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**Molecular characterization of *ALDRXv4*, an aldose reductase orthologue isolated from *Xerophyta viscosa*, in response to abiotic stress**

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**A dissertation submitted in fulfilment of the requirements for the degree of Master in Science in the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, South Africa**

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## Abbreviations

14-3-3 protein	A signaling molecule acting by kinase modulation and protein-protein interactions
ABA	abscisic acid
ABRE	ABA-responsive element (PyACGTGGC)
<i>ALDRXv4</i>	the gene encoding an aldose reductase orthologue isolated from <i>Xerophyta viscosa</i>
AP	ascorbate peroxidase
APS	ammonium persulfate
AR	aldose reductase
Bp	base pairs
bZIP	A family of transcription factors with basic region and Leucine-zipper motif
cDNA	complementary DNA
CDPK	calcium-dependent protein kinase
<i>COR</i>	A family of genes encoding <i>cold responsive</i> polypeptides
dCTP	deoxycytidine triphosphate
DEPC	diethylpyrocarbonate
DRE	dehydration-responsive element (TACCGACAT)
DW	dry weight
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immuno-sorbent assay
$F_v/F_m$	quantum efficiency
FW	fresh weight
GR	glutathione reductase
h	hours
HPLC-PAD	high performance liquid chromatography-pulsed amperometric detection
HNE	4-Hydroxynonenal
IgG	immunoglobulin G

IPKS	isoleucine, proline, lysine, serine motif
$k_{cat}$	catalytic constant of an enzyme
$K_m$	Michaelis-Menten constant
LEA	late-embryogenesis abundant protein
MAPK	mitogen activated protein kinase
MAPKKK	A protein kinase that phosphorylates MAPKK
min	minutes
MYB	A family of transcription factors with tryptophan cluster motif
MYC	A family of transcription factors with basic-helix loop-helix (bHLH) and Leucine-zipper motif
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate hydrogenase
O/N	overnight
ORE	osmotic response element
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PSII	photosystem II
rDNA	ribosomal deoxyribonucleic acid
ROS	reactive oxygen species
RWC	relative water content
S6K	ribosomal S6 protein kinase
SDS	sodium dodecyl sulfate
SNF1	sucrose non-fermenting1
SOD	superoxide dismutase
SOS	A family of genes encoding <i>salt overly sensitive</i> polypeptides
TBS	tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TIM	triosephosphate isomerase

T <sub>m</sub>	melting temperature
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume per volume
WW	wet weight
w/v	weight per volume

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## Abstract

The leading factor that reduces crop yield is abiotic stress. The world's rising population and decline in available arable land has led to the exploration of methods enabling the utilisation of traditionally non-arable land. The resurrection plant *Xerophyta viscosa* is a possible source of genes that may confer abiotic stress tolerance to sensitive species. A putative aldose reductase orthologue, *ALDRXv4*, was isolated from *X. viscosa* utilizing the method of 'complementation by functional sufficiency' in osmotically stressed *E. coli*. Expression of *ALDRXv4* in both leaf and root tissue was investigated by northern and western blot analyses. Gene expression in the leaves was investigated in response to salinity, low temperature, abscisic acid and dehydration with subsequent rehydration and in roots under dehydration conditions. In leaf tissue *ALDRXv4* expression did not appear to alter in response to salinity or low temperature, but was found to be upregulated in response to desiccation stress and to be potentially ABA responsive. *ALDRXv4* was detected in plants during dehydration and subsequent rehydration and throughout the ABA treatment. In the roots *ALDRXv4* was expressed during dehydration, but the protein was detected at full turgor and levels decreased as the stress persisted. AR catalyses the reduction of D-glucose to sorbitol. HPLC analysis identified the presence of sorbitol in both hydrated phloem exudates and root tissue. It is proposed that *ALDRXv4* may be induced in the stress response in *X. viscosa*, either resulting in the synthesis of compatible solutes or having a detoxification function. The production of sorbitol may result in osmoprotection or be involved in plant signalling between different organs. This may aid in enabling this plant to tolerate desiccation.

# **CHAPTER ONE**

## **INTRODUCTION**

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## Introduction

By the year 2050 the world's population is expected to be nearly double that of its current figure (Herrera-Estrella, 2000). Ninety percent of the additional population will inhabit developing countries. The challenge to feed an ever-growing population must be undertaken whilst considering that the next 50 years will see less arable land available due to deforestation, overgrazing and soil deprecation from excessive cultivation of the land (Kishore and Shewmaker, 1999). African agricultural productivity per unit of cultivated land is the lowest in the world, with this continent having among the poorest soils (Wambugu, 1999). In addition there will be a reduction in non-renewable resources used in the production of fertilizers and cattle feed and less fresh water worldwide, particularly in African countries where perennial water sources are scarce (Kishore and Shewmaker, 1999). Areas such as the Middle East also face these issues, however these countries can afford to import food, whereas sub-Saharan African countries cannot (Dysan, 1999). In order for these areas to become self-sustainable and not have to rely on foreign food aid, an increase in productivity is needed.

Among the leading factors that restrict agricultural productivity worldwide are abiotic stresses, such as those associated with temperature, drought and salinity (Cherry *et al.*, 1999). A study by Askew (1995) reported that drought and soil acidity could be responsible for over 80% of yield loss in South Africa. A prediction for the food shortage that this region could experience was determined, based on the average cereal yield from 1989 to 1991. In the year 2025, the average yield per year has been calculated to be approximately 1.5 tons per hectare, which could result in a shortage of 88.7 million tons of cereal (Dysan, 1999). Certain first world countries, such as those in Europe, Oceania and Northern America, should produce surplus cereal yields. However even if distribution problems were dealt with a global shortage of 68.5 million tons is still predicted.

There are many ways in which agricultural production can be improved, such as the use of fertilizers, improving pest control, soil and water conservation, and the use of improved crop varieties, developed either by traditional means or by utilizing genetic engineering (Herrera-Estrella, 2000). Of all these options, the use of biotechnology to

develop transgenic crops is likely to be the most promising solution for developing countries, if introduced into their systems properly.

Most crop plants have not been selected for their capability to tolerate stress, therefore their ability to endure such conditions is normally restricted (Grover *et al.*, 2001). If crops that can tolerate these stresses are to be cultivated it is imperative to conduct research on those plants that have evolved to possess such traits.

### **1.1 Plant Water Deficit and Stress Responses**

Many crop plants are vulnerable to even minimal dehydration stress and very few organisms can endure dehydration to the air-dried state (Mundree and Farrant, 2000). Water is central in the functioning of these organisms. It is required as a biological solvent and electron donor in biochemical reactions, as an intra- and intercellular transport medium and as an evaporative coolant (Bohnert *et al.*, 1995). Water is also vital in maintaining plant cell structure (Meryman, 1974).

The response to water deficit in the majority of higher plants can result in a variety of changes, and can have detrimental effects. These include physiological alterations at the whole plant and cellular level, reductions in photosynthetic activity and heightened reactive oxygen species (ROS) production (Hideg *et al.*, 2003).

The plant stress response is not well understood, as complex regulatory and signalling cascades dictate the expression of stress responsive genes (Bray, 1993). Not enough is known about the means with which plants detect drought or the functioning and identity of the various response mechanisms that are activated (Scott, 2000). The current understanding of the signal transduction cascades and protein products thought to be involved is shown in Figure 1. Activation of these pathways results in the expression of genes believed to function in stress tolerance and those that regulate the signal transduction pathways involved in the stress response (Shinozaki and Yamaguchi-Shinozaki, 1997).

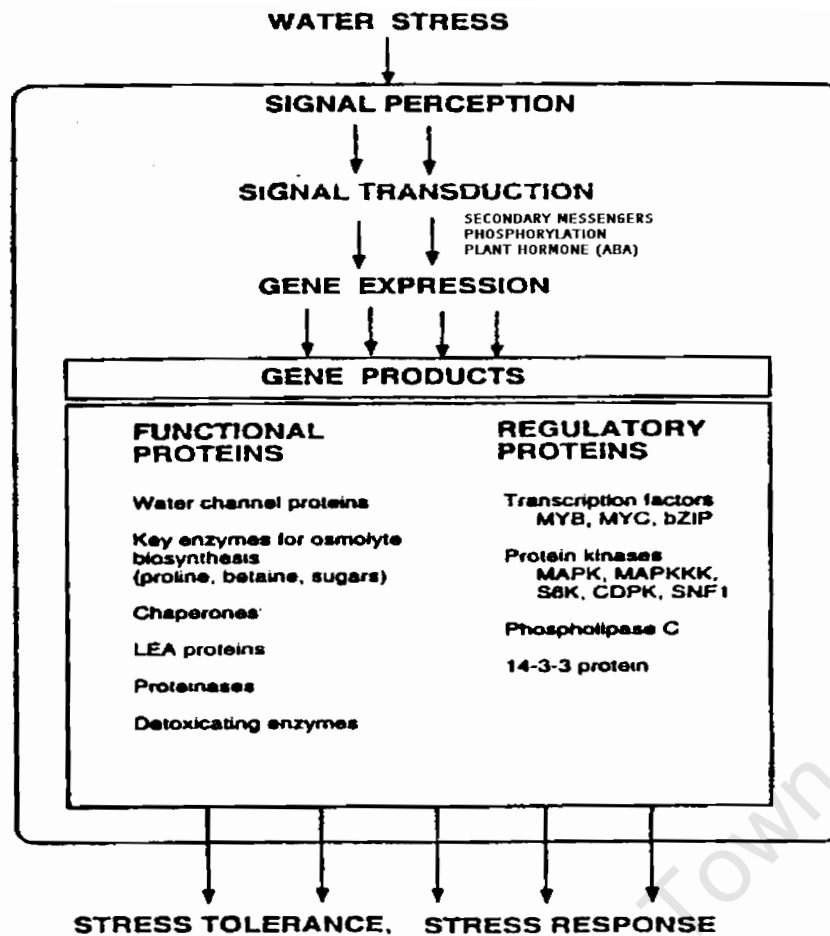


Figure 1. The signal transduction cascades and gene products induced due to water deficit stress. The gene products expressed are generally either functional proteins, which are involved in tolerating the stress, or regulatory proteins that are involved in signal transduction and regulating gene expression (modified from Shinozaki and Yamaguchi-Shinozaki, 1997).

## 1.2 Resurrection Plants

There are plants that contain desiccation-tolerant vegetative tissue. They include certain algae, bryophytes, lichens, ferns, and a unique group of angiosperms known as “resurrection” plants (Oliver *et al.*, 1998). These have the ability to survive extremes of dehydration and consequent rehydration from the air-dried state (Gaff, 1971). Their ability to tolerate desiccation enables them to colonise niches on rocky outcrops where water and shade are limiting (Sherwin and Farrant, 1998). Several resurrection plants are being studied in order to understand what enables them to have this “resurrectional” ability.

### 1.2.1 Mechanisms of Desiccation Tolerance

All resurrection plants employ a combination of mechanisms that protect and repair the cellular contents to avoid or cancel out the effects of dehydration and consequent rehydration on the plant cells (Sherwin and Farrant, 1996). However, there are two general strategies utilised which enables division of angiosperm resurrection plants into groups, being either poikilochlorophyllous or homoiochlorophyllous. Poikilochlorophyllous species lose their chlorophyll upon dehydration. This breakdown of certain pigments occurs in tandem with the dismantling of the plants' thylakoid membranes, and is done in order to prevent the formation of free radicals during water stress. Examples of these include *Xerophyta viscosa* and *Eragrostis nindensis* (Mundree *et al.*, 2002). Homoiochlorophyllous species retain their chlorophyll, but shield it from possible photosynthetic activity by curling or folding the leaves so that some leaves are shaded from and are thus protected (Sherwin and Farrant, 1996). *Craterostigma wilmsii* and *Myrothamnus flabellifolius* are examples of such plants (Sherwin and Farrant, 1998; Farrant 2000). Recovery from desiccation stress in homoiochlorophyllous species is more often than not faster than that in poikilochlorophyllous plant species, due to reassembly of cellular machinery (Sherwin and Farrant, 1996).

Resurrection plants use different mechanisms in order to survive up to 95% water loss and this survival is believed to be due to these plants having the ability to:

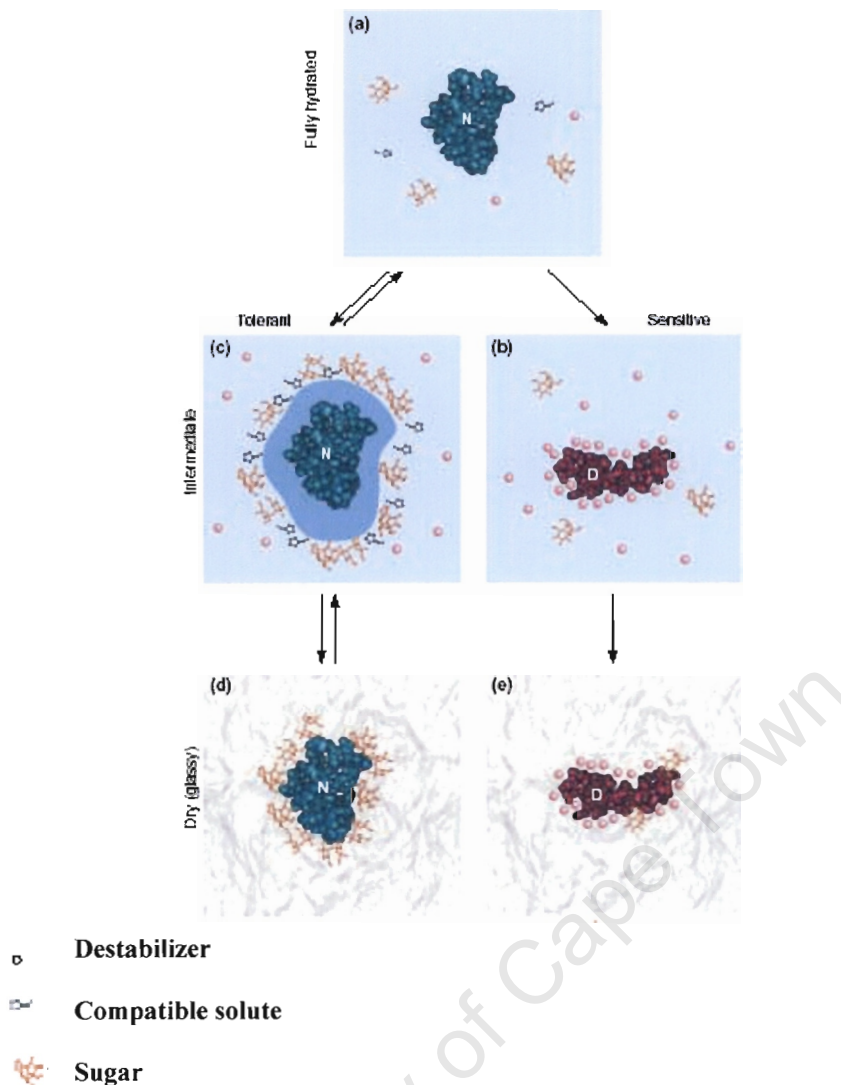
- minimize mechanical damage caused by loss of turgor in the plant cells,
- preserve the integrity of the membranes and macromolecules within the cell by the accumulation of compatible solutes and certain water stress proteins,
- reduce free radicals and other toxins accumulating and damaging the cellular contents as metabolic activity starts to break down (Vertucci and Farrant, 1995).

It is important that plant cell turgor is not disrupted in order to maintain the structure of the plasmalemma, which is attached to the cell wall via plasmodesmata (Mundree and Farrant, 2000). In conditions where the plant is free from desiccation stress, the cell possesses a water-filled vacuole that maintains this turgor. In desiccation intolerant species the vacuole will lose water and decrease in size during dehydration.

This can result in shrinkage and tearing of the plasmalemma as it pulls away from the plant cell wall. Hydrolytic enzymes, which are normally found between the plasmalemma and the plant cell wall, are released into the cell, which can result in damage to cellular macromolecules. In extreme cases, as the cell loses turgor the cell wall collapses. This is termed mechanical stress (Levitt, 1960).

Compatible solutes reduce the mechanical damage caused by cellular dehydration. These compounds, also known as osmoprotectants, are soluble compounds that have no net charge at physiological pH (McNeil *et al.*, 1999). These compounds are not toxic when found in high concentrations in the cell. The accumulation of compatible solutes, particularly sugars, is important in stabilizing membranes, protecting proteins, contributing to cellular osmoregulation (Whittaker *et al.*, 2001), filling vacuoles to minimize mechanical stress (Farrant 2000; Mundree and Farrant, 2000) and they raise the osmotic pressure in the cytoplasm (McNeil *et al.*, 1999). These compounds interact with macromolecules through hydrogen bonding, thus preventing protein denaturation and increasing the  $T_m$  of organelle and cell membranes (Hoekstra *et al.*, 2001a). When the cellular water level drops below a critical level, osmoprotectants also form part of the glassy matrix of the cytoplasm, preventing hazardous interactions by reducing molecular mobility. Interactions between osmoprotectants and proteins at various stages of hydration are illustrated in Figure 2.

Plant cells undergoing photosynthesis can easily be damaged during desiccation due to the presence of high light (Sherwin and Farrant, 1998). When water is limiting the guard cells surrounding the stomata become flaccid, resulting in their closure (Raven and Johnson, 1990). This action prevents the loss of more water. In the absence of water, excitation energy is absorbed by chlorophyll and transferred to oxygen (Sherwin and Farrant, 1998). This can lead to the formation of oxygen free radicals, which react directly with cellular constituents, such as proteins, amino acids and nucleic acids, and can result in the peroxidation of lipid membranes (Ramanjulu *et al.*, 2003). Lipid peroxidation can result in a chain reaction generating chemically reactive cleavage products, such as alkanes, aldehydes, ketones and hydroxyl acids.



**Figure 2. Compatible solute and protein interactions at various stages of water content. (a) In fully hydrated cells the structure of the native protein (N) is favored. As water is lost from the cell a glassy matrix forms and results in increased interaction between cellular macromolecules. (b) In sensitive cells the lack of accumulated compatible solutes and sugars can result in destabilization molecules to bind proteins, causing them to denature (D). (c) In tolerant cells the presence of osmoprotectants results in exclusion of destabilization molecules, therefore preserving the protein in the native form. Compatible solute binding results in preferential hydration of the protein surface, as visualized by the blue ring. (d) As water is lost the ‘hydration ring’ is replaced by sugars, which stabilize the proteins native structure through hydrogen bonding. The arrows indicate reversibility during dehydration and rehydration (Hoekstra *et al.*, 2001b).**

Resurrection plants prevent this damage caused by surplus excitation energy in different ways. Some, such as *C. wilmsii*, employ specific leaf movements in order to prevent certain regions of their leaves being exposed to light, while others, such as *X. viscosa*, dismantle their photosynthetic apparatus in order to prevent light chlorophyll interactions (Sherwin and Farrant, 1998). Another mechanism of avoiding excess light damage is by the production of certain pigments, such as carotenoids or anthocyanins, which absorb or reflect light. Most resurrection plants also produce enzymes that quench free radicals, such as ascorbate peroxidase (AP), glutathione reductase (GR) and superoxide dismutase (SOD). Proteases and heat shock proteins, with potential chaperonin function are also synthesized in order to promote repair and degrade proteins modified by oxidation (Ingram and Bartels, 1996; Zhu, 2002). Late-Embryogenesis Abundant (LEA)/ dehydrin-type proteins describe a group of proteins initially notified as products of genes abundantly expressed in seeds during their final stage of acquisition of desiccation tolerance (Baker *et al.*, 1988; Ingram and Bartels, 1996) and have since been identified in vegetative tissue in response to osmotic stress (Bray, 1993). An association between survival and accumulation of these proteins under dehydration conditions has been reported, with these proteins having a variety of proposed functions including renaturation of unfolded proteins, macromolecular protection, ion sequestration (Dure 1993), water binding and osmoprotection (Ingram and Bartels, 1996).

### **1.3 *Xerophyta viscosa***

*X. viscosa* Baker (Family Velloziaceae) is a monocotyledonous angiosperm with 'resurrectional' abilities. Most resurrection plants grow in dry environments, such as those found in southern America, western Australia and southern Africa (Scott, 2000). *X. viscosa* is endemic to southern Africa, Madagascar and southern America. It grows in rocky outcrops, mainly in shallow soil (Hartung *et al.*, 1998).

#### **1.3.1 Mechanisms of Desiccation Tolerance**

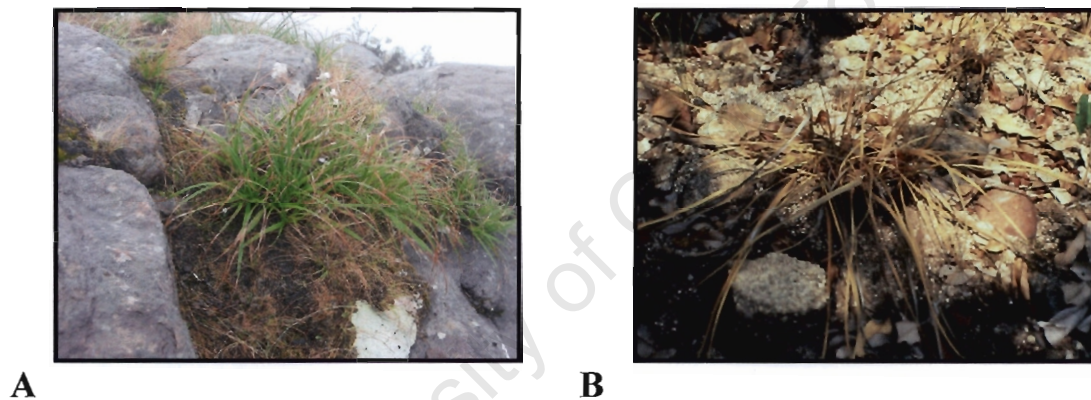
Research on *X. viscosa* has concluded that this species tolerates desiccation stress in a number of ways. *X. viscosa* cells, under normal conditions, contain a large central vacuole which, when undergoing dehydration, fragments into many smaller ones (Mundree and Farrant, 2000). As water leaves the cell, these smaller vacuoles fill with

non-aqueous material, therefore retaining the cells' turgor and preventing mechanical stress on the cell membrane. The identity of this non-aqueous material is not known. The predominant soluble sugar that accumulates in the leaves of all resurrection plants studied during dehydration is sucrose (Ghasempour *et al.*, 1998; Scott, 2000), and sucrose accumulation is believed to be important for acquiring desiccation tolerance. During dehydration *X. viscosa* shows a dramatic increase in sucrose, from around 120  $\mu\text{mol/g DW}$  to about 230  $\mu\text{mol/g DW}$ , between 89% and 10% relative water content (RWC) (Mundree and Farrant, 2000; Whittaker *et al.*, 2001). This sugar, as well as other compounds such as sorbitol, anthocyanins, various other compatible solutes and even proteins, may be the material which replaces water in these vacuoles (Mundree and Farrant, 2000). This phenomenon of fragmenting and packing of these vacuoles with non-aqueous substances is also seen in other resurrection plants and desiccation-tolerant seeds when undergoing dehydration (Farrant and Sherwin, 1997).

Dehydration can also result in plants being subjected to light-related damage, such as the formation of free radicals (Sherwin and Farrant, 1998; Van Breusegem *et al.*, 2001). As already mentioned, in order to avoid free radical damage in the plant cells, *X. viscosa* destroys its chlorophyll and disassembles its thylakoid membranes into small vesicles (Mundree and Farrant, 2000). Due to this practice photosynthesis in *X. viscosa* does not take place when the cell has less than 55% RWC. Termination of photosynthesis occurs before the photosynthetic apparatus is completely disassembled and while other cellular metabolism is still taking place. It has been proposed that this is a specifically controlled mechanism done in order to prevent free radical formation during dehydration.

In order to decrease the leaf surface area exposed during dehydration, *X. viscosa* leaves fold in half along the midrib (Figure 3) (Sherwin and Farrant, 1998). The leaves become yellow as the plant breaks down its chlorophyll, and then become dark purple due to the accumulation of protective pigments. Certain pigments have been suggested to be involved in light damage avoidance, for example carotenoids and anthocyanins. However in *X. viscosa* the levels of carotenoids are seen to drop during desiccation and only return to normal levels once the leaves are fully rehydrated (Mundree and Farrant, 2000). This could be due to the fact that *X. viscosa* dismantles

its photosynthetic apparatus, as carotenoids are one of the chief pigments used during photosynthesis (Sherwin and Farrant, 1998). However, an increase in anthocyanin content during dehydration has been recorded in the leaves (Mundree and Farrant, 2000). Anthocyanins are thought to protect chlorophyll, act as filters to avoid excess light being absorbed and have anti-oxidant properties (Smirnoff, 1993). Anthocyanin content also remains high during rehydration and is thought to have the above-mentioned functions during reassembly of the thylakoid membranes and production of chlorophyll (Sherwin and Farrant, 1998). The leaf surfaces of the *X. viscosa* plant that lie away from the centre of the plant develop a sticky reflective covering which may be present in order to reduce the light absorbed by the leaf and also may have a role in temperature control.



**Figure 3. *X. viscosa* Baker in (A) the hydrated state and (B) the dehydrated state. Note the curling in of the leaves in order to decrease the leaf area exposed to light.**

Anti-oxidant enzymes are also produced during dehydration and rehydration. Enzymes such as peroxiredoxins are synthesized in response to stress conditions (Mundree *et al.*, 2002). AP and GR activity increases during dehydration and return to control levels during rehydration (Sherwin and Farrant, 1998). These enzymes could protect against harm caused by light or respiration-induced free radical formation (Mundree and Farrant, 2000). SOD extracted from *X. viscosa* leaf material over a range of dehydration and rehydration values was found to have an increase in activity during drying and returned to control levels during rehydration.

### 1.3.2 Rehydration Processes

Rehydration processes are also an important factor in desiccation tolerance, as this is the period when desiccation-induced damage has to be repaired and metabolic activity must be restored (Sherwin and Farrant, 1996).

*X. viscosa* has been shown to recover fully and regain complete metabolic activity after 120 h of rehydration (Sherwin and Farrant, 1998). It has also been shown that in *X. viscosa* a certain amount of damage to the cell does occur during desiccation, however this is repaired during rehydration. Electrolyte leakage during the dehydrated state indicates that the integrity of the plasma membrane is not maintained, suggesting an alteration in membrane structure at this stage (Sherwin and Farrant, 1996). However this modification to the plasma membrane is repaired or reversed upon rehydration, as leakage rates return to control levels upon rehydration, indicating recovery of these cells. The chloroplasts, which are dismantled in times of water stress, become completely functional when the plant is rehydrated and the cells' chlorophyll content, which too degrades during desiccation, reaches normal levels after 120 h (Sherwin and Farrant, 1998).

According to some criteria *X. viscosa* is considered a “modified” desiccation tolerant plant, as it survives desiccation only when drying occurs over several hours and complete recovery only occurs after 24 h of rehydration (Oliver *et al.*, 1998). These “modified” resurrection plants are thought to survive desiccation due to their protection mechanisms induced during dehydration, with repair mechanisms playing a lesser, but accompanying role. This is in contrast to “fully” desiccation tolerant species that can survive rapid drying rates, such as the moss *Tortula ruralis*. These plants rely chiefly on the mechanisms induced during rehydration to repair cellular damage, with constantly expressed protection mechanisms allowing it to survive swift desiccation.

It is necessary to improve our understanding of mechanisms of desiccation tolerance in resurrection plants and identify the genes and gene products involved in desiccation tolerance, in both dehydration and rehydration stress, in order to genetically engineer these qualities into desiccation-intolerant crop plants.

## 1.4 Abiotic Stress Tolerance

As described above, many putative mechanisms of tolerating desiccation have been described in resurrection plants. However other abiotic stresses, such as salinity, low and high temperature, create practical problems such as significant loss in crop yields and restrictions to locations where crop and horticultural species can be successfully cultivated (Thomashow, 1998). Strong relationships between desiccation, salinity and high and low temperature tolerances have been observed (Cushman and Bohnert, 2000; Grover *et al.*, 2001; Zhu, 2002). This is because most of these stresses ultimately result in dehydration and oxidative stress at the cellular level by inhibiting water absorption and transport (Shi *et al.*, 2002).

### 1.4.1 Salinity Tolerance

In the plant cell excess salt results in ion toxicity and an overload of salt in the medium causes hyperosmotic stress that impairs water absorption and transport. These effects on the cell can result in secondary stresses such as nutritional imbalance and oxidation (Zhu, 2000). In order to survive these stresses it is imperative that a cell regain ion and water homeostasis (as cited by Shi *et al.*, 2002). Salinity stress therefore results in osmotic stress being applied on the cell. A universal response to osmotic stress in all cells studied is the accumulation of osmolytes (McCue and Hanson, 1990).

In most studies *Arabidopsis thaliana* is used as the model system. This species is generally not particularly salt tolerant, however recent studies have shown that salt-sensitive plants also have salt tolerance genes and *A. thaliana* mutants have been isolated that are tolerant to elevated levels of NaCl, KCl, LiCl, K<sub>2</sub>SO<sub>4</sub> and mannitol (Zhu, 2000). Results of this nature are encouraging, as they illustrate that plants sensitive to certain stresses do have the necessary genes required for tolerance, indicating that it is the mechanism of regulating these genes that is important.

There are five salt tolerance genes in *A. thaliana*: *SOS* (*Salt Overly Sensitive*) 1 through *SOS5*. Elucidation of the signaling cascades of these genes has been initiated (Zhu, 2002). Excess intra- or extracellular Na<sup>+</sup> prompts the *SOS* pathway, causing a cytosolic Ca<sup>2+</sup> signal (Zhu, 2000), which results in alterations in expression and

activity of cellular ion transporters, such as  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{H}^+$  transporters (Zhu, 2002). It is postulated that a change in turgor indicates an osmotic stress, which may activate SOS-independent protein kinases, initiating osmotic stress signaling cascades (Zhu, 2001). These cascades may trigger alterations in gene expression and/or activity of osmolyte biosynthesis enzymes and osmolyte transport systems (Zhu, 2002).

Although these SOS genes have been identified (Shi *et al.*, 2000; 2002) no comprehensive mechanism for salt tolerance has yet been elucidated. These studies have however resulted in a few important discoveries; firstly that although many salt-responsive genes point out damage due to salt stress, these genes are not necessarily involved in tolerance, and secondly that many of the genes involved in salt stress tolerance are not induced by the stress (Zhu, 2000). This applies to other genes and gene products involved in abiotic stress tolerance in plants. Associations between oxidative detoxification and multiple stress tolerance have been noted. Plants that have been genetically modified to overexpress reactive oxygen scavenging osmolytes have been shown to have increased tolerance to salt, cold and heat stress. This further emphasizes the link between tolerance to these above-mentioned stresses, and their association to oxidative and desiccation stress.

#### **1.4.2 Low Temperature Tolerance**

Studies on *A. thaliana* have shown that freeze-induced dehydration can have a variety of effects that lead to cellular damage, such as the precipitation of numerous molecules and denaturation of proteins (Thomashow, 1998). Preventing or reversing cold-induced denaturation of proteins, precipitation of these molecules and reducing the direct physical damage that results from the accumulation of intercellular ice are possible mechanisms that can contribute to cold tolerance.

Freezing tolerance in plants is often developed during gradual exposure to low, non-freezing temperatures. This is known as “cold acclimation” (Thomashow, 1998) and enhanced freezing tolerance is found after this and treatment with high light intensities (Wanner and Junttila, 1999). This latter response has been attributed to increased photosynthetic carbon fixation due to the presence of intense light. Carbon fixation is necessary for the accumulation of sucrose and other compatible solutes, which ameliorate against the low temperature stress.

Another mechanism that has been found to contribute to cold tolerance is the stabilization of membranes against cold-induced damage (Thomashow, 1998). Simple sugars, such as sucrose, accumulate in the cell during cold acclimation and appear to function in stabilizing the cells' membranes, sequestering toxic ions and protecting proteins against denaturation (Steponkus, 1984). Other osmoprotectants that are expressed at high levels in the cell during stress may also fulfill this function. The accumulation of these soluble sugars during cold stress corresponds to the degree of freezing which the plant cell endures, indicating a direct relation between sugar accumulation and freezing tolerance (Wanner and Junttila, 1999).

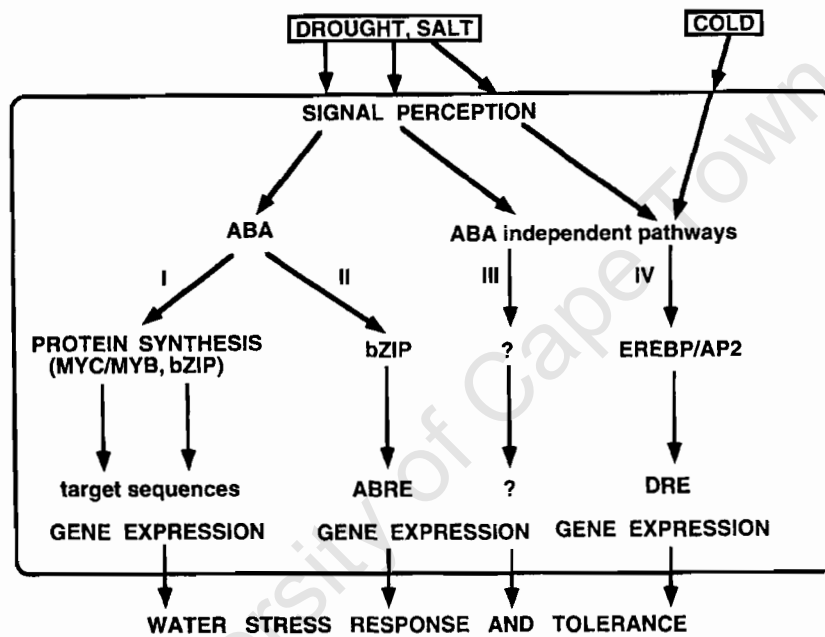
Many *A. thaliana* genes have been identified that could be involved in cold acclimation. These include a number of genes encoding *COR* (*cold responsive*) polypeptides, homologs of LEA proteins (Thomashow, 1998) and heat shock proteins (as reviewed by Xin and Browse, 2000). However the role of most of these genes in freezing tolerance remains unclear (Wanner and Junttila, 1999). Some, such as *COR15a* are expressed in response to both cold and dehydration stresses, illustrating the relationship between responses to these stresses.

### **1.5 Abscisic Acid**

The seeds of most higher plants have a stage in which they develop desiccation tolerance (Ramanjulu and Bartels, 2002). The phytohormone abscisic acid (ABA) is thought to be involved in many processes associated with this development (Bartels *et al.*, 1991; Hoekstra *et al.*, 2001a). Studies have shown that the ability of vegetative tissues in many plants to develop dehydration tolerance increases with the application of ABA (Shinozaki and Yamaguchi-Shinozaki, 1997). This is also true for a plants ability to tolerate salinity and cold stress. ABA production is activated by the onset of an osmotic or cold stress, which can be seen by the dramatic increase in ABA accumulating in the leaves of resurrection plants such as *Xerophyta humulis*, *M. fabellifolia* and *Craterostigma* species during water stress (as cited by Scott, 2000). The production of this hormone results in the induction and regulation of various genes (Shinozaki and Yamaguchi-Shinozaki, 1997; Bray, 2002). Although ABA has been seen to be required for alterations in gene expression for certain stress-

response genes, many genes have been identified that are induced by osmotic stress but not by ABA (Bray, 2002).

It has been postulated that there are a number of possible pathways that may result in regulation of stress-response genes (Figure 4) (Shinozaki and Yamaguchi-Shinozaki, 1996), including ABA-dependent and independent pathways. Although the ABA-independent ones do not require the presence of this hormone for expression during stress, the genes induced in these pathways respond to exogenous ABA. According to this hypothesis cold stress results in the activation of only one of these pathways, whereas water and salinity stress activate all four proposed pathways (Figure 4). However it is possible that interactions and overlap between these pathways exist (Bray, 2002).

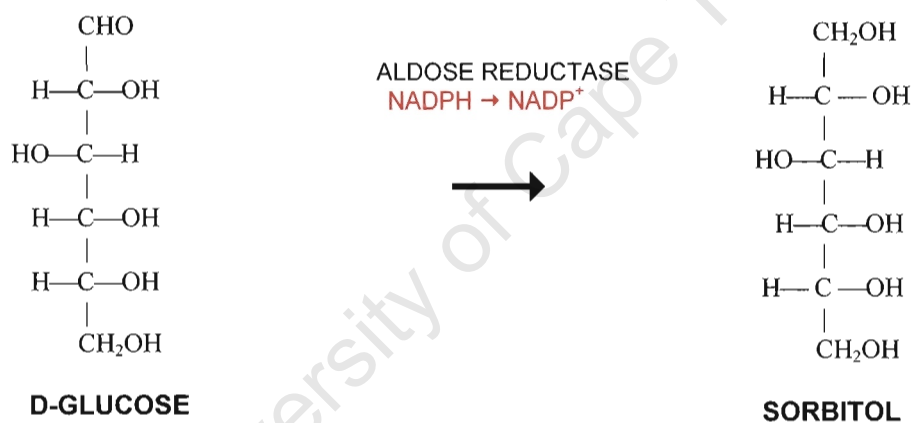


**Figure 4.** The proposed signal transduction pathways involved in the response to abiotic stresses that ultimately lead to alterations in gene expression. Pathways I and II are abscisic acid (ABA) dependent and pathways III and IV are ABA independent. Overlap and interactions between these pathways is suspected (Shinozaki and Yamaguchi-Shinozaki, 1997).

## 1.6 Aldose Reductase

### 1.6.1 General Characteristics

Aldose reductase (AR) is an enzyme having an  $\alpha/\beta$ -barrel, or triosephosphate isomerase (TIM) barrel structure (Ondrechen *et al.*, 2001) and is part of the aldo-keto reductase superfamily (Bohren *et al.*, 1989). These are cytosolic, monomeric enzymes, with a broad substrate specificity, that catalyze the NADPH-dependent reduction of aldo-sugars and a variety of aromatic and aliphatic aldehydes to their related alcohols. Specifically, AR is an enzyme found in the polyol pathway, a pathway by which non-phosphorylated sugars and sugar alcohols are interconverted. In this pathway AR catalyses the reduction of glucose to sorbitol (Figure 5; Ko *et al.*, 1997).



**Figure 5. Reduction of D-glucose to sorbitol, the reaction catalyzed by aldose reductase. This reaction is NADPH dependent, and the oxidation of NADPH to NADP<sup>+</sup> is indicated in red.**

### 1.6.2 AR Isolated from Animal Cells

Sorbitol is a compatible osmoprotectant, which helps sustain cell volume and electrolyte contents during hyperosmosis without disrupting the cellular protein structures (reviewed by McNeil *et al.*, 1999). Rabbit renal medullary cells, situated in the central region of the kidney that are subject to steep osmotic gradients, are rich in AR and transcription of this enzyme with subsequent increases in levels of sorbitol

occur during extracellular hyperosmolality (Ferraris *et al.*, 1996; Kultz *et al.*, 1997). Increased expression of this enzyme during hyperosmolality has also been found in cells such as Chinese hamster ovary cells (Hyndman *et al.*, 1997) and Chang liver cells (Ko *et al.*, 1997).

The *in vivo* function of AR is still under dispute. This enzyme has been suggested to be involved in maintaining osmotic balance and cellular volume by catalyzing the reduction of glucose to sorbitol (Ko *et al.*, 1997). Due to the fact that aldose and aldehyde reductases have high reactivity with many aldehydes, AR has also been suggested to have a detoxification role (Roncarati *et al.*, 1995).

The expression of AR in human cells is osmoregulated, indicating that this enzyme is inducible, and increased expression occurs during both dehydration and rehydration (Ko *et al.*, 1997). It has also been found that in the testis AR, together with sorbitol dehydrogenase, catalyze the reaction converting glucose to fructose, having sorbitol as an intermediate. Fructose is an important energy source in these cells.

Glucose is not the preferred substrate of AR (Cao *et al.*, 1998). This is illustrated by looking at a few  $k_{cat}/K_m$  values, which represent the catalytic efficiency of the enzyme with respect to that particular substrate (Garrett and Grisham, 1995). For example with glucose as a substrate  $k_{cat}/K_m = 2.8 \times 10^2 \text{ M}^{-1}\text{min}^{-1}$ , whereas with 4-hydroxynonenal (HNE) as a substrate  $k_{cat}/K_m = 4.6 \times 10^6 \text{ M}^{-1}\text{min}^{-1}$  and methylglyoxal  $k_{cat}/K_m = 1.8 \times 10^7 \text{ M}^{-1}\text{min}^{-1}$  (Cao *et al.*, 1998). HNE is a toxic lipid aldehyde produced during oxidative stress and methylglyoxal is a toxic by-product of glucose metabolism (Cao *et al.*, 1998; Oberschall *et al.*, 2000). This indicates that AR may have other functions during oxidative stress, besides its role in the production of sorbitol. This enzyme may also be involved in detoxifying the above-mentioned damaging molecules produced during cellular metabolism (Cao *et al.*, 1998). This is supported by the fact that various members of the aldo-keto reductase superfamily can detoxify HNE and reduce methylglyoxal to a non-toxic form (Vander Jagt *et al.*, 1995) and further supported by a study on giant cell arteritis (GCA), an inflammatory disease, where AR directly functions as an HNE-detoxifying enzyme (Rittner *et al.*, 1999).

Keightley *et al.* (2004) developed an oxidative stress resistant human cell line that showed a 4-fold increase in AR levels in comparison to control lines. Induction of this enzyme in control cells resulted in protection against two toxic aldehydes; acrolein, an aldehyde in the same class as HNE which are products of lipid peroxidation, and glycolaldehyde, which results from amino acid oxidation. AR expression appears to result in better protection against acrolein than glycolaldehyde toxicity, with respect to both cell survival rate and catalytic efficiency.

AR expression in rabbit renal medullary cells and human liver cells is induced during dehydration through an osmotic response element (ORE) in the 5' flanking region of the AR gene (Ferraris *et al.*, 1996; Ko *et al.*, 1997). A similar model of activating osmotic response elements can be seen in bacteria, where hyperosmotic stress results in transcription from the *proU* operon (Ferraris *et al.*, 1996). This operon encodes for the transport system necessary for accumulating betaine, an osmolyte. *proU* is also osmoregulated by an upstream activating region (as cited by Ferraris *et al.*, 1996). In human liver cells experimental evidence suggests that AR expression is regulated by an increase in salt levels in the medium (Ko *et al.*, 1997). This supplies further evidence that AR expression is induced by desiccation stress, as salt stress ultimately results in cellular dehydration.

### 1.6.3 AR Isolated from Plant Cells

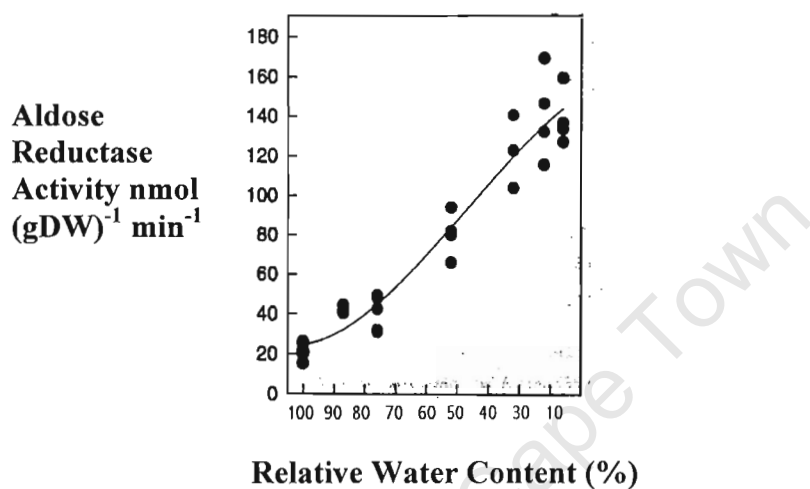
The first putative AR to be reported in plants was isolated from mature barley (*Hordeum vulgare*) embryos by Bartels *et al.* (1991). The gene encoding a potential AR was found to be part of a small subset of genes induced at the start of desiccation, and thus believed to be involved in the acquisition of desiccation tolerance in barley seeds. Many genes expressed at this stage are found to be involved in seed maturity, but some have been found to have a role in osmotic stress tolerance, which is the proposed role of this isolated AR orthologue. This gene appears to encode transcripts that respond to ABA and are repressed by gibberellic acid. It is thought that a similar protein is present in *Craterostigma plantagineum*. This is because a protein of similar molecular weight was detected when extracts from mature *C. plantagineum* dehydrated leaves were probed with the antibodies raised against the barley AR (Bartels *et al.*, 1991).

Putative aldose/aldehyde reductase genes have since been isolated from a number of different plant species. An AR orthologue associated with freezing tolerance has been isolated from cultured bromegrass (*Bromus inermis* Leyss) cells (Lee and Chen, 1993). Li and Foley (1995) reported the isolation and identification of an AR gene from dormant wild oat (*Avena fatua*) embryos which is ABA and dehydration stress inducible. Two aldose/ketose reductase genes were isolated from foxglove (*Digitalis purpurea*) leaves, which were developmentally regulated and showed upregulation under dehydration, salt, heat and wounding stresses (Gavidia *et al.*, 2002); and an AR related protein has been identified in alfalfa (*Medicago sativa*) cells subjected to oxidative stress (Oberschall *et al.*, 2000). This alfalfa gene was transformed into tobacco plants and the transformants showed increased tolerance to oxidative stress caused by heavy metal, paraquat and dehydration treatment. They also showed enhanced recovery following rehydration. Some lines showed a lower concentration of toxic aldehydes and the recombinant alfalfa AR was shown to react with HNE. A potential link between dehydration and UVB response was found in these plants (Hideg *et al.*, 2003). Pre-exposure to a non-lethal, reversible water stress resulted in enhanced tolerance to a subsequent UVB treatment, implying an association between these stresses at the ROS scavenging stage. These results are encouraging for the use of AR to be able to aid in conferring drought tolerance to transgenic crops.

#### **1.6.4 AR Isolated from *X. viscosa***

An AR orthologue (*ALDRXv4*) has been identified in *X. viscosa* Baker (Mundree *et al.*, 2000). In comparison to that isolated by Bartels *et al.* (1991) this transcript is not embryo specific, nor developmentally regulated, but isolated from vegetative tissue. Isolation was carried out utilizing the method of 'complementation by functional sufficiency' in osmotically stressed cells. This proteins' ability to confer tolerance in osmotically stressed conditions is illustrated by the fact that *Escherichia coli* (*srl::Tn10*) containing the *ALDRXv4* cDNA clone survived in medium containing 1.25 M sorbitol, whereas the strain transformed with vector lacking the cDNA clone did not. The *ALDRXv4* clone is 1144 bp with an open reading frame of 960 bp, encoding a protein of 319 amino acids, with a calculated  $M_r$  of 35.667. It contains the tetrapeptide IPKS, which is highly conserved between ARs (Bohren *et al.*, 1991). In this motif the lysine residue has been found to be involved in NADPH binding.

Expression studies of *ALDRXv4* have shown that the encoded protein is not found in fully hydrated *X. viscosa* leaves, but is present in leaves having RWCs of 85%, 37% and 5% (Mundree *et al.*, 2000). This finding correlates with enzyme activity analysis, which showed that AR activity increases as the RWC in the leaves drops (Figure 6). The highest mean values were obtained for leaves having between 32% and 15% RWC.



**Figure 6. Aldose reductase activity in *X. viscosa*. This figure illustrates the correlation between the increase in enzyme activity and decrease in RWC (Mundree *et al.*, 2000).**

Although the AR protein was detected at 5% RWC, the mRNA transcripts were not (Mundree *et al.*, 2000). This could be related to the fact that cellular respiration in *X. viscosa* ceases at less than 15% (Mundree and Farrant, 2000), at which stage transcription of the gene could have stopped, but the protein may still be preserved at this low moisture content.

### 1.7 Aims of this Dissertation

The aim of this work was the molecular characterization of *ALDRXv4*. The expression patterns of this gene in response to salinity, low temperature, ABA and dehydration, with subsequent rehydration, were investigated through northern and western blot analyses. The presence of sorbitol was investigated in leaves, phloem and roots. It is hoped that an increased understanding of the molecular functioning of *ALDRXv4* and

its gene product will aid in the generation of transgenic plants with improved tolerance to abiotic stresses.

University of Cape Town

**CHAPTER TWO**  
**MATERIALS AND METHODS**

University of Cape Town

# Materials and Methods

## 2.1 Plant Material and Treatments

Mature *X. viscosa* plants were collected from the Cathedral Peak nature reserve in the Drakensburg mountains (Kwazulu Natal, South Africa). Plants were potted and grown under greenhouse conditions as described by Sherwin and Farrant (1996).

In order to determine whether salinity induces *ALDRXv4* expression a plant was watered with 1 l 150 mM NaCl, every 24 h, over a period of 96 h. The plant was maintained in a controlled environment chamber [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) 25°C; 8 h dark] and was equilibrated to the stated conditions for one month prior to the treatment. Samples were taken at 0, 6, 12, 24, 48, 72 and 96 h. To impose a low temperature stress a plant was kept at 4°C over a period of 120 h. For the duration of the treatment the plant was maintained under environmental conditions of 16 h light ( $160 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) 8 h dark. Samples were taken at 0, 6, 12, 24, 48, 72, 96 and 120 h. To determine whether ABA had an effect on *ALDRXv4* expression the leaves and soil of a plant was sprayed with 1 l 100  $\mu\text{M}$  ABA (resuspended in 100% ethanol), every 24 h, over a period of 96 h. The plant was equilibrated in a controlled environment chamber [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) 25°C; 8 h dark] one month prior to the experiment. Samples were taken at 0, 6, 12, 24, 48, 72 and 96 h subsequent to application of ABA. For a leaf dehydration and rehydration treatment nine *X. viscosa* plants were held without water for approximately 13 days, until an average leaf RWC of 7% was maintained for each plant. They were kept in a controlled environment chamber [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) 25°C; 8 h dark]. The plants were equilibrated to this environment for one month preceding the treatment. Leaf samples were taken at full turgor and after 5, 8, 9, 11 and 13 days without water. Sampling again took place at 18, 42 and 66 h after watering. Material from three replicates was pooled for each sampling point. Root dehydration treatments were conducted by withholding water from two *X. viscosa* plants for approximately 16 days. The plants were equilibrated and maintained under greenhouse conditions, having an average light intensity of  $1000 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ . Samples were taken at full turgor, 10 and 16 days without water. Material from the two replicates was pooled for each sample.

## 2.2 Physiological Assays

### 2.2.1 Relative Water Content

RWC was determined on leaf samples, using a minimum of three replicates per sampling point. The following weights were determined:

Fresh weight (FW) is the weight of the tissue at the time of sampling.

Dry weight (DW) is the weight of the tissue after drying at 80°C for a minimum of 48 h.

Wet weight (WW) is the weight of the tissue at full turgor, after being submerged in distilled water for 24 h.

RWC was calculated using the formulae previously described by Barrs and Weatherly (1962).

$$\text{Water content A (WCA)} = \frac{\text{FW} - \text{DW}}{\text{DW}}$$

$$\text{Water content B (WCB)} = \frac{\text{WW} - \text{DW}}{\text{DW}}$$

$$\text{RWC} = \frac{\text{WCA}}{\text{WCB}} \times 100$$

RWC could not be determined for roots as bound particulate matter interfered with the determination of the above weights. Sampling was conducted according to the drying rate of the leaves.

### 2.2.2 Chlorophyll Fluorescence

The quantum efficiency ( $F_V/F_M$ ) of photosystem II was determined using a portable fluorometer (OS 500, Optiscience, USA), according to Sherwin and Farrant (1996). A minimum of four replicates were measured at each sampling point. Leaves were dark acclimated for 10 min prior to measurement. A saturating light, with an intensity of approximately 4 mmol photons  $\text{m}^{-2} \text{s}^{-1}$  having a duration of about 1 s, was utilized to determine initial and maximum fluorescence,  $F_0$  and  $F_M$  respectively.  $F_V$  was calculated by subtracting  $F_0$  from  $F_M$  and  $F_V/F_M$  was thus determined.

These data were determined for the plants treated with high salinity, low temperature and ABA, but not those exposed to a dehydration and rehydration treatment. This is

because the effect on photosynthesis of these two treatments had previously been determined (Sherwin and Farrant, 1996; Mundree and Farrant, 2000).

## **2.3 Gene Expression Analysis**

### **2.3.1 RNA Isolation**

*X. viscosa* leaf and root samples were flash frozen in liquid nitrogen and stored at -80°C. In order to remove bound particulate matter, the root material was rinsed in distilled water for no longer than 3 seconds. Unfortunately the effect of this rinsing on the relative water content of this material could not be accounted for. Total RNA was isolated from this tissue using the TRIZOL<sup>®</sup> LS Reagent (Life Technologies, USA). Isolation was carried out according to the manufacturer's instructions, with the following amendments. Plant material was ground using liquid nitrogen and the cells were lysed by agitation on a Vortex Genie 2 (Scientific Industries, USA) in 1 ml TRIZOL<sup>®</sup> LS Reagent. A high salt precipitation [0.8 M tri-sodium citrate, 1.2 M NaCl, 50% (v/v) isopropanol] at -80°C was performed in replacement to the standard 100% isopropanol precipitation for the isolation of root RNA in order to remove polysaccharides. RNA was resuspended in 0.5% (w/v) sodium dodecyl sulphate (SDS) in diethyl pyrocarbonate (DEPC) treated water and stored at -80°C.

### **2.3.2 Synthesis of the *ALDRXv4* Probe**

The cloned *ALDRXv4* gene was isolated from *X. viscosa* as previously described (Mundree *et al.*, 2000). This cDNA insert in pBluescriptSK+ (Stratagene, La Jolla, CA) was supplied by SG Mundree. The pSK-*ALDRXv4* construct was isolated from *E. coli* cells using the High Pure Plasmid Isolation Kit (Roche, Germany), according to the manufacturer's instructions. The correct insert size was confirmed by incubation with *EcoRI* and *XhoI* restriction endonucleases. In a 20 µl reaction, 500 ng of the pSK-*ALDRXv4* construct was incubated with 10 units each of *EcoRI* and *XhoI* O/N at 37°C (results not shown). The correct insert sequence was confirmed by nucleotide sequence analysis (results not shown). The *ALDRXv4* insert was isolated by restriction with *XhoI* and *XbaI*. In a 30 µl reaction, 120ng of plasmid DNA was incubated with 15 units each *XhoI* and *XbaI* for approximately 5 h at 37°C. Restriction fragments were electrophoresed in a 1% agarose gel stained with ethidium bromide, excised from the gel using a sterile blade under short wavelength ultraviolet

light and purified using the High Pure PCR Product Purification Kit (Roche, Germany), following the manufacturer's protocol. DNA was quantitated both spectrophotometrically (Sambrook *et al.*, 1989), and visually on a quantitative agarose gel using lambda DNA of known concentrations. The *ALDRXv4* gene was radiolabelled with [ $\alpha$ - $^{32}$ P]-dCTP using a Megaprime labeling kit (AP Biotech, U.K), according to the manufacturer's instructions.

### 2.3.3 Northern Blot Analysis

Approximately 8  $\mu$ g RNA was electrophoresed in a 1.2% (w/v) agarose gel. Equal loading of RNA was determined by ethidium bromide staining of the gel. 10  $\mu$ l of 100% Formamide (Saarchem, S.A.), 1.3  $\mu$ l of 100% formaldehyde (Saarchem, S.A.) and 10X MOPS buffer [0.2 M 3-(N-morpholino) propanesulfonic acid (Mops), 0.05 M NaAC and 0.001 M EDTA, pH 7.0] was added to each sample prior to loading. The RNA was transferred to a Hybond-XL nylon membrane (AP Biotech, U.K.) utilizing a downward transfer method modified from Koetsier *et al.* (1993). This method was modified by the use of 20X SSC (1X SSC consists of 3 M NaCl, and 0.3 M tri-sodium citrate) as transfer buffer and the use of 20X SSC-soaked sponges on top of the agarose gel in replacement of a wick. The RNA was allowed to transfer for 3 h. RNA was cross-linked onto the membrane using a Hoefer<sup>TM</sup> UVC 500 Crosslinker (Amersham Biosciences, UK).

The membranes were blocked at 65°C in hybridization buffer [1 M NaHPO<sub>4</sub>, 0.5 M EDTA, 20% (w/v) SDS and 10% (w/v) bovine serum albumin] for 2 h, with agitation, in a hybridization oven (Amersham biosciences, UK). Hybridization of the *ALDRXv4* probe was conducted for approximately 18 h at 65°C with agitation. The membranes were washed with wash buffer A [0.5% (w/v) SDS and 2X SSC] and, when required, wash buffer B [0.1% (w/v) SDS and 0.5X SSC]. In the case of the membranes containing RNA isolated during the NaCl, low temperature and dehydration/rehydration treatments, the membranes were exposed to high performance autoradiography film (Amersham Biosciences, UK) in a light proof cassette at -80°C. The autoradiographs were developed manually. The membrane containing RNA isolated during the ABA treatment was exposed to a storage phosphor screen (Kodak, USA) in a light proof cassette at room temperature. Images

were developed using the Bio-Rad Molecular Imager<sup>®</sup> FX and analyzed utilizing Quantity One quantitation software (Bio-Rad, USA).

All membranes were subsequently probed with a constitutively expressed gene, 18S rDNA. The 18S rDNA was radiolabelled with [ $\alpha$ -<sup>32</sup>P]-dCTP using PCR. The PCR reaction was performed using the following protocol: (1) 95°C for 5 min (2) 95°C for 1 min (3) 55°C for 0.5 min (4) 72°C for 1.5 min (5) 72°C for 5 min. Steps 2-4 were repeated 30 times. The membranes were blocked as described above. Hybridization was conducted for 3 h at 65°C and the membranes were washed with wash buffer A and B. All membranes were exposed to a storage phosphor screen (Kodak, USA) in a light-proof cassette at room temperature and developed as described above.

#### **2.3.4 Protein Isolation**

*X. viscosa* plant material was flash frozen and stored at -80°C. Total protein was isolated according to Swidzinski *et al.* (2004), with the following amendments to the protocol. 0.2 - 0.3g of leaf and 0.1g of root material was ground in liquid nitrogen and allowed to thaw, with agitation, in chilled extraction buffer [0.5 M Tris-HCl, pH 7.5; 10 mM EDTA; 1% (v/v) Triton X-100, 2% (v/v)  $\beta$ -Mercaptoethanol]. Following the phenol precipitation, the protein pellet was resuspended in Laemmli buffer [0.625 M Tris-HCl, pH 6.8; 2% (w/v) SDS; 10% (v/v) glycerol; 5% (v/v)  $\beta$ -Mercaptoethanol]. Protein was stored at -20°C.

#### **2.3.5 Enzyme Linked Immuno-Sorbent Assay**

Polyclonal antibodies were raised against the purified ALDRXv4 protein in rabbits and supplied courtesy of Alice Maredza. Recombinant ALDRXv4 protein was diluted in a range from 1:400 to 1:204800 in phosphate-buffered saline [PBS (1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 137 mM NaCl, pH 7.4)]. ALDRXv4 antibody was diluted in a range from 1:400 to 1:12800 in blocking buffer [1X PBS; 3% (w/v) skimmed milk powder]. The various recombinant protein dilutions were inoculated into the wells of a Nunc-Immuno<sup>™</sup> 96 MicroWell<sup>™</sup> Plate, Maxisorp<sup>™</sup> surface (Nunc, Denmark) and incubated at 4°C O/N. The protein was washed three times with 1X PBS and incubated with blocking buffer for 2 h at room temperature. These samples were washed again three times with 1X PBS and incubated O/N at 4°C

with the various ALDRXv4 antibody dilutions. The recombinant protein-antibody complex was washed three times with 1X PBS and incubated with anti-rabbit IgG alkaline phosphatase conjugate (1:10 000 dilution) for 2.5 h at 37°C. This was washed three times with 1X PBS. Detection was done utilizing  $\rho$ -Nitrophenyl phosphate tablets (Sigma Fast<sup>TM</sup>, Germany), according to the manufacturers recommendations. Absorbance at 405 nm was determined using a Titertek Multiskan<sup>®</sup> Plus MK II (Flow laboratories, UK).

### 2.3.6 Western Blot Analysis

Proteins were separated according to size by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). All stacking gels were 5% acrylamide [5% (w/v) acrylamide, 0.125 M tris, pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), 0.01% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED)] and separating gels were 12% acrylamide [12% (w/v) acrylamide, 0.375 M tris, pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.04% (v/v) TEMED]. Electrophoresis was performed in either a Hoefer Mighty Small<sup>TM</sup> or Hoefer Mini VE (Amersham Biosciences, UK) apparatus. For each set of samples duplicate gels were electrophoresed. The proteins in one gel were stained with Coomassie blue stain [45% (v/v) methanol, 10% (v/v) tri-chloroacetic acid, 1% (w/v) Coomassie brilliant blue R250] to determine equal loading. The proteins in the second gel were transferred to nitrocellulose membrane (Osmonics, USA) in transfer buffer (48 mM tris, 40 mM glycine, 1.3 mM SDS, 20% methanol) using a western transfer apparatus (Bio-Rad, USA). Membranes were stained with Ponceau stain [2% (w/v) Ponceau, 1.1 M sulfosalicylic acid, 1.8 M tri-chloroacetic acid] to ensure successful transfer. Immunodetection was performed by blocking the membrane with blotto [1X tris-buffered saline (TBS) (0.5 mM NaCl, 50 mM tris-HCl, pH 7.2); 5% (w/v) milk powder] for 2 h. The membrane was incubated O/N at 4°C, with agitation, with diluted ALDRXv4 antibody (1:5000). The membrane was washed three times for 10 min in wash buffer [1X TBS; 0.2% (v/v) Triton X-100; 0.05% (v/v) Tween-20] and incubated with secondary antibody (anti-rabbit IgG, alkaline phosphatase conjugate, diluted 1:20 000) for 2 h with agitation. The membrane was again washed three times for 10 min in wash buffer. The protein-antibody complex was visualized by chromagenic detection, utilizing NBT/BCIP (nitro blue tetrazolium chloride/ 5-

bromo-4-chloro-3-indolyl phosphate toluidine salt) tablets (Roche, Germany), according to the manufacturers' instructions.

### 2.3.7 High Performance Liquid Chromatography

*X. viscosa* plant material was freeze-dried and ground in liquid nitrogen and extractions carried out according to Tapernoux-Lüthi *et al.* (2004) with the following modifications. The samples were extracted twice with 1.5 ml 80% ethanol and twice with 1.5 ml 20% ethanol. All supernatants were pooled and volumes adjusted to 6 ml with sterile distilled water.

Desalting columns were prepared as follows: one millilitre-Mobicol spin columns (MoBiTec, Germany) were fitted with a 10 µm filter and layered with 150 µl Biorad AG 1-X8 (HCO<sub>3</sub><sup>-</sup>-form, 200-400 mesh), 100 µl Polyklar AT (insoluble PVPP) and 50 µl Biorad AG 50W-X8 (H<sup>+</sup>-form, 200-400 mesh). The columns were centrifuged with 400 µl sterile distilled water for 3 min at 3000xg. Fifty microlitre aliquots of each sample were column-deionized and dephenolized by centrifugation at 3000xg for 4 min at 4°C. The columns were rinsed twice with 325 µl sterile distilled water at 3000xg for 4 min at 4°C and all eluants pooled. The desalted samples were dried in a vacuum concentrator centrifuge (Univapo 100 ECH, UniEquip GmbH, Germany) and resuspended in sterile distilled water for carbohydrate analysis by high performance liquid chromatography (HPLC) using a pulsed amperometric detection system (PAD) as previously described (Tapernoux-Lüthi *et al.*, 2004).

### 2.3.8 Phloem Sap Analysis

Carbohydrate analysis of *X. viscosa* phloem sap was carried out by HPLC examination of phloem exudates. Phloem sap was collected by modification of an exudation technique previously described for dicotyledonous plants (King and Zeevart, 1974). Leaves were excised at their base and submerged in exudation buffer (5 mM Na<sub>2</sub>EDTA, 5 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.0). The leaves were cut once again whilst in the buffer and transferred to 2 ml microfuge tubes containing 500 µl exudation buffer. This buffer was replaced every 2 h over an 8 h period. Throughout the exudation process leaves were maintained in a high relative humidity chamber. Samples were

centrifuged through pre-rinsed syringe filters, fitted with a 0.45  $\mu\text{m}$  nylon filter, at 3000xg for 4 min at 4°C in order to remove particulate matter. Exudates were analyzed by HPLC-PAD without desalting.

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## **CHAPTER THREE**

### **RESULTS**

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# Results

## 3.1 Physiological Assays

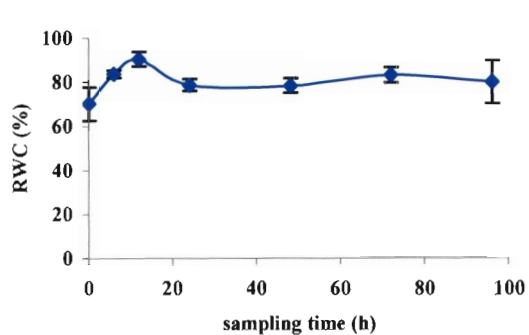
### 3.1.1 RWC

For the duration of the 150 mM NaCl, 4°C and 100 µM ABA treatments the RWC of the *X. viscosa* plants was maintained above 70% (Figure 7 A – C). This ensured that the plants were well hydrated throughout the duration of these stress treatments. The plants exposed to the dehydration/rehydration treatment were initially at full turgor. After eight days without water the RWC decreased to approximately 40%, after 11 days the RWC was approximately 15% and this decreased to 7% after 13 days (Figure 7D). The RWC increased from 7% to 16, 26 and 40% after 18, 42 and 66 h respectively following the addition of water (Figure 7E).

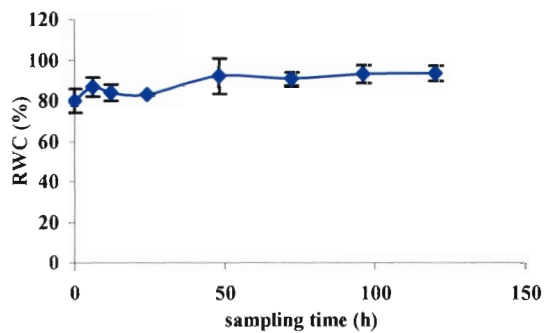
### 3.1.2 Chlorophyll Fluorescence

The changes in quantum efficiency ( $F_v/F_M$ ) are shown in Figure 8 (A – C). During the salinity stress the samples initially demonstrated  $F_v/F_M$  values of 0.73 to 0.67. After 72 h this decreased, finally dropping to approximately 0.2 after 120 h (Figure 8A). Plants exposed to low temperature conditions maintained  $F_v/F_M$  values above 0.74 throughout the 120 h treatment (Figure 8B). Throughout the ABA treatment the quantum efficiency remained above 0.72 (Figure 8C).

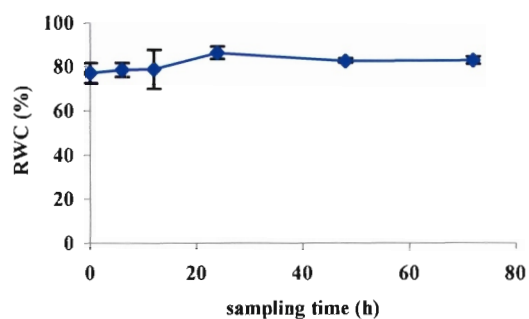
These data were not determined for the *X. viscosa* plants exposed to the dehydration and subsequent rehydration treatment, as this has been previously published (Sherwin and Farrant, 1996; Mundree and Farrant, 2000).



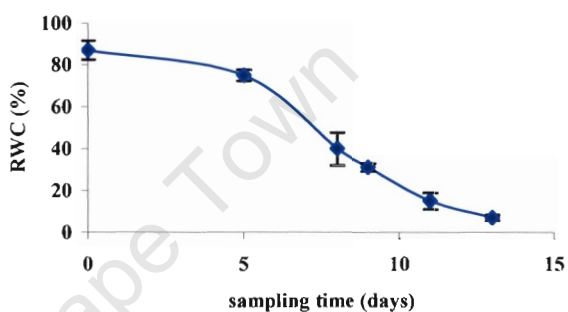
A



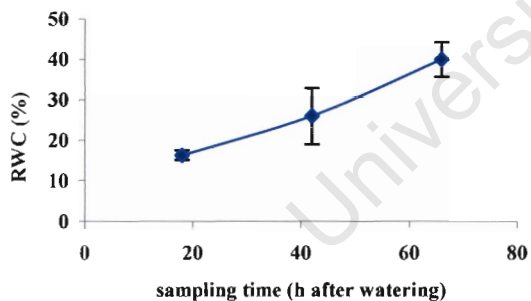
B



C

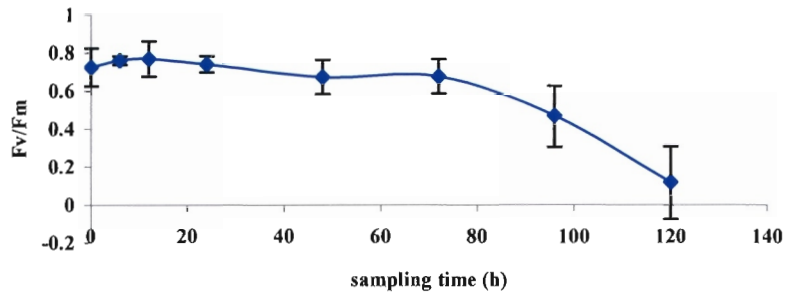


D

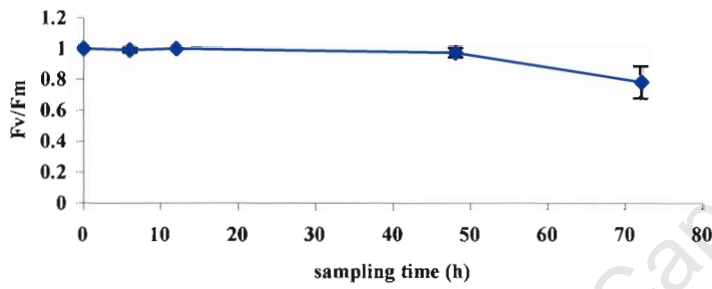


E

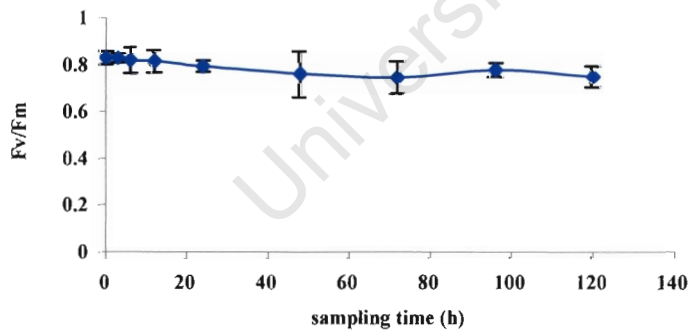
**Figure 7. RWC of *X. viscosa* plants exposed to (A) 150 mM NaCl, (B) low temperature (4°C), (C) 100 mM ABA, (D) dehydration and (E) rehydration treatments.**



A



B



C

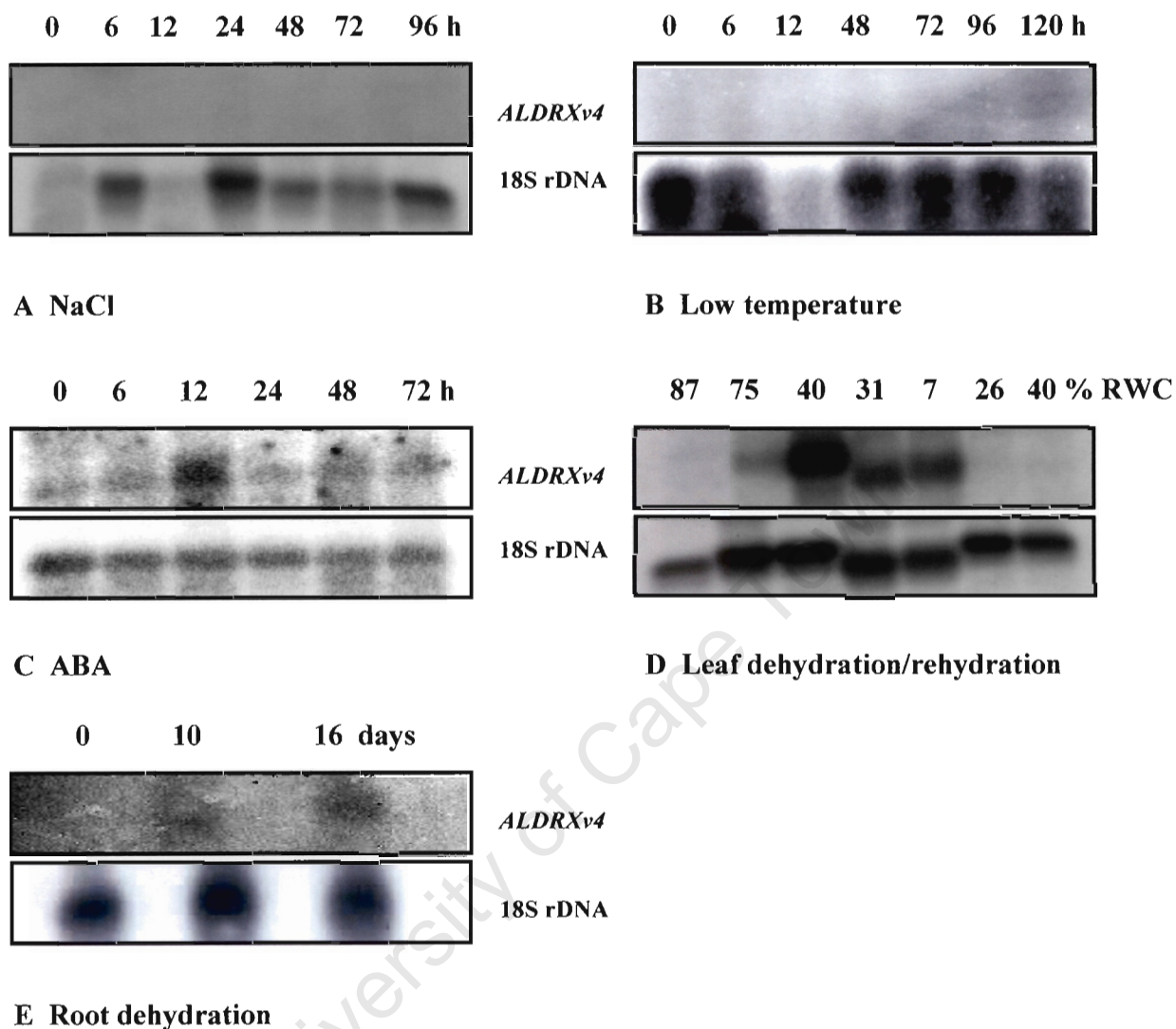
Figure 8. Chlorophyll fluorescence of *X. viscosa* plants subjected to (A) 150 mM NaCl, (B) low temperature (4°C) and (C) 100 mM ABA treatments. Where error bars are not visible, the size of the error is smaller than that of the data point.

## 3.2 Gene Expression Analysis

### 3.2.1 Northern Blot Analysis

The 150 mM NaCl and temperature stress did not result in a relative increase in the level of *ALDRXv4* transcripts present (Figure 9A and B). The 100  $\mu$ M ABA treated samples showed a potential increase in the level of *ALDRXv4* transcripts present at 12 h, but this was transient as no relative increase was seen after 24 h (Figure 9C). The leaf dehydration/rehydration stress showed a relative increase in the number of transcripts present as material dried to 75% RWC. This was amplified at 40% RWC, but decreased slightly at 31 and 7% RWC (Figure 9D). No transcript was evident after watering. Transcripts were evident in both the 10 and 16 day samples of the root dehydration treatment (Figure 9E). Unfortunately due to large amounts of background signal on the membrane, the presence of *ALDRXv4* transcript at full turgor could not be determined.

18S rDNA hybridisation showed relatively equal amounts of total RNA transferred onto the ABA and dehydration/rehydration membranes (Figure 9C and D). There appeared to be varying amounts of RNA present on the membrane containing RNA isolated from the salinity stress (Figure 9A) and less RNA transferred in the 12 h sample of the membrane containing RNA isolated from the low temperature treatment (Figure 9B).

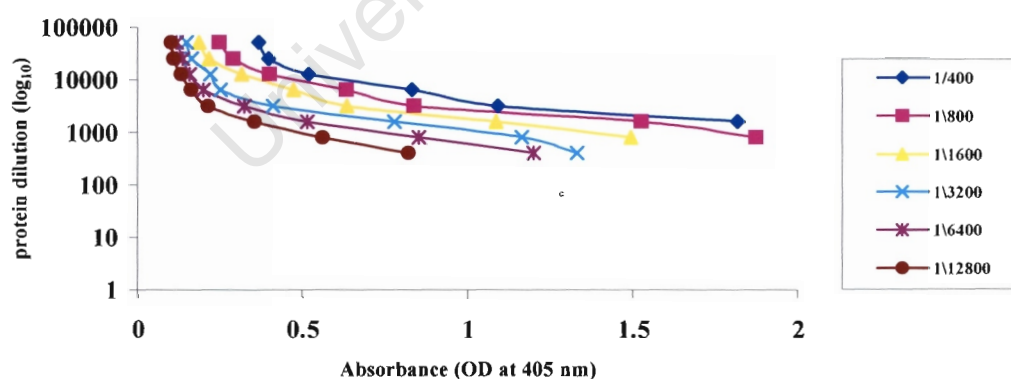


**Figure 9.** Northern blot analyses of total RNA isolated from the leaf tissue of *X. viscosa* plants exposed to (A) 100 mM NaCl, (B) low temperature (4°C), (C) 100  $\mu$ M ABA, (D) dehydration/rehydration and (E) root material exposed to dehydration treatments. The numbers above the NaCl, low temperature and ABA images indicate sampling times, in hours. The numbers above the dehydration/rehydration image indicate percentage RWC and those above the root dehydration image denote sampling time, in days. Where applicable, transcripts of approximately 1.1 kb were visualized.

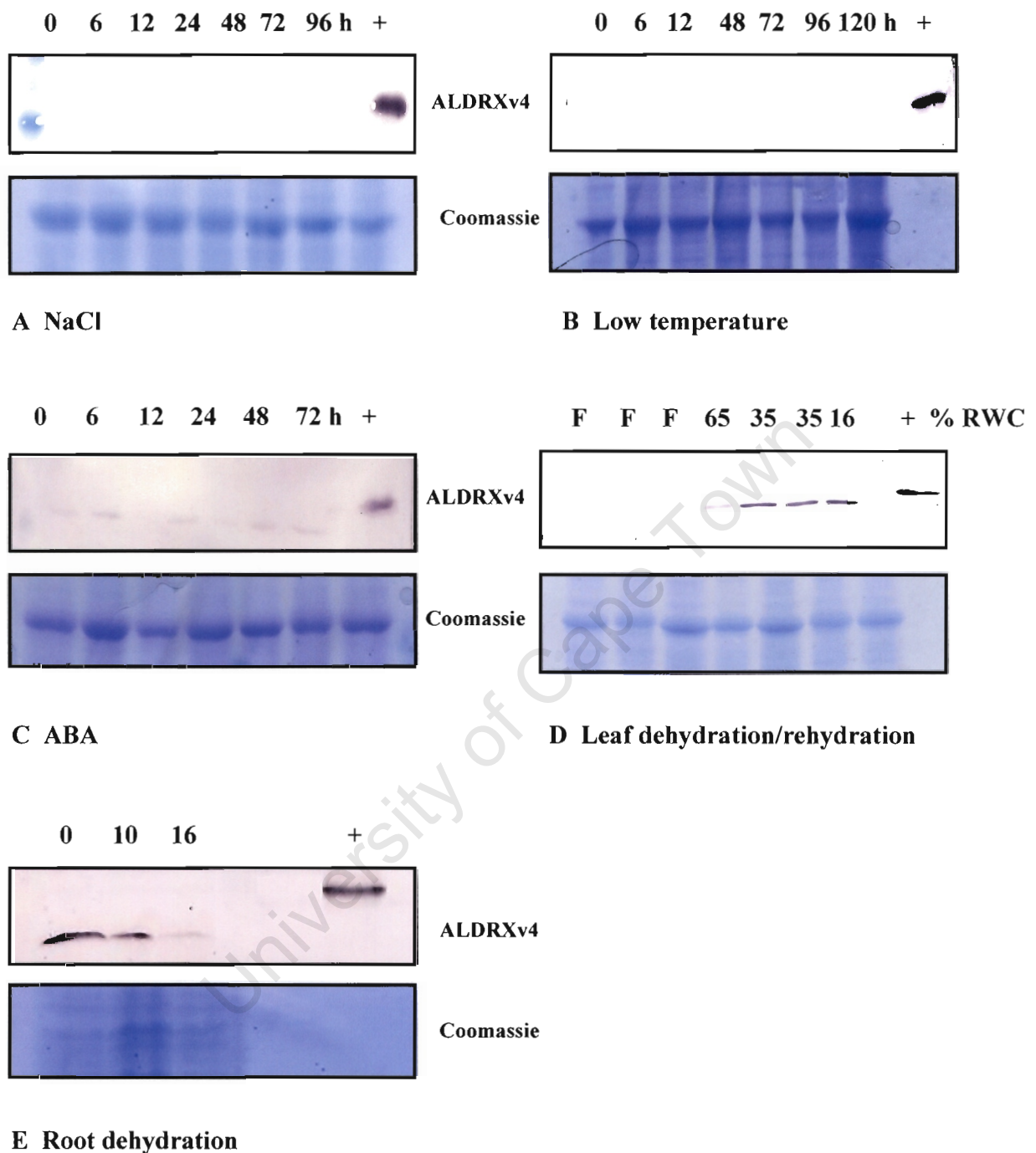
### 3.2.2 Western Blot Analysis

The ELISA determined that the optimal antibody concentration, for the range being tested, was a 1 in 400 dilution (Figure 10). As most of the dilutions yielded feasible results a 1 in 5000 dilution was tested by western blot analysis and chosen for subsequent assays for economical reasons.

The 150 mM NaCl and low temperature samples analysed exhibited no relative increase in protein expression throughout the treatments (Figure 11A and B). The samples exposed to ABA showed low amounts of ALDRXv4 protein present at 0 and 6 h. The level of this protein present on the membrane appeared to decrease at 12 h, but was again present at 24 h, with this expression maintained through to 96 h of the treatment (Figure 11C). During the leaf dehydration and rehydration stress ALDRXv4 was not expressed at full turgor. There was a relative increase in the amount of protein present at 65% and 35% RWC and this appeared to be maintained 18 h after watering (Figure 11D). In the dehydrated root samples ALDRXv4 was present at full turgor, with decreasing amounts of protein in the 10 and 16 day samples (Figure 11E). Coomassie stained gels indicated that comparatively equal concentrations of protein were loaded onto each polyacrylamide gel.



**Figure 10. Enzyme linked immuno-sorbent assay (ELISA) to determine optimal ALDRXv4 antibody dilution for western blot analysis. The 1 in 400 dilution yielded the best binding, however all dilutions tested yielded feasible results.**



**Figure 11.** Western blot analysis of total protein extracted from *X. viscosa* leaf tissue exposed to (A) 150 mM NaCl, (B) 4°C, (C) 100 μM ABA, (D) dehydration/rehydration and (E) root dehydration stress. The numbers above the NaCl, low temperature and ABA images indicate sampling time in hours. The

numbers above the dehydration/rehydration image indicate percentage RWC, where F denotes full turgor and 16% RWC is after 18 h of rehydration. The numbers above the root dehydration denote sampling time, in days. Hybridization of the recombinant ALDRXv4 protein is indicated by the + symbol. The recombinant ALDRXv4 protein contains a histidine tag, and so has a higher molecular weight than that of the endogenous ALDRXv4 protein. Gels were stained with Coomassie blue to determine whether equal amounts of protein were loaded. Where applicable, protein of approximately 34 kDa was visualized.

### 3.2.3 HPLC Analysis of the Sorbitol Content in Leaves, Phloem Tissue and Roots

As sorbitol is a putative product of AR activity, the content of this alditol was analyzed in the leaves, phloem exudates and roots of *X. viscosa* plants. No sorbitol was detected in either hydrated or dehydrated leaf tissue (results not shown). Sorbitol was present in the phloem sap of hydrated plants (Figure 12). HPLC analysis of hydrated root material showed that sorbitol was present in variable amounts (Figure 13).

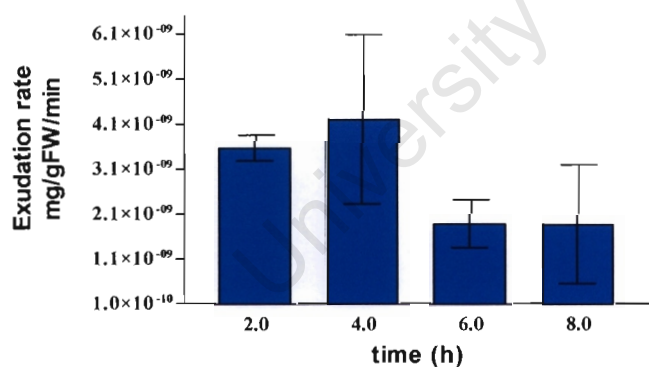
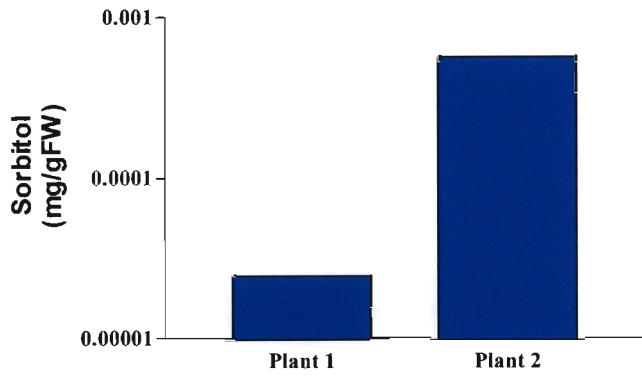


Figure 12. Sorbitol content in phloem exudates of hydrated *X. viscosa*.

Concentration of sorbitol was determined by HPLC and each time point represents two replicates.



**Figure 13. HPLC-PAD analysis of the sorbitol content in the roots of two hydrated *X. viscosa* plants.**

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## **CHAPTER FOUR**

### **DISCUSSION**

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## Discussion

The most common abiotic stresses that affect crop productivity worldwide are high salinity, low temperature and dehydration (Rabbani *et al.*, 2003). The ability of a plant to tolerate these stresses involves intricate physiological and cellular responses (Bohnert *et al.*, 1995; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray 1997). Due to this genetic complexity the utilization of traditional plant breeding methodology is not practical for generating stress tolerant varieties. Molecular biology has to come to the fore with regards to generation of stress tolerant crops. However an important prerequisite for molecular manipulation is an understanding of plant physiology.

### Salinity Treatment

Chlorophyll fluorescence measures the functionality of photosystem II (PSII) phytochemistry, therefore reflecting the status of PSII (Maxwell and Johnson, 2000). Damage to this system is often the first symptom of stress in a cell. The quantum efficiency ( $F_V/F_M$ ) value is the variable to maximum fluorescence ratio, yielding the quantum efficiency of open PSII centres. This value decreases as photoinhibition increases, and so reflects any damage or shut down experienced by the reaction centres. Healthy reaction centres typically display  $F_V/F_M$  values of 0.7 and above. In this study, excessive NaCl appeared to compromise electron transport in the chloroplasts. Figure 8A illustrates that the  $F_V/F_M$  values obtained at 96 and 120 h of the stress did yield considerable standard deviation from the mean readings. However the upper limits of this standard deviation indicated photoinhibition, which can be attributed to the increase of sodium and chloride ions in the environment. An increase in ROS due to the presence of these ions is a reported phenomenon and salt stress can cause secondary stresses, such as nutritional imbalance and oxidation (Zhu, 2000; 2001). As stated previously, *X. viscosa* breaks down chlorophyll in order to prevent light stress, limiting oxidative stress under dehydration conditions (Sherwin and Farrant, 1998). The decrease in quantum efficiency may be due to a controlled breakdown of photosynthetic apparatus, as occurs during drying, or just a consequence of oxidative damage. However photoinhibition does indicate that the  $\text{Na}^+$  and  $\text{Cl}^-$  ions were taken up into the plant cells, as no other stress was imposed on the plant. Ultrastructure studies using electron microscopy could give a better indication as to the intracellular response to an ionic stress. In *X. viscosa* electron microscopy

images have visualized vesiculated thylakoid membranes and pigment analysis shows a decrease in chlorophyll content as the cell loses water (Sherwin and Farrant, 1998; Mundree and Farrant, 2000). These phenomena have also been reported in another member of the *Xerophyta* genus, *X. humulis*, which in addition showed reassembly of thylakoid membranes upon rehydration (Farrant *et al.*, 1999). This suggests that during dehydration this break down was a controlled process. Western blot analysis using antibodies specific to known PSII proteins, such as the D1 protein, could be carried out on plant material at various stages of salinity stress in order to see if there is a reduction in these protein levels.

Total RNA and protein extracted from the plants were analyzed by northern and western blot analyses, respectively, in order to determine whether *ALDRXv4* transcripts increased or the protein was expressed in response to the imposed stress. RNA isolated from salinity stressed plants was transferred to the membrane in variable quantities, resulting in inconclusive northern analysis. However, high salinity did not appear to have an effect on the relative level of transcripts present (Figure 9A), nor the amount of protein recognized by the ALDRXv4 antibody (Figure 11A). Coomassie blue staining was utilized to determine equal amounts of protein loaded, as the isolated proteins could not be quantified using the Bradford quantification assay. This is because the proteins were extracted with and resuspended in buffers containing  $\beta$ -mercaptoethanol, which interferes with the assay.

Hypertonicity can result in loss of intracellular water that can cause cell shrinkage or a decrease in turgor pressure (Burg *et al.*, 1996). This can result in inorganic ion uptake in order to regain cell volume and pressure, but this too can be toxic over the long term. Therefore compatible solutes are synthesized in order to replace these inorganic ions (Yancey *et al.*, 1982). The absence of *ALDRXv4* transcripts or its gene product indicates that this gene may be regulated by a different means to its putative orthologues in other organisms that are expressed under high salinity conditions. Oberschall *et al.* (2000) reported that transgenic tobacco expressing the alfalfa AR had improved tolerance to high salinity, foxglove AR increased expression under salt stress (Gavidia *et al.*, 2002) and hypertonicity stimulated AR gene transcription in epithelial cells derived from rabbit renal inner medulla (Uchida *et al.*, 1989). By placing these epithelial cells in a variety of media, it was deduced that this increase in

transcription was due to an elevated intracellular ion concentration as opposed to increased osmolality. This increase in salt resulted from a reduction in cell volume, however it was the resulting change in ion concentration, not the change in cell volume that stimulated expression.

### **Low Temperature Treatment**

Despite the significant reduction in the amount of RNA present in the 12 h sample, as visualized by 18S rDNA hybridization, low temperature did not seem to alter the expression of *ALDRXv4* (Figure 9B), nor result in a change in the amount of *ALDRXv4* protein detected (Figure 11B). Cellular water content illustrates that this plant did not experience a dehydration stress (Figure 7B), therefore any alterations that may have existed would have been due to the low temperature environment, implying that *ALDRXv4* probably does not have a role in cold acclimation of *X. viscosa*. However the results showing that this stress did not affect electron transport in the chloroplasts (Figure 8B) and the fact that *X. viscosa* is an extremophile, do pose the question as to whether or not 4°C is a stress for this plant. Experiments in which *X. viscosa* plants were exposed to a -20°C stress or acclimatized at 4°C, with subsequent exposure to -20°C were carried out. However -20°C appeared to be too severe a stress, resulting in disruption of cellular structures, as visualized by electron microscopy images (not shown, S. Peters MSc thesis, 2005). A less severe, but still freezing, stress should be undertaken in order to decisively determine whether or not this putative AR is low temperature responsive.

### **Abscisic Acid Treatment**

The potential increase in the abundance of *ALDRXv4* transcripts at 12 h after exposure to exogenous ABA can be seen in Figure 9C, however 18S rDNA hybridization showed low levels of RNA present on the membrane, and low levels of the *ALDRXv4* transcript were present throughout the treatment. As basal levels of *ALDRXv4* transcript were not evident in the other plants investigated by northern blot analyses, this poses questions about a potential memory effect in the plant used for analysis. The natural history of the plants analyzed is not known, and although these plants were collected from similar areas, microenvironments can affect different plants experiences. Western blot analysis also showed low levels of *ALDRXv4* protein

present at the outset of the treatment (Figure 11C) and no increase in these protein levels was apparent throughout exposure to ABA. The RWC of the plant remained above 70% (Figure 7C) and photosynthetically healthy cells were evident throughout the duration of the stress (Figure 8C), implying that any physiological changes or alterations in expression were due to the imposed stress and not due to an unintended dehydration stress. Repetition of this treatment may indicate whether or not ABA regulates *ALDRXv4* expression.

### **Dehydration and Rehydration Treatment**

Net photosynthesis previously determined for dehydrating *X. viscosa* showed a decrease in photosynthetic activity as the RWC decreased (Mundree and Farrant, 2000). The rehydration process showed that the chloroplasts recovered completely, with the quantum efficiency having returned to control levels within 72 h of watering (Sherwin and Farrant, 1996). It was therefore postulated that dehydration and subsequent rehydration in this plant is a controlled procedure. Various mechanisms employed by *X. viscosa* to avoid and prevent dehydration-induced damage have previously been shown and support this (Sherwin and Farrant, 1995; 1996; 1998; Mundree and Farrant 2000). The rate of dehydration and rehydration (Figures 7D and E) was also consistent with previous findings (Mundree *et al.*, 2000; Mowla *et al.*, 2002; Garwe *et al.*, 2003).

Alterations in the amount of *ALDRXv4* transcript, and protein, in leaf tissue under dehydration conditions has been previously published (Mundree *et al.*, 2000); however this was not investigated during rehydration. Although it has been postulated that modified-desiccation tolerant species rely more heavily on inducible protection mechanisms, rather than cellular repair (as reviewed by Oliver *et al.*, 1998), the fact that poikilochlorophyllous species re-synthesize the photosynthetic apparatus is a sign that some kind of repair takes place during rehydration. It was therefore important to investigate the regulation of this transcript or its product during the rehydration process. In accordance with previous findings transcript was present during dehydration, but there was no apparent transcript present during rehydration in leaf tissue (Figure 9D). This result correlates with the fact that no transcript was previously seen at 5% RWC (Mundree *et al.*, 2000), which could be attributed to

cellular respiration shutting down at 15% RWC (Mundree and Farrant, 2000). The presence of the protein 18 h after watering (Figure 11D) could be due to either stable maintenance so that it was able to function and prevent rehydration-induced damage, or it was a consequence of dehydration-induced protection.

The transcript and protein levels in the roots of two *X. viscosa* plants were analyzed during dehydration, as these are destructive assays and no more plants could be sacrificed due to a limitation on the number of plants available. Samples were taken at 0, 10 and 16 days after withholding water, which corresponds to approximately full turgor, 20 and 7% RWC according to leaf dehydration rates. Due to background radiation on the membrane it could not be determined conclusively if *ALDRXv4* transcript was present in the time 0 sample (Figure 9E). However the *ALDRXv4* protein was visualized at this sampling time (Figure 11E). The fact that transcript remained, but the amount of protein decreased as the plant lost water could indicate that in the roots *ALDRXv4* translation ceases as this tissue dehydrates. Transcript may be stored for protein synthesis upon rehydration. Alternatively *ALDRXv4* may be involved in signaling, with synthesized protein being translocated. Under dehydration stress shoot injury primarily results from an altered balance between water uptake in the roots and transpiration rates in the leaves (Saunders and Markhart, 2001). Therefore it is expected that in tolerant plants some kind of signaling between these organs must occur to initiate stomata closure in the shoots. Signaling between the sink and shoot tissue under stress conditions has been reported extensively. Water stress in leaves, as a result of low temperature, was signaled to the root tissue, via the phloem sap in *Phaseolus vulgaris* (Vernieri *et al.*, 2001). Root sourced signaling has also been reported under dehydration and high salinity conditions utilizing chemical signals such as fluctuations in ABA and pH in xylem sap.

HPLC analysis was only conducted in plants exposed to dehydration, as this yielded the most conclusive results utilizing northern and western blot analysis. Sorbitol not detected in either the hydrated nor the dehydrated leaf material (results not shown) may have been due to the fact that analysis of this particular alditol by HPLC on that apparatus had not been sufficiently optimized. Sorbitol levels detected in phloem exudates from hydrated plants (Figure 12) were approximately 10 times lower than

other carbohydrates, such as hexose sugars and sucrosyl compounds, measured in the leaves (results not shown). Therefore sorbitol could have been present in the leaf tissue, and potentially have had alterations in concentration under dehydration conditions, but HPLC detection methods can result in compounds present at low levels being overshadowed by more prominent compounds. The extraction procedure would need to be modified so that only alditols, and not carbohydrates present in comparatively large quantities, are isolated and measured in order to detect alterations in the leaves. Sorbitol was probably detected in hydrated phloem exudates (Figure 12), as other translocation sugars, such as sucrose, raffinose and glucose, were present in comparatively similar amounts (results not shown). The presence of the above sugars was not determined in root material, but the ability to detect sorbitol in roots (Figure 13) may be due to the above reasons. The sampling of both the phloem exudates and the root tissue in both plants was conducted simultaneously, avoiding differences in the sorbitol levels detected in these tissues due to circadian variation. Variations may be due to extraction and detection methods not being optimal, as this protocol was originally designed for dicotyledonous plants, or due to variable levels between different *X viscosa* plants. Unfortunately more than two plants could not be tested, as no more plants were available for analysis. HPLC analysis was unable to detect sorbitol in the dehydrated root material. This again could be due to sorbitol not being present during dehydration, insufficient optimization of the extraction and detection methodology or the presence of other compounds in far greater concentration. In *C. plantagineum* sucrose has been found to accumulate in dehydrated roots in concentrations similar to its accumulation in dehydrated leaves of other poikilochlorophyllous plants (Norwood *et al.*, 2003). Again these detection methods need to be optimized, in order to ascertain whether there were variations in the levels in these tissues during dehydration or it was due to experimental procedure.

The detection of sorbitol in the phloem and roots under hydrated conditions does indicate that this alditol could be translocated. It is hypothesized here that the presence of sorbitol in both root and phloem exudates at full turgor is due to ALDRXv4 activity in the root tissue. This alditol may be transported to the leaves or other plant structures from the roots. Compatible solutes have been previously isolated from translocation and sink tissues. Significant increases in inositol and ononitol levels were detected in phloem exudates of the ice plant

(*Mesembryanthemum crystallinum*) following a high salt stress (Nelson *et al.*, 1998). These solutes are believed to function as signaling molecules between the leaves and the roots and facilitate long distance sodium transport in this plant (Nelson *et al.*, 1999). The ice plant's regulatory pathways may differ to other species, as it is a halophile, however the utilization of alditols as regulatory molecules may be consistent. In another resurrection plant, *C. plantagineum*, 2-octulose was isolated in the phloem and in low concentrations in hydrated roots (Norwood *et al.*, 2000). The authors postulated that this compatible solute was probably transported from leaves to roots and may be metabolized into other carbohydrates during translocation. In this plant total root carbohydrate content decreased by over 50% under dehydration conditions, suggesting that carbohydrates may be translocated to other plant organs (Norwood *et al.*, 2003).

If sorbitol was present in dehydrated leaves and roots, but levels were too low for detection this may suggest a few possibilities. Sorbitol may not need to accumulate in large quantities to be effective as an osmoprotectant. Hoekstra *et al.* (2001a) review cases in which sugars need not accumulate significantly to confer desiccation tolerance. Alternatively, AR main role may be to act as a detoxification enzyme, as identified by Cao *et al.* (1998), Rittner *et al.* (1999) and Oberschall *et al.* (2000), or AR may not play a significant role in the stress response of these plants. It is likely, but not necessary, that genes expressed under stress conditions aid cellular tolerance to dehydration (Bray, 1993). Alternatively, expression may result from injury to the plant at the onset of the stress or the gene may be expressed, but the gene product does not assist stress tolerance. The fact that the *ALDRXv4* clone was able to confer tolerance to osmotically stressed *E. coli* (*srl::Tn10*) cells (Mundree *et al.*, 2000) and that induced expression of AR homologues isolated from alfalfa (Oberschall *et al.*, 2000) and human cell lines (Keightley *et al.*, 2004) offered improved tolerance to osmotic stress supports the idea that *ALDRXv4* may have an active role in aiding stress tolerance. This could be tested in desiccation sensitive plants transformed with *ALDRXv4* under the control of a constitutive promoter. Analysis of the *ALDRXv4* promoter region could yield improved understanding of the regulation of this gene and could aid in identifying whether osmotic detection in this resurrection plant involves novel elements or stress response elements similar to that identified in other organisms. This could be performed by synthesizing reporter constructs containing

various 5' upstream flanking regions of *ALDRXv4* and exposing plants transformed with these constructs to an osmotic stress. Electrophoretic mobility shift assays, using the rat AR ORE as a probe, have previously been used to demonstrate increased binding of *trans*-acting elements to genomic DNA once exposed to a hypertonic environment (Ferraris *et al.*, 1996). Experiments of this nature substantiate findings that specific promoter regions of DNA are involved in osmotic regulation.

*ALDRXv4*'s potential role in tolerance needs to be further investigated. Significant alterations in sorbitol levels may not occur if this enzyme functions as a detoxification enzyme. It may have a dual function in both a detoxification and compatible solute synthesis capacity or have a role in signaling the presence of a stress. Methods to test this hypothesis include determining *ALDRXv4*'s catalytic efficiency with toxic aldehydes, such as HNE or methylglyoxal. A lipid peroxidation assay kit could be used to measure any alterations in lipid peroxidation-derived reactive aldehydes in osmotically stressed cells or transgenic lines repressing and overexpressing this gene. The presence and localization of *ALDRXv4* in the leaf and root tissue of stressed and non-stressed *X. viscosa* could also be confirmed by immunocytochemistry.

In conclusion, the levels of *ALDRXv4* transcript and its gene product were found to increase in response to dehydration and the protein remained during rehydration in leaf tissue. By contrast in the roots *ALDRXv4* appeared to be present at full turgor and decreased as these cells lost water, with the transcript remaining. This gene is potentially ABA responsive, but no transcript or protein was detected under high salinity or low temperature. It is proposed that at full turgor sorbitol may be transported into the phloem from the root to the leaf tissue. Under dehydration conditions sorbitol may be synthesized in the leaves and the roots, but transported from the root tissue elsewhere where it is thought to have a compatible solute, signaling or detoxification function.

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