm; CW, cell wall; N, nucleus; PM, plasma membrane; V, vacuole. Magnification: A, 64000X; B, 38000X; C, D, 32000X.

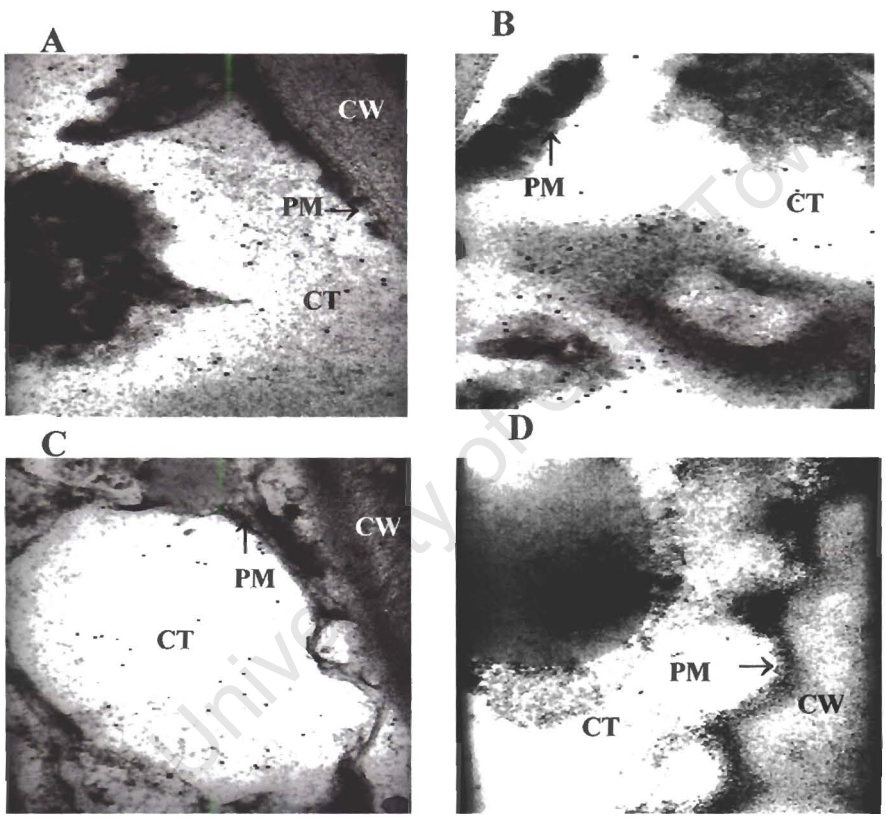


Figure 5.4 Electron micrographs of dehydrated (5% RWC) leaf sections of *X viscosa* labelled with PLA-100 antibody (A, B and C) and with pre-immune serum (D). AS, intercellular air space; C, chloroplast; CT, cytoplasm; CW, cell wall; N, nucleus; PM, plasma membrane; V, vacuole. Magnification: A, B, 43000X; C, 32000X; D, 22000X.

5.4 Discussion

This study was conducted to determine the subcellular localization of the XVDH protein. The antibody PLA-100 was commercially prepared against the highly conserved Lys-rich (KIKEKLPG) domain located at the C-terminus of the protein (Close *et al.* 1993). At 100% RWC there was evidence that most of the protein was localised along the cell walls with some in the cytoplasm. Normally this protein is not expressed in a fully hydrated state but its expression is induced by stresses such as dehydration, cold, and ABA. The same plant from which the samples were taken had previously been exposed to other treatments but was allowed to recover from these stresses and maintained under normal glasshouse conditions for three weeks prior to being used for this study. It is possible that the protein which was expressed in the same plant during the earlier stresses was still present in the leaf tissues.

At 37% RWC the protein was found to be distinctly associated with the plasma membrane rather than the cytosol (Fig. 5.3 A, B & C). The hydropathic plot of XVDH revealed that it is mostly hydrophilic, with one hydrophobic domain at the N-terminus. This suggests that the protein is anchored within the plasma membrane while the remainder remains non-membrane bound. At 5% RWC the localization of the protein was found in both the membrane and cytosol in almost equal amounts (Fig. 5.4 A, B & C).

Previous studies have found dehydrins localised in the cytoplasm of desiccated leaf cells in *C. plantagineum*. In contrast, results from WCOR410 which is a cold-regulated dehydrin, revealed that it accumulates at high levels in the vicinity of plasma membranes and not in the cytosol during dehydration (Danyluk *et al.* 1998). Results from this study are different from these findings. The fact that *XVDH* encodes a protein that accumulates in both the cytosol and plasma membrane could be explained by it being induced by cold and other stresses. Another reason could be that the subcellular location of dehydrins is greatly influenced by tissue type (Close *et*

CHAPTER 6

GENERAL CONCLUSIONS

Plants respond to dehydration and/or water deficit through changes in gene expression depending on the developmental stage of the plant as well as the tissue type in question. These changes may include induction of specific genes and the synthesis of specific proteins as well as repression of a number of other genes. In order to identify genes induced during dehydration a cDNA library constructed from dehydrated (85%, 37% and 5% RWC) *X. viscosa* leaves was differentially screened by hybridizing duplicate replicates of 192 randomly selected cDNAs, with mRNA isolated from hydrated (100%) and dehydrated (37% RWC). Of 192 cDNAs, 30 were up-regulated and 20 were down-regulated during dehydration. Three cDNAs that were up-regulated during dehydration were chosen for further analysis and characterization.

The identities of the sequenced cDNAs were determined by searching the data banks, using the BLAST network service. The cDNAs showed highest identities with LEA, dehydrin and lectin-like proteins and were labeled *XVLEA, XVDH* and *XVLEC.* All were found to be truncated at their 5' ends. The presence of the cDNAs in the *X. viscosa* genome was shown.

X. viscosa was exposed to different stresses *viz.* dehydration-rehydration, cold, heat, salinity, ABA, MeJa and ethylene treatments. *XVLEA* and *XVDH* were found to be expressed in great abundance during dehydration (37% RWC) and rehydration (4%, 32% and 72% RWC). In addition *XVDH* was found to be induced by other stresses *viz.* cold, heat, ABA, ethylene and MeJa treatments. No induction by NaCl was found. Previous studies have shown that most of the genes induced by drought, salinity and cold have different stress signals transmitted separately in plant cells to activate DRE-dependent

transcription of *rd29A* gene (Liu *et al.* 1998). This gene has been found to be regulated by drought and high salinity via an ABA-mediated pathway. In this study, although *XVDH* gene was induced by ABA, its expression during salt stress could not be achieved. Since ABA is thought to be produced under these conditions (Nelson *et al.* 1994), this area needs further investigation in order to determine expression levels associated with drought and salt stress.

Lastly, the immunolocalization of the XVDH encoded protein was determined. At 100% RWC there were a few gold particles associated with the membrane, an unexpected finding since the protein does not accumulate under normal conditions. It is possible that as the plant was rehydrated and stress was removed, the protein remained in the tissue due to its slow turnover. XVDH was found mainly in the plasma membranes at 37% RWC. This is probably due to the presence of a hydrophobic domain which is probably associated with the plasma membrane (Kyte & Doolittle 1982). At 5% RWC, XVDH was equally distributed between the membrane and the cytoplasm. This could be explained by possible leakage within the membrane structure since at that RWC there is folding of membranes and dismantling of macromolecular structure (Sherwin & Farrant 1996; Farrant *et al.* 1999, 2000). Previous studies have revealed the localization of dehydrin in the cytoplasm and nuclei (Close 1996) and plasma membranes in a cold regulated dehydrin from wheat (Danyluk *et al.* 1998). In order to determine the localization of XVDH it would be better to generate the antibodies specifically against this protein and not use the commercially prepared ones which react against all dehydrins.

Further work is required to obtain a full length sequence of *XVDH* the gene in a crop plant and determine its relationship to the plant resistance to abiotic stresses which impose cellular dehydration. Full length cDNAs of *XVLEA* and *XVLEC* are also needed to further characterize the structure and function.

APPENDIX A: BUFFERS, SOLUTIONS AND MEDIA

A.1 MEDIA

A.1.1 Standard growth media (LB)

Constituent	Final Concentration	Per litre
Tryptone	1.0 %	10 g
Yeast extract	0.5 %	5 g
NaCl	0.5 %	10 g

pH to7.5, add distilled water to final volume and autoclave

A.1.2 LB Agar (LA)

Constituent	Concentration in stock	Per litre
Tryptone	1.0 %	10 g
Yeast extract	0.5 %	5 g
NaCl	1.0 %	10 g
Agar	1.5 %	15 g

pH to 7.5, add distilled water to final volume and autclave.

A.2 BUFFERS AND SOLUTIONS

A.2.1 Miniprep of *E.coli* plasmid isolation solutions

A.2.1.1 Solution 1

Constituent	Final concentration	Per 100 ml
Tris-HCl pH 8.0	25 mM	25 ml of 1 M stock
Glucose	50 mM	50 ml 1 M stock
EDTA	10 mM	20 ml of 0.5 M stock

Autoclave glucose separately, make up to 100 ml with distilled water.

A.2.1.2 Solution 2

Constituent	Final concentration	Per 10 ml
SDS	1.0 %	50 µl of 20 % stock
NaOH	0.2 N	40 µl of 5 M stock

Make it up to 10 ml with distilled water, must be prepared fresh every day.

A.2.1.3 Solution 3

Constituent	Per 200 ml
Potassium acetate	60 ml of 5 M stock
Glacial acetic acid	11.5 ml of 2 M stock

A.2.2 DNA electrophoresis solutions

A.2.2.1 20X Tris-borate-EDTA (TBE) buffer

Constituent	Final concentration	Per 1 litre
Tris-HCl	216 mg	216 g
Boric acid	110mg	110 g
EDTA	25 mM	80 ml of 0.5 M stock

Make up to 1 litre with distilled water.

A.2.2.2 Sample buffer

Constituent	Final concentration	Per 25 ml
Bromophenol blue		62.5 mg
Sucrose		10 g
EDTA	20 mM	1 ml of 0.5 M stock

Autoclave.

A.2.2.3 RNase A

Constituent	Final concentration	Per 10 ml
RNase A	10 mg.ml	100 mg
Tris-HCl (pH 7.5)		10 mM
NaCl		15 mM

Heat to 100^0 C for 15 minutes, allow to cool slowly to room temperature. Dispense into aliquotes and store at -20^0 C.

A.2.2.4 Extraction buffer

Constituent	Final concentration	Per 100 ml
Tris-Hcl (pH 8.0)	100 mM	10 ml of 1 M stock
EDTA (pH 8.0)	50 mM	10 ml of 0.5 M
NaCl	500 mM	ml of 5 M stock
β-mercaptoethanol	10 mM	150 µl added directly

Add the first 3 and add β-mercaptoethanol separately in each sample under the fume hood.

A.2.2.5 Restriction enzyme digests of DNA

Constituent	Amount per 20 µl	Amount per 50 µl
DNA	x µl	x µl
Buffer	2 µl	5 µl
BSA acetylated	2 µl	5µl
Enzyme	2 µl	5 µl
Water	x µl	x µl
Total	**20 µl**	**50 µl**

For a 20 µl reaction in a double digests enzyme add 1 µl of each enzyme, For 50 µl reaction add 2,5 µl of each of the enzymes. Incubate at 37^0 C for 2 hours to overnight.

A.2.2.6 Preparation of Pst 1-digested λ ladder

Constituent	Per 50 µl reaction
λ DNA	30 µl
Buffer H	5 µl
BSA acetylated	5 µl
Pst 1	5 µl
Distilled water	5 µl
Total	**50 µl**

Mix the constituents gently by tapping with an index finger. Spin it briefly to settle it down and incubate at 37 ^{0} C for overnight. Make up to 4x 50 µl reactions and after incubation combine them together. Add about 20 µl of gel loading buffer. Load about 10-15 µl of the ladder in a well.

A.3.2 General solutions and buffers

A.3.2.1 Phenol

Constituent	Per 500 g
Crystallized phenol	500 g
Tris-HCl	6 ml of 1 M stock pH 7.6
8-Hydroxyquinoline	0.6 g
NaOH	7.5 ml of 2 M stock
Distilled water	130 ml

Leave it at room temperature for overnight to liquefy, adjust pH to 7.6 and store it at -20^{0} C.

A.3.2.2 EDTA

Constituent	Final concentration	Per litre
EDTA	0.5 M	186.1 g
NaOH	approximately 4 %	Approximately 20 g

Add 800 ml of water and adjust the pH with NaOH to 8.0. Make the volume up to 1 litre and autoclave.

A.3.2.3Tris-HCl

Constituent	Final concentration	Per litre
Tri-HCl	1 M	121 g

Dissolve Tris-HCl in water and adjust pH with HCl. Autoclave

A.3.2.4 Sodium acetate

Constituent	Final concentration	Per 250 ml
Sodium acetate	3 M	61.53 g

Dissolve it in 250 ml of water. Adjust pH to 4.8 with glacial acetic acid, autoclave

A.3.2.5 Potassium acetate

Constituent	Final concentration	Per 250 mg
Potassium acetate	5 M	

Autoclave

A.3.2.6 Ethidium bromide

Constituent	Final concentration	Per 10 ml
Ethidium bromide	10 mg/ml	0.1 g

Wrap the container with aluminium foil to prevent light from entering.

A.3.2.7 Tris-EDTA (TE) buffer

Constituent	Final concentration	Per 1 litre
Tris-HCl	10 mM	10 ml of 1 M stock
EDTA	1 mM	200 µl of 0.5 M stock

Final pH should be adjusted to 7.6.

A.3.2.8 20 % SDS

Constituent	Final concentration	Per 200 ml
SDS	20 %	40 g

Do not autoclave, heat it to dissolve it properly.

A.3.2.9 Glucose

Constituent	Final concentration	Per 200 ml
Glucose	1 M	40.04 g

Autoclave.

A.3.2.10 NaOH

Constituent	Final concentration	Per 200 ml
NaOH	5 N	40 g

Do not autoclave, use sterile distilled water.

A.3.2.11 Sodium chloride (NaCl)

Constituent	Final concentration	Per 500 ml
NaCl	5 M	146.1 g

A.3.3 RNA electrophoresis solutions

A.3.3.1 10X MOPS

Constituent	Final concentration	Per 500 ml
MOPS	200 mM	20.93 g
NaAc	50 mM	3.402 g
EDTA	10 mM	1.8612 g

Make up with RNase-free ingredients and DEPC-treated water. Adjust pH to 7.0 with glacial acetic acid (RNase-free)

A.3.3.2 DEPC-treated water

Constituent	Final concentration	Per 1 l
DEPC	0.1 %	1 ml

Add 999 ml of deionised water, keep under the fume hood until the reaction has stopped and autoclave.

A.3.3.3 Sample buffer

Constituent	Per 1.5 ml
Deionized formamide	750 µl
Formaldehyde	240 µlof 37 % solution
MOPS	150 µl of 10X stock
Glycerol	200 µl 50 % stock
Ethidium bromide	10 µl of 10 mg/ml stock
Bromophenol blue	Add 10 % stock dropwise until dark blue

A.3.4 Southern hybridization solutions

A.3.4.1 Pre-hybridization buffer

Constituent	Per 100 ml
BSA	10 ml of 10 % stock
EDTA (pH 8.0)	200 ml of 0.5 M stock
NaH_2PO_4	50 ml of 1M stock
SDS	35 ml of 20 % stock

Make up to 100 ml with deionized water.

A.3.4.2 20X SSC

Constituent	Per 1 litre
NaCl	175 g
tri-Na Citrate	88.2

Add 800 ml of deionized water, adjust the pH to 7.4. Make up to 1 litre and autoclave.

A.4 Hormones and salt treatments

A.4.1 Abscisic acid (ABA)

Constituent	Final concentration	Per 100 ml
ABA	100 µM	2.643 g
TWEEN 20	0.02 %	200 µl

Make up a 1 mM stock solution (i.e. 10x stock solution)

= 26.43 mg in 100 ml dH_2O

Do a 1:10 dilution (10 ml; ABA solution in 90 ml dH_2O) filter sterilize

Add 200 µl Tween 20, store at 4^0 C.

A.4.2 Methyl jasmonate (MeJA)

Constituent	Final concentration	Per 100 ml
MeJA	100 µM	22.43 µl

Add 22.43 µl into 100 ml dH_2O, store at 4^0 C.

A.4.3 NaCl (Sodium chloride)

Constituent	Final concentration	Per 500 ml
NaCl	100 mM	10 ml of 5M stock

A.5 Antibiotics

A.5.1 Ampicillin

Constituent	Final concentration	Per 10 ml
Ampicillin	100 µl/ml	100 mg/ml

Filter sterilize and store in aliquotes at -20^0 C.

A.6 Immunolocalization protein buffer

A.6.1 10X PBS stock

Constituent	Per 100 ml
NaCl	8 g
KCl	0.2 g
$Na_2HPO_4\_2H_2O$	1.44 g
KH_2PO_4	0.2 g

Dissolve in 60 ml of water, pH to 7.4 and make up to 100 ml.

A.6.2 1X PBS buffer

Constituent	Final concentration	Per 10 ml
BSA	1%	1 ml of 10% stock
PBS	1%	1 ml of 10X stock

Tween 20 working concentration = 50µl/ml

A.6 Immunolocalization protein buffer

A.6.1 10X PBS stock

Constituent	Per 100 ml
NaCl	8 g
KCl	0.2 g
$Na_2HPO_4\_2H_2O$	1.44 g
KH_2PO_4	0.2 g

Dissolve in 60 ml of water, pH to 7.4 and make up to 100 ml.

A.6.2 1X PBS buffer

Constituent	Final concentration	Per 10 ml
BSA	1%	1 ml of 10% stock
PBS	1%	1 ml of 10X stock

Tween 20 working concentration = 50µl/ml

APPENDIX B: METHODS AND PROTOCOLS

B.1 Mini-preparation isolation of *E. coli* plasmid DNA

1. Grow 5 ml LB broth cultures in an antibiotic of selection for overnight.
2. Spin 1.5 ml of culture in a 1.5 ml eppendorf for 40 seconds. Discard the supernatant and dispense another 1.5 ml culture and spin again. Discard all the supernatant.
3. Resuspend the pellet in a 0.1 ml of Solution 1. Make sure the cell pellet is properly lysed.
4. Keep the tubes at room temperature for 5-10 minutes.
5. Add 0.2 ml of solution 2. Mix it very gently by inverting the tube a few times and keep the tubes on ice for 2 minutes.
6. Add .150 ml of pre-chilled solution 3 and mix the resulting white flocculent gently too. This is a step that will precipitate the proteins or rather DNA. Keep them on ice for another 2 minutes.
7. Spin the tubes down for 5 minutes in a microcentrifuge at room temperature
8. Remove the supernatant and transfer to new 1.5 ml tube.
9. Add RNase A 10 mg/ml (10 µl), mix gently place them at on a heating block at 37^0 C for 30 minutes.
10. Add 450 µl of phenol and vortex the tubes for 40 seconds.
11. Spin the tubes down for 5 minutes at room temperature.
12. Very carefully, take the upper aqeous layer and transfer it into new microtube. Dispose the phenol layer properly.
13. Add 225 µl of phenol and same amount of chloroform-isoamyl alcohol. Vortex for 40 seconds and spin them down for another 5 minutes.
14. Transfer the upper layer into the next set of tubes and add 450 µl chloroform-isoamyl alcohol. Vortex and spin down for another 5 minutes. Transfer the upper layer only to the next set of tubes.
15. Add 45 µl of 3M NaAC to each tube, 450 µl or more of absolute ethanol to fill the tube.Shake the tubes gently by inverting a tube a few times.
16. Keep this at -20^0 C for at least 2 hours to overnight to precipitate the DNA.

17. Take the tubes out of the refrigerator and spin them down for 15 minutes at 4^0 C. The idea at this stage is that DNA is going to precipitate at the bottom of the tube.
18. Discard the supernatant and leave the precipitated DNA at the bottom. Add 70 % ethanol and spin down for 5 minutes . The importance of this step is to clean away the residual absolute ethanol and salts as far as possible.
19. Discard the ethanol using a pipette and air dry the pellet for 5 minutes.
20. Resuspend the pellet DNA in 20 µl TE buffer and leave it for 10 minutes at room temperature and then store at 4^0 C.

B.1.1 Restriction endonuclease digestion

1. Restriction digests of DNA were carried out using one of the four restriction buffers according to the salt requirements of the particular enzyme.
2. Multiple restrictions with different enzymes were combined together in a single digest provided the buffers are compatible.
3. The incubation temperature for all the buffers used were at 37^0 C.
4. 1-3 µg of DNA was digested in a volume of either 20 or 50 µl using 2 units of restriction enzyme per 1 µg of DNA (table A. 2.2.5).
5. Digests are usually performed for 2 hours to overnight.
6. For electrophoretic analysis, digestions are usually stopped by the addition of DNA sampling loading buffer.

B.1.2 Agarose gel electrophoresis

1. A horizontal submerged gel system for electrophoresis was used .
2. Agarose gel concentrations varied from 0.8-1.2 % depending on the sizes of the fragments.
3. The gel was stained during preparation by adding ethidium bromide (10 mg/ml).
4. DNA was loaded into wells in a volume between 20-50 µl containing 2-5 µl gel loading buffer.
5. Gels were run at constant voltages of 80-100 V for 1-2 hours or at 30 V for overnight in the case of large DNA gels for transfer and for Southern blot analysis. DNA fragment sizes were estimated against λ DNA Pst 1 marker loaded and ran on the same gel.

B.1.3 Electroelution of DNA restriction fragments from agarose gels

1. Digest sufficient DNA with the appropriate enzymes. Electrophorese on ethidium bromide stained agarose gels.
2. Excise the appropriate DNA band using the scapel and slide into dialysis tubing (pre-rinsed 2 X with 1 X TBE electrophoresis buffer and double knotted at one end.
3. Fill the tubing with 500 µl TBE electrophoresis buffer, seal and electroelute at 100 V for 30-60 minutes, depending on the size of the fragment. Look for the presence of DNA in the gel using short wavelength to see if electroelution is complete.
4. After electroelution is complete, reverse polarity of electrodes for 30 seconds at 100 V.
5. Open one end of the tubing and collect the eluted DNA in TBE and transfer it to new 1.5 eppendorff tube.
6. Concentrate DNA by isopropanol precipitation and resuspend in TE buffer.

B.1.4 Phenol-chloroform treatment and isopropanol precipitation of DNA

1. The volume of the reaction was made up to 180 µl with TE.
2. Added to it was 20 µl of phenol emulsified by shaking.
3. 1 Volume of $CHCl_3$/isoamyl alcohol (24:1) was added and emulsified by shaking.
4. The mixture was then spun down for 5 minutes and the aqueous top layer was recovered.
5. 1/10 volume of 5 M $NaClO_4$ and an equal volume of isopropanol was added. Mixed well, left on ice for another 5 minutes and then spun for 10 minutes.
6. The pellet was washed with 70 % ethanol and spun down for 5 minutes with the tube re-orientated.
7. The ethanol was decanted and the pellet air dried.
8. The pellet was then resuspended in TE buffer.

B.2 Maxi-preparation and isolation of *E. coli* plasmid DNA

1. Grow the cells in 200 ml culture with antibiotic of selection for overnight.
2. Pellet the cells by centrifugation in GSA tubes at 6 K for 10 minutes at 4^0 C. Remove all the supernatant.
3. Resuspend the cells well in solution 1. Leave it at room temperature for 5 minutes.
4. Add 4 ml of solution 2 , mix gently by inverting the tube. Leave it at room temperature for 5 minutes.
5. Add 6 ml solution 3, invert the tube gently and leave it for 5 minutes on ice.
6. Centrifuge at 10 K for 45 minutes. This leaves the supernatant to be loaded into the columns.
7. Equilibrate the columns with 2 ml of N2 solution which is added to the column directly. Repeat this 2 times.
8. Load the supernatant onto the column/cartridge and discard the flow-through.
9. Wash the cartridge with 4 ml of buffer N3. Repeat this twice and discard the flow-through.
10. Elute the plasmid DNA with 2.0 ml of N5 and dispense this in 2.5 ml eppendorf tubes.
11. Precipitate the purified plasmid DNA with 0.7 volumes of isopropanol.
12. Centrifuge at 10 000 rpm for 15-20 minutes.
13. Discard the supernatant and wash the DNA pellet with 70 % ethanol.
14. Dry the pellet and resuspend it in TE buffer. Store it at -20^0C.

B.3 Isolation of genomic DNA

1. Take and cut 4-5 young leaves into ± 1.5 cm long pieces, soak them in distilled water for 2-3 minutes to remove any insect that might have stuck.
2. Quick freeze them into liquid nitrogen and grind it into a fine powder in a sterile mortar and pestle.
3. Transfer the powder in a 30 ml SS34 tubes and fill the tube to about 500 ml of the powder. Never allow the specimen to thaw out during grinding and transfer.
4. Add 15 ml of extraction buffer (EB) and 150 µl of β-mercaptoethanol.
5. Add 1 ml of 20 % SDS, mix thoroughly by vigorous shaking. Incubate the tubes at 65^0 C for 10 minutes.

6. Add 5.0 ml of 5 M potassium acetate. Shake vigorously to break the cell wall to release DNA and incubate on ice for 20 minutes.
7. Spin the tubes at 12 000 rpm for 20 minutes. Carefully take off the supernatant and transfer to another set of new SS34 tubes containing 10 ml isopropanol. Gently mix and incubate the tubes at -20^0 C for 30 minutes to 2 hours.
8. Pellet the DNA at 10 000 rpm for 15 minutes. Gently pour off the supernatant and dry the pellet by inverting the tubes on paper towels for 10 minutes.
9. Re-dissolve the DNA pellet with 1 ml of TE buffer and transfer 500 ml of DNA in 1.5 eppendorff tubes.
10. Add 10 µl of RNase A and incubate at 37^0 C for 30 minutes.
11. Precipitate the DNA using Phenol; phenol-chloroform; chloroform, add 50 ml of sodium acetate and fill it up with absolute ethanol. Precipitate it for overnight.
12. Spin down the tubes for 15 minutes at 4^0 C.
13. Decant the supernatant and wash the pellet with 70 % ethanol and spin the tubes for 5 minutes.
14. Remove the ethanol and air dry the pellet
15. Dissolve the pellet in each tube with 50 µl TE buffer and store it at -20^0 C.

B.4 Protocol for RNA isolation fron *X. Viscosa* leaves

1. Take 4-5 young leaves and quick freeze them in a liquid nitrogen. Grind them into fine powder.
2. Transfer the powder using a spatula in a 1.5 ml eppendorff tubes and fill to about 500 µl of the powder. Never allo the specimen to thaw when grinding or transfering.
3. To each tube add about 1 ml of Trizol reagent and vortex vigorously.
4. Store the homogenates for 5 minutes at room temperature (RT) to permit complete dissociation of nucleoprotein complex.
5. Add 200 µl of chloroform, mix gently by inverting the tubes for 15 minutes.
6. Store the resulting mixture at RT for 2-15 minutes. Centrifuge it at 12 000 x g for 15 minutes at 4^0 C.
7. Transfer the aqueous phase to a fresh tube.
8. Precipitate RNA from aqueous phase by adding 500 µl of isopropanol.

9. Store the samples at RT for 10 minutes and centrifuge at 12 000 g for 8 minutes at 4^0 C.
10. Remove the supernatant and wash the pellet once with 75 % ethanolwith subsequent centrifugation at 7 500 g for 5 minutes at 25^0 C.
11. Discard the ethanol and air dry the pelletat RT.
12. Dissolve the pellet in DEPC-treated water and incubate at 55-60^0 C.

B.5 Preparation of slot blot analysis and transfer of DNA

1. Cut the nylon membrane and Whatmann's filter papers to "slot blot apparatus" size and use gloves whenever working with these.
2. Denature 1 µl of DNA made up to total of 50 µl plasmid DNA. Achieve this by adding NaOH and EDTA to a final concentration of 0.4 M NaOH and 10 mM EDTA pH 8 to a final volume of 100 µl. Heat the samples at 65- 100^0 C for 10 minutes to ensure complete denaturation.
3. Pre-wet the nylon membrane by placing the membrane gently at a 45^0 angle into a tray of sterile distilled water.
4. Assemble the slot blot apparatus, placing the pre-wetted filter paper first and the pre-wetted nylon membrane on top.
5. Load the samples (100 µl) into the respective slots. Apply a vacuum once all the slots have been filled with samples.
6. After the samples have filtered through each well or slot, turn the vacuum off. Add 100 µl of 0.4 M NaOH .
7. Remove the slot blotted membrane and cross-link DNA onto membrane using the short wavelength UV light for 5 minutes or a cross-linker.
8. Store the membranes safely between filter papers in a sealed plastic bag until further use.

B.6 Preparation of slot blot and transfer of RNA

1. Pre-wet the blotting by placing it gently at a 45^0 angle into a tray of wetting solution. Always wear golves when handling blotting membranes.
2. Assemble the apparatus.

3. 5 µg of RNA made up to 100 µl should be heated at 65^0 C to denature it prior to loadind it onto the wells.

4. Load the samples (100 µl) into the respective slots. Apply a vacuum once all the slots have been filled with samples

5. After the samples have filtered through each well, turn the vacuum off. Add 100 µl of DEPC-treated water and apply a vacuum again.

6. Remove the slot blotted membrane and crosslink the RNA using short wavelength.

7. Store the membranes safely between filter papers in a sealed plastic bag until further use.

B.8 Transformation of competent *E. coli* cells

1. Thaw frozen competent cells at room temperature until just molten and incubate on ice for 10 minutes, or use freshly prepared cells. Aliqoute about 20 µl of cells into a pre-chilled microtube.
2. Add up to 10 ng DNA in maximum volume of 10 µl, incubate on ice for 20 minutes.
3. Heat shock the cells at 42^0 C water-bath for 30 seconds and immediately return on ice for 2 minutes.
4. Add 80-100 µl of LB broth and shake for 50-60 minutes at 37^0 C.
5. Spin the cells for 30 seconds in micro-centrifuge, pour off the supernatant and leave in anything between 5-50 µl and resuspend the cells.
6. Plate onto LA plates with selective antibiotic and do dilutions if necessary.
7. Incubate the plates upside down at 37^0 C for overnight.

B.9 Random primed labeling with ^{32}P[dCTP]

1. Take 25 ng DNA to an eppendorff (insert) and make it up to 9 µl with distilled water.
2. Denature the DNA by heating for 5-10 minutes at 95^0 C and subsequent cooling on ice.
3. Add the following to an eppendorff vial on ice and make up to a final volume of 20 µl.

9 µl of denatured DNA

1 µl of dGTP

1 µl of dATP

1 µl of dTTP

2 µl of Rxn mix

1 µl of Klenow enzyme

5 µl of dCTP radioactively labeled

4. Incubate for 30 minutes at 37 ^{0} C.
5. Prepare a spun column
6. Prepare 20 µl of DNA labeled sample by adding 10 µl of tracking dye and 70 µl of STE buffer to make up to volume of 100 µl.
7. Take 1µl of this sample prior to passing it through a column and add it to the 2 ml scintillation fluid..
8. Pass it through a column with a resevor to collect the probe. Centrifuge this column for 8-10 minutes.
9. Take another 1 µl and add it to the 2 ml scintillation fluid, Determine the specific activity prior and after passing through a column.
10. Denature the probe again by heating at 95^{0} C and add the probe to the membranes in a hybridization buffer.
11. Leave the labeling for 18-24 hours at 65^{0} C.
12. Wash the membranes with low stringency buffer in a shaker at the same hyb temperature for 10 minutes. Following this high stringency wash is needed at the same temperature and same time. Careful not to wash all the probe.
13. Place the membrane in sealed plastic bag in an auto-radiograph cassette containing enhancer screens. Insert the X- ray film over the bag containing the membrane. Fix this at -70^{0} C for a few hours or days depending on the radioactivity bound to the membrane.
14. Thaw the cassette for 30 minutes at RT and develop it manually in a dark room.

B.10 Fixation and embedding and thin sectioninig

1. Cut up the specimens into small pieces.
2. Fix the tissue for overnight in 2.5% gluteraldehyde in PBS pH 7.4.
3. Wash in PBS (pH 7.4) twice for 5 min.
4. Postfix in osmium (1% in PBS)for 1 hour
5. Wash in PBS 2x 5 min.
6. Wash with water 2x 5min.
7. Fix in dehydration series:30%, 50%, 70%, 80%, 90%, 95% ethanol 1x 5 min in each.
8. Wash in 100% ethanol, then 100% acetone 2x 10 min in each.
9. Transfer into 50% resin 50% acetone and leave for overnight or longer.
10. Grdually replace acetone with resin usually for 2 days or more.
11. Embed the samples in resin blocks and solidify by placing samples in resin blocks and solidify by placing in oven at 60^0C for 16 hours.
12. Thin sectioning was done using the Reichert Ultracut S (Leica) ultramic

B.11 Immunogold labelling

1. Float on 15µl drop of water for 5 min.
2. Float on 0.02M glycine diluted in PBS for 3 min.
3. Float the grids on 1%BSA/1X PBS 2x for 3 min.
4. Dilute antibody in 1:50 and float your grids in it for 16 hours.
5. Wash them with 1% BSA/1X PBS + tween 20 5x for 2 min.
6. Float them in 1:100 of gold 10nm (with secondary antibody) in 1% BSA/1X PBS for ± 2 hours.
7. Washes 5x for 2 min in 1%BSA/1X PBS + tween 20.
8. Fix with 1% gluteraldehyde in PBS for 3 min
9. Wash the grids in water 5x for 2 min.
10. Stain with uramyl acetate for 10 min.
11. Wash with water 5x for 30 seconds.
12. Stain with lead acetate for another 10 min.
13. Wash thoroughly with drops of water.
14. Dry the grids, view and photograph in a JEOL 200CX transmission electron microscope.

APPENDIX C

COMPLETE NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCES OF CLONES

C.1 NUCLEOTIDE SEQUENCE OF *XVLEA*

ID *XVLEA* PRELIMINARY; DNA; 770 BP.
SQ SEQUENCE 770 BP; 248 A; 143 C; 235 .G; 144 T;

```
1      ATTCGGCACG AGGTTCACGG AACGACGGTG GATGAAACCA AAGAGAGGGT GAATGCTGCG
61     ATTCCGACCA TGGATGAAAC CAAGGAGAAG GTGAGCCCCG CCATGCAGGC GGCGAAGGAG
121    AAGAAAGAGG CTTGGTCTGA ATGGGTGGAA GATAAGCTAG AAGGATTGGG GCTTAAGATG
181    GAGAATAAAA TGGCGGCCGC AGACGACACC ACGGACAAAG CTGCAGAGGT TGCCATGAAG
241    GCCAAGGACT CTGCTTCAGG AGCTGCTGAA ACCTCATCTG AGAAGGCAGG AGAGAGCAAG
301    AACACCGTGA AAGACAAGGC GGCGAAGTCC GCGGACTGCA TGTCCGGCAA GGCCGAGGAT
361    GTGAAGGGGA AAACCACCGA AACCGCCGGG AAAGCATCAG AGGACGCCGG CGGGTCTACG
421    AGAGCGCCAA GCAGAAGATG GAAGAAGCCT ACGCTGCTGC CAGGGACAAG CTGGCTGAGA
481    ATTCTAAGGC AAGCTATGAA ACTGCGAAGG ATAAGATGTC CCAGGCGACT GGTGCCAAGG
541    CGGGCGAAGG GGAAGTCCGA GGAGCTGTAT ACAATCTCTC AGGCTATGTT TTTGAAATCA
601    TACGGAAACT ATATACGGAG GAGGGATCGG ATTTCGTCGA TTAATAATAT TTTCCTACTC
661    TGTAATATAT TATGTTATAT TTTCCTTTAT AAATTGTGTT GTGTTGTGTA TTGTGTGAGT
721    CGCGAAAGGA AATGAACATT CTAGTTCTTA AAAAAAAAAA AAAAAAAAA
```

C.1.1 AMINO ACID SEQUENCE OF *XVLEA* IN ITS PROPER FRAME

Translation of *XVLEA*(1-769)
Universal code
Total amino acid number: 254, MW=28501
Max ORF: 1-732, 244 AA, MW=27225

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1          ATTCGGCACGAGGTTCACGGAACGACGGTGGATGAAACCAAAGAGAGGGTGAATGCTGCG
1           I  R  H  E  V  H  G  T  T  V  D  E  T  K  E  R  V  N  A  A

61         ATTCCGACCATGGATGAAACCAAGGAGAAGGTGAGCCCCGCCATGCAGGCGGCGAAGGAG
21          I  P  T  M  D  E  T  K  E  K  V  S  P  A  M  Q  A  A  K  E

121        AAGAAAGAGGCTTGGTCTGAATGGGTGGAAGATAAGCTAGAAGGATTGGGGCTTAAGATG
41          K  K  E  A  W  S  E  W  V  E  D  K  L  E  G  L  G  L  K  M

181        GAGAATAAAATGGCGGCCGCAGACGACACCACGGACAAAGCTGCAGAGGTTGCCATGAAG
61          E  N  K  M  A  A  A  D  D  T  T  D  K  A  A  E  V  A  M  K

241        GCCAAGGACTCTGCTTCAGGAGCTGCTGAAACCTCATCTGAGAAGGCAGGAGAGAGCAAG
81          A  K  D  S  A  S  G  A  A  E  T  S  S  E  K  A  G  E  S  K

301        AACACCGTGAAAGACAAGGCGGCGAAGTCCGCGGACTGCATGTCCGGCAAGGCCGAGGAT
101         N  T  V  K  D  K  A  A  K  S  A  D  C  M  S  G  K  A  E  D

361        GTGAAGGGGAAAACCACCGAAACCGCCGGGAAAGCATCAGAGGACGCCGGCGGGTCTACG
121         V  K  G  K  T  T  E  T  A  G  K  A  S  E  D  A  G  G  S  T

421        AGAGCGCCAAGCAGAAGATGGAAGAAGCCTACGCTGCTGCCAGGGACAAGCTGGCTGAGA
141         R  A  P  S  R  R  W  K  K  P  T  L  L  P  G  T  S  W  L  R

481        ATTCTAAGGCAAGCTATGAAACTGCGAAGGATAAGATGTCCCAGGCGACTGGTGCCAAGG
161         I  L  R  Q  A  M  K  L  R  R  I  R  C  P  R  R  L  V  P  R

541        CGGGCGAAGGGGAAGTCCGAGGAGCTGTATACAATCTCTCAGGCTATGTTTTTGAAATCA
181         R  A  K  G  K  S  E  E  L  Y  T  I  S  Q  A  M  F  L  K  S

601        TACGGAAACTATATACGGAGGAGGGATCGGATTTCGTCGATTAATAATATTTTCCTACTC
201         Y  G  N  Y  I  R  R  R  D  R  I  S  S  I  N  N  I  F  L  L

661        TGTAATATATTATGTTATATTTTCCTTTATAAATTGTGTTGTGTTGTGTATTGTGTGAGT
221         C  N  I  L  C  Y  I  F  L  Y  K  L  C  C  V  V  Y  C  V  S

721        CGCGAAAGGAAATGAACATTCTAGTTCTTAAAAAAAAAAAAAAAAAAAA
241         R  E  R  K  *  T  F  *  F  L  K  K  K  K  K  K
```

C.2.1 NUCLEOTIDE SEQUENCE OF *XVDH*

```
ID   XVDH      PRELIMINARY;   DNA;  1255 BP.
SQ   SEQUENCE   1255 BP;   379 A;   313 C;   334 G;   224 T;  5 OTHER;

1       TTCGGCACGA GACAACAATA CCAATCCAAC GATCTCAGCA AAAGGCAATT GAGAGTCTTT
61      CTTTGGTTTG TTGTAAAATC TAAGATGGAA GGTTTCGGGA ACCAAGACCA ACTCCGCCGC
121     AACGACCGCA CCAGCGAGCA CACCGCGCCT GGCCAGGGCA TGCACCCTAC CCAGCAGGGA
181     ACTGGCAAAG GCACCAACGA GTTCGCCCCT ACCGGCCAGG GTGTCTTCGG CGGCCAGCAA
241     CACCACCAGA ATAAACAGAA GGGAATGGGC ACTGGTGCCG GCATTACCGA CAAGCTCCAC
301     CGCTCCAACA GCTCCAGCTC TAGTTCTGAG AGTGATGGAG AAGGAGGGAG GAGGAAGAAG
361     GGAATTAAGG AGAAGATCAA GGAGAAAATC CCAGGCCAGC ACAACCAAGG AGCCACCGGC
421     CACCAGGGCT TCACTCAGAA CAAGCAAGGC TATGGAGCCA CTGGGCAGCA CGGGCAGCAG
481     GAAGGAATGA TGGACAAGTT CAAAGACAAC CTTCCTGGGA ATCACTTAAC CTGTCATCTA
541     TCACTAGGGT TGTATGAATA TATATGTATG CTTTCAACCA GGGTGCTGGC TGCTGTGTGT
601     ACCGTGGTGT TTTTCTTTTT TCTTGTTTAT TTTGTGTGTG TGTGTGTGTG TGTGAATAAA
661     TCATGTGTGT GTGTCCGTGA ATGCACATTA AGGCTGTCTA TGCCCTGTAA CATTATGCCT
721     GCTATATATA TATGAATGCA CTGTAATTCA CGTAAAAAAA AAAAAAAAAA
```

C.2.2 AMINO ACID SEQUENCE OF *XVDH* IN ITS PROPER FRAME

```
Translation of XVDH(1-770)
Universal code
Total amino acid number: 244, MW=28345
Max ORF: 369-554, 62 AA, MW=6938

1         TTCGGCACGAGACAACAATACCAATCCAACGATCTCAGCAAAAGGCAATTGAGAGTCTTT
1            R  H  E  T  T  I  P  I  Q  R  S  Q  Q  K  A  I  E  S  L

61        CTTTGGTTTGTTGTAAAATCTAAGATGGAAGGTTTCGGGAACCAAGACCAACTCCGCCGC
20        S  L  V  C  C  K  I  *  D  G  R  F  R  E  P  R  P  T  P  P

121       AACGACCGCACCAGCGAGCACACCGCGCCTGGCCAGGGCATGCACCCTACCCAGCAGGGA
40        Q  R  P  H  Q  R  A  H  R  A  W  P  G  H  A  P  Y  P  A  G

181       ACTGGCAAAGGCACCAACGAGTTCGCCCCTACCGGCCAGGGTGTCTTCGGCGGCCAGCAA
60        N  W  Q  R  H  Q  R  V  R  P  Y  R  P  G  C  L  R  R  P  A

241       CACCACCAGAATAAACAGAAGGGAATGGGCACTGGTGCCGGCATTACCGACAAGCTCCAC
80        T  P  P  E  *  T  E  G  N  G  H  W  C  R  H  Y  R  Q  A  P

301       CGCTCCAACAGCTCCAGCTCTAGTTCTGAGAGTGATGGAGAAGGAGGGAGGAGGAAGAAG
100       P  L  Q  Q  L  Q  L  *  F  *  E  *  W  R  R  R  E  E  E  E

361       GGAATTAAGGAGAAGATCAAGGAGAAAATCCCAGGCCAGCACAACCAAGGAGCCACCGGC
120       G  N  *  G  E  D  Q  G  E  N  P  R  P  A  Q  P  R  S  H  R

421       CACCAGGGCTTCACTCAGAACAAGCAAGGCTATGGAGCCACTGGGCAGCACGGGCAGCAG
140       P  P  G  L  H  S  E  Q  A  R  L  W  S  H  W  A  A  R  A  A

481       GAAGGAATGATGGACAAGTTCAAAGACAACCTTCCTGGGAATCACTTAACCTGTCATCTA
160       G  R  N  D  G  Q  V  Q  R  Q  P  S  W  E  S  L  N  L  S  S

541       TCACTAGGGTTGTATGAATATATATGTATGCTTTCAACCAGGGTGCTGGCTGCTGTGTGT
180       I  T  R  V  V  *  I  Y  M  Y  A  F  N  Q  G  A  G  C  C  V

601       ACCGTGGTGTTTTTCTTTTTTCTTGTTTATTTTGTGTGTGTGTGTGTGTGTGTGAATAAA
200       Y  R  G  V  F  L  F  S  C  L  F  C  V  C  V  C  V  C  E  *

661       TCATGTGTGTGTGTCCGTGAATGCACATTAAGGCTGTCTATGCCCTGTAACATTATGCCT
220       I  M  C  V  C  P  *  M  H  I  K  A  V  Y  A  L  *  H  Y  A

721       GCTATATATATATGAATGCACTGTAATTCACGTAAAAAAAAAAAAAAAAAA
240       C  Y  I  Y  M  N  A  L  *  F  T  *  K  K  K  K  K
```

C.3.1 NUCLEOTIDE SEQUENCE OF *XVLEC*

```
1       ACGAGGTTCC ATGTTGTAGC TTACCTTCAG CAGATTTGGT GGTTCGAGGT TAGTGGAGAG
61      GTAGACTTCC CATTCCCTCA GGGCACATAT AGCCTCTTCT TCAGGCTTCA TCTGGGTCGT
121     CCATCAAAGA GGCTGGGGAG AAGGACCTAC AACCCTGACC ACATCCACGG GTGGGATATT
181     AAACCAGTGA GGTTCCAGCT CTCTACATCC GATGATCAGC AGGCTCTATC GAAATGTTAT
241     TTGGATGATC CTGGCAGTTG GATGTATTAT CGAGTAGGCG ATTTTGTTGT TGGGAACTCG
301     TATGCATCTA CAAAGCTGAA GTTTTCCATG ACGCAGATCG ATTGCACTCA TACAAAAGGG
361     GGTCTCTGTG TGGATTCTGT GGTAATATAC CCCAAGGGAA GTAAGCAGGA GAAGGCACTT
421     TCCGCATATG CGTAGCATTG TAGTTAGCTT AGGTGAAGTG AATATAATCA AGTACAACGG
481     GATAGTTTTG GTTTCTCCGT AGGCTACTTG CATTGAAGAT ATCTTGTACA GATCAGTTGC
541     ATCATAGGAA AATTTTGTAT GTACATTTTA TGCTGTATTC TCTCATAACT CTTCAGAATG
601     AAATACAACC ACCCGTAGAG GCTTGGATTG TAAAAAAAAA AAAAAAAAA
```

C.3.2 AMINOACID SEQUENCE OF *XVLEC* IN ITS PROPER FRAME

Translation of *XVLEC*(1-649)
Universal code
Total amino acid number: 209, MW=24283
Max ORF: 1-432, 144 AA, MW=16547

```
1         ACGAGGTTCCATGTTGTAGCTTACCTTCAGCAGATTTGGTGGTTCGAGGTTAGTGGAGAG
1          T  R  F  H  V  V  A  Y  L  Q  Q  I  W  W  F  E  V  S  G  E

61        GTAGACTTCCCATTCCCTCAGGGCACATATAGCCTCTTCTTCAGGCTTCATCTGGGTCGT
21         V  D  F  P  F  P  Q  G  T  Y  S  L  F  F  R  L  H  L  G  R

121       CCATCAAAGAGGCTGGGGAGAAGGACCTACAACCCTGACCACATCCACGGGTGGGATATT
41         P  S  K  R  L  G  R  R  T  Y  N  P  D  H  I  H  G  W  D  I

181       AAACCAGTGAGGTTCCAGCTCTCTACATCCGATGATCAGCAGGCTCTATCGAAATGTTAT
61         K  P  V  R  F  Q  L  S  T  S  D  D  Q  Q  A  L  S  K  C  Y

241       TTGGATGATCCTGGCAGTTGGATGTATTATCGAGTAGGCGATTTTGTTGTTGGGAACTCG
81         L  D  D  P  G  S  W  M  Y  Y  R  V  G  D  F  V  V  G  N  S

301       TATGCATCTACAAAGCTGAAGTTTTCCATGACGCAGATCGATTGCACTCATACAAAAGGG
101        Y  A  S  T  K  L  K  F  S  M  T  Q  I  D  C  T  H  T  K  G

361       GGTCTCTGTGTGGATTCTGTGGTAATATACCCCAAGGGAAGTAAGCAGGAGAAGGCACTT
121        G  L  C  V  D  S  V  V  I  Y  P  K  G  S  K  Q  E  K  A  L

421       TCCGCATATGCGTAGCATTGTAGTTAGCTTAGGTGAAGTGAATATAATCAAGTACAACGG
141        S  A  Y  A  *  H  C  S  *  L  R  *  S  E  Y  N  Q  V  Q  R

481       GATAGTTTTGGTTTCTCCGTAGGCTACTTGCATTGAAGATATCTTGTACAGATCAGTTGC
161        D  S  F  G  F  S  V  G  Y  L  H  *  R  Y  L  V  Q  I  S  C

541       ATCATAGGAAAATTTTGTATGTACATTTTATGCTGTATTCTCTCATAACTCTTCAGAATG
181        I  I  G  K  F  C  M  Y  I  L  C  C  I  L  S  *  L  F  R  M

601       AAATACAACCACCCGTAGAGGCTTGGATTGTAAAAAAAAAAAAAAAAAA
201        K  Y  N  H  P  *  R  L  G  L  *  K  K  K  K  K
```

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