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Isolation of the aldose reductase gene (*XvAld1*) from the resurrection plant *Xerophyta viscosa*, and characterisation of the gene product and transgenic plants expressing the gene

Alice T Maredza

University of Cape Town

Thesis Presented for the Degree of

Doctor of Philosophy

Department of Molecular and Cell Biology

University of Cape Town

September 2007

DECLARATION

I hereby declare that this thesis entitled:

Isolation of the aldose reductase gene (*XvAld1*) from the resurrection plant *Xerophyta viscosa*, and characterisation of the gene product and transgenic plants expressing the gene

is my own work and has not been previously submitted in its entirety or in part at any university for another degree.

_____

Signature

_____

Date

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"If we knew what it was we were doing, it would not be called research…" (Albert Einstein, 1879 - 1955)

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DEDICATION

For my dad, you always believed in me and pushed me to beat my own records. My mother, you taught me love; that it is a bottomless well, that it is the commandment of God and my step-mother, who for a long time was the only mother I knew. My elder brother Ephraim, forever my role model; you have always looked out for me. My sister-in-law Zhwie, you brought so much love, life and friendship to our family. My younger brothers; Machel, Garikai, Tapiwa and Blessing, and my younger sisters Farai, Belinda and Bianca; you have inspired me more than you realise, and your love keeps me going. For the new generation; my nephew Tirivashe (my first child), you taught me unconditional love; my son Anesu, who makes every effort worth the while; and my niece Mutsawashe (chiyevo). And for Tich, for the encouragement and the hard times, the pain and the joy…

This work is dedicated to my whole family, especially to the memory of my late cousins, Edward and Edgar; always full of life. Their lives were painfully cut short. And my departed grandfather - conscripted to toil in Joni, he learnt the value of education and made it his legacy.

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List of abbreviations

µg	microgram
µM	micromolar
°C	degrees Celsius
µg	microgram(s)
µl	microlitre(s)
µm	micrometer(s)
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ABRE	ABA-responsive element
ALR	aldehyde reductase
AR	aldose reductase
AREB	ABRE-binding proteins (also ABF)
ARF	auxin response factors
ARR	family of response regulators
a_w	water activity
BLAST	basic logarithm alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
bZIP	A family of transcription factors with basic region and Leucine-zipper motif
CaMK	calmodulin-like kinase
CARE	cis-acting response element
CBL	calcineurin B-like
cDNA	complementary deoxyribonucleic acid
CDPK	calcium-dependent protein kinase
CE	coupling element
cm	centimeter
CRT	C-repeat motif found in cold and drought responsive gene promoters
Da	Dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanidine triphosphate
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	desoxy-nucleotide triphosphate
DRE	dehydration response element
DREB	dehydration response element binding protein
DTT	dithiothreitol
dTTP	deoxythiamidine triphosphate
DW	dry weight
EDTA	ethylenediaminetetraacetate
EREBP	ethylene-responsive element binding protein
ERF	ethylene response factor
ERK	external signal-regulated kinase
FTW	full turgor weight

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F_V/F_M	quantum efficiency
FW	fresh weight
g	gram(s)
GA	gibberrelic acid
GDP	guanosine diphosphate
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GSH	reduced glutathione
His	histidine
HNE	4-hydroxynon-2-enal
hr	hour
HSP	heat shock protein (s)
HXK	hexokinase
IgG	immunoglobulin G
IPKS	isoleucine, proline, lysine, serine motif
IPTG	isopropyl-β-D-thiogalactoside
JA	jasmonic acid
JERE	jasmonic acid and elicitor responsive elements
kb	kilobase(s)
LB	Luria Bertani
LEA	late embryogenesis abundant
LTRE	low temperature-responsive element
M	molar concentration
MAPK	mitogen activated protein kinase
MAS	marker assisted selection
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mM	millimolar
mRNA	messenger RNA
MYB	A family of transcription factors with a tryptophan cluster motif
MYC	A family of transcription factors with basic-helix loop-helix (bHLH) and Leucine-zipper motifs
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Ni-NTA	nickel-nitrilotriacetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
PNA	plant nutrient agar
PSII	photosysytem II
RE	response element(s)
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	room temperature

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RT-PCR	reverse transcription-polymerase chain reaction
RWC	relative water content
SA	salicylic acid
SAUR	small Auxin-Up RNA
SDS	sodium deodecyl sulphate
Ser	serine
SOS	A family of genes coding for a salt overly sensitive phenotype
SSC	saline sodium citrate
TAIL-PCR	thermal asymmetric interlaced PCR
TBA	thiobarbituric acid
TBE	tris borate EDTA
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TF(s)	transcription factor(s)
Tris	tris-(hydroxymethyl)-aminomethane
Triton X-100	poly(ethylenglycolether)n-octylphenol
UTR	untranslated region
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
WP	water potential
WRKY	domains containing W, R, K and Y amino acids
WT	wild type
YFP	yellow fluorescent protein

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ABSTRACT

The *Xerophyta viscosa* aldose reductase cDNA (*XvAld1*) was isolated from a dehydration library. Gene transcripts that are upregulated during stress are normally involved in protection and/ or adaptation, leading to stress tolerance. The genomic organisation of *XvAld1* was characterised using Southern blot analysis and DNA sequencing. The results revealed more than one copy of the gene with a complex banding pattern that was partially resolved by sequencing. The sequencing of PCR-amplified genomic clones showed that the gene is organised into nine exons and eight introns spanning ~2.9 kb. The observed nucleotide differences between the sequenced clones could reflect polymorphisms between different copies of the gene. An 870-bp clone of the 5′ untranslated region, matching the 5′ leader sequence on the *XvAld1* cDNA was analysed for *cis*-acting response elements. Many of the sequence motifs matched those for hormonal regulation, organ specific expression, dehydration, high or low temperature responses, light and phytochrome responsiveness, wounding, as well as G-box, CAAT and TATA-boxes.

For further characterisation, the XvAld1 protein was expressed in *E. coli* and affinity purified using the histidine tag. Western blot analysis demonstrated the expression and purification of a 40.5-kDa monomeric protein. In order to extend our understanding of the stress-responsiveness, the induction of *XvAld1* in *X. viscosa* by various treatments was investigated using quantitative real time PCR. The mRNA levels at 66% RWC were five orders of magnitude higher than under well-watered conditions or mild dehydration. The high expression levels of *XvAld1* mRNA were maintained until the leaves reached an air-dried state. Western blot analysis showed correlation between mRNA and protein levels with increasing desiccation. The response of *XvAld1* to hormonal cues was determined by separately exposing plants to a single dose of 100 μM abscisic acid, 1 ppm ethylene, 100 μM jasmonic acid and 100 μM salicylic acid. The *XvAld1* mRNA, determined in leaf tissues from the hormone treatments, was marginally induced over three days of sampling. XvAld1 protein was not detectable by western blot analysis of similarly treated samples. To investigate if the low induction was caused by the method of treatment or whether the expression was not induced by exogenous ABA, an additional experiment involving repeated exposure to 100 μM ABA was performed. Western blot analysis showed increased protein expression at 12 and 48 hr after the first ABA application,

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demonstrating that *XvAld1* is responsive to prolonged exogenous treatment with the hormone. Localisation studies, carried out using both N- and C-terminal fusions to fluorescent proteins and immunocytochemistry, detected the protein in the cytoplasm and vacuoles and the intracellular space between cell wall and cytoplasm.

Arabidopsis thaliana and *Digitaria sanguinalis* plants were transformed with *XvAld1* cDNA driven by the cauliflower mosaic virus 35S and the maize ubiquitin promoters. Most of the putative transgenic *A. thaliana* and *D. sanguinalis* plants carried the *XvAld1* transgene. The majority of the plants expressed high levels of *XvAld1* mRNA. Western blot analysis showed high protein expression in leaves, stems and siliques of transgenic *A. thaliana* plants. Protein levels in *D. sanguinalis* transgenic plants were comparatively low. All the regenerated *D. sanguinalis* (both the transformed and the wild type plants) were sterile, setting nonviable seeds. In contrast, transgenic *A. thaliana* plants were fertile and phenotypically similar to untransformed controls. Southern blot analysis showed simple integration patterns and low copy numbers of transgene.

Selected transgenic *A. thaliana* plants, constitutively expressing *XvAld1*, were exposed to various abiotic stresses. The phenotypes, physiological and biochemical properties of the transgenic plants under stress were evaluated and compared to similarly stressed wild type (WT) and vector transformed (VT) control plants. The expression of *XvAld1* in *A. thaliana* enhanced tolerance to salt, osmotic and dehydration stresses. All the transgenic plants performed better than the WT on 75 mM mannitol. Similar trends were observed on 14% (w/v) PEG and NaCl. However, increasing the osmotic stress to 100 and 150 mM mannitol or NaCl reduced the growth of the transgenic plants.

The dehydration tolerance of potted plants was assessed based on phenotypic assessment and survival after resumption of watering. Transgenic plants showed significantly greater tolerance compared to WT and VT controls. There was no difference between the relative water contents of transgenic and control plants under dehydration. In contrast, the water potentials of line Xv8 leaves were more negative than the rest of the plants. Despite this, a larger percentage of line Xv8 plants exhibited the tolerance phenotype compared to the WT and other transgenic lines. Transgenic plants accumulated lower levels of lipid peroxidation-derived aldehydes compared to WT and VT implying that XvAld1 protects against oxidative damage.

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CHAPTER 1

Literature review

1.0 INTRODUCTION

The limitations to crop productivity are geographically determined by diseases, poor quality of soils (over-cultivation, accumulation of salts and heavy metals, acidity or alkalinity), availability of water (drought or flooding), and extremes of temperature (Boyer, 1982). In developing countries, many small-scale and subsistence farmers have no access to agricultural resources that farmers in developed countries use to mitigate the adverse effects of these environmental conditions (Alexandratos, 1999). In sub-Saharan Africa, the Green Revolution has bypassed resource poor farmers who have no economic means to improve their yields per hectare, which are the lowest in the world (Dyson, 1999). Notwithstanding all the other factors, erratic and inadequate rainfall is one of the most devastating problems faced by resource-poor farmers in sub-Saharan Africa. Drought can cause complete loss of crops, depending on the timing and severity of the dry spells. As these farmers depend on agriculture for both food and income, crop failure usually leads to malnutrition and/ or famine.

Stress tolerance is a multigenic trait in plants and the mechanisms are not fully understood (Cushman and Bohnert, 2000). In addition, the yield potential, which is the ultimate goal of crop improvement, is also a multigenic trait. Breeding for abiotic stress tolerance, while preserving or improving the yield potential, has had limited success (marker assisted selection - MAS, Holmberg and Bulow, 1998; Ribaut and Hoisington, 1998; Ribaut and Ragot, 2007). Genetic engineering technologies are vital in complementing conventional breeding to develop crops that can withstand biotic and abiotic stresses. These new technologies hold promise for improving yields under stress, while requiring minimal changes in agronomic practises (Delmer, 2005). The successful implementation of these new technologies depends on integrating an appreciation of the mechanisms involved in stress tolerance into the crop improvement efforts.

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1.1 Abiotic stress

The abiotic stresses affecting plants include high light intensity, mechanical damage, water availability, nutrient deficiencies and heavy metal poisoning, extremes of temperature and the osmotic environment. Stress conditions are usually sporadic and the accompanying adverse effects normally overlap. Stresses such as dehydration, extremes of temperature and the presence of high concentrations of solutes in the environment ultimately lead to water deficit (Holmberg and Bulow, 1998). The scarcity of environmental water for extended periods can have deleterious effects on the growth and development of plants. Water is indispensable for cellular reactions and for the maintenance of cell integrity and function. When the environmental water becomes limiting, many plants use various drought-avoiding strategies to limit water loss to the environment and keep protoplasmic water contents within a narrow range. If the duration of the drought is prolonged, irreparable cell damage and eventual cell death occurs. Different plant species have varying capacities to withstand or avoid dehydration stress.

1.2 Desiccation tolerance

The term 'desiccation stress' is used to describe an extreme form of stress when plants lose most of their protoplasmic 'free water' (Gaff, 1971; 1997). Tolerators of this water deficit stress can survive only on the water associated with the cell matrix; 'bound water'. Dehydration tolerance is likely to have been an important determinant in the evolution of many terrestrial plant species (Zhu, 2002), as they are constantly exposed to variable conditions of water availability. Ironically, water deficit remains one of the most acute environmental stresses affecting living cells. Plant species possess different levels of dehydration tolerance. Water stress levels that are lethal to some plant species may be easily tolerated by others. Many plants are homoiohydric, avoiding losing cellular water by utilising various conservation strategies. These strategies include limiting transpiration (Sperry et al., 2002) and deepening of roots. Nonetheless, most plants produce desiccation tolerant tissues at some developmental stage. Desiccation tolerant tissues in plants include seeds, pollen and spores (reviewed by Ingram and Bartels, 1996; Oliver and Bewley, 1997a; Alpert, 2000; 2005). A number of fungi and lower plants such as mosses, ferns and a few flowering plants also exhibit desiccation tolerance in vegetative tissues (Alpert, 2000; 2005).

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1.3 Resurrection plants

Angiosperms that are capable of withstanding desiccation in vegetative tissues are known as 'resurrection plants' (Gaff, 1971). Resurrection plants grow in ecological niches where water is limited and rainfall patterns are sporadic; such as the arid areas of sub-Saharan Africa, South America and Australia (Gaff, 1971; 1977; 1987). The plants tend to grow in shallow, sandy soils and rocky outcrops (Sherwin and Farrant, 1995; Alpert, 2005; 2006). These flowering resurrection plants have a remarkable ability to recover from the air-dried state, regaining normal metabolism within hours of rehydration (Bewley, 1979; Sherwin and Farrant, 1996). In addition, resurrection plants can often withstand other abiotic stresses such as extremes of temperature (Meyer and Santarius, 1998) and ultra-violet (UV) radiation (Takacs et al., 1999). Such extremophiles are valuable models for studying environmental stress responses. Additionally, these plants are a rich source of unique genes or regulatory mechanisms that can be used to improve stress tolerance in susceptible crop plants (Ramanjulu and Bartels, 2002). Resurrection plants use morphological, physiological and molecular strategies to maintain cellular homeostasis under adverse environmental conditions (Sherwin and Farrant, 1998; Bartels and Salamini, 2001).

When non-resurrection plants are subjected to water deficit, they suffer disruption of physiological and metabolic processes, and of cellular structures, sometimes leading to cell death. In vegetative tissues, cell reduction as a result of water loss leads to mechanical injury (Mundree and Farrant, 2000). Unregulated cellular processes, particularly photosynthesis and metabolism generate reactive oxygen species (ROS, Hendry, 1993; Smirnoff, 1993; Allen, 1995). Additionally, the loss of the cellular bound water disrupts membrane integrity and the structure and function of macromolecules.

There are two strategies of desiccation tolerance that resurrection plants utilise, namely, protection against damaging effects of desiccation and repair of damage upon rehydration (Sherwin and Farrant, 1998; Mundree and Farrant, 2000; Bartels and Salamini, 2001). Little damage to tissues is observed following lengthy periods of desiccation (Scott, 2000), indicating that many angiosperms employ the protection mechanism (Ingram and Bartels, 1996; Oliver et al., 1998; Mundree and Farrant, 2000; Cooper and Farrant, 2002). The short time it takes for the plants to recover from desiccation is compelling evidence for little damage but may also reflect an elaborate repair system. However, some resurrection plants take longer to recover

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compared to others, showing that the mechanisms involved are complex and may vary between species (Bewley and Oliver, 1992; Farrant et al., 1999). These strategies are not mutually exclusive, and in reality different resurrection plant species employ varying levels of both protection and repair.

1.3.1 Protection mechanisms in resurrection plants

There is no common strategy for desiccation tolerance among resurrection plants (Alpert, 2000; Farrant, 2000; Farrant et al., 2003; Alpert, 2005; 2006). Resurrection plants are found in many different species and families and as a result, the mechanisms are likely to have evolved separately (Oliver et al., 1998; 2005). On the other hand, there is also recent compelling evidence for uniformity in the mechanism of drought tolerance, suggesting a common ancestor at one stage during evolution. Oliver et al. (2000) hypothesised that desiccation tolerant angiosperms evolved around the mechanisms found in seeds. Illing et al. (2005) tested this hypothesis and showed that many of the genes upregulated during the drying of vegetative tissues are similar to those involved in desiccation tolerance of orthodox seeds. They supported the notion that mechanisms of drought tolerance have been co-opted into the vegetative tissues, and are regulated by environmental cues rather than developmental signals associated with seed development. The evolution of the differences observed in vegetative tissues of various resurrection plant species probably evolved after the events that incorporated seed drought tolerance into vegetative tissues (Oliver et al., 2005; Farrant et al., 2007).

During dehydration, light has one of the most damaging effects on vegetative tissues. In the absence of water, light-chlorophyll interactions damage the photosynthetic apparatus and induce production of ROS (Smirnoff, 1993; Sherwin and Farrant, 1998; Farrant et al., 1999; Shen et al., 1999). The accumulation of anthocyanins, xanthophylls and other pigments has been proposed to protect against light-induced damage by shielding photosynthetic machinery from excess light (Eickmeier et al., 1993; Mundree and Farrant, 2000). The pigments may also participate in the quenching of ROS.

In order to limit the deleterious effects, a common feature of some resurrection plants under desiccation is the loss or masking of chlorophyll (Tuba et al., 1993a; 1993b; 1994; Sherwin and Farrant, 1998; Farrant, 2000; Farrant et al., 2007). The prevention of photodamage also often involves shielding and/or dismantling of

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photosynthetic machinery (Tuba et al., 1998; Farrant et al., 2003). Angiosperm resurrection plants are classified as 'homoiochlorophyllous', if chlorophyll and chloroplast structure is retained during desiccation, and 'poikilochlorophyllous', if chlorophyll is lost and thylakoids are dismantled (Gaff, 1977; Bewley, 1979; Sherwin and Farrant, 1996; Tuba et al., 1996; Sherwin and Farrant, 1998; Farrant et al., 1999). Some plants take the middle ground, only partially losing their chlorophyll (Navari-Izzo et al., 1999; Whittaker et al., 2001). In dehydrated poikilochlorophyllous angiosperms, photosynthesis ceases and plants remain quiescent until rehydration. These plants, such as *Xerophyta viscosa* and *X. humilis*, take longer to resurrect as they have to synthesise photosynthetic pigments and reconstitute chloroplasts (Tuba et al., 1993a; Tuba et al., 1993b; Sherwin and Farrant, 1996; Dace et al., 1998). On the other hand, homoiochlorophyllus species recover quickly because they retain chlorophyll during desiccation (Larson, 1988; Smirnoff, 1993; Sherwin and Farrant, 1998; Alamillo and Bartels, 2001). *Craterostigma wilmsii* and *Myrothamnus flabellifolius* utilise leaf folding and protective pigments to shield the photosynthetic machinery from damage.

Cell structure and subcellular components are reorganised to limit damage to cells. Leaf folding, curling or rolling are all used to lessen light-induced damage and water loss (Farrant, 2000; Farrant et al., 2003). At the ultrastructural level, cell wall folding (Vander Willigen et al., 2004; Vicre et al., 2004; Moore et al., 2006), segmentation of vacuoles and packing of the cytoplasm (Farrant et al., 1999; Farrant, 2000; Mundree and Farrant, 2000) are proposed to reduce the mechanical strain on the cell, caused by loss of turgor. The vacuole constitutes the largest part of the cell volume and is a major determinant of cell size and shape. It is used to store water, resources, toxic materials and waste products. Vacuoles become subdivided under stress and are associated with the sequestration of ions and the regulation of cell osmotic potentials (Shen et al., 1999; Mundree and Farrant, 2000). Protective compounds, which may be sugars or sugar derivatives (Koster and Leopold, 1988; Williams and Leopold, 1989; Bianchi et al., 1991; Bianchi et al., 1993; Shen et al., 1999; Mundree and Farrant, 2000), or amino acids (Ghasempour et al., 1998; Vander Willigen et al., 2004) can accumulate to high levels within the cell. Protein synthesis is a major requirement for recovery from desiccation (Dace et al., 1998; Oliver et al., 1998; Wood et al., 2000; Cooper and Farrant, 2002). Furthermore, gene products such as late embryogenesis abundant (LEA) proteins, aquaporins and antioxidants are

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synthesised in response to water stress (Dure, 1993; Sgherri et al., 1994; Mundree and Farrant, 2000). The modes of protection engendered by these molecules will be discussed in the following sections.

1.3.2 Molecular mechanisms resulting in adaptive responses

The mechanisms of stress tolerance are regulated at the genetic and molecular levels. For example, the degreening and regreening of poikilochlorophyllous resurrection plants is a controlled mode of protection from desiccation damage rather than a consequence of it. Molecular regulation of stress tolerance raises several questions regarding the adaptive responses. How are stresses converted to molecular responses? For instance, how is the need to dismantle thylakoid membranes perceived, relayed to the cell and then executed? The expression of genes is controlled at transcriptional, posttranscriptional, translational and posttranslational levels. Cells have to perceive and properly respond to changes in the intra- and extra-cellular environments to optimise growth and development. The regulation of each gene and the encoded protein is unique. The control involves the coordination of a particular subset of regulatory components to ensure that the right amount of gene product is produced at the right time. Expression profiling studies have revealed changes in mRNA and protein levels in plants under stress. Plant biologists have coined the term "dehydrin" to describe a subset of proteins sharing similar sequence motifs and whose expression is upregulated during dehydration (Bartels et al., 1990; Oliver and Bewley, 1997b; Bockel et al., 1998). Similarly, the translation of a subset of proteins is favoured during rehydration (Wood and Oliver, 1999; Oliver et al., 2000). Most of these proteins are likely to be involved in the repair of damage following desiccation. Investigating how these expression patterns are controlled is a subject of extensive research (Ingram and Bartels, 1996; Wood and Oliver, 1999; Mundree et al., 2002; Bernacchia and Furini, 2004; Bartels, 2005; Mundree et al., 2006). An understanding of the complexity and interplay of stress-responsive genes and signalling components, and the different response strategies utilised by different plant species will help to shape technologies that are used to improve stress tolerance in sensitive species.

1.3.3 Using *X. viscosa* as a model for studying abiotic stress tolerance

The resurrection plant *X. viscosa* is indigenous to sub-Saharan Africa. This monocot tolerates extremes of desiccation; dehydrates to the air-dried state and

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resumes full physiological activities within 72 hours of rehydration (Mundree and Farrant, 2000). *X. viscosa* is poikilohydric and poikilochlorophyllous, losing all chlorophyll during desiccation and accumulating anthocyanins (Sherwin and Farrant, 1996). Our research group is studying desiccation tolerance in *X. viscosa* with the ultimate aim of integrating the genetic material for the improvement of drought tolerance in cereal crops. To improve our understanding of stress tolerance, a cDNA library was constructed from dehydrated *X. viscosa* leaves. The regulation of *X. viscosa* genes by various stresses was investigated (Mundree et al., 2002; Mundree et al., 2006). Functional characterisation of some of the gene products has also been attempted (Mundree et al., 2000; Mowla et al., 2002; Garwe et al., 2003; Marais et al., 2004; Garwe et al., 2006; Govender, 2006; Peters et al., 2007). The stress-responsive expression and the possible adaptive roles of *XvAld1*, an aldose reductase isolated from *X. viscosa*, are investigated in this thesis.

1.4 Changes of gene expression during stress

Changes in gene expression have been more widely studied in non-tolerant plants compared to desiccation tolerant species. Analysis of stress-responsive gene expression in Arabidopsis has revealed that the transcripts fall into two classes (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki et al., 1999). The first group codes for proteins that are directly involved in protective functions during stress, leading to adaptation and tolerance. The second group codes for proteins that regulate the expression of other genes. Figure 1.1, adapted from Shinozaki et al. (1999), illustrates the various classes of gene products involved in adaptation to stress. Since many strategies have been shown to improve stress tolerance in plants, it follows that many gene products are involved in collectively conferring the protection and repair required for survival under stress. Tolerance depends on the genetic make-up of a species and the expression patterns of the available stress-regulated genes. However, Zhu et al. (2000) argued that not all genes that are upregulated serve adaptive roles in stress tolerance, but may be a result of cellular injury. The proposed functions of some of the gene families will be reviewed.

1.5 Factors controlling gene expression in response to stress

While the expression profiles of stress-responsive genes have been widely investigated in a few of the resurrection plants, little is known about how these genes

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are regulated. The molecular characterisation of gene expression is vital for understanding the proposed functions of the gene products. In addition, gene expression and protein function occurs in the cell milieu containing other gene products. Investigating how a gene or gene product is affected by, and interacts with the cellular environment will provide a better understanding of molecular, biochemical and physiological control mechanisms. Many stress-responsive genes have only been characterised with respect to whether or not they are expressed during stress. The regulatory effects of signalling molecules during stress are relatively well understood in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1993; Shinozaki et al., 1999; Yamaguchi-Shinozaki and Shinozaki, 2005). The corresponding signalling pathways in resurrection plants have not been characterised, and comparative reports are few (Munnik et al., 1998; O'Mahony and Oliver, 1998; Villalobos et al., 2004). In addition, the interaction of these components with the stress-responsive genes is rarely investigated.

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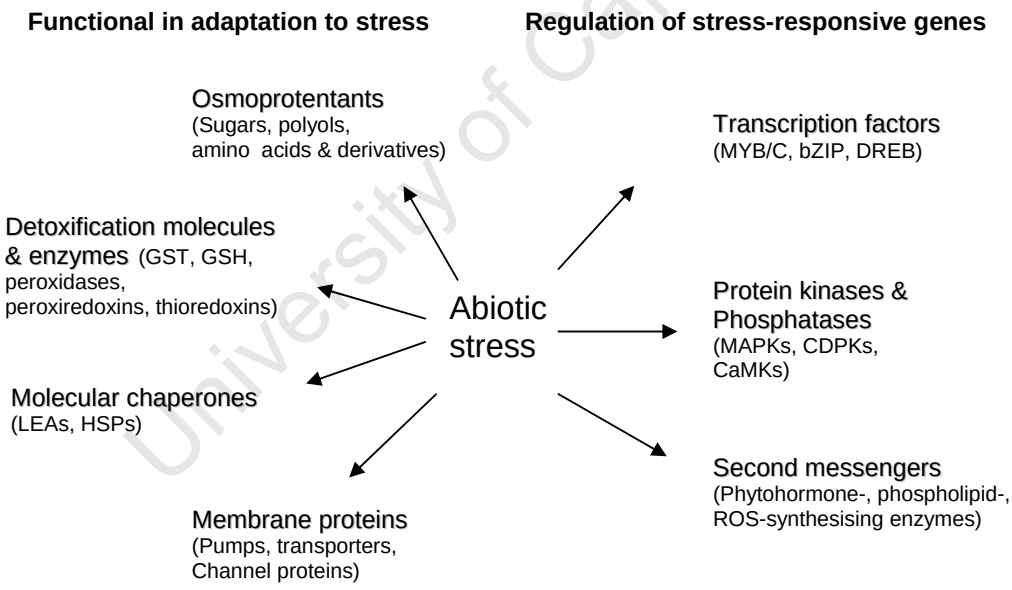

Figure 1.1 The categories of genes that may be involved in stress tolerance
The families of genes whose products function in the protection and/ or repair of cellular components are presented on the left. The expression of these protective genes is controlled by several signalling molecules, exemplified by gene families on the right. Adapted from Shinozaki et al. (1999).

The mRNAs for many protein classes involved in signal transduction are upregulated in response to different environmental and developmental stimuli. These transcripts code for various proteins such as protein kinases and phosphatases (Urao et

al., 1994; Hwang and Goodman, 1995; Mizoguchi et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 2000; Takahashi et al., 2007), phospholipids-hydrolysing enzymes (Hirayama et al., 1995; Frank et al., 2000), calcium-binding proteins, G proteins, transcription factors (Urao et al., 1993; Kusano et al., 1995; Choi et al., 2000; Abe et al., 2003; Tran et al., 2004; Tran et al., 2007), enzymes involved in the metabolism of reactive oxygen species (ROS), and diverse membrane-associated proteins such as ion-channel proteins (Shinozaki and Yamaguchi-Shinozaki, 1996; 1997). Figure 1.2 summarises the components and molecular processes that are involved from perception of stimulus to the adaptive responses. Understanding the interactions between stress-responsive genes and these signalling components will advance the technologies used to improve stress tolerance in plants.

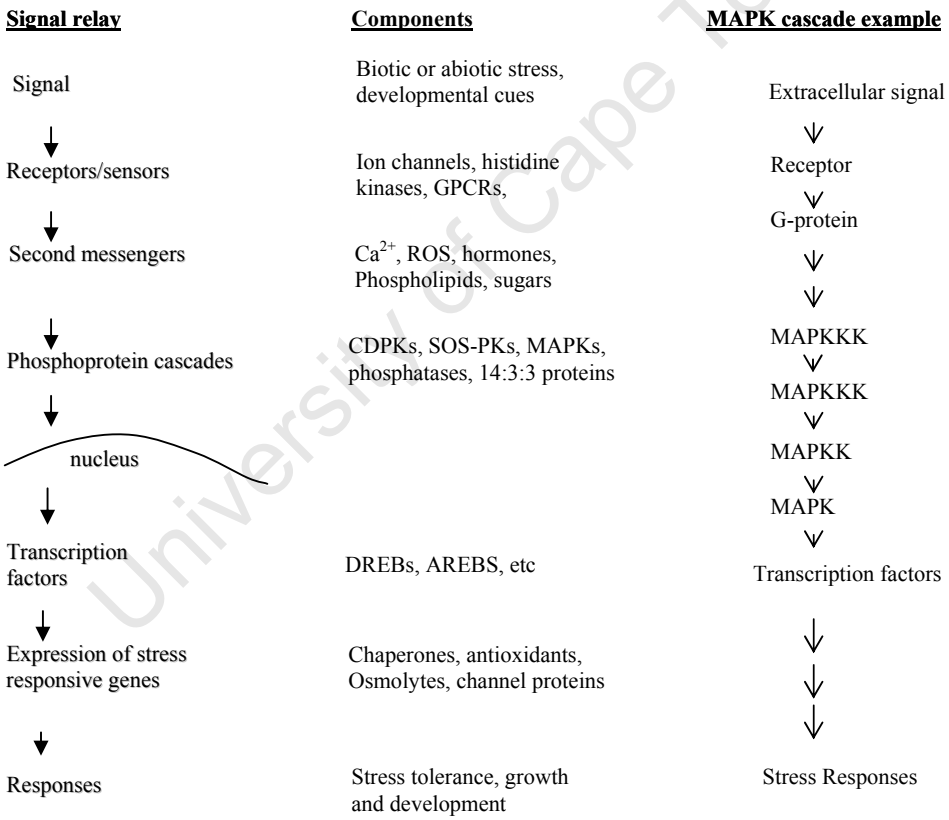

Figure 1.2 Schematic representations of signal transduction pathways, the components, and an example of the MAPK cascade. Extracellular signals are perceived at membrane located receptors that may be coupled to G proteins. The G proteins then activate a kinase module such as the MAPK. The activated MAP kinases may regulate other signalling components in the cytoplasm (e.g. phospholipases, other kinases, cytoskeletal proteins) or may translocate into the nucleus to regulate transcription factors. Adapted from Xiong et al. (2002).

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1.5.1 Signal transduction in plants

Signal transduction has been extensively explored in animal systems. The animal components have served as useful reference points for identifying and characterising related transduction components and pathways in plants. The transduction of perceived signals involves the integration of networks that convey a variety of internal and external stimuli, leading to the regulation of many genes and proteins. Cellular processes are tightly controlled by the coordination of these interacting and finely tuned pathways that maintain the biological function of the organism.

Many plant signal transduction components have been identified (for reviews see Trewavas and Malho, 1997; Boudsocq and Lauriere, 2005). However, although the molecular properties of many of these components have been elucidated, what is still lacking is an understanding of how these pathways are connected. The complexity of signal transduction pathways is oversimplified and often presented as an isolated linear chain from signal perception to response (Fig. 1.2). In reality, transduction pathways are a complex integrated network of receptors, cascades and response mechanisms that culminate into dynamic regulatory machinery.

Plants, as sessile organisms, are shaped by their environment. The external stresses, together with cellular metabolites and developmental processes form the plethora of signals that are perceived and responded to (Gilroy et al., 1990). These signals are detected by sensors or receptors located on the plasma membranes and cell walls; vantage positions to sense external cues. However, some receptors are found in the cytoplasm, cytoskeleton, nucleus and nuclear membranes (Reiser et al., 2003; Dharmasiri et al., 2005; Ueguchi-Tanaka et al., 2005; Razem et al., 2006; Liu et al., 2007). Studies by Wood et al. (2003) also implicate cytosolic proteins such as antioxidant enzymes as primary sensors of stress.

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1.5.2 Protein kinases

Reversible phosphorylation of serine (Ser), threonine (Thr) and/or tyrosine (Tyr) residues by kinases and phosphatases is a common posttranslational control mechanism. This obligate partnership between protein kinases and phosphatases regulates many cellular processes by switching proteins between the active and inactive states. This function is invaluable in transduction cascades where phosphorylation is used to relay signals through the cells. Many identified protein kinases possess receptor-like properties, and are capable of sensing changes in

environment. Receptor-like kinases (RLK) were among the first signal transduction components to be characterised in plants (Harper et al., 1991; Stone and Walker, 1995). A typical RLK features an extracellular, a membrane-spanning and an intracellular Ser/Thr protein kinase domain (Morillo and Tax, 2006). RLKs are the largest family of receptors found in plants, with more than 600 and 1000 proteins in the Arabidopsis and rice genomes respectively (Shiu et al., 2004). The structure of RLKs indicates that these proteins can perceive external stimuli and transduce messages into the cytoplasm. Tamura et al. (2003b) identified a membrane-bound RLK in tobacco that is possibly sensitive to membrane architecture changes and may play a role in signalling for osmotic stress tolerance.

Guanosine triphosphate- (GTP) binding proteins (G proteins or GTPases) are well known molecular switches that regulate the functions of proteins involved in many diverse processes, including signal transduction (Stryer and Bourne, 1986; Milburn et al., 1990; Schmidt and Hall, 1998). GTPases may be membrane-bound proteins coupled to a receptor on the cell surface; that is, G protein-coupled receptor (GPCR, Pierce et al., 2002; Tohgo et al., 2003). The transfer of a signal from the receptor to a G protein may trigger GTP-binding and the activated protein will then transduce signals across the membranes (see Fig. 1.1). O'Mahony and Oliver isolated and characterised a desiccation-responsive small GTP-binding protein from the resurrection plant *Sporobolus staphianus* (O'Mahony and Oliver, 1998), demonstrating that these signalling components are functionally involved in stress tolerance.

The activation of a kinase receptor triggers the transduction of signals by protein kinase cascades. One such cascade system is the histidine kinase two-component system consisting of a sensor domain and a response regulator component. The identification of putative cytokinin and ethylene osmosensors (Sakai et al., 1998; Urao et al., 1999; Urao et al., 2000; Hwang and Sheen, 2001; Inoue et al., 2001), and the Arabidopsis response regulators (ARR; Lohrmann and Harter, 2002) supports the function of the two-component system in plants. In yeast, the mitogen activated protein kinase (MAPK) cascade operates downstream of the histidine kinase. A similar cascade is also proposed to operate in higher plants (Nakagami et al., 2005). MAPKs relay, integrate and amplify signals. They have been implicated in the transduction of external stimuli such as mechanical signals, wounding and osmotic stress (Meyerowitz, 1997; Pitzschke et al., 2006; Pitzschke and Hirt, 2006; Torres et

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al., 2006; Zhang et al., 2006) and many plant MAPKs are related to external signal-regulated kinases (ERKs) found in animals. In addition, MAPKs are implicated in signalling cascades initiated by hormones, developmental cues and other biotic factors. There are three distinct classes of MAP kinases (Hirt, 2000). MAPKs are activated by MAPK kinases (MAPKKs), bifunctional kinases that are in turn activated by MAPK kinase kinases (MAPKKK, Kultz, 1998; Widmann et al., 1999). Upstream of the MAPKKKs are MAPKKKK or G proteins which couple kinases to the receptors (illustrated in Fig. 1.2, Marshall, 1996).

The activities of some kinase enzymes are regulated by interaction with calcium ions. Proteins such as calmodulin (CaM) act as calcium sensors by undergoing changes in protein conformation that alter the activity of partner protein, usually kinases (Luan et al., 2002). On the other hand, calcium-dependent protein kinases (CDPKs) are transponders whose conformational changes upon Ca^{2+} binding induce altered activity within the same protein (not in an interacting partner). CDPKs are a diverse group of kinases in plants (Harmon et al., 2000; Asano et al., 2005). These kinases are differentially regulated by Ca^{2+}, depending on the divergence of their Ca^{2+}-binding motifs (Harper et al., 2004). CDPKs contain a C-terminal CaM-like regulatory domain (the Ca^{2+} sensor) coupled with a kinase domain that elicits a response (Harmon et al., 2000). CDPKs contain several EF (elongation factor) hand domains for binding Ca^{2+} (Harper et al., 1991; Nagae et al., 2003; Chandran et al., 2006). In *X. viscosa*, Conrad (2005) identified two abiotic stress-responsive Ca^{2+} binding proteins, a CaM homologue (XvCaM) containing three EF hands, and a Ca^{2+} binding protein (XvEF) with one EF hand.

While CDPKs possess CaM-like C-termini, calmodulin-dependent protein kinases (CaMKs) and protein kinase C are calcium-regulated kinases that require CaM for activity (Patil et al., 1995; Liu et al., 1998b). Calcineurin B-like (CBL) proteins are named after their similarity to both the regulatory subunit of calcineurin and the neural Ca^{2+} sensor in animals (Kudla et al., 1999). CBLs target a group of kinases, CBL-interacting protein kinases (CIPKs), that share similarities with the yeast sucrose non-fermenting (Snf) protein kinases (Shi et al., 1999). Using *A. thaliana* mutants, Liu and Zhu (1998) identified a cDNA clone whose gene product, salt overly sensitive 3 (SOS3), was predicted to contain three Ca^{2+}-binding domains. SOS3 is a CBL4-type protein kinase known to regulate gene expression under salinity stress (Halfter et al., 2000) by acting as a salt sensor (Liu and Zhu, 1997; 1998). SOS3

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contains a domain similar to the B-subunit of calcineurin B (Halfter et al., 2000; Ishitani et al., 2000; Sanchez-Barrena et al., 2005).

1.5.3 Transcription factors

One of the major control points for gene expression is the initiation of transcription. Signal transduction pathways lead to the nucleus where *de novo* gene expression is initiated. Transcription is activated by *trans*-acting transcription factors (TFs) that bind to specific DNA sequence motifs in the promoter regions of a gene. The TFs contain a variety of structural motifs that specifically bind to target DNA sequences. The TFs, upon binding to promoter sequences, direct transcriptional activation and consequently, gene expression. The activities of TFs are regulated by specific environmental conditions, developmental stages or in specific tissues, among other factors. The characterisation of plant TFs has helped to identify stress response-specific signal transduction pathways (Fig. 1.3; reviewed by Shinozaki et al., 2003) that are grouped according to the involvement of abscisic acid (ABA).

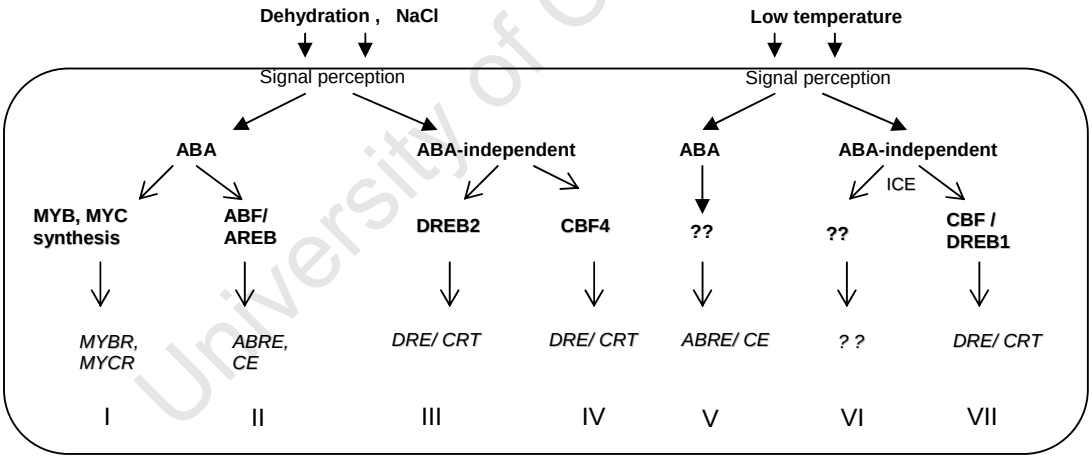

Figure 1.3 The ABA-dependent and independent regulatory pathways in response to drought, salt and cold The transcriptional regulators involved and the specific DNA sequences that interact with the regulatory proteins are discussed below.

1.5.3.1 ABA-dependent gene expression

The roles of ABA in plant growth and development include controlling seed development and germination, and mediating abiotic stress responses (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow, 1998; Oliver et al., 2000; Nambara and Marion-Poll, 2005). Researchers have demonstrated that

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endogenous ABA levels increase during water deficit. In addition, treating plants with ABA results in improved stress tolerance (Chandler and Robertson, 1994). However, the physiological functions of ABA in stress tolerance have been better elucidated by characterising TFs involved in ABA-mediated gene expression. Analysis of stress-responsive genes revealed that the expression of some genes is ABA-dependent, some are induced independently of ABA, and yet others are partially regulated by ABA (Shinozaki and Yamaguchi-Shinozaki, 1997; Seki et al., 2001; Seki et al., 2002a; Seki et al., 2002b). The expression patterns and promoter regions of the stress-inducible *rd29* genes have helped to dissect ABA-dependent and -independent regulation pathways (Yamaguchi-Shinozaki and Shinozaki, 1993).

Several drought- and cold-inducible genes contain potential ABA-responsive elements (ABREs) in their promoter regions (Bray, 1993). The ABRE motif consists of the sequence; PyACGTGGC (where Py is a pyrimidine) and is a *cis*-acting response element (CARE) in ABA-regulated gene expression (Giraudat et al., 1994). Functional variations of the ABRE motif have been identified in several promoters (Guiltinan et al., 1990; Mundy et al., 1990). These include the G-box element, which contains the ACGT core motif of ABREs (Menkens et al., 1995). Shen and Ho, (1995) demonstrated that the ACGT motif determines the binding specificity of the basic leucine zipper (bZIP) TFs that are involved in the regulation of some ABA-responsive genes (Jakoby et al., 2002; Kang et al., 2002; Abe et al., 2003; Finkelstein et al., 2005). The ABRE-binding proteins (AREBs), also called ABRE-binding factors (ABFs), are activated by posttranslational modifications, possibly phosphorylation (Uno et al., 2000; Yoshida et al., 2002; Fujita et al., 2005). The requirement for a coupling element (CE) that functions in synergy with ABREs for effective ABA-dependent gene expression (Shen et al., 1996; Hobo et al., 1999; Shen et al., 2004) was demonstrated by showing that a single copy of ABRE failed to induce transcription.

Analysis of the drought-inducible *rd22* from *A. thaliana* showed that the gene was not only regulated by ABA but that its expression was also dependent on protein synthesis (Iwasaki et al., 1995). The *rd22* promoter did not contain any ABREs, but had conserved motifs for MYC and MYB DNA-binding proteins. The MYC and MYB proteins are transcriptional regulators that contain basic helix-loop-helix (bHLH) motifs and recognise specific *cis*-elements in promoters of ABA-responsive genes (Abe et al., 1997; Massari and Murre, 2000; Bailey et al., 2003; Toledo-Ortiz et

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al., 2003). Several homologues have been isolated and characterised in plants, and their involvement in ABA-dependent adaptation to abiotic stress has been demonstrated (Nelson et al., 1994; Abe et al., 1997; Abe et al., 2003). These results show that there are two ABA-dependent signalling pathways; one involving MYB and MYC type of transcriptional regulators (Fig. 1.3, Pathway I) and the other regulated by AREBs/ABFs (Pathway II).

1.5.3.2 ABA-independent gene expression and crosstalk

Analysis of ABA-deficient (*aba*) and -insensitive (*abi*) mutants showed upregulated expression of some genes under dehydration and low temperature (Nordin et al., 1991; Yamaguchi-Shinozaki and Shinozaki, 1993; Gosti et al., 1995). These observations showed that some stress-responsive genes do not require ABA for their expression. Expression analysis of two Arabidopsis genes found on the same locus showed that *rd29A* does not require ABA for transcription under low temperature and dehydration, while *rd29B* is responsive to ABA but not low temperature (Yamaguchi-Shinozaki and Shinozaki, 1994). The *rd29A* promoter sequence contained a dehydration-responsive element (DRE), with a nine base pair core motif, TACCGACAT. A similar motif containing the seven base pair core of the DRE (ACCGACA) was identified as a low temperature responsive element or C-repeat (CRT, Nordin et al., 1993; Baker et al., 1994; Wang et al., 1995). A shorter five base pair core sequence (CCGAC) first identified in *Brassica napus* (White et al., 1994; Jiang et al., 1996) was designated the low temperature-responsive element (LTRE, Shinwari et al., 1998). The corresponding DRE or CRT-binding factors (DREBs/CBFs) mediate gene expression under cold and drought or salinity (Stockinger et al., 1997; Liu et al., 1998a). These results support the existence of other signalling pathways that function independent of ABA. Pathway III is regulated by DREBs, and requires posttranslational activation of the TFs (Liu et al., 1998a; Liu et al., 1999). Pathway IV requires *de novo* synthesis of CBFs for interaction with DRE/CRT motifs (Gilmour et al., 1998). Unlike the ABREs, DREs do not require synergy with other elements to function in ABA-independent regulation of gene expression (Narusaka et al., 2003). Narusaka et al. (2003) reported that CBF and DREB2 (ABA-independent TFs) could function cooperatively with ABREs (ABA-dependent TFs), demonstrating crosstalk between ABA-dependent and -independent pathways. Although DREs could be activated in the absence of ABA, the involvement

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of ABA resulted in improved expression of *rd29A* under osmotic stress. An ethylene response element binding protein (EREBP/AP2) was also shown to interact with DRE motifs (Stockinger et al., 1997; Liu et al., 1998a). Many drought-inducible genes contain both DRE and ABRE motifs in their promoters (Yamaguchi-Shinozaki and Shinozaki, 2005; 2006) and DREs can act as CEs for ABREs.

DREB1 functions in cold-responsive gene expression whereas DREB2 regulates genes that respond to drought and heat (Liu et al., 1998a; Sakuma et al., 2006a; Sakuma et al., 2006b). These results demonstrate that, despite the reported crosstalk, cold and dehydration signalling operate through distinct pathways. The CBF transcription factor is regulated by several components (Massari and Murre, 2000; Chinnusamy et al., 2003). One of the pathways is ABA-dependent (Pathway V) and involves interaction with ABREs. The other two pathways are ABA-independent (Thomashow, 1999). Pathway VI does not require *de novo* protein synthesis while Pathway VII requires synthesis of CBFs. Some components involved in the transcriptional activation of cold-responsive genes are unknown (Pathways V and VI, denoted by question marks).

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1.5.3.3 The involvement of other phytohormones in controlling gene expression

Ethylene gas promotes fruit ripening, leaf senescence and abscission, flowering, cell elongation, abiotic stress, and may support or inhibit seed germination (Ecker, 1995; Johnson and Ecker, 1998; Wang et al., 2002). EIN3 is a transcription factor (Chao et al., 1997; Solano et al., 1998) that regulates ethylene response factors (ERFs), also known as ethylene response element-binding proteins (EREBPs). EREBPs bind to GCC box motifs found in promoters of many pathogen-responsive genes (Berrocal-Lobo et al., 2002; Chen et al., 2002).

Auxins regulate plant developmental and physiological processes such as embryogenesis, organogenesis, and shoot and root growth (Woodward and Bartel, 2005). Three gene families, GH3s, SAURs (Small Auxin-Up RNAs) and Aux/IAA are rapidly induced following auxin application (Hagen and Guilfoyle, 2002). Auxin-responsive elements (AuxREs) are found in promoters of genes that are regulated by auxins (Ulmasov et al., 1995). Auxin response factors (ARFs) are transcriptional regulators controlling the expression of auxin-responsive genes (Ulmasov et al., 1997; Tiwari et al., 2003). More details on auxin signalling have unravelled since the

identification of an auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

Salicylic acid (SA) is the key signalling molecule produced in response to biotic stresses. SA-inducible gene expression is well characterised using *WRKY* genes (Dong et al., 2003). The WRKY proteins are transcription factors that contain domains with WRKY amino acids. These TFs recognise and bind to W-box cis-acting response elements (T/CTGACC/T, Maleck et al., 2000; Ulker and Somssich, 2004). Many genes belonging to the WRKY superfamily are involved in different physiological and developmental processes, including abiotic stresses, senescence, and seed germination (Eulgem et al., 2000; Xie et al., 2005; Zhang and Wang, 2005).

Jasmonic acids (JAs) are involved in many processes that include fruit ripening, root growth, responses to wounding, insect damage, pathogens and abiotic stresses (Turner et al., 2002; Devoto and Turner, 2003). MAPK cascades are implicated in JA signalling (Ellis and Turner, 2001; Takahashi et al., 2007). Some JA-responsive TFs, also called GCC-box binding proteins, possess sequence similarity to ethylene response binding factors (ERFs; Ohme-Takagi and Shinshi, 1995; Fujimoto et al., 2000). The TFs bind to jasmonic acid and elicitor responsive elements (JEREs) found in promoters of JA-responsive genes (van der Fits and Memelink, 2001).

Gibberellic acid (GA), widely regarded as a growth-promoting hormone, regulates processes such as seed germination, stem elongation, leaf expansion, and flower and fruit development (reviewed by Swain and Singh, 2005). ABA and GA are antagonistic, with the former inhibiting growth and the latter promoting it (Xie et al., 2006)**.** Many of the studies on GA signal transduction pathways were done using cereal aleurone cells as a model system. Promoters of α-amylases and other genes expressed in the aleurone contain GA response elements (GAREs) with the common motif TAACAAA (Skriver et al., 1991; Gubler and Jacobsen, 1992; Gubler et al., 1995; Cercos et al., 1999; Gubler et al., 2002).

1.5.3.4 Sugar signalling

Sugars not only fuel metabolism but also serve as signalling molecules. They possess hormone-like properties and regulate many aspects of growth, development and cellular processes including transcription, protein synthesis and stability, and the activities of many enzymes (Smeekens, 2000). Sugar sensing and signalling help to maintain cellular homeostasis, growth and development under fluctuating

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environmental conditions (Roitsch, 1999; Moore et al., 2003; Yanagisawa et al., 2003). Carbon metabolism is greatly affected by abiotic stresses and is interlinked with hormone signalling (Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002). The regulation of sugar concentrations is intricately connected to photosynthesis, light quality and many other abiotic stresses (Nakashima et al., 1998; Rolland et al., 2006; Rook et al., 2006). One of the best characterised receptor is the hexokinase (HXK) which interacts with many signalling components to promote or repress processes involved in carbohydrate metabolism (Rolland et al., 2002; Blasing et al., 2005; Moreno et al., 2005). HXK-mediated signalling converges with ABA, ethylene, auxin and cytokinin signalling components as demonstrated by several mutant phenotypes that show altered sensitivities to both hormone and sugar signals (Leon and Sheen, 2003; Gibson, 2005). Studies on dehydration of *S. staphianus* and *X. viscosa* showed that the activities of HXK in these plants increased during dehydration (Whittaker et al., 2001).

Many sugar-inducible genes contain WRKY binding motifs in their promoters. These response elements are also found in α-amylase genes and many genes involved in disease resistance (Maleck et al., 2000). G-box and related sequences are found in many sugar-responsive genes (Martinez-Garcia et al., 2000). WRKY and G-box motifs are also found in promoters that are responsive to SA and ABA respectively, showing that sugars, hormones and pathogen defence signalling pathways may share components (Rolland et al., 2002).

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1.6 Genes involved in adaptation and protection

Changes in gene expression and the protein complement of a range of gene families have been observed. Many researchers have attempted to link expression levels to observed stress tolerance. However, the physiological roles of many gene products associated with adaptation and tolerance are largely unknown.

1.6.1 Molecular chaperone proteins

Late embryogenesis associated (LEA) proteins accumulate during embryo development or in response to abiotic stresses. Many classes of LEA proteins have been isolated. They are characterised by specific amino acid motifs, hydrophilicity, heat stability, and existence in a generally unfolded state (Dure, 1993). LEA proteins are thought to play a role in protecting macromolecules in the cytoplasm (Blomstedt

et al., 1998; Mowla et al., 2006). Another class of molecular chaperones are the heat shock proteins (HSPs). These proteins are strongly induced by heat stress and their accumulation in plants and other organisms imparts thermotolerance (Chen and Vierling, 1991; Feder and Hofmann, 1999). Understanding the regulation of HSPs is complicated by the fact that some of the proteins are essential for normal growth and development, and tolerance to other stresses apart from heat (Sun et al., 2002; Sun and MacRae, 2005; Kotak et al., 2007). However, the evidence that small HSPs can bind to partially unfolded proteins, utilise ATP to refold and activate denatured proteins (Horwitz, 1992; Jakob et al., 1993; Nakamoto and Vigh, 2007) and modulate membrane fluidity (Balogi et al., 2005) has demonstrated the involvement of some HSPs in the protection of cellular components during stress.

1.6.2 Control of ion homeostasis: pumps, transporters and channel proteins

The influx and transport of ions within a cell is important for plant growth and development as well as signal transduction. Ca^{2+} transients, used as a signalling strategy, are well characterised in guard cells (Assmann and Shimazaki, 1999). Ion-transporting proteins are found on plasma membranes and on intracellular membranes of organelles. A diverse array of these membrane-localised transporters is involved in ion influx, efflux and compartmentation (Pardo et al., 2006), utilising a variety of strategies to maintain or re-establish ion homeostasis. Ionic environments cause osmotic stress, cytotoxicity and oxidative stress as a result of the disruption of cell homeostasis and biochemical processes (Mittler, 2002). Although the activities of many membrane transporters are not well characterised, there is valuable information on co-transporters such as Ca^{2+}/H^+, Na^+/H^+, H^+/K^+, and Na^+/K^+ exchangers (Blumwald, 2000; Blumwald et al., 2000; Maser et al., 2002; Yokoi et al., 2002; Apse et al., 2003; Hepler, 2005). A stress-responsive vacuolar ATPase (V-ATPase) subunit c″ homologue was isolated from *X. viscosa* (Marais et al., 2004). XvVHA-c″1 was able to complement subunit c yeast knockout mutants grown under salt stress. Another *X. viscosa* protein, XvSap1, an intrinsic membrane protein homologous to K^+ transporters, was shown to improve membrane stability under heat stress as measured by electrolyte leakage (Garwe et al., 2003; 2006). Both these proteins were induced several abiotic stresses, especially dehydration.

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1.6.3 Antioxidants

Low levels of ROS are essential for normal cell function, redox balance and signalling (Mittler et al., 2004; Davletova et al., 2005b; Bailey-Serres and Mittler, 2006; Gapper and Dolan, 2006). ROS can lead to oxidative stress and ultimate cell death if allowed to accumulate (Apel and Hirt, 2004; Valko et al., 2004). Cells contain an array of molecules that can detoxify ROS and prevent damage to cellular components (Halliwell, 2006; Kwak et al., 2006). ROS scavengers include pyridine nucleotides (NADH and NADPH), ascorbate, glutathione (GSH), anthocyanins and tocopherol (Dietz, 2003; Foyer and Noctor, 2005). Antioxidant enzymes are present in cells even under non-stress conditions. The antioxidative capacity of cells determines their survival under stress. Detoxification enzymes include several peroxidases, glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), peroxiredoxins, thioredoxins and different reductases (Noctor and Foyer, 1998; Smirnoff, 2000; Smirnoff and Wheeler, 2000; Mittler, 2002; Dietz, 2003; Foyer and Allen, 2003; Wood et al., 2003; Davletova et al., 2005a). Accumulation of antioxidants has been documented for many resurrection plants (Farrant, 2000; Mundree and Farrant, 2000; Farrant et al., 2003). In *X. viscosa*, two peroxiredoxin genes that are differentially regulated by stress were isolated. *XvPer1* is a novel antioxidant enzyme that accumulates in vegetative tissues (Mowla et al., 2002) whereas related proteins are seed specific (Haslekas et al., 1998; Stacy et al., 1999). *XvPrx2* is capable of detoxifying ROS, including toxic peroxides (Govender, 2006). Another class of antioxidant molecules includes osmolytes (Hare et al., 1998; Chen and Dickman, 2005). Enzymes involved in the biosynthesis of several classes of osmolytes have been isolated in many plants.

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1.6.4 Osmolytes

Osmolytes are low molecular weight organic compounds that can accumulate to high concentrations in cells without perturbing cellular functions (Yancey et al., 1982; Sakamoto and Murata, 2002). Due to their non-disruptive properties, osmolytes are also called 'compatible solutes' (Shen et al., 1999). Osmolytes comprise a wide range of organic compounds that includes amino acids and derivatives, quaternary ammonium compounds, polyhydric alcohols or 'polyols', both cyclic (cyclitols) and straight chain (alditols), and non-reducing sugars such as sucrose and trehalose (Shen

et al., 1999; Garg et al., 2002; Armengaud et al., 2004). The accumulation of these metabolites is a predominant characteristic of resurrection plants, micro-organisms and animals under osmotic stress (reviewed by Serraj and Sinclair, 2002). The osmoprotectants can stabilise cell structures by interacting with both hydrophobic and hydrophilic domains of macromolecules thereby maintaining their native conformations and functions (Sheveleva et al., 1998; Shen et al., 1999; Sakamoto and Murata, 2002; Xiong and Zhu, 2002). Bryant et al. (2001) however argue that specific interactions are less important than the effects of the solutes on phase transitions.

The survival of plants under limiting water levels is presumably linked to the presence of osmolytes that sustain cell activities by aiding water retention. The metabolites are energy rich, serving as an alternative source of carbon and energy (Pattanagul and Madore, 1999; Shen, et al., 1999). Osmoregulation or osmotic adjustment is achieved by the synthesis of compatible solutes in response to prolonged water deficit. The accumulation of osmolytes leads to a decrease of the cell osmotic potential. The affected cells can sustain turgor pressure as well as absorb water from dry environments (Bray, 1993; Ingram and Bartels, 1996; Sakamoto and Murata, 2002). Osmolytes are also proposed to participate in detoxifying ROS (Shen et al., 1997b; Yoshiba et al., 1997; Pattanagul and Madore, 1999; Shen et al., 1999). In addition, the altered metabolism of carbohydrates during desiccation is linked to the accumulation of osmolytes, the majority of which are sugars and sugar alcohols (Shen et al., 1997b; Pattanagul and Madore, 1999).

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1.6.4.1 Sugars and polyols

Sucrose is the dominant carbohydrate that accumulates in higher plants under desiccation stress (Scott, 2000; Whittaker et al., 2001; Cooper and Farrant, 2002). Sucrose is thought to protect dehydrated cells by stabilising cell membranes and proteins through the maintenance of inter- and intra-cellular hydrogen bonding (Ingram and Bartels, 1996; Ingram et al., 1997; Scott, 2000). The hydrogen bonding probably preserves the integrity of structural lipids, and maintains the functions of proteins and the activities of enzymes by protecting functional groups (Scott, 2000). In *X. viscosa*, glucose and fructose accumulate in drying leaves but are metabolised to sucrose as desiccation continues (Whittaker et al., 2001), implying that the accumulation of this sugar is involved in the acquisition of desiccation tolerance. Some higher plants are thought to use hexitols (for example, sorbitol and mannitol)

instead of sucrose as the major photosynthetic products and for carbohydrate translocation (Swedlund and Locy, 1993).

Some plants naturally accumulate polyols to counter the adverse effects of the environment. One strategy used by plants subjected to chronic hyperosmosis is the accumulation of sorbitol (Ahmad et al., 1979). Polyols have been identified in axes of germinating soybean (*Glycine max*; Kuo et al., 1990), maize (*Zea mays*) silks and seeds (Shaw and Dickinson, 1984; Krall et al., 1989), celery (*Apium graveolens*, Fox et al., 1986; Davis et al., 1988) and in several fruit trees (Moing et al., 1997; Noiraud et al., 2001). It has been suggested that polyols provide osmotic adjustment in plants growing under low water potentials. Moreover, these sugar alcohols may serve as reducing power and carbon source in stressed cells (Tao et al., 1995). Plants that naturally synthesise polyols accumulate large quantities which may be adequate for the proposed function in osmotic adjustment. In contrast, transgenic strategies engineering genes for polyol synthesis in plants have attained low levels of metabolite accumulation, yet improved tolerance is achieved. The compounds are also thought to function as free radical scavengers, reducing damage to cellular structures and macromolecules (Shen et al., 1997b; 1997a; Zhu, 2002). Although the evidence for these functions is mainly correlative, plants grown under osmotic stress conditions have been shown to accumulate polyols in the cytoplasm (Ahmad et al., 1979; Mundree and Farrant, 2000; Deguchi et al., 2006). Concomitant with the altered carbohydrate metabolism is the increase in the activity of aldose reductase, implying the involvement of this enzyme in protection mechanisms.

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1.6.4.2 Aldose reductase

Aldose reductase (AR) enzymes are traditionally known for their function in the polyol pathway (Fig. 1.4), catalysing the conversion of glucose to sorbitol (Srivastava et al., 2005). Not much is known about the physiological roles of AR in osmotic stress tolerance in plants. The proposed effects of the metabolites include; the shifts in the balance of redox molecules, NADP(H) and NAD(H), the osmoprotective roles of sorbitol and the control of carbohydrate metabolism through fructose-6-phosphate. Plant genes that are homologous to AR have been isolated from various species including some resurrection plants (Negm, 1986; Bartels et al., 1991; Lee and Chen, 1993; Li and Foley, 1995; Roncarati et al., 1995; Mundree et al., 2000; Gavidia

et al., 2002). AR may have multiple physiological functions that may interact with defence mechanisms against osmotic and oxidative stress.

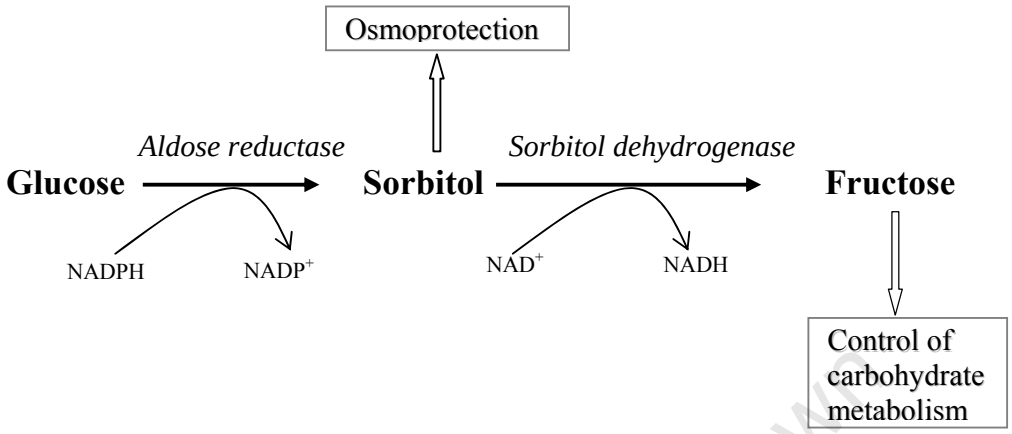

Figure 1.4 A schematic representation of the polyol pathways
Aldose reductase is the first enzyme in the polyol pathway; reducing glucose to sorbitol and utilising NADPH as a cofactor. The balance of the nicotinamide cofactors may cause a shift in the redox state of the cell. The sorbitol may accumulate in cells, leading to osmoprotection or osmotic adjustment. Alternatively, the sorbitol is metabolised to fructose by sorbitol dehydrogenase. Fructose may be utilised as an energy source and/ or serve as a second messenger for the control of carbohydrate metabolism. Adapted from Srivastava et al. (2005), a model for plants.

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1.7 AKR proteins

AR enzymes belong to the aldo-keto reductase (AKR) superfamily. The AKR superfamily is one of three oxidoreductase superfamilies. The other two families are the medium-chain and the short-chain dehydrogenases (Oppermann et al., 2003). AKR superfamily proteins are found in all forms of life, including mammals, amphibians, reptiles, plants, yeast, protozoa and bacteria. The AKR enzymes are characterised by their multiplicity for a wide range of carbonyl substrates that include aliphatic and aromatic aldehydes, monosaccharides, carbohydrates, steroids, prostaglandins and xenobiotics (Welle et al., 1991; Takahashi et al., 1993; Bruce et al., 1994; Penning et al., 1996; Cao et al., 1998; Bauman et al., 2004; Petrash, 2004). However, some members of the oxido-reductase superfamily can catalyse saturated carbonyl bonds (alkenal reductases, Mano et al., 2002). The diversity of substrates may be indicative of both housekeeping functions and specialised physiological roles played by AKRs to mitigate the effects of toxic products of biotic and abiotic stresses.

A nomenclature for AKR proteins, based on amino acid sequence identity, superseded the old system that was based on substrate specificity (Jez et al., 1997b).

The old naming system failed to cope with the wide substrate specificities of AKR enzymes. The naming became confusing as one enzyme ended up being assigned multiple names, for example the human liver 3α-hydroxysteroid dehydrogenase (HSD) type II (E.C. 1.1.1.213; Khanna et al., 1995a) was also called dihydrodiol dehydrogenase (DD) type X (E.C. 1.3.2.20; Deyashiki et al., 1994), and chlordecone reductase (E.C. 1.1.1.255; Winters et al., 1990). Using the new nomenclature, delineation into families occurs at about 40% amino acid sequence identity. Within a family, proteins with amino acid sequence identity greater than 60% constitute a subfamily. The new names contain the root symbol "AKR" for Aldo-Keto Reductase, an Arabic number designating the family, a letter indicating the subfamily when multiple subfamilies exist and an Arabic numeral representing the unique protein sequence.

To date more than a hundred proteins belonging to 15 families have been identified, with a similar number regarded as "potential members" [AKR Homepage: www.upenn.edu/akr; (Hyndman et al., 2003)]. Despite a lot of research into this superfamily, the physiological functions of most of the proteins are still not clear. The human ARs are the most studied of the AKR proteins. The ARs are implicated in the development of diabetic neuropathy and retinopathy (Bohren et al., 1989; Wallner et al., 2001; Srivastava et al., 2005; Srivastava et al., 2006). Researchers have intensified efforts into finding AR inhibitors to reverse polyol accumulation-associated problems in diabetics. However, the use of inhibitors has resulted in many pathological complications, indicating that ARs are responsible for other functions besides the synthesis of osmolytes.

Many plant AKRs belong to family 4 of the superfamily, comprising chalcone polyketide, codeinone and aldehyde reductases (Jez and Penning, 2001; Hyndman et al., 2003). Using the new nomenclature, the *X. viscosa* aldose reductase (*XvAld1*) was classified as an aldehyde reductase (ALR) belonging to family 4, subfamily C and is the fourth protein in the subfamily (*AKR4C4*; Jez and Penning, 2001).

1.7.1 AKRs as osmolyte synthase enzymes

In animals, renal cells are the best model for the control of gene expression in hyperosmotic environments. Renal cells are routinely exposed to high and variable levels of salt (NaCl) as a result of their function in urine concentration and excretion. These cells have been extensively studied to identify genes and the mechanisms

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regulating their expression under hypertonic conditions. The accumulation of osmolytes is a delayed response to osmotic stress. Sorbitol, inositol, glycine, betaine and glycerophosphorylcholine (GPC) are the major compatible solutes in the renal cells (Uchida et al., 1989; Kwon et al., 1991; Moriyama et al., 1991; Robey et al., 1991; Uchida et al., 1991; Zablocki et al., 1991; Lopez-Rodriguez et al., 2004). Sorbitol is synthesised from glucose by aldose reductase (AR, Garcia-Perez and Burg, 1991a; 1991b; Smardo et al., 1992) and GPC from phosphatidylcholine (Bagnasco et al., 1986; Balaban and Burg, 1987).

Hyperosmotic stress upregulates the transcription of AR mRNA and translation into the protein, resulting in increased activity of the enzyme (Garcia-Perez et al., 1989). The increased enzyme activity is correlated with the reduction of glucose and the accumulation of sorbitol (Uchida et al., 1989; Smardo et al., 1992). The activity of AR in animals is not restricted to renal cells but is also found in liver cells, ocular lens, small intestines, and erythrocytes (Cao et al., 1998). The functions of AR in some of these cells are not well understood.

The characterisation of plant AR-like enzymes has been influenced by the osmoprotective function attributed to the kidney AR and the presumed involvement of the mammalian ARs in diabetic complications. Researchers have traditionally concentrated on the polyol pathway as the major biochemical function of AR-like enzymes. Recently, studies have started treating the wide substrate specificity of AKR enzymes as indicative of a wide range of functions.

1.7.2 AKRs are involved in detoxification

The broad and overlapping substrate specificities of the closely related AKR enzymes make them ideal for detoxification functions. The characterisation of many AR-related enzymes was originally driven by the interest in diabetic complications. As a result, many of the enzyme characteristics are based on hyperglycaemic environments. Experimental evidence shows that many AKRs cannot efficiently use simple sugars as substrates (Roncarati et al., 1995; Mano et al., 2002; Crabbe, 2003; Hideg et al., 2003; Matsunaga et al., 2006) except when the sugar levels are above physiologically relevant concentrations. The roles of AKRs under euglycaemic conditions are largely unknown. When plants are exposed to environmental stress conditions, photosynthesis may be limited and energy becomes limiting. The levels of glucose in these cells should therefore be low, leaving little glucose for channelling

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into polyol synthesis. Of potential physiological significance, however, is the reduction of cytotoxic aldehydes such as 4-hydroxynon-2-enal (HNE). HNE has been shown to react with cellular macromolecules and high levels are toxic to cells.

Oberschall et al. (2000) identified an AR homologue from alfalfa. Transgenic tobacco plants overexpressing AR were tolerant to paraquat, an oxidative stress-inducing agent. ROS are known to destroy lipid membranes and other cellular macromolecules. The accumulation of products of lipid peroxidation is used as a measure for lipid damage (Srivastava et al., 2000; Burczynski et al., 2001). The alfalfa AR could also reduce HNE *in vitro*. The transgenic plants, apart from showing increased tolerance to salt and heavy metals, had lower levels of HNE compared to the wild type controls (Oberschall et al., 2000; Hideg et al., 2003). These observations imply a possible role for AR as a detoxification enzyme. The enzyme itself may detoxify cytotoxic aldehydes and related substrates (Srivastava et al., 2001; Mano et al., 2002; Srivastava et al., 2002; Srivastava et al., 2004; Spite et al., 2007) or the osmolyte products of AR enzyme activities may be involved in quenching ROS (Shen et al., 1997b; 1997a). The results from the alfalfa AR are corroborated by investigations involving animal AKRs. The AR-mediated reduction of free aldehydes and glutathione-HNE conjugates has been demonstrated (Srivastava et al., 1995; Srivastava et al., 1998; Rittner et al., 1999; Dixit et al., 2000; Ramana et al., 2000). Other substrates that may be detoxified include methylglyoxal (Vander Jagt et al., 1992; Aguilera and Prieto, 2001; Vander Jagt and Hunsaker, 2003), and exogenous substrates (xenobiotics) such as pollutants and therapeutic drugs (Kolb et al., 1994; Lee et al., 2002). The lack of stereospecificity of some AKR enzymes (Crabbe, 2003) may be an evolutionary characteristic that tailored these enzymes to detoxify toxins of both endogenous and exogenous origin.

The studies by Oberschall et al. (2000) and Hideg et al. (2003) have provided evidence that AR may provide protection through mechanisms other than the accumulation of osmoprotectants (Bartels, 2001). These results also supported previous observations that simple sugars are unlikely to be the physiological substrates for ARs. The continued search for physiologically relevant substrates may result in the assignment of physiological functions to many AKR proteins in plants.

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1.7.3 Possible roles of AKR enzymes in signalling

Some evidence suggests that AKR-induced metabolic changes may be associated with signalling functions. In animals, these enzymes have been linked to cytokine-mediated (Ramana et al., 2003a) and tumour necrosis factor- (TNF) α-mediated (Chandra et al., 2003; Ramana et al., 2003b; Ramana et al., 2007) signalling pathways as well as many cascades involving protein kinase C (PKC, Henry et al., 1999; Varma et al., 2003). In plants, the signalling roles of AR-related enzymes have not been investigated. However, researchers are beginning to realise the regulatory functions of many carbohydrate metabolites, some of which are products of osmolyte synthase enzymes (Avonce et al., 2004; Rolland et al., 2006; Paul, 2007).

1.8 Objectives of the study

The overall objective of this study was to determine whether *XvAld1*, a stress-responsive gene isolated from the resurrection plant *X. viscosa*, could confer stress tolerance when transformed into susceptible plants. To contribute towards the overall aim, we focused on a number of specific objectives.

To determine the genome organisation of the *XvAld1* gene, the genomic DNA sequence was isolated by PCR using gene specific primers. In addition, thermal asymmetric interlaced (TAIL)-PCR was used to amplify the 5′ untranslated region, utilising a combination of gene specific and degenerate primers. Sequencing of the 5′ untranslated and protein coding genomic regions and comparison with the cDNA sequence could reveal the mechanisms involved in transcriptional control of the *XvAld1* expression. Computational analysis of these sequences was used to identify putative promoters together with *cis*-acting response elements, which constitute another level of transcriptional control. The upregulation of *XvAld1* mRNA by various stresses and hormonal cues was investigated using real time PCR to improve on previous work that employed the less sensitive dot blots.

The *XvAld1* cDNA was cloned into an expression vector and overexpressed in *E. coli* cells. The recombinant protein was utilised to produce polyclonal antibodies after immunising rabbits. In addition, the protein was used for in vitro assays to characterise the biochemical properties of the *XvAld1* protein.

The functions of the XvAld1 protein could be inferred from its subcellular localisation in *X. viscosa* and in onion epidermal cells. Green fluorescent protein

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(GFP) and yellow fluorescent protein (YFP) fusions in onions and immunocytochemistry in hydrated and dehydrated *X. viscosa* sections were analysed using fluorescence microscopy and XvAld1 polyclonal antibodies, respectively.

The accomplishment of the above would advance our understanding of the *XvAld1* gene and protein, while transformation of two model plants, *A. thaliana* and *Digitaria sanguinalis*, with the *XvAld1* cDNA would answer whether the protein confers stress tolerance in susceptible plants. *A. thaliana* and *D. sanguinalis* plants were transformed using Agrobacterium-mediated and transformation biolistic methods, respectively. Putative transgenic plants were analysed to confirm integration of the transgene into the host genome, and the expression of both mRNA and protein. To achieve this, PCR, reverse transcriptase (RT)-PCR, real time RT-PCR and northern blot analysis were used in combination to screen for transgenic plants, monitor expression levels and confirm the heritability of the transgene. In addition, western blot analysis confirmed the accumulation of the protein and its distribution in different plant tissues.

To assess improved stress tolerance in transgenic plants expressing the XvAld1 protein, physiological and biochemical analyses of seedlings in tissue culture and mature potted plants were carried out. Various osmotica, at a range of concentrations, were used to impose water deficit to seedlings in tissue culture. Mature plants were stressed by withholding water for specified periods. Statistical analyses were used to test the significance of any observed trends in stress tolerance.

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CHAPTER 2

Characterisation of the aldose reductase from *X. viscosa*: genome organisation, functional motifs and comparison with other AKRs

2.0 SUMMARY

The *Xerophyta viscosa* aldose reductase (XvAld1) deduced amino acid sequence was aligned and compared with sequences from other plant and animal AKR proteins. The alignment showed sequence conservation within the $(\alpha/\beta)_8$ barrel regions, a characteristic of aldo-keto reductases. Major sequence variations were observed in the loop regions and the non-core forming α-helices and β-sheets. Computational analyses for post-translational modifications predicted N-glycosylation, phosphorylation by many kinases, and several motifs for targeting the protein to various subcellular compartments. Southern blot analysis revealed a complex banding pattern that was partially resolved by sequencing of genomic clones. The Southern blot results are indicative of the presence of more than one copy of the gene, or the existence of a multifamily of related genes. A genomic clone isolated by PCR revealed that the *XvAld1* gene is organised into nine exons and eight introns spanning ~2.9 kb, excluding the 5′ and 3′ untranslated regions (UTRs). The nucleotide differences between the sequenced clones could neither be attributed to sequencing errors nor to polymorphisms because of limited sequence information on *X. viscosa*. The 5′-UTR was amplified using thermal asymmetric interlaced PCR and sequenced. An 870-bp clone, that matched the 5′ leader sequence on the *XvAld1* cDNA, was analysed for documented *cis*-acting response elements (CAREs). Many of the identified CAREs matched those for hormonal regulation, organ specific expression, dehydration and high or low temperature responses, light and phytochrome responsiveness, wounding, as well as G-box, CAAT and TATA-boxes. For further characterisation, the XvAld1 protein was expressed in *E. coli* and purified using nickel-nitrilotriacetate affinity column for histidine tagged proteins. Polyclonal antibodies against the purified recombinant XvAld1 protein were generated by immunising rabbits. Western blot analyses using monoclonal antibodies against the histidine tag as well as the polyclonal antibodies against XvAld1 demonstrated the expression and purification of a 40.5-kDa monomeric protein.

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2.1 INTRODUCTION

The *X. viscosa* aldose reductase, *XvAld1*(AF133841; Mundree et al., 2000), is a member of the aldo-keto reductase (AKR) superfamily. XvAld1 is a 36-kDa monomeric protein (Mundree et al., 2000). The physiological roles of many AKR proteins are largely unknown. In order to gain insights into the possible biological functions of this enzyme, the DNA and protein sequences were analysed.

Southern blot analysis was used to estimate gene copy number and the genomic organisation of AKR genes in *X. viscosa*. Genomic structures of genes give information on control of gene expression and how the genes of interest are related to similar or neighbouring genes. The genomic sequences, including the 5′-untranslated region (UTR), were isolated and analysed. Little is known about how the expression of *XvAld1* is regulated in *X. viscosa*. The significance of the genome organisation and selected cis-acting elements will be discussed.

Multiple sequence alignments of XvAld1 with other plant AKRs were performed to determine the relatedness of the proteins and possibly infer structural and functional similarities. Post translational modifications (PTM) play a pivotal role in the functions of some proteins. Bioinformatics analysis was used to predict the possible post translational modifications that XvAld1 could undergo.

Protein-protein interactions are fundamental processes in all biological systems because they facilitate the biochemical functions of many gene products. Investigating the interacting partners of XvAld1 could give clues of the possible physiological functions of the *X. viscosa* protein. Based on insights gained from studying AKR proteins in animals, the significance of the predicted XvAld1 interacting partners will be discussed.

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2.2 MATERIALS AND METHODS

2.2.1 Plasmids

pSK-*XvAld1* (Mundree et al., 2000), the plasmid containing the *X. viscosa* aldose reductase cDNA was used as the template for PCR unless otherwise stated. pGEM™-T Easy (Promega Corporation, USA) or pDrive (Qiagen, Germany) TA cloning vectors were used for cloning of PCR products. The pJET plasmid (GeneJET™ PCR Cloning Kit; Fermentas, Germany) was used for blunt end cloning of PCR products. The pProEX HT™ System (Invitrogen Life Technologies, USA) was used for expression of the *XvAld1* recombinant protein.

2.2.2 Transformation protocol

Aliquots (50 µl) of competent cells of the desired strain were mixed with 5 µl of the ligation reaction or purified plasmid and incubated on ice for 20 min. The cells were heat shock treated by incubating the tubes at 37°C for 5 min. The tubes were quickly put back on ice for another 5 min. A millilitre of Luria Bertani (LB; Appendix A) media was added and the tubes were incubated at 37°C for up to 2 hr. Appropriate volumes of the transformed cells were plated on agar plates supplemented with the appropriate antibiotic and incubated overnight at 37°C.

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2.2.3 DNA oligonucleotides

Unless otherwise specified, all oligonucleotides (listed in Appendix B) were synthesised on a Beckman 1000M DNA synthesiser (Beckman Instruments Inc., USA; Synthetic DNA Laboratory, University of Cape Town).

2.2.4 DNA extraction

DNA was isolated using an adaptation of the Dellaporta et al. (1983) method. Approximately 1 g of leaf tissue was ground to a fine powder in liquid nitrogen. DNA was extracted from the ground leaf material using 15 ml of extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 10% β-Mercaptoethanol). The DNA was precipitated using 0.5 volumes of isopropanol, centrifuged at 12 000 x g for 20 min and the pellet was resuspended in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). The DNA was purified by successive extractions with equal volumes of phenol, phenol-chloroform (1:1) and chloroform-isoamyl alcohol (24:1). The DNA

was again precipitated, pelleted and resuspended in TE. DNA was quantified using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Alternatively, DNA for PCR analysis was isolated from about 100 mg of leaf material using Edwards' method (1991). The quality of the extracted DNA was checked by electrophoresis on ethidium bromide-stained 1% (w/v) agarose gel using 0.5 x TBE (45 mM Tris-HCl, 45 mM borate, 1 mM EDTA). DNA was viewed under ultra violet (UV) light. Gel pictures of nucleic acids were obtained using the GDS 2000 gel documentation system (UVP Ltd, UK) or the Bio-Rad Molecular Imager Gel Doc XR system and the Quantity One 1-D analysis software (Bio-Rad Laboratories, Germany).

2.2.5 Southern blot analysis

Ten microgram aliquots of DNA were separately digested with *Eco*RI, *Eco*RV, *Pvu*II, *Xba*I and *Eco*RI/ *Xba*I at 37ºC and separated on 1% agarose gels at 45V for 4 hr or overnight at 20V. All the restriction enzymes were obtained from Roche Diagnostics (Germany), Fermentas Life Sciences (Germany) or New England Biolabs (USA). The DNA was blotted onto Hybond™–N^+ or Hybond™–XL membranes (Amersham Biosciences, UK) by capillary transfer (Sambroock et al., 1989) or the downward gravity transfer method (Koetsier et al., 1993).

XvAld1 cDNA, used as probe, was labelled with radioactive [α-^{32}P]-dCTP (Amersham Biosciences, UK) using PCR. The reaction consisted of 20 – 100 ρg of *XvAld1* cDNA, 1 x PCR reaction buffer, 1.5 mM $MgCl_2$, 1.5 mM dNTP mix (containing equal concentrations of dATP, dGTP and dTTP), 50 µCi [α-^{32}P]-dCTP, 200 ρM each *XvAld1* gene specific primers, 1 unit/reaction of Super-therm Taq DNA polymerase (JMR Holdings, UK) and sterile distilled water to 50 µl. The *XvAld1* sense (XvAld1A-F; 5′-CGGCACGAGAAGCTACAGAGA-3′) and antisense (XvAld1B-R; 5′-CGAAGACCTGGATGTTCTCC-3′) primers correspond to positions 1 – 21 and 873 – 892 of the *XvAld1* cDNA sequence (AF133841) respectively.

The Hybaid PCR Sprint thermal cycler (Thermo Hybaid, UK) was used for amplification under the following conditions: 95°C for 5 min; 25 cycles of 95°C for 30 sec, 58°C for 1 min, and 72°C for 10 min; and a final extension step at 72°C for 10

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min. Unincorporated [α-^{32}P]-dCTP was removed by passing the PCR product through a Sigma Post-Reaction Clean-Up Column (Sigma-Aldrich, Germany).

Blots were prehybridised at 65°C for 30 min to 4 hr in prehybridisation buffer (1% BSA, 1 mM EDTA, 0.5 M PB, 7% SDS). Hybridisation with the labelled *XvAld1* probe was carried out at 65°C for 16 hr. The blots were subsequently washed twice with Wash Buffer A (2 x SSC, 0.5% SDS) at 65°C for 10 min and more stringently with Wash Buffer B (0.5 x SSC, 0.1% SDS) at 65°C for 5 min. The number of washes in Wash Buffer B was determined empirically by monitoring the residual radioactivity on the membrane after each wash using a hand-held Geiger counter (Amersham Biosciences, UK). Washing was stopped when the count was between 20 and 50 counts per minute (cpm). After washing, the membranes were exposed to high-performance autoradiography film (Hyperfilm™ MP; Amersham Biosciences, UK) in a light proof cassette and kept at -80°C for up to a week. The autoradiograms were developed manually.

Alternatively, *XvAld1* sense mRNA probe was prepared by *in vitro* transcription using non-radioactive DIG-RNA labelling mix (Roche, Germany). Briefly, the *XvAld1* coding sequence was PCR amplified from 1 ng of plasmid pSK-*XvAld1* using M13 forward (5′-TTCCCAGTCACGACGTTG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers that flank the T7 promoter and the *XvAld1* gene. The Phusion DNA polymerase kit (Finnzymes, Finland) was used for PCR amplification to produce blunt-end fragments suitable for the *in vitro* transcription. The PCR products were separated by electrophoresis on an ethidium bromide-stained 1% (w/v) agarose gel. The *XvAld1* DNA band was excised from the gel and purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK), following the manufacturer's protocol. The *in vitro* transcription reaction consisted of sterile water (up to 20µl), 1 x DIG-RNA labelling mix (1 mM rATP, 1 mM rCTP, 1 mM rGTP, 0.65 mM rUTP, 0.35 mM DIG-11-UTP, pH 7.5; Roche, Germany), 1 x T7 transcription buffer (Promega, USA), 200 ng *XvAld1* PCR product (template DNA) and 40U T7 RNA polymerase enzyme mix (Promega, USA). The *in vitro* transcription reactions were carried out according to instructions in the RiboMAX™ Large Scale RNA Production Systems-SP6 and T7 kit (Promega, USA). The reaction samples were incubated for 2 hr at 37°C before treatment with 2 U per reaction of RQ1 RNase-Free DNase (Promega, USA) at 37°C for 15 min. The unincorporated

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nucleotides were removed by extraction with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1; Promega, USA). The labelled mRNA transcript (aqueous phase) was precipitated by adding 0.1 volume of 3 M sodium acetate pH 5.2 and 3 volumes of absolute ethanol. The tubes were incubated at -20^{o}C for 20 min and centrifuged at 12 000 x *g* for 15 min. The RNA pellet was washed with 1 ml of 70% ethanol. The pellet was dissolved in 20 µl of RNase-free water by incubating the tubes at 65^{o}C for 3 min. The integrity of the mRNA transcripts was checked on an ethidium bromide-stained 1% (w/v) agarose gel. The concentration of the DIG-labelled mRNA was measured spectrophotometrically while the incorporation of DIG-11-UTP was determined according to the DIG RNA labelling Kit (Roche, Germany). The efficiency of DIG-labelling was assessed using a spot assay, by comparing serial dilutions of the *XvAld1* RNA with DIG-labelled control RNA (Roche, Germany), as outlined by the manufacturer.

Nucleic acids were blotted onto positively charged nylon membranes (Hybond-N^{+}; Amersham Biosciences, UK) and UV-cross-linked as previously described. Pre-hybridisation was carried out by incubating in DIG Easy Hyb buffer (Roche, Germany) at 50°C for 30 min. The DIG-labelled RNA probe was denatured by heating at 95°C for 10 min, and placing immediately back on ice. The pre-hybridisation buffer was drained and replaced with fresh DIG Easy Hyb buffer containing 20 ng/ml of the denatured sense *XvAld1* RNA probe. Hybridisation was carried out at 50°C for 12 - 16 hr in a sealed plastic bag. All hybridisation and washing steps were performed with continuous agitation. The hybridisation solution was drained and two low stringency washes were carried out at RT each for 5 min in Wash Buffer 1 (2 x SSC, 0.1% SDS). High stringency Wash Buffer 2 (0.1 x SSC, 0.1% SDS) was pre-warmed and was used for two 15 min washes at 65°C. Another wash for 5 min was performed in DIG washing buffer (Roche, Germany) for 2 min.

After blocking the membranes in 1 x DIG blocking buffer (Roche, Germany) for 30 min, anti-DIG-AP (alkaline phosphatase) was diluted 1:20 000 in 1 x DIG blocking buffer and incubated for 30 min to bind with the DIG-labelled nucleic acids. Two washes at RT for 15 min were carried out in DIG washing buffer to remove unbound antibody conjugate. Equilibration for 2 min in detection buffer was performed, followed by chemiluminescent detection using a 1:20 000 dilution of CDP-Star substrate (Roche, Germany). Incubation times were determined empirically.

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2.2.6 TAIL-PCR for 5′-UTR isolation

DNA was isolated from well-watered *X. viscosa* leaves using the Dellaporta method (1983) as previously outlined. Thermal asymmetric interlaced (TAIL)-PCR was carried out as described by Liu and Whittier (1995) with modifications. As the sequences upstream of the *XvAld1* gene (AF133841) are unknown, a set of degenerate primers: AD1; 5′-TG(A/T)GNAG(A/T)ANCA(G/C)AGA-3′, AD2; 5′-AG(A/T)GNAG(A/T)ANCA(A/T)AGG-3′, AD3; 5′-CA(A/T)CGICNGAIA(G/C)G AA-3′, and AD4; 5′-TC(G/C)TICGNACIT(A/T)GGA-3′ was used for forward primers, where "I" and "N" denote "deoxyinosine" and "any deoxynucleotide" respectively. The three nested gene-specific reverse primers used were within 120 bp upstream of the *XvAld1* start codon. The reactions were set up such that each of the four degenerate forward primers was paired up with a gene specific reverse primer as described below.

The first TAIL-PCR reactions were performed using 40 ng of *X. viscosa* DNA, 3 µM each of the degenerate primers, 0.5 µM of the gene specific reverse primer (ALD-PR1 5′-GCGTTGCCGGCCTTGTCA-3′), 0.25 mM dNTP mix, 1x Phusion GC Buffer, 3% dimethylsulfoxide (DMSO) and 0.02 U/µl Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). The PCR product was diluted 1:1000 and 1 µl of the diluted product was used for the second TAIL-PCR mix (as outlined above) except that ALD-PR2 (5′-CCAGACTTCCACGTCCCGAGC-3′) was used as reverse primer. The third PCR reaction was carried out using 1 µl of a 1:1000 dilution of the second PCR product as template and the reverse primers ALD-PR3 (5′-GGATTGAGTGCCCGCTGAGGA-3′) and P120 (5′-TGAGTGCCCGCTGAGGAGCTTG-3′). Unless specified, PCR was carried out using a Gene Amp 9700 thermocycler (Applied Biosystems, USA). The cycling conditions, adapted from Nakayama et al. (2001), are briefly outlined below. The first TAIL-cycling consisted of 5 high-stringency cycles of 98°C for 25 sec, 63°C for 30 sec and 72°C for 1 min; one low stringency cycle of 98°C for 25 sec, 30°C for 30 sec and 72°C for 1 min and 15 cycles of alternating high and reduced stringency cycling thus: 2 cycles of 98°C for 25 sec, 63°C for 30 sec and 72°C for 1 min (high stringency) and 1 cycle of 98°C for 25 sec, 44°C for 30 sec and 72°C for 1 min (low stringency). A final extension step at 72°C for 7 min was included. Cycling conditions for the second and third PCR reactions were the same. Each cycle consisted of two

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high stringency cycles of 98°C for 25 sec, 63°C for 30 sec, 72°C for 1 min and one low stringency cycle of 98°C for 25 sec, 44°C for 30 sec and 72°C for 1 min. The three alternating cycles of high and reduced stringency were repeated 15 times.

PCR products from the third PCR reaction were analysed by agarose gel electrophoresis. DNA bands of interest were excised from the agarose gel under long wavelength UV. The DNA was purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK) following the manufacturer's instructions. The blunt-ended PCR products were cloned into the pJET plasmid (Fermentas, Germany). Alternatively, 3′-A overhangs for TA cloning were added to the blunt ended PCR product by incubating the purified DNA with 0.25 mM dATP and 1 unit of Supertherm Taq DNA polymerase (JMR Holdings, UK) at 72°C for 20 min. The PCR products were cloned into the pGEM™-T Easy vector (Promega Corporation, USA) following the manufacturer's protocol. The ligated plasmids were transformed into competent DH5α *E. coli* cells using the heat-shock method as previously described. Colonies of putative transformants were screened by PCR using M13 forward (5′-TTCCCAGTCACGACGTTG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers. The PCR reaction consisted of 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 x SuperTherm buffer, 1 U/reaction Super-therm Taq DNA polymerase (JMR Holdings, UK) in a 25 µl reaction. PCR was carried out using the following conditions; 2 min at 94°C; 30 cycles of 94°C for 30 sec, 54°C for 1 min, 72°C for 1 min and finally 72°C for 7 min.

Plasmid DNA was isolated from overnight cultures of PCR positive colonies using EZ-10 Spin Column Plasmid DNA Kit (Bio Basic Inc., Canada). M13 forward and reverse primers were used for sequencing both strands of the insert DNA using a 3130 Genetic Analyser DNA capillary sequencer (Applied Biosystems, USA). Sequencing reactions were set up using BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA). Fragments longer than 1 kb were sequenced by primer walking. The sequences were analyzed *in silico* for *cis*-acting elements using the tools provided by the following databases for plant promoters: PlantCARE [http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/; (Rombauts et al., 1999; Lescot et al., 2002)], PLACE [http://www.dna.affrc.go.jp/PLACE; (Higo et al., 1999); and Softberry NSITE-PL [http://www.softberry.com/berry.phtml]. The results were screened manually for response elements associated with stress tolerance.

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2.2.7 Isolation of the *XvAld1* genomic DNA using PCR

Different combinations of the following gene specific primers were used for PCR amplification of the aldose reductase genomic sequence; sense primers XvAld1A-F; 5′-CGGCACGAGAAGCTACAGAGA-3′) and XvAld1-BF (5′-CGCATGCACCGTGTTTTGCTG-3′), and antisense primers XvAld1-AR (5′-CTTCACCGTCCCAGAGTTCAG-3′) and (XvAld1B-R; 5′-CGAAGACCTGGATGTTCTCC-3′). The *XvAld1* genomic sequence was amplified from 100 ng of *X. viscosa* genomic DNA in a 50 µl reaction containing 200 µM dNTPs, 0.5 µM each of the gene specific primers, 5% DMSO, 1 x Phusion GC buffer and 0.02 U/ µl Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Finland).

The PCR product was excised from ethidium bromide-stained 1% (w/v) agarose gel and purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK). The 3′-A overhangs were added and fragments were cloned into the pGEM™-T Easy vector (Promega Corporation, USA) as previously described. The *XvAld1* genomic fragment was sequenced by primer walking. Internal primers (Xv4F-Gen2: 5′-GATAACCACTATAGTGCTTCATTCATCG-3′; Xv4R-Gen2: 5′-GGATATTATTGATTTTCTTCATTTTCTTTCC-3′; and Xv4R-GenPR2: 5′-CCATTTCCCTCCACACTCCTCCTATGTCG-3′) were designed from the well resolved sequences of the genomic DNA fragment and used for further sequencing.

2.2.8 *XvAld1* sequence analyses

The BLAST programme of the National Centre for Biotechnology Information (NCBI) was used to search databases for sequence similarities. The gapped BLAST and PSI-BLAST (Altschul et al., 1997) were used for protein database searches. Nucleotide and amino acid multiple sequence alignments were constructed using CLUSTAL W version 1.4 (Thompson et al., 1994) function in the BioEdit programme (Hall, 1999). DNAMAN (Version 5.2.10, Lynnon BioSoft) was used to construct the homology tree from multiple sequence alignment files. The following five databases were used to search for interesting motifs: ELM database (Puntervoll et al., 2003) linked to the SMART/Pfam databases (Schultz et al., 1998; Letunic et al., 2006), Softberry [http://softberry.com/cgi-bin/programs/ploc/psite.pl], Prosite [http://ca.expasy.org/cgi-bin/prosite/; (Gattiker et al., 2002; Sigrist et al., 2002)], Minimotif Miner [MnM, http://sms.engr.uconn.edu/servelet/; (Balla et al., 2006)], and NetPhosK 1.0 [http://www.cbs.dtu.services/NetPhosK; (Blom et al., 2004)].

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2.2.9 Cloning and heterologous expression of the XvAld1 protein

The *X. viscosa* aldose reductase coding sequence was amplified from a cDNA clone in pSK-*XvAld1*. The *Sal*I-F primer; 5′-CGTCGACCC**ATG**GCGCATGCACCG TG-3′, was designed to contain a *Sal*I restriction site (underlined) and a start codon (in bold). The *Xba*I-R primer; 5′-CTCTAGACCCTAGCATGGCTCTCCAG-3′, contains the *Xba*I restriction site (underlined).The reverse primer was designed such that the priming site is in the 3′-UTR, beyond the stop codon. The PCR reaction consisted of 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 x PCR reaction buffer, 1 U/reaction of Expand High Fidelity Enzyme Mix (Roche Diagnostics, Germany) and 1 ng of plasmid DNA template (pSK-*XvAld1*) in a 25 µl reaction. PCR was carried out using the following conditions; 95°C for 2 min followed by 2 cycles of 95°C for 30 sec, 54°C for 30 sec and 72°C for 1 min, another 25 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 2 min and a final extension step at 72°C for 7 min.

The PCR product was digested with *Sal*I and *Xba*I and ligated in-frame into the pProEX HTa™ Prokaryotic Expression System (pProEXa, plasmid map in Appendix D; Invitrogen Life Technologies, USA) digested with the same enzymes. The ligated plasmid was transformed into competent DH5α *E. coli* cells, plated onto LB agar supplemented with 100 µg/ml of ampicillin and grown at 37°C overnight. Putative transformants were screened by PCR using the gene specific primers and conditions mentioned above. The sequence was confirmed to be in-frame and free of mutations by sequencing both strands using the automated Sanger dideoxy terminator method (MegaBACE 500; Molecular Dynamics, USA).

For protein expression, the *E. coli* cells containing the expression vector were grown at 37°C in LB broth containing 100 µg/ml ampicillin to an optical density (OD_{600}) of 0.6 - 0.8. Protein expression was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubation at 37°C was continued for another 3 hr. Alternatively, cells were grown overnight at 37°C in a self inducing medium (Overnight Express™ Instant TB medium; Novagen, USA) containing 10% glycerol and supplemented with 100 µg/ml ampicillin. The cells were harvested by centrifugation at 5000 x *g* for 10 min at 4°C.

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2.2.10 Affinity purification

The histidine-tagged recombinant protein was purified using metal-chelate affinity-chromatography on Ni-NTA (nickel-nitrilotriacetate; Qiagen, Germany) column. The batch-wise purification method adapted from Polayes and Hughes (1994) was used. All protein purification steps were carried out at 4°C. Briefly, 0.3 g of cells were lysed by resuspending in 1 ml of lysis buffer [50 mM Tris-HCl pH 8.5 (at 4°C), 10 mM 2-mercaptoethanol (β-ME), 100 µg/ml lysozyme and 1 mM PMSF (phenylmethylsulfonyl fluoride)] and sonicated to ensure efficient lysis. After centrifuging for 20 min at 8000 x *g* to remove cell debris, 500 µl of the crude extract was mixed with 200 µl of 50% slurry of Ni-NTA resin. Samples were agitated for 15 min, the matrix was centrifuged for 2 min at 5000 x *g* and the supernatant was discarded. The matrix was washed with 3 x 1 ml of Buffer A [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 20 mM imidazole, 10 mM β-ME and 10% glycerol], agitated for 5 min during every wash cycle. The protein sample was eluted three times with 200 µl of Buffer C [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 100 mM imidazole, 10 mM β-ME and 10% glycerol] by incubating the resin with agitation for 5 min and centrifugation at 3000 x *g* for 2 min.

The eluted protein fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The fractions were pooled and desalted using Amicon Ultra centrifugal filter columns (Millipore Corporation, USA) with 10-kDa molecular weight size exclusion according to the manufacturer's recommendations, until the desired volume and concentration was reached. After desalting, the protein was quantified by Bradford's method (1976), using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Germany). Dilutions of a bovine serum albumin standard (Pierce, USA) were used for calibration. The protease inhibitor cocktail, Complete EDTA-free (Roche Diagnostics, Germany) was added to all purified fractions. The purified XvAld1 protein at a concentration of 1 mg/ml was used for raising antibodies. Two New Zealand rabbits were bled for pre-immune sera. The two rabbits were then injected with 1 ml of antigen emulsion sample on days 1, 5, 10, 15 and 20 using the protocol by Rybicki (1979). Rabbits were bled on day 30 and every 2 weeks thereafter until a high titre antiserum was obtained.

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2.2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Proteins were separated according to size by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). Stacking gels [5% (w/v) acrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate (APS), 0.01% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED)] and resolving gels [12% (w/v) acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.04% (v/v) TEMED] were used. Acrylagel 5 solution [40% (w/v) (BDH, England)] was used as the acrylamide stock solution.

Electrophoresis was performed using either a Hoefer Mighty Small™ or Hoefer Mini VE™ (Hoefer Inc., USA) apparatus and 30 mA constant current until the dye front was eluted. Samples for SDS-PAGE were prepared by mixing 5 x SDS loading buffer (0.225 M Tris-HCl pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 0.25 M DTT or 10% β-ME) with the appropriate volumes of protein extracts. Equal protein concentrations, as determined by the Bradford method (1976) were used. Equal loading was confirmed by staining the gels with Coomassie solution [0.05% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) ethanol, 10% (v/v) glacial acetic acid] or PageBlue™ protein staining solution (Fermentas Life Sciences, Germany). Alternatively, Ponceau S solution [0.5% (w/v) Ponceau S stain (Sigma-Aldrich, Germany), 1% (v/v) glacial acetic acid] was used to stain nitrocellulose membranes. For all protein gel electrophoresis, the Low Range Prestained SDS-PAGE Standards (Bio-Rad, Germany) or PageRuler™ Prestained Protein Ladder (Fermentas Life Sciences, Germany) were used.

2.2.12 Purification of antibodies by polyethylene glycol precipitation

Polyclonal antibodies were purified using a method adapted from Polson et al. (1964). One volume of antiserum was mixed with 2 volumes of borate buffered saline pH 8.6 (BBS; 35 mM Boric acid, 37.5 mM NaCl). Crushed polyethylene glycol (PEG) 6000 [14% (w/v)] was added to the antiserum and dissolved by gentle inversion. The solution was centrifuged at 12 000 x g for 10 min at 4°C. The pellet was redissolved in the original volume of antiserum and 14% (w/v) PEG 6000 was again added and dissolved. The antibodies were pelleted as before and dissolved in half the original antiserum volume using PBS containing 60% glycerol. Purified polyclonal antibodies were stored in aliquots at -20°C.

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2.2.13 Western blot analysis

SDS-PAGE gels were equilibrated in transfer buffer [25 mM Tris; 192 mM Glycine; 0.1% (w/v) SDS; 20% methanol] following electrophoresis. The separated proteins were transferred onto Nitrobind 0.45 micron nitrocellulose membrane (Osmonics, USA) using the Bio-Rad Mini Trans-Blot® Transfer System (Bio-Rad, Germany) for 90 min at 300 mA. The membrane was removed from the blotting apparatus and was washed for 5 min in TBS/Tween/Triton buffer [TBSTT; 50 mM Tris pH 7.5; 150 mM NaCl; 0.05% (v/v) Tween 20; 0.2% (v/v) Triton X-100]. All the washing and incubation steps were carried out at RT with agitation. To verify uniform transfer and equal loading of proteins, the membrane was stained in Ponceau S stain [0.1% Ponceau S (Bio Basic Inc., USA); 5% acetic acid] a stain that binds reversibly to proteins. Thereafter, the membrane was washed with water to destain. The membrane was washed thrice for 10 min in TBSTT and incubated for 1 hr in blocking solution [3% BSA in TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl)], followed by three washes each for 10 min in TBSTT. The membrane was incubated for 2 hr in primary antibody [1:1000 dilution of tetra-His mouse monoclonal IgG (Qiagen, Germany) in blocking buffer] and thereafter washed thrice for 10 min in TBSTT buffer. The membrane was incubated for 1 hr in secondary antibody [1:5000 dilution of goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich, Germany) in blocking buffer] and washed thrice for 10 min in TBSTT buffer.

For the chromogenic detection, the membrane was stained with alkaline phosphatase solution [one tablet of NBT/BCIP (nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate toluidine salt) ready to use tablets (Roche Diagnostics, Germany)] until the signal was clearly visible (ca. 5-15 min). The reaction was stopped by rinsing the membrane in water. Alternatively, a 1:7 500 dilution of the XvAld1 polyclonal antibody raised in rabbits and 1:20 000 dilution of the Immunopure® Peroxidase conjugated goat anti-rabbit secondary antibody (Pierce, USA) were used. The washing and incubation steps were carried out as outlined above. The aldose reductase protein was detected using the SuperSignal® West Pico Chemiluminescent Substrate Kit (Pierce, USA) following the manufacturer's instructions. The western blots were exposed to CL-Xposure™ film (Pierce, USA) for empirically determined incubation periods. The films were developed manually in developer and rapid fixer solutions (AGFA, Belguim).

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2.3 RESULTS

The *XvAld1* cDNA sequence, isolated from an *X. viscosa* dehydration library (Mundree et al., 2000), was 1140-bp and contained a 960-bp open reading frame which codes for a protein of 320 amino acids (not shown).

A Kyte and Doolittle (1982) hydropathy plot was used to make predictions about the structure of the full protein sequence of XvAld1 (Fig. 2.1). The profile gives a similar distribution between positive and negative scores suggesting that the XvAld1 protein is globular and soluble, with no transmembrane or membrane anchored segments. The possibility that XvAld1 folds into a globular structure was supported by results from the ELM (Eukaryotic Linear Motif) resource [http://elm.eu.org/basicELM/].

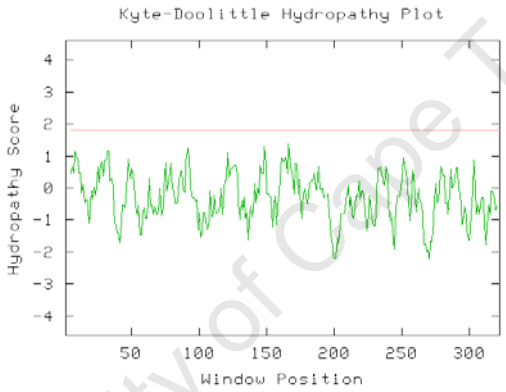

Figure 2.1 Kyte and Doolittle hydropathy plot for the *X. viscosa* aldose reductase
A window of nine amino acids was used for the calculations on a full-length protein sequence input. The red line indicates a threshold beyond which peaks are predicted to be transmembrane domains.

2.3.1 Posttranslational modifications

The functions of many proteins are affected by posttranslational modifications (PTMs). Several databases were used to scan XvAld1 for possible modifications on amino acid residues. Using NetPhos 2.0 [http://www.cbs.dtu.dt/services/NetPhos/; (Blom et al., 1999)] to determine potential phosphorylation sites, it is apparent that many XvAld1 amino acid residues could be phosphorylated. The identities, positions and prediction scores for these amino acids are given in Figure 2.2. Using a cut-off value of 0.5, eight serine, two threonine and one tyrosine residues were predicted to be phosphorylated.

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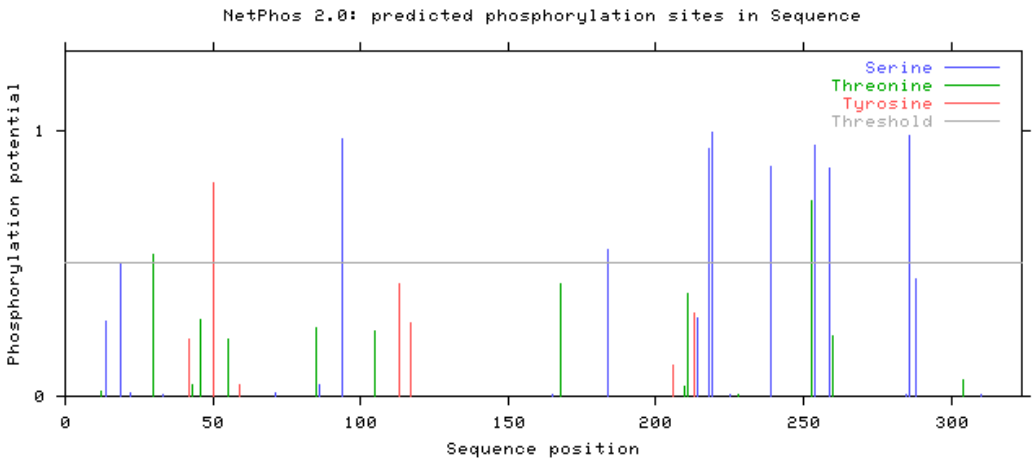

Figure 2.2 Prediction of phosphorylation sites using NetPhos 2.0 and a threshold value of 0.5

A summary of predicted PTMs and putative ligand binding motifs identified on the XvAld1 sequence is shown in Table 2.1. Use of the ELM database predicted that XvAld1 consists of two globular domains with two small regions of disorder at the beginning and in the middle of the protein. The prediction is in agreement with the results from the Kyte and Doolittle plot. Most of the motifs detected were potential phosphorylation sites and possible ligand-binding sites for protein kinases. All the listed motifs have a high probability of surface exposure and are unlikely to be buried in the hydrophobic portions of the protein (Blom et al., 2004; Balla et al., 2006). A ligand-binding site for interaction with cyclins is an absolute requirement for the phosphorylation of a cyclin-dependent kinase (CDK) substrate motif on the same protein (Adams et al., 1996; Schulman et al., 1998). Interestingly, both the ligand-binding and the phosphorylation motifs are present on XvAld1, meaning that CDK activity is possible. Other putative phosphorylation sites are specificity motifs for casein kinases I and II (CKI and CKII), calmodulin-dependent kinase II (CaMKII), extracellular signal-regulated kinases (ERK), mitogen activated protein kinases (MAPK), cyclin AMP-dependent protein kinases (or protein kinase A; PKA), protein kinase C (PKC), proline-directed protein kinase (PDPK), phosphorylase kinases (PK), ataxia telengiectasia mutated (ATM) kinases, glycogen synthase kinases (GSK), and ribosomal S6 kinases (RSK). The different classes of kinases are involved in the transduction of signals relating to various aspects of cell physiology, including cell cycle regulation, responses to hormonal cues and environmental stress tolerance.

Several other ligand-binding recognition sites for protein-protein interactions were identified. These include interaction motifs for forkhead associated (FHA)

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proteins, tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) and major TRAF2-binding consensus, motifs for interaction with Class IV WW (named for their specific recognition of proline-rich motif) domains and WD40-binding sites. The binding of the protein ligands is phosphorylation dependent, suggesting that XvAld1 may be specifically targeted as a substrate for various kinases.

2.3.2 Protein-protein interaction and protein sorting motifs

Other potential protein-protein interaction motifs (Table 2.1) include two phosphate binding motifs, a clathrin-binding motif and a di-leucine-binding motif. The di-leucine motif is known to bind to receptors involved in endocytosis. Interestingly, four tyrosine-based endocytosis internalisation motifs were also predicted on XvAld1. This taken together implies that XvAld1 may be actively transported from the cytoplasm to other microbodies by endocytic vesicles. The presence of a clathrin-binding motif on XvAld1 supports the participation of receptor-mediated endocytosis in subcellular sorting processes involving the aldose reductase. In addition, a type 2 peroxisomal targeting signal, a Golgi targeting signal and 3 motifs for targeting proteins to microbodies were predicted on XvAld1 (Table 2.1). The Golgi targeting motif is similar to the one found on Arf1, a GTPase protein involved in Golgi-budding and known to associate with clathrin-coated vesicles (Zhu et al., 1998; Bigay et al., 2003). The XvAld1 amino acid sequence also contains three possible N-glycosylation sites and a potential N-myristoylation site (Table 2.1). Protein myristoylation involves the covalent attachment of myristate, a 14-carbon saturated fatty acid, to an N-terminal glycine of a particular protein. This myristoyl attachment provides hydrophobicity that may be important for protein-protein interactions and acts as a lipid anchor that changes the cellular function of the modified protein (Gordon et al., 1991; Farazi et al., 2001).

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Table 2.1 Posttranslational modifications and functional motifs identified by *in silico* analysis

Name and function	Motif Pattern	Position	Notes	Compartment
Phosphorylation				
Lig_Cyclin_1[a,d]	[RK].L.{0, 1} [FYLIVMP]	80, 174, 221, 292	Ligand-binding site that interacts with cyclin facilitating increased	Nucleus, cytosol
Mod_CDK[a,d]	…([ST])P.[KR]	91	phosphorylation by cyclin/cdk complexes. Presence of a CDK substrate motif on the same protein is a requirement for activity.	
Mod_CK_I[a,d,e]	S..([ST])…	19, 165, 225, 285	CK-I phosphorylation site	Nucleus, cytosol
Mod_CK_II[b,c,d,e]	[ST]..[DE]	105, 219, 260, 286, 310	CK-II phosphorylation site	
Mod_PKA_2[a,d,e]	.R.([ST])…	250	PKA phosphorylation site	Cytosol
Mod_PKC[b,c,d,e]	[ST].[RK]	14, 30, 85, 168, 219	PKC phoshorylation site	
Mod_ProDKin_1[a]	…([ST])P..	91, 211, 236	Proline-Directed Kinase phosphorylation site	Cytosol, nucleus
Lig_WW_4[a,d]	…[ST]P.	91, 211, 236	Phospohorylation-dependent Class IV WW domains interaction motif, also ERK phosphorylation, MAPK phosphorylation	Nucleus, cytosol
Lig_FHA_1[a,d]	T..[ILA]	30, 253	FHA domain interaction motif dependent on threonine phosphorylation	Nucleus, cytosol, PM
Lig_TRAF2_1[a,d]	[PSAT].[QE]E	105	Major TRAF2-binding consensus motif	Cytosol
Lig_TRAF6[d]	P.E..[FYWHDE]	132	TRAF6 binding motif	
Mod_ATM[d,e]	[ST]Q, [ST]..Q	12, 239	ATM kinase phosphorylation motif	
Mod_RSK[d]	[KR]..[ST]	16, 251	RSK phosphorylation motif	
Mod_GSK_3[d]	[ST]…S	214	GSK3-alpha phosphorylation motif	
Lig_WD40[d]	S…S	214	WD40-binding motif when both serines are phosphorylated	
Mod_CaMKII[d,e]	R..[ST]V	33, 251	CaMKII phosphorylation site	
Mod_PK[d]	[K/R]..S[IV]	251	Phosphorylase kinase site	
Targeting/sorting				
Endocytic_2[a,d]	Y..[LMVIF]	42, 50, 113, 213	Tyrosine-based endocytosis internalisation motif	Cytosol, PM, clathrin-coated vesicles
Microbodies[b]	[STAGCN][RKH] [LIVMAFY]	86, 129, 234	Microbodies C-terminal targeting signal	
PTS2 targeting[d]	[RK][LVI]….. [HQ][LA]	235	Type 2 peroxisomal targeting signal	Peroxisomes
Golgi targeting[d]	M..M	131	Similar to motif targeting Arf1 to Golgi	
Other modifications				
N_glycosylation[b,c,d]	N{P}[ST]{P}	166, 237, 302	N-linked glycosylation	ER
Myristoylation[b,c]	G[EDRKHPFY W]..[STAGCN].P	29	N-myristoylation	
Other protein interactions				
Di-leucine motif[d]	[DE]..LL	220	Di-Leu acidic motif for receptor endocytosis	
Clathrin-binding motif[d]	DLL	222	Binds to clathrin heavy chain	
PO_4-binding motif[d]	D..G	35, 142	Phosphate-binding motif in GDP and GTP BPs	

[a] ELM database (Puntervoll et al., 2003);[b] Softberry prosite patterns; [c] Prosite (Gattiker et al., 2002; Sigrist et al., 2005)
[d] Minimotif Miner (Balla et al., 2006); [e] NetPhosK 1.0 (Blom et al., 2004)
"[ ]" – the various amino acids allowed, "{}" – preference not strict, "." – "any amino acid is permitted" ATM – ataxia telengiectasia mutated; Arf – ADP ribosylation factor; BP – binding protein; CaMKII – calmodulin-dependent kinase II; CDK – cyclin-dependent kinase; CKI/II - casein kinase I/II; ER – endoplasmic reticulum; ERK – extracellular signal-regulated kinase; FHA – forkhead associated; GDP/ GTP – guanidine di-/tri-phosphate; GSK – glycogen synthase kinase; MAPK – mitogen activated protein kinase; PKA – protein kinase A (cyclin AMP-dependent protein kinase); PKC – protein kinase C; lig – ligand; mod – modification; PM – plasma membrane; PO_4 – phosphate; PTS2 – Type 2 peroxisomal targeting; RSK – ribosomal S6 kinase; TRAF – TNF (tumour necrosis factor) receptor-associated factor; WW and WD40 domains – domains for protein-protein interactions

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N-glycosylation, the addition of a glycan moiety, is linked to accurate protein folding, stability and sorting (Wormald et al., 1991). Both the myristoylation and glycosylation processes occur in the endoplasmic reticulum (ER) lumen. The modifications, ligand-binding motifs for protein-protein interactions and the consensus sequences for protein sorting discussed above indicate that although XvAld1 is soluble and cytoplasmic, it is possibly specifically targeted to other microbodies within the cell.

2.3.3 Sequence comparison with other AKRs

The BLAST tool (Altschul et al., 1997) was used to search protein databases for sequences similar to that of XvAld1. Since XvAld1 belongs to a superfamily of proteins with conserved sequences and structure, many hits were returned. A multiple sequence alignment was constructed using sequences selected from plant proteins already classified as aldo-keto reductases (AKRs; AKR Homepage), potential AKRs or proteins predicted to be "AKR-like".

The multiple sequence alignment (Fig. 2.3) shows that the *Hordeum vulgare*, *Avena fatua* and *Bromus inermis* proteins are the closest relatives, showing ~66% amino acid sequence identity to XvAld1. These proteins have been reclassified as aldehyde reductases (ALRs; Jez and Penning, 2001) because aldoses are not efficiently reduced by these enzymes (Roncarati et al., 1995). The multiple sequence alignment is in agreement with the conserved $(\alpha/\beta)_8$ scaffold at the core of the AKR proteins (illustrated at the top of the aligned sequences). The alignment indicates that many of the conserved residues are involved in coenzyme (c) or substrate/active site (a) interaction. These include the invariant or strictly conserved residues boxed in pink (Fig. 2.3). Of the eleven boxed residues, only L-82 (numbered with respect to the XvAld1 protein) is variable and is found as an isoleucine in rat and a valine in the legume *Sesbania rostrata*. Other notable conserved motifs include the N-terminal LxxxGxxxPxxGxG which is in the form; LxxGxxxPxxGxG from L17 of XvAld1 and the GxxxxDxAxxY present from G49. Another signature pattern, containing the conserved histidine of the KYDH catalytic tetrad (Hoog et al., 1994), is Lx(9)DxxxxH found in XvAld1 as Lx(8)DxxxxH from L106 to the catalytic histidine 120.

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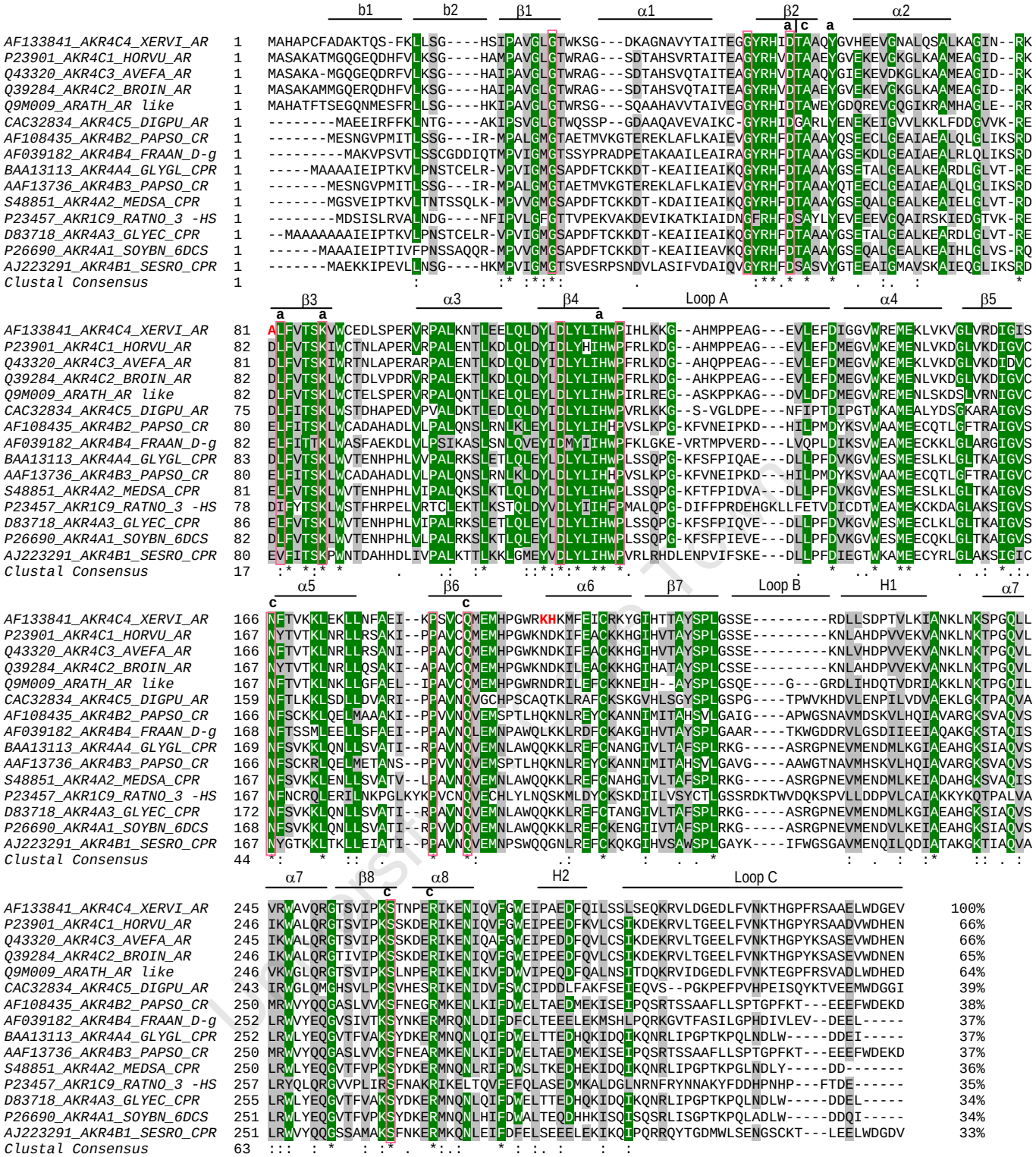

Figure 2.3 Multiple sequence alignment of plant aldo-keto reductases

Sequences were obtained from the Aldo-keto Reductase Homepage, and from the GenBank. Multiple alignments were performed using Clustal W version 1.4. The rat 3α-HSD was used as reference for locating the positions of the common $(\alpha/\beta)_8$ three dimensional fold (Jez et al., 1997b). Residues boxed in pink are invariant or strictly conserved. The characters '*', ':' and '.' represent 'identical', 'conserved substitutions' and 'semi-conserved substitutions' respectively. The residues in green represent blocks with greater than 85% sequence identity. The letters 'a' and 'c' above the alignment denote active site residues and amino acids involved in cofactor binding, respectively. H1, H2 and b1, b2 are α-helices and β-sheets not forming the $(\alpha/\beta)_8$ core of the AKR barrel. The accession numbers, AKR names (where applicable) and the species names (on the left) are given in full in Appendix C.

The rat 3α-HSD (3α-hydroxysteroid dehydrogenase) was incorporated into the alignment to enable the structural comparison (adapted from Jez et al., 1997a). As expected, the core structure of the protein contains substantial sequence conservations. Sequence variations are observed in the loop regions (A, B, and C) and the non-core forming helical and β-sheet structures, labelled "h" and "b" respectively. The loop regions, proposed to determine substrate specificity, are less varied among the plants ALRs (the first 4 proteins) and the *A. thaliana* ALR-like protein.

The three residues in red indicate the positions where the XvAld1 GenBank sequence (AF133841) consistently differs from the amino acid sequence deduced from the cDNA clone in the pSK plasmid. The first mutation is caused by a change from GCT to GAT at position 242 of the nucleotide sequence. The two amino acid differences at positions 296 and 297 are caused by a change from CG to GC (C296GG297C) of the nucleic acid sequence. Although the altered amino acids are not strictly conserved among AKR proteins, the protein synthesised from the cDNA clone gives amino acid residues that perfectly match the other plant proteins. The change from 'A' to D-81 (A81D) and from 'KH' to N-196 and D-197 (K196NH197D) makes the XvAld1 sequence well aligned with the other plant AKR sequences.

A dendogram depicting the relationships among these proteins (Fig. 2.4), demonstrates that the XvAld1 protein is closely related to the *H. vulgare*, *Bromus inermis*, and *Avena fatua* proteins. However, the *X. viscosa* protein forms its own arm of the tree while the other three monocot species clade together (group in green). The protein from *A. thaliana* (a dicot), though it appears to be closely related to the *X. viscosa* aldose reductase, shows less sequence homology to XvAld1 compared to the proteins from monocots. The proteins from *Rattus norvegicus* (rat) and *Homo sapiens* (human) AKRs were included in order to assess the robustness of the classification method. These two proteins, which clade together here, are among the most characterised of family 1 AKRs. The β subunits of potassium channel (Shaker) proteins share amino acid sequence similarity with AKR proteins. The protein used to root the tree is the first Shaker protein (from rat) for which aldo-keto reductase activity was demonstrated using a recombinant protein (Weng et al., 2006). The animal AKRs are highlighted in grey.

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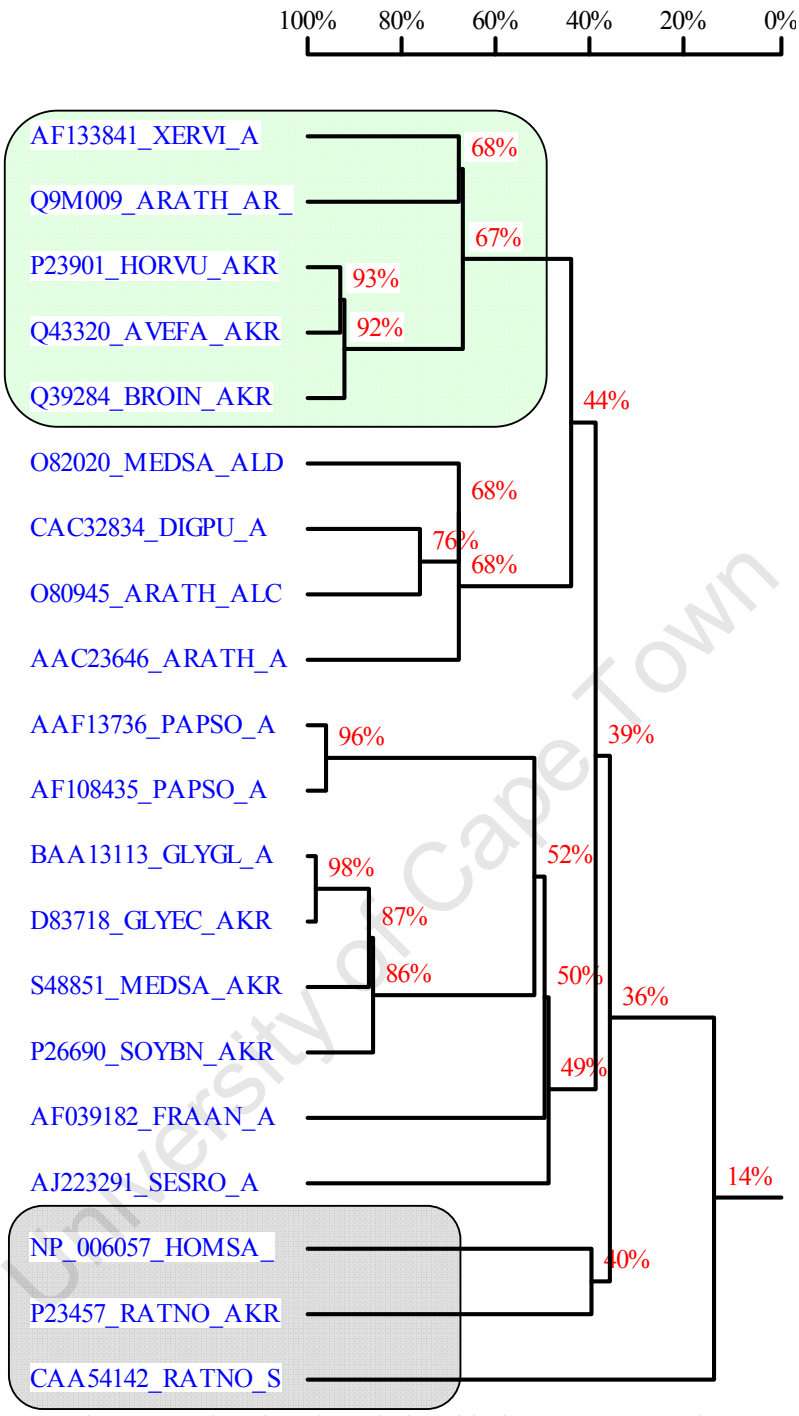

Figure 2.4 Homology tree showing the relationship between some plant AKRs
The *X. viscosa* AR is closely related to the wheat (*Hordeum vulgare*; Bartels et al., 1991), the smooth brome grass (*Bromus inermis*; Lee and Chen, 1993) and the wild oats *(Avena fatua*; Li and Foley, 1995) aldose reductases and the *A. thaliana* aldose reductase-like protein boxed in green. The human and rat aldose reductases included here are among the most characterised of the AKR proteins. The tree was rooted using a rat Shaker protein with demonstrated aldo-keto reductase activity (Weng et al., 2006). The AKRs in grey are of animal origin. See Appendix C for full names and accession numbers of the proteins used.

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2.3.4 Southern blot analysis

To investigate the genetic organisation of the *XvAld1* gene in *X. visco*sa, Southern blotting was performed (Fig. 2.5).

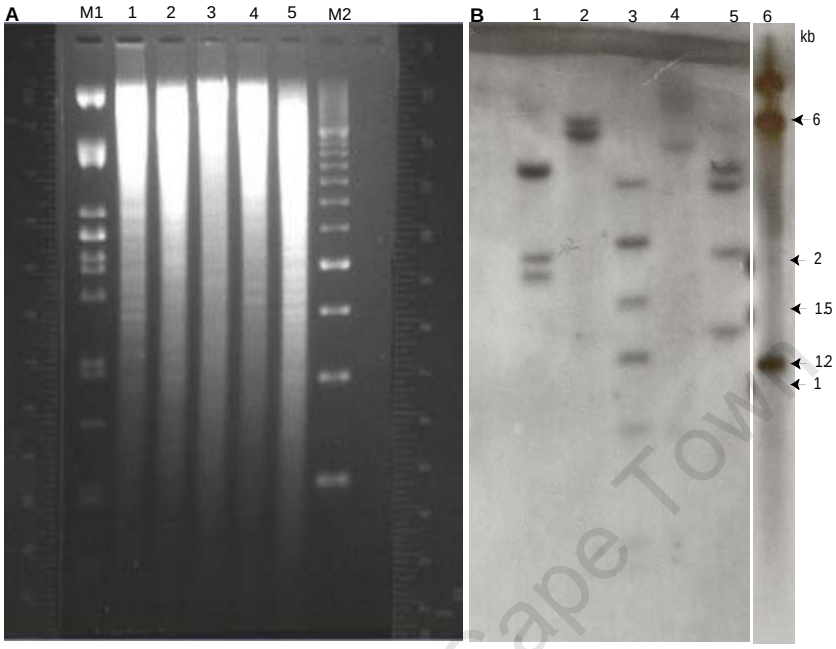

Figure 2.5 Southern blot analysis of the *XvAld1* gene in *X. viscosa*
A. Ten micrograms of genomic DNA were digested with restriction enzymes as single or double digests. The digested DNA was separated on 0.8% (w/v) agarose gel. **B**. An autoradiogram of the DNA in (A) on a nylon membrane and probed with radiolabelled *XvAld1* full length coding sequence. The labels represent DNA digested with; lane 1, *Eco*RI; lane 2, *Eco*RV; lane 3, *Pvu*II; lane 4, *Xba*I; lane 5, *Eco*RI/*Xba*I and lane 6, 100 ρg of a purified 1.2-kb *XvAld1* PCR product that was used as a positive control. Lanes M1 and M2 represent *Pst*I digested λ DNA ladder and an O'Gene Ruler™ 500 bp DNA ladder (Fermentas, Germany) respectively.

Digestion with *Eco*RI produced three prominent bands (Fig. 2.5B, lane 1). Two of the bands are ~2 kb in size and the larger band is ~4 kb. It is reasonable to suggest that the intense band in lane 1 is a doublet that is resolved into two bands by the double digest in lane 5 (*Eco*RI and *Xba*I). One of the fragments remains intact (~4 kb) and the other has an internal *Xba*I site that produces a smaller than 4-kb band and another undetectable fragment. Of the four bands obtained when genomic DNA was digested with *Eco*RI and *Xba*I (lane 5), two of the larger bands (the top and third largest band) correspond to the *Eco*RI single digest. The smallest *Eco*RI band appears to contain an internal *Xba*I site, cleaving the DNA into larger fragment of ~1.3 kb and a smaller one that is faint.

The *XvAld1* cDNA sequence contains no *Eco*RV or *Xba*I restriction sites, yet digestion of genomic DNA with these enzymes produced two prominent bands each

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(lanes 2 and 4). All the bands are larger than the cDNA, at least 5 kb in size. The two bands in lane 2 (*Eco*RV digestion) may both be doublets, judging from the thickness and similarity with the intensity of the *Eco*RI ~4 kb band.

The *Pvu*II digest produced at least six bands (lane 3). The *Pvu*II banding pattern is unusual because the *XvAld1* cDNA does not contain any *Pvu*II restriction sites. At least two of the six bands are smaller than 1.2 kb, the size of the *XvAld1* cDNA. This raised the possibility that the *XvAld1* gene is interrupted by several introns. To investigate this possibility, the *XvAld1* genomic sequence was amplified from *X. viscosa* genomic DNA. Several attempts using a variety of long template DNA polymerases produced no amplicons. As a result, it was presumed that the target sequence had a high GC content. The Phusion High Fidelity DNA Polymerase, which has a buffer specially formulated for templates with high GC content, was then used to amplify the genomic sequence.

2.3.5 Genomic DNA amplification and sequencing

A PCR product of approximately 3 kb (Fig. 2.6, lane 4) was amplified from the *X. viscosa* genomic DNA. The results from sequencing and BLASTn searches indicated that the *XvAld1* gene consists of nine exons interspaced with eight introns. Figure 2.7A is a schematic representation of the structure of the *XvAld1* genomic DNA. The nucleotide sequence used in Figure 2.7B is a consensus of three sequences from the two genomic clones. The beginning and end sequences of the genomic clones were obtained using the M13 primers. After the first round of sequencing, new sets of primers, shown by arrows and italics, were designed for primer walking until the whole 2.9-kb clone was sequenced. The sequences in black represent the exons. The intronic sequences in blue were delineated by aligning the genomic consensus sequence with the cDNA sequence in the GenBank and by comparison with conserved intron-exon junctions found in eukaryotic genes (Breathnach and Chambon, 1981; Brown, 1986). The intronic sequences (in blue) are characterised by clusters of poly-A and poly-T sequences. The translation start and stop codons are shaded blue.

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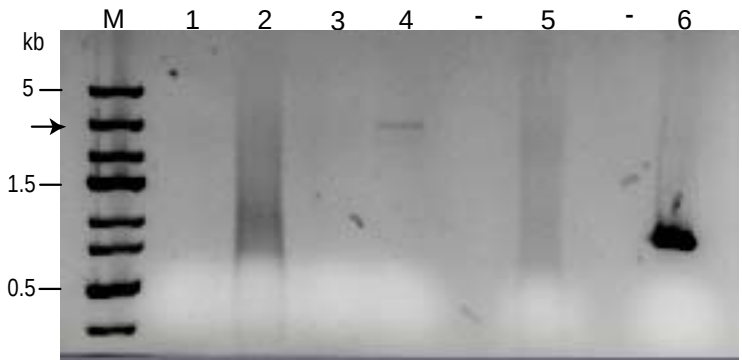

Figure 2.6 PCR amplification of the *XvAld1* genomic sequence from *X. viscosa* genomic DNA. Different primer combinations; XvAld1-AF/AR; XvAld1-BF/BR; XvAld1-AF/BR; and XvAld1-BF/AR (lanes 1 – 4 respectively) were used. Lane M: the Fermentas DNA ladder plus, lane 5: no template control, lane 6: cDNA clone (pSK-*XvAld1*) used as positive control. The arrowhead highlights the position of the 3 kb marker band which is of a similar size to the PCR product (lane 4). '-' denotes blank lanes.

Five of the eight exon-intron splice junctions conform to the classical GT/AG donor and acceptor consensus sequences respectively (Table 2.2), found in many eukaryotic genes (Brown, 1986; Jackson, 1991). Introns 3 and 4 contain the less common "TG" 5′ donor splice junction and intron 6 contains a "CT" donor sequence. Violations of the GT consensus have been reported for a number of splice junctions in plant genes (Brown, 1986; Jackson, 1991). The 3′ intron splice sites all conform to the conserved AG splice acceptor sequence. All the splice junctions, except for intron 3 junctions, are phase 0 splice sites. Phase 0 splice sites fall between translation codons (Ruvinsky et al., 2005), reducing the probability of frame-shift mutations during transcription.

Of the four gene-specific primer combinations used for PCR amplification, only one set produced a product. This primer set (XvAld1-BF and XvAld1-AR; sequences in green in Fig. 2.7B) did not amplify the beginning and the end of the gene coding sequences. As a result, the genomic clone is truncated at the 5′ and 3′ ends of the gene (boxed sequences including the start and stop codons; Fig. 2.7B). Figure 2.8 represents the three genomic sequences obtained from the genomic clones, 7b and 7c. Initially, two clones were sequenced and ambiguous sequences were resolved by re-sequencing genomic clone 7c. However, some of the ambiguities were unresolved, raising the possibility of polymorphisms within the aldose reductase genomic sequences.

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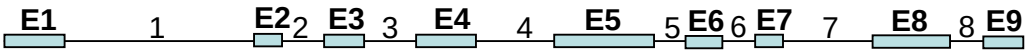
A



B

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ATGGCGCATGCACCGTGTTTTGCTGAGGCGAAGACACAgAGCTTCAAGCTCCTCAGCGGG     60
CACTCAATCCCCGCAGTTGGGCTCGGCACgTGGAAGTCGGGCGACAAGGCCGGCAACGCC    120
GTGTACACTGCCATCACTGAGGTACCTACCTTTCTTCTTCAGATTTAaGTTCGTCCTTGA    180
TAAACGTTGCGTGCATTTCTTGGGATAATTTGAGAATTTGTTTGATTTGATAGTTGATAC    240
GGAAAAATGCCTAAGTTAAACGCAATGTTGCGAATTGGTTCTTTGCTTTCCGCTCTGGAG    300
AAATTTGTGAATTGTTTTAGCGTTTGCAGACACTTGGTTAAAGATCTACACATATGACCT    360
TAACTACGTGGGAAGCACCATAGACCGGTGTCCATGTCCAACTTGGCCAATGGTTGGATT    420
AGGGGTGGATTTCTCCTCATCTTTAAACAAAGAAAAAACGAAAACAGAGTTGGTTCTCTG    480
TTTCcGAGGACAAGATTTATgAATTGAAACCCTAATTTCGGATATTATTGATTTTCTTCA    540
TTTTCTTTCCCGATTACATTTGCATGAAGTAGTAAATTATTTCTATGACTCCAAACTTAT    600
GTCACGTTGACCGTGAATGTGCATTAACCAAAAAAAAAAAAAAATTATTGGATTGTTGTG    660
TCAATTACTTACCCTTCACAAATTCTCACCGGATGTTGTCAATATTTGCATGCAATTCAT    720
ATAGAGCTGGCGTTTATTTTGATTAAGTtTAGTGAAAATAGTTATGTTTACATTCCTGGA    780
TAGTATGCCGAGGCTGATGCATAGTTTTTCTAATATAGCTAATACCTTAAATTCTCGGAA    840
AAAAAAACATTGATGGATAGTATGCTATTCTCAATTCTTTACATAGAACTGCAAACTTCT    900
TCGTTCTATCATTTGTCTTCAGATGATGAATTAACACATTCTTACATTCAGGGGGGATAC    960
AGGCACATTGATACTGCAGCACAATATGGAGTCCATGAAGAGGTAAAAATTACAGACTCC   1020
GATTCATGATTTCCTCAAAACAACAAtTGGTATACAGTGATTTAATGGAGATTTTGTCTT   1080
CTTGGTTATTATGCAGGTTGGCAATGCTCTTCAGGCTGCTTTGAAAGCGGGGATCAATAG   1140
GAAAGATTTTTTCGTCACATCGAAAATATGGTAAGAATTTCAAGAAAATACAATCCACCT   1200
CATTTTTTCTCTAAAAAAAAGAACTCCAACAAAAATTGATTGTCATTATGTTCGTTGCAT   1260
AGATAGAATTTTAGAGCTCAGATCTATTCTGCgAGATTTGCCCGGTTGAATTTACTGGAT   1320
TTCCGCTTATGATATTGTATGACATAATACATGCACATTTGAACGCTCAGGTGCGAAGAT   1380
TTATCACCTGaAAGAGTTCGACCTACATTGAAAAATACACTTGAgGAGCTACaACTGGAT   1440
TAcCTTGATCTTTACCTGGTATGCTCTATATATATTTTTTTGAGAAGTCATCCACAAAAT   1500
AAATTGCGATGAAGGGTTTTGACAAAGACCCGACCAGAGTGaAAAAAATAAAAGgCACGC   1560
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GTGTGGAGGGAAATGGAGAAGCTCGTGAAAGAAGGGCTTGTTAGAGATATTGGTATCTCT   1860
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TGCCAGGTAAGCCACTATGTCGACGACTATTTGTTGCTCTTATTTCAACGCATGTTTGAG   1980
TAAATTGTTCTTGGACTTGAAATCAGATGGAGATGCACCCGGGTTGGAGAAACGACAAGA   2040
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TCGATGAATGAAGCACTATAGTGGTTATCCACGTCATATTTCTCCAAAAAAAATTAAATA   2340
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ATTCTGGTGAGATGGGCTGTTCAAAGAGGAACTAGTGTCATCCCAAAGTCCACCAACCCG   2640
GAGAGGATAAAGGAGAACATCCAGGTCTTTGGGTGGGAGATTCCTGCAGAGGACTTCCAG   2700
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ATTGTGGACCAAGAAAATCTGAAATGCTTTACTAACTGGTTAACCTTACTGAAATGCAGA   2820
AGAGAATCTTGGATGGTGAGGATTTCTTCGTCAACAAAACCCATGGCCCGTTCAGGAGCG   2880
CTGCTGAACTCTGGGACGGTGAAGTCTAA
```

Figure 2.7 The structure and sequence of the *X. viscosa* aldose reductase gene

A: A schematic representation of the *XvAld1* gene structure. The positions and relative lengths of the exons (E1 to E9) and introns (numbered 1 8 8) are shown. Intron 1 is the longest (810 bp). Introns 2 - 8 are 94, 200, 261, 80, 89, 320 and 95 bp respectively. The sizes of exons 1 - 9 are; 141, 51, 74, 88, 207, 72, 60, 177 and 90 bp respectively. **B:** The *XvAld1* nucleotide sequence excluding the 5′ and the 3′ untranslated regions (UTRs). The intronic sequences are in blue and exons are in black. The intron-exon junctions were determined by aligning the genomic sequence with the *XvAld1* cDNA sequence (Accession number AF133841). The italicised nucleotides with blue arrows over them represent sequences used for primer walking during sequencing of the genomic DNA. M13 forward and reverse primers were used for the initial sequencing. The underlined sequences are complementary to the antisense primers used to amplify the 5′-UTR. The sequences in green boldface represent the primers used to amplify the ~3 kb *XvAld1* genomic DNA PCR product. The nucleotides highlighted in red are where the genomic DNA differs from the GenBank cDNA *XvAld1* sequence. The three bold and underlined nucleotides in red (mismatches "A" and "CG") are responsible for the three amino acid difference between the protein translated from the cDNA clone and the deduced protein sequence deposited in the GenBank. Sequences in lowercase denote nucleotide differences between the two *XvAld1* genomic clones.

Table 2.2 Patterns of intron splice junctions in the *X. viscosa* genomic DNA

Intron number	5′ exon-intron junction[a]	3′ intron-exon junction[b]	Intron size (bp)
1	ACT GAG/gtacct	attcag GGG GGA[c]	810
2	GAA GAG/gtaaaa	atgcag GTT GGC	94
3	A ATA TG/gtaaga	gctcag G TGC GA	200
4	TAC CTG/gtatgc	ttatag ATT CAC	261
5	TGC CAG/gtaagc	aatcag ATG GAG	80
6	ACA ACT/gtaagt	ctgcag GCT TAT	89
7	CTG AAG/gtgagt[c]	ttacag ATA GCA	320
8	GAA CAG/gtaaaa	atgcag AAG AGA	95

[ab] Exons are represented by capitalised letters and intronic sequences are in lowercase
[c] The nucleotide sequence in red represents a mismatch between the genomic and the cDNA sequences

The sequences from genomic clone 7c (denoted DNA1 and DNA2) are nearly identical, except for differences between six nucleotides arising from four deletions and one misread ('N') in the DNA1 sequence, and one deletion in the DNA2 sequence. The six nucleotide differences appear to be sequencing errors in all cases, as confirmed by comparison with the genomic clone 7b (Fig. 2.8). The 25 nucleotide difference between the two genomic clones (7b and 7c) could also be sequencing errors. These include the truncation of clone 7b by four nucleotides at the 5′-end and a five nucleotide insertion at the 3′-end. In addition, clone 7b contains 16 nucleotide differences with clone 7c (both DNA1 and DNA2), in places other than the six sequencing errors mentioned above. These differences result in 99.1% nucleotide identity between the clones.

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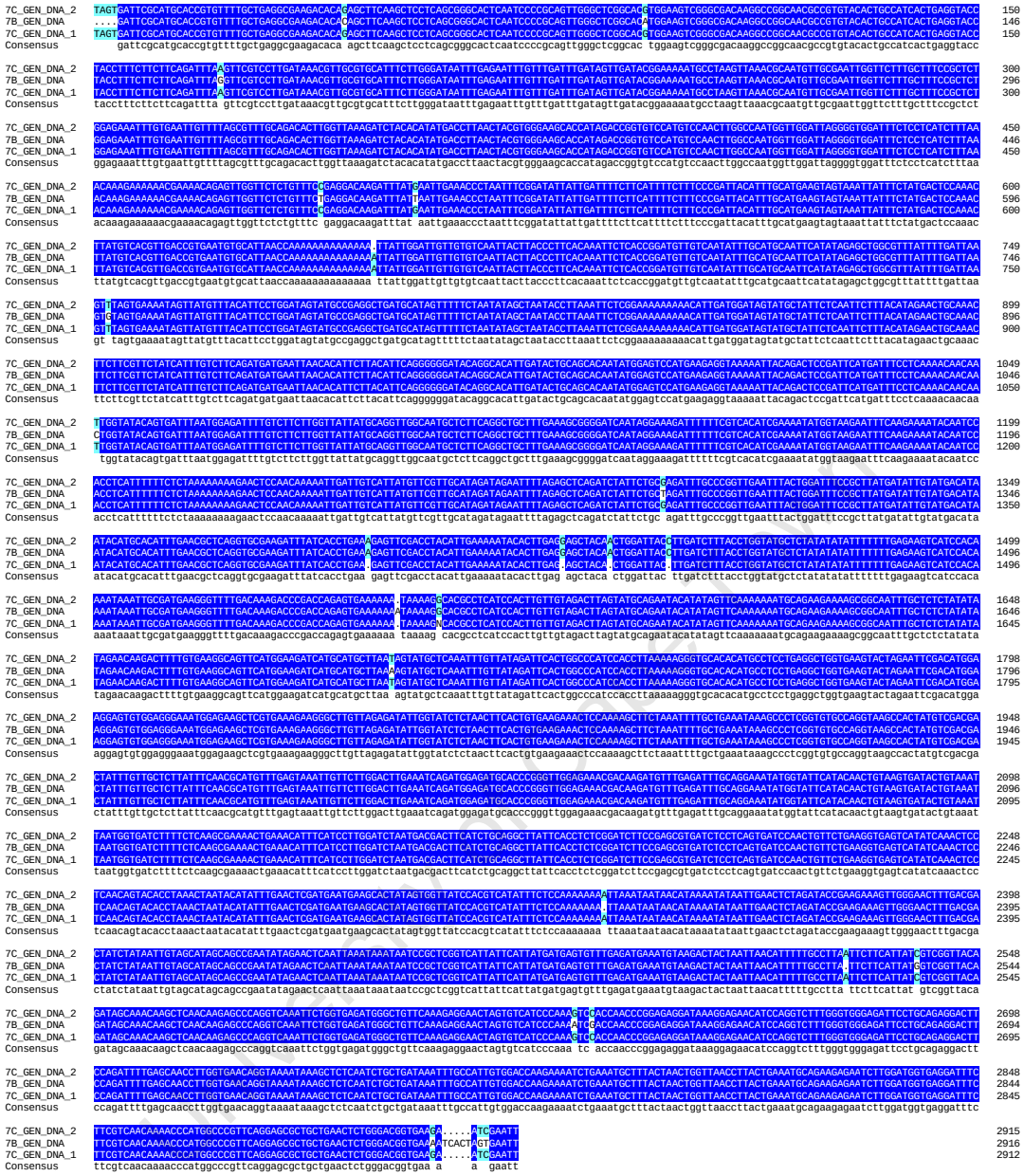

Figure 2.8 Sequence alignments of the *XvAld1* genomic clones
Two clones containing the genomic sequence (clones 7b and 7c) were selected for sequencing. Twenty-five nucleotide differences were observed between the two clones. Genomic clone 7c was re-sequenced (7c_GEN_DNA2) in an attempt to resolve the differences. The nucleotide sequences of the genomic clones (7b and 7c), are 99.1% identical. The gaps show mismatched sequences.

2.3.6 Southern blot analysis of genomic clones

Southern blot analysis of the pGEM-T Easy plasmids containing the PCR amplified genomic clones (7b and 7c) was performed to resolve the complex banding pattern obtained from the chromosomal genomic DNA Southern blot analysis (Fig. 2.9A). The

plasmid used contains two *Eco*RI sites flanking the insert (see Appendix D for pGEM-T Easy vector information). *XvAld1* cDNA also contains an *Eco*RI site at position 493 of the coding sequence (position 1857 in the genomic clone). Digestion with *Eco*RI produced the three expected fragments (lane 1; Fig. 2.9A); the 3-kb plasmid backbone, and the ~1.8 and ~1.2 kb *XvAld1* genomic sequences (lane 1; Fig. 2.9B), consistent with only one *Eco*RI site in the genomic clones. Therefore, the three or more bands obtained (Fig. 2.5B; lane 1) cannot be explained by the presence of introns.

There are no *Eco*RV, *Hind*III and *Xba*I restriction sites in the pGEM-T Easy plasmid. The *XvAld1* cDNA also contains no sites for *Eco*RV and *Xba*I and one *Hind*III site. Only intact plasmid (several forms of circular DNA) was detected for the *Eco*RV digest in lane 2 (Fig. 2.9A and B). However, the plasmid is linearised by digestion with either *Hind*III or *Xba*I (lanes 4 and 5 respectively; Fig. 2.9A and B), suggesting that the genomic sequence has gained one *Xba*I site while the same *Hind*III site in the cDNA is maintained.

The *Eco*RI and *Xba*I double digest produced two bands that were shared with the *Eco*RI digest (compare Figs. 2.9 A and B, lanes 1 and lanes 6). The largest band (plasmid backbone, Fig. 2.9A lanes 1 and 6) is not detected by the cDNA probe and is absent from Figure 2.9B (lanes 1 and 6). There is a faint ~3 kb fragment possibly a result of incomplete digestion of the genomic DNA insert with *Eco*RI (Fig. 2.9B, lane 6). The more than 1.5-kb middle band is maintained in both the gel picture and the Southern blot. The smallest band in the *Eco*RI digest (Fig. 2.9B, lane 1) is digested to a doublet (lane 6). A faint band (~1.5-kb) possibly resulted from incomplete digestion with *Xba*I (Fig. 2.9B, lane 6). *Xba*I does not cut within the *XvAld1* cDNA sequence. However, results from the *Xba*I and *Eco*RI/ *Xba*I digests suggest that an *Xba*I site exists within the *XvAld1* genomic DNA sequence.

*Pvu*II contains two restriction sites in the pGEM-T Easy vector and none in the *XvAld1* cDNA. The gel picture of the *Pvu*II digest produced the two expected bands, the larger band containing the insert flanked by some plasmid sequences and a smaller band consisting of the plasmid backbone (Fig. 2.9A, lane 3). Probing with the *XvAld1* cDNA produced a prominent band larger than 3 kb as expected but also some non-specific hybridisation with smaller bands. The ~2.5 kb faint band is caused by non-specific hybridisation with high concentrations of plasmid DNA. The identity of the smaller bands, also visible in the gel picture, could not be determined.

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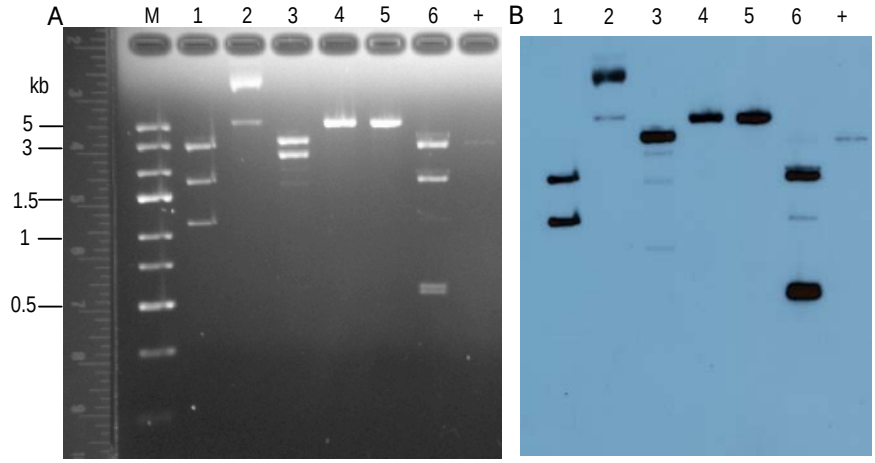

Figure 2.9 Southern blot analysis of the *XvAld1* genomic sequence
One microgram aliquots of the pGEM-T Easy vector containing the aldose reductase genomic clone were digested with *Eco*RI, *Eco*RV, *Pvu*II, *Hind*III, *Xba*I and *Eco*RI/*Xba*I (lanes 1 – 6 respectively). The digested DNA was separated on 0.8% (w/v) agarose gel stained with ethidium bromide (Panel A) and transferred to nylon membrane. The membrane was probed with *in vitro* transcription DIG-labelled *XvAld1* sense RNA (1 ng/ml) and exposed to X-ray film (Panel B). The full-length genomic DNA fragment obtained by PCR amplification (100 ng) was used as a positive control (+). M: DNA ladder plus (Fermentas, Germany).

2.3.7 Amplification and analysis of the 5′-UTR

The 5′-UTR sequences were amplified using TAIL-PCR which employs degenerate sense primers and gene specific antisense primers. The prominent TAIL-PCR products (Fig. 2.10) were cloned into the pGEM-T Easy vector and sequenced using M13 primers. Larger products were sequenced by primer walking. An 870-bp fragment that overlapped with the 5′ leader sequence of the *XvAld1* cDNA was selected for promoter analysis. The sequence was analysed for regulatory elements using several promoter analysis databases [PlantCARE, (Rombauts et al., 1999); Softberry NSITEP; and PLACE (Higo et al., 1999)]. Table 2.3 summarises some of the interesting cis-acting response elements (CAREs) that were selected based on the proposed functions of *XvAld1* and our knowledge of the expression patterns of *X. viscosa* genes. It is interesting to note that cis-acting motifs for many cellular processes were identified on the 870-bp fragment. These included many hormone response elements specifically for ABA, auxins, cytokinins, ethylene, gibberellic acids (GA), jasmonic acids (JA) and salicylic acid (SA).

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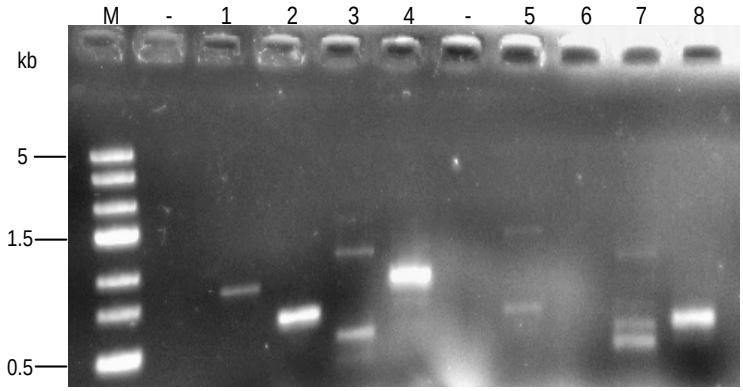

Figure 2.10 Amplification of the *XvAld1* 5′-UTR using TAIL-PCR
Nested primers were used to amplify the 5′-UTR of the *XvAld1* gene using *X. viscosa* genomic DNA as template for the first round of PCR and diluted PCR product for the second and third rounds. The tertiary PCR product was separated on 1% (w/v) agarose gel. All the prominent DNA bands were excised from gel, purified and the fragments cloned into pGEM-T Easy vector

Some ABA response elements (ABRE3 or G-box) have been shown to be more efficient when they occur in repeats on a promoter or when associated with coupling element (CE) sequences (Shen and Ho, 1995). Interestingly, there are numerous ABRE, ABRE3 and ABRE-like motifs as well as G-box motifs containing the ABRE core sequence on the *XvAld1* 5′-UTR. However, no coupling elements were identified. Abiotic stress CAREs identified included several dehydration, a heat shock, a low temperature, and several light-regulated and several nutrient responsive motifs. Interestingly, the only osmotic stress motif found was the hypo-osmotic and proline response element. Most of the nutrient responsive motifs are sugar responsive and are similar to motifs found in promoters of many genes involved in carbohydrate metabolism (Lu et al., 1998; Rook et al., 2006).

A large number of response elements directing tissue specific expression were also identified. Among them were guard cell, meristem, mesophyll, pollen, root, stem, vascular and seed storage motifs. Of particular interest are the numerous motifs that are similar to cis elements commonly found in promoters of genes for seed storage proteins such as glutelin, napin and prolamins (Ezcurra et al., 1999; Lamacchia et al., 2001).

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Table 2.3 Cis-acting response elements identified on the *XvAld1* promoter fragment

Response element	Motif	Position[a]	Notes	References
HORMONES				
ABA responsive				(Shen et al., 1996)
ABREs	ACGTSSSC	-372	Cooperative activation with coupling elements or multiple ABREs. Some ABRE-like motifs not ABA responsive	(Hattori et al., 2002)
ABRE-related	ACGTG	591, -537, -375		(Narusaka et al., 2003)
ABRE-core	ACGTGKC	591, -373, 752, 555		(Simpson et al., 2003)
G-box related	CACGTY	-597, 609, 448, 328		
ABF (as-1-like)	**core**[b]	818		
Auxin				(Li et al., 1994)
NDE/SAUR	CATATG	367, 584, -595	Small Auxin-Up RNA in soybean, auxin REs found in many unrelated genes	(Xu et al., 1997)
DR5	CTCTGTT	822		(Hagen and Guilfoyle, 2002)
ARFAT	TGTCTC	594		
Cytokinin				
ARR1AT	NGATT	14, 39, 173, 265, 446, 656, -288, -554, -675	Cytokinin response factor	(Sakai et al., 2000)
Ethylene				
EIN3	**core**[b]	558	Binds to ethylene responsive elements	(Ogawa et al., 2005) (Ogawa et al., 2007)
Gibberellic acid				
CARE	CAACTC	-361	Gibberellic acid RE, may act co-ordinately with pathogenesis REs	(Gubler et al., 1995)
GADOWN	ACGTGTC	591		(Cercos et al., 1999)
GARE	TAACAGA	338, 711, -750		(Ogawa et al., 2003)
Pyrimidine Box	CCTTTTT	90, 314		(Sutoh and Yamauchi, 2003)
Jasmonic acid				
T/G Box	AACGTG	590, -537	Jasmonate and elicitor RE	(Boter et al., 2004)
JERE	AGACCACC[c]	59, 738		
Salicylic acid (SA)			SA REs and pathogenesis elements act cooperatively	(Shah and Klessig, 1996)
SARE	TTCGACCTCC[c]	275, 776, 130		
Abiotic stress				
Antioxidant defence				
CORE	**core**[b]	-786	Coordinate RE	(Chen and Singh, 1999)
OCS	**core**[b]	131, 289, 322, 431, 538	Octopine synthase RE	(Garreton et al., 2002)
As-1	TGACG	30, 273, 322, 448, 836, -190, -776, -818	Activation sequence-1 Ocs and as-1 responsive to JA, auxin, SA and oxidative stress	(Tsukamoto et al., 2005)
Dehydration				
CBFHv, DRE	RYCGAC	241	C-repeat and G-box core motifs in dehydration and cold responsive genes	(Urao et al., 1993)
ACGTAtERD1	ACGT	376, 532, 538, 591		(Abe et al., 1997)
MYB2 Consensus	YAACKG	558		(Xiao and Xue, 2001)
MYB Core	CNGTTR	-338, -558		(Simpson et al., 2003)
MYC Consensus	CANNTG	353, 367, 486		
Temperature				
Box 1	CCAAT	391, -123	Heat shock responsive	(Rieping and Schoffl, 1992)
LTRE Core	CCGAC	529	Low temperature responsive	(Kim et al., 2002)
Light regulated (LR)				
GT1 consensus	GRWAAW	160, 766, -101,-267	Motif in many LR genes, Light-regulated gene expression Phytochrome RE Sequences over-represented in light inducible promoters	(Terzaghi and Cashmore, 1995)
I-Box, G-Box	GATAA	160, 709, 766		(Nakashima et al., 2006)
RE-alpha	AACCAA	472		(von Gromoff et al., 2006)
SORLIPs	GCCAC	373		
Circadian	CAAN(4)ATC	375		
PRE consensus	SCGAYNRN(15)HD	132, 565, -438,-481		
Nutrients				(Quinn et al., 2000)
CURE Core	GTAC		Copper and oxygen RE	(Rubio et al., 2001)
P1BS	GNATATNC	86, 534	Phosphate starvation	(Satoh et al., 2002)
SURE Core	GAGAC	478	Sulphur RE	(Maruyama-Nakashita et al., 2005)
Proline	ACTCAT	723, -595, -616, 775	Hypo-osmolarity and proline RE	

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Sugar response				
SURE	TTGAC	112	Sugar responsive elements (SURE)	(Hwang et al., 1998)
WBOXHv	TGACT	-427	Sugar starvation	(Sun et al., 2003)
Amylase CGACG	CGACG	530	Motifs found in promoters of carbon metabolism genes, show cooperativity with light REs	(Grierson et al., 1994)
DOF Core	AAAG	402, 425, 731, 771		
RBS consensus	AATCCAA	554		
Biotic stress				
Pathogens/wounding				
BIHD1	TGTCA	117, -502	Pathogenesis and wounding responsive genes	(Elliott and Shirsat, 1998)
GT1	GAAAAA	-315	GT1 is pathogen and salt RE	(Park et al., 2004)
WRKY	TGAC	113, 502, 516, -428	Wounding activating region (WAR) in Brassica	(Yamamoto et al., 2004)
WAR	**core**[b]	487, -721		(Nishiuchi et al., 2004)
WBox	TGACY	113, 516, -427		(Luo et al., 2005)
Tissue specificity				
Guard cells				
TAAAG motif	TAAAG	-321	Regulation of guard cell specific expression	(Plesch et al., 2001)
Meristems				
TE2F2	ATTCCCGC	-5	Meristem tissue specific expression	(Kosugi and Ohashi, 2002)
Mesophyll		453, 602, 755, -21,	Mesophyll-specific expression	(Gowik et al., 2004)
CACT motif	YACT	-142, -233, -336, -778		
Pollen				
GTGA motif	GTGA	501, -606	Pollen specific expression	(Bate and Twell, 1998)
pollen1	AGAAA	423, -181, -646,-658		(Rogers et al., 2001)
Root				
NODConsensus1	AAAGAT	771	Nodulation consensus, nodulin specificity elements in soybean. Root specific expression motif	(Stougaard et al., 1990)
NODConsensus2	CTCTT	227, 642, 662, -420, -577		
ROOT motif	ATAAT	121, 566, -479		
RY-repeat legumin box	CATGCAY	788, -268		
Seed storage genes				
E-box motif	CANNTG	353, 367, 486	Cis elements common in promoters of seed storage protein genes eg napin, glutelin, prolamin	(Fujiwara and Beachy, 1994)
RY repeat (*napA*)	CATGCA	788		(Stalberg et al., 1996)
SEF4 motif	RTTTTTR	299, 305		(Ezcurra et al., 1999)
Box 1, Box 2 (glutelin)	CATGT (variable)[c]	-515, -591, 93, -404, 55	Endosperm specificity palindrome	(Lamacchia et al., 2001) (Vickers et al., 2006)
vascular				
BS1	AGCGGG	-25	Vascular-specific expression	(Lacombe et al., 2000)
General				
CAAT Box	CAAT (variable)[c]	253, 287, -111, -123	Common promoter motif	
TATA Box 3, 4, 5	TATA (variable)[c]	46, 666, 99	Transcription initiation RE	(Grace et al., 2004)
Enhancers, EEC	GANTTNC	266, 361, 385, -8, 424	EEC enhancer elements	(Kucho et al., 2003)
QAR	AACGTGT	590, -536	Quantitative activator region	(Elliott and Shirsat, 1998)
UAS and PE	core	98, 585, 595, -199	Upstream activating sequences, affect many different REs	
Other				
CGCG Box	VCGCGB	3	REs in promoters of CaM and DNA binding proteins	(Yang and Poovaiah, 2002)
ABRE-Cal	MACGYGB	590, -536	ABRE motif in Ca^{2+} responsive genes	(Kaplan et al., 2006)

[a] Nucleotides not numbered with respect to the transcriptional start site, "-" denotes antisense strand
[b] The core sequence motif was not determined
[c] Several functional motif patterns have been identified
Degenerate nucleotide abbreviations; B – t/c/g; D – a/t/g; K – g/t; M – a/c; R – g/a; S – c/g; V – a/c/g; W – a/t; Y – c/t and N – any nucleotide

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2.3.8 Cloning of XvAld1 into the pProEX expression vector

The *XvAld1* coding sequence, cloned into the expression vector, pProEX HTa, was sequenced to confirm that it was in the correct reading frame and to check for mutations. Sequencing results confirmed that the pProEXa5-*XvAld1* contained the aldose reductase without mutations and cloned in-frame with the poly-histidine tag. The sequenced clone (pProEXa5-*XvAld1*) was used for subsequent protein expression studies.

2.3.9 Expression of the *X. viscosa* aldose reductase in *E. coli*

A small scale protein expression experiment was carried out to determine if the recombinant protein was expressed in *E. coli* cells. Equivalent amounts of cell samples were lysed and the crude protein extracts separated on SDS-PAGE as shown in Figure 2.11. The calculated molecular weight of the recombinant protein was 40.5 kDa, resulting from the polyhistidine tag and several amino acid residues comprising the spacer region and the protease cleavage site (refer to Appendix D for detailed plasmid information). No protein of the expected size was visible in the uninduced sample (lane 1). However, progressive accumulation of 40.5-kDa recombinant protein is evident from one hour after induction (lanes 2 - 4). It is interesting to note that the 40.5-kDa recombinant protein co-migrates with the 45-kDa molecular weight standard in lane M.

After demonstrating successful expression of the recombinant protein, a large scale protein expression experiment was carried out. Purification of the expressed protein was performed using the Ni-NTA affinity purification resin. A batch-wise purification method (Polayes and Hughes 1994) was used. The His-tagged XvAld1 was the only protein visible on the Coomassie stained gels (data not shown). However, to further demonstrate the purity of the protein, immunodetection was carried out using anti-histidine monoclonal antibodies. The antibodies recognise the poly-histidine tag attached to the N-terminus of the recombinant protein.

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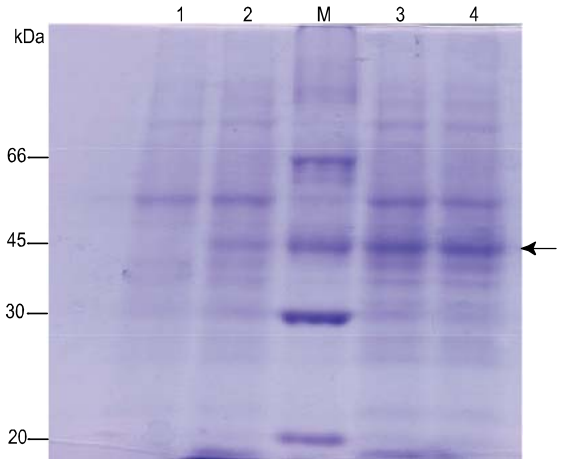

Figure 2.11 Coomassie stained gel showing expression of the His-tagged *X. viscosa* aldose reductase in *E. coli*. The *E. coli* cells were grown at 37°C in LB supplemented with 100 µg/ml ampicillin until OD_{600} of 0.5 – 0.8 was reached. Protein expression was induced by adding 1 mM IPTG and the cells were cultured for a further 3 hr. Aliquots of the broth were collected before induction (lane 1) and at 1, 2 and 3 hr after induction (lanes 2 – 4 respectively). Equal concentrations of the crude protein extracts were mixed with 5 x SDS loading buffer and separated on 10% SDS-PAGE. The arrow indicates the band corresponding to the 40.5-kDa recombinant protein which comigrates with the 45-kDa molecular weight marker. The protein accumulates over the 3 hr incubation and is absent in the uninduced sample (lane 1). The molecular weights of the standards in lane M are indicated on the left.

The purified poly-histidine-tagged recombinant protein was probed with poly-histidine monoclonal antibody (Fig. 2.12). The detected bands were specific for a single protein, except for the crude protein sample which showed smearing, probably due to overloading or cross-interaction of the antibody with other poly-histidine proteins in the *E. coli* lysate. The purification batch in lane 1 showed two forms of the recombinant protein, probably a result of differential protein modification.

2.3.10 Immunodetection using the XvAld1 polyclonal antibodies

Polyclonal immunoglobulins (IgG) were precipitated from the antiserum using polyethylene glycol (PEG). The purified polyclonal antibodies were used for immunodetection of varying concentrations of recombinant protein separated by SDS-PAGE and blotted onto nitrocellulose membrane. The western blot in Figure 2.13 demonstrated that the XvAld1 polyclonal antibodies are specific for the *X. viscosa* aldose reductase since no contaminating *E. coli* proteins were detected.

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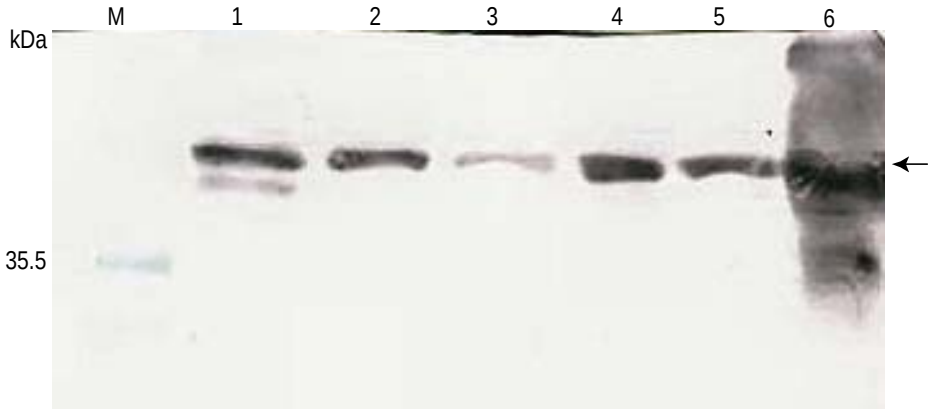

Figure 2.12 Immunodetection of purified His-tagged XvAld1 recombinant protein using anti-histidine monoclonal antibodies. Lanes 1-5; batch-wise purified recombinant protein, lane 6; crude cell lysate containing the recombinant protein and lane M; 35.5-kDa prestained molecular weight marker visible on the blot. The arrow highlights the 40-kDa recombinant protein band. The NBT/BCIP chromogenic substrate (Roche, Germany) was used to develop the immunoblot.

Figure 2.13 Immunodetection of recombinant XvAld1 protein using polyclonal antibodies. Varying concentrations of the affinity-purified recombinant protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with 1:5000 dilution of the polyclonal antibody and 1:20 000 dilution of the peroxidase-linked secondary antibody. Lane 1 – 5: 150, 75, 38, 20 and 10 ng of recombinant protein respectively.

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2.4 DISCUSSION

The AKR superfamily is a family of oxido-reductase enzymes that is receiving increasing reports in the literature. Hundreds of protein sequences and enzymatic properties have recently been determined (Hyndman et al., 2003). However, the physiological functions of many AKRs remain unresolved. Mundree et al. (2000) have postulated that the ameliorative properties of the *X. viscosa* aldose reductase may be due to synthesis of osmoprotectants, or may result from the protein participating in maintaining the structural integrity of macromolecules under abiotic stress. In the absence of biochemical and physiological evidence pointing to the functions of AKR proteins in stress tolerance, *in silico* analyses were performed to assist in predicting the possible functions of the XvAld1 protein.

2.4.1 Predicted posttranslational modifications and functional motifs

Computational analysis of the XvAld1 amino acid sequence revealed that the protein is soluble. This prediction was confirmed when the recombinant protein expressed in *E. coli* was purified from the soluble fraction. The functional properties of proteins are often regulated by post-translational modifications (PTMs). These modifications may greatly increase the complexity of the protein by causing structural changes and modulating protein activities, stability, subcellular localisation and/or interactions with other proteins.

It is reasonable to conclude that the XvAld1 protein may require post-translational modifications for full function, since potential phosphorylation, N-myristoylation and N-glycosylation sites were predicted using several *in silico* prediction tools. In addition, motifs for targeting to microbodies were found, including specific peroxisomal localisation consensus sequences. Nuclear and Golgi-ER export signals were also identified. Several tyrosine-based endocytotic internalisation signals and a DLL motif known to bind to clathrin heavy chains indicate that AR may be actively transported from the cytosol to other subcellular compartments. N-glycosylation motifs have been detected on other AKR proteins but the presence of glycan moieties could not be experimentally verified (Schade et al., 1990). AKR proteins are generally soluble and cytosolic. However, the aflatoxin B1 aldehyde reductase from rat was shown to be associated with the Golgi apparatus (Kelly et al., 2002), and AR (also from rat) was detected in mitochondrial fractions (Varma et al., 2003).

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The presence of a cyclin docking site on the XvAld1 protein shows that the aldose reductase may specifically interact with CDK for phosphorylation. Other ligands for specific protein-protein interactions and motifs for phosphorylation by other protein kinase families including PKA, PKC, CaMKII, ERK and MAPK were also identified. Many signalling proteins contain structural modules that specifically interact with ligands, usually peptide sequences. In addition, protein phosphorylation may also play a role in regulating protein-protein interactions. The identification of both ligand-binding and phosphorylation motifs on XvAld1 suggests complex regulation of the functional properties of the protein.

Possible phosphorylation of XvAld1 is particularly interesting because of the significance of this modification in signalling. Protein kinase C (PKC) has been implicated in the phosphorylation of some AKR proteins (Henry et al., 1999; Nakamura et al., 1999; Nishikawa et al., 2000). Nakamura et al. (1999) demonstrated that selective inhibition of PKC in diabetic rats produced the same effect as aldose reductase specific inhibitors. In addition, inhibition of PKC activation prevented sorbitol accumulation (Nishikawa et al., 2000). These studies show that there is a specific interaction between PKC and aldose reductase. Varma et al. (2003) demonstrated using a recombinant protein that AR can be phosphorylated by PKC isoforms. This phosphorylation is thought to be responsible for the translocation of the AR into mitochondrial membranes (Varma et al., 2003).

Knowledge of potential PTMs of a protein may increase the understanding of the molecular and/or physiological processes in which the protein may be involved. However, validation of the PTMs of interest using experimental methods is vital. This is because a protein exists in a 3-dimensional structure, and only motifs that are exposed to the surface of the protein are thought to interact with the cellular environment. Many PTM programs over-predict, failing to take into account the probability of surface exposure [SPS – surface prediction score; (Balla et al., 2006)]. *In vivo*, PTMs may be prevented by structurally inaccessible motifs or if the protein is not found in the predicted subcellular compartment. To account for surface exposure, all phosphorylation sites considered (Table 2.1) have at least one motif with SPS of 1. All sites considered have SPS greater that 0.5. Subcellular localisation of XvAld1 protein needs to be determined experimentally. Filtering methods used for PTM predictions may also over-predict, underestimating PTMs by removing true predictions (Blom et al., 2004).

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2.4.2 Multiple sequence alignments

Multiple sequence alignments of the XvAld1 sequence with selected plant AKR sequences revealed that the protein shares significant similarities with the other proteins, particularly in the regions that determine the conserved $(\alpha/\beta)_8$-barrel structure. The alignment demonstrates that the sequences that determine the invariable secondary and tertiary structure are conserved. In addition, the motifs that are important for enzyme function, for example catalytic and cofactor binding residues, are either invariable or conserved.

The three plant ALRs with which XvAld1 shares the subfamily 4C and the *A. thaliana* aldose reductase-like protein possess significant sequence similarities in the C-terminal loops. The C-terminal end of the proteins, containing three loops (loops A, B and C) is variable among AKRs in both size and sequence. These variable loops have been implicated in determining AKR enzyme substrate specificities (Jez et al., 1997a; Jez et al., 1997b; Ma and Penning, 1999). The similarities in this region imply that the four plant ALRs share common functions and substrate preferences.

Sequencing of the *XvAld1* cDNA cloned into pSK consistently showed a three nucleotide difference with the aldose reductase sequence deposited in the GenBank (AF133841). These differences translated to a three amino acid disparity between the deduced amino acid and the sequence of the recombinant protein. The three amino acid difference could be a result of sequencing errors in the GenBank sequence. Although the changes A→D and KH→ND are maintained in the sequence of the genomic clone, there still is the possibility of the existence of another gene variant, which possibly has the same sequence as the GenBank clone. This possibility would mean that the nucleotide differences are a result of genetic polymorphisms as the altered residues are not strictly conserved. Polymorphic differences between a number of AKR proteins have been reported. The protein sequences of two aldo-keto reductase cDNA clones from *Digitalis purpurea* leaves have 98.4% amino acid sequence identity but one possessed three times higher specific activity for glyceraldehydes, glucose and fructose (Gavidia et al., 2002). The two dihydrodiol dehydrogenases (DD) from human liver have a six amino acid difference (Hara et al., 1996). DD1 has prostaglandin F synthase activity and low affinity for lithocholic acid, a bile acid. In contrast, the other isozyme (DD2) exhibited high affinity for the same

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bile acid. Some polymorphisms found in AKR genes are silent, causing no differences in amino acid sequences of the homologues (Ho et al., 1999).

Allelic variations or sequencing errors may occur between members of superfamily proteins. Jez and Penning (2001) recommended that proteins with greater than 97% amino acid sequence identities be regarded as alleles of the same gene. An exception would occur when different enzyme activities are demonstrated, if the cDNAs have distinct 3′-UTR sequences or if the genes are on different chromosomal locations. The *XvAld1* genomic sequence contained many instances where the two sequenced clones differed (Fig. 2.8). The clones showed 91.2% nucleotide sequence identity. The consensus sequence of the genomic clones also differed from the GenBank cDNA sequence (nucleic acids in red; Fig. 2.7B). The nucleotide sequence was 96.14% identical to the *XvAld1* cDNA sequence in the GenBank and translated sequence showed 94.67% identity. These observations, although supporting the possibility of the existence of polymorphic forms, are not conclusive as they are based on limited genomic information. More research involving more rigorous sequencing needs to be done before these differences can be conclusively attributed to genetic polymorphisms. In addition, the presence of multiple genes has to be confirmed by characterising the 3′-UTRs and using that sequence to probe total RNA instead of using the coding sequence. The chromosomal locations of the AKR genes in *X. viscosa* also remain to be determined.

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2.4.3 Genomic organisation and structure of the *XvAld1* gene

The Southern blot analysis (Fig. 2.5B) demonstrates the existence of other *XvAld1*-related genes in *X. viscosa*. Since the sizes of the *Eco*RV and *Xba*I bands are larger than 1.2 kb, the estimated size of the full length *XvAld1* cDNA sequence, it is reasonable to assume that there are at least two copies of *XvAld1* in *X. viscosa*. The *Pvu*II digest produced at least 6 bands when probed with the full length cDNA sequence. At least two of the bands are smaller than the size of the cDNA sequence, yet *Pvu*II does not have any sites within the coding sequence. This raised the possibility that the *XvAld1* gene may contain several introns.

Amplification of a ~3 kb genomic sequence from *X. viscosa* genomic DNA and sequencing of the genomic clones revealed that *XvAld1* gene contains nine exons and eight introns (Fig. 2.7). The genomic organisation of the *XvAld1* gene had not been previously reported. The existence of intronic sequences in eukaryotic gene

sequences is common (Breathnach and Chambon, 1981; Brown, 1986) and most of the AKR genes examined to date contain several introns. Sequencing of the genomic clones did not resolve the complex banding pattern produced by *Pvu*II digestion, since none of the introns contained the expected *Pvu*II restriction sites. Southern blot analysis of the genomic clones also yielded a map with *Pvu*II digested DNA showing an unexpected banding pattern. *In silico* restriction enzyme analysis of the genomic sequence showed no *Pvu*II sites in the 2.9-kb fragment. However, both sequencing and Southern blot analysis on the genomic clones confirmed the presence of an *Xba*I site in intron 7 at position 2437. The complex banding patterns obtained from Southern blotting using both the chromosomal genomic DNA and PCR amplified genomic DNA digested with *Pvu*II raised the possibility that the enzyme may be exhibiting anomalous restriction properties or star activity, when a restriction enzyme displays altered specificity under suboptimal conditions. Alternatively, the multiple *Pvu*II sites may be a characteristic of other *X. viscosa* AR genes still to be characterised.

The genomic organisation of AKR genes in terms of gene copy numbers and intron-exon patterns has been reported in a number of studies. The intron-exon numbers and sizes seem to be somewhat conserved. The wheat aldose reductase genomic sequence consists of nine exons (Roncarati et al., 1995) as does the *X. viscosa* aldose reductase. The genes span 2.3 and 2.9 kb respectively, excluding the 5′ and 3′-UTRs. Sequence information on *A. thaliana* homologues from The Arabidopsis Information Resource (TAIR) webpage [http://www.arabidopsis.org/; (Rhee et al., 2003)] shows nine genomic sequences on five different loci, comprising five, six, seven, or eight exons. The variability of the intron/exon numbers in the Arabidopsis genome is interesting, given the conservation of genomic organisation shown in mammalian organisms.

In mice, of the nine genes found in a cluster on chromosome 13 (Vergnes et al., 2003), seven contain nine exons (Lou et al., 1994; Nishizawa et al., 2000). The other two genes contain ten exons as previously reported (Usui et al., 1994; Lin et al., 1999)). On chromosome 6, another five AKR and AKR-like genes are located in close proximity. The genomic structures revealed that three of the genes each consisted of ten exons spanning ~18 kb (Ho et al., 1999). The remaining two genes did not contain introns, and were therefore regarded as pseudogenes. The organisation of AKR genes into ten exons is also found in some mouse ARs (Lin et al., 1999) and in a rabbit AR

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gene spanning 15 kb (Ferraris et al., 1994). The possibility that the rabbit gene formed part of a cluster was not investigated, and Southern blot analysis was not done. Four human AKR genes are also clustered closely on chromosome 10 (Khanna et al., 1995a; Khanna et al., 1995b; Rheault et al., 1999; Nishizawa et al., 2000). Like the mouse genes, the human hydroxysteroid dehydrogenases (HSDs) and closely related sequences had a similar intron-exon organisation and high sequence homologies. Each of the genes comprised nine exons spanning between 14 and 25 kb. The human 5β reductase consists of nine exons spanning more that 42 kb (Charbonneau and The, 2001). Another intronless copy was regarded as a pseudogene because it contains multiple stop codons. In human tumour cells, a multiple gene family locus of three aflatoxin B1 aldehyde reductase genes and two pseudogenes exist in a cluster on chromosome 1 (Praml et al., 1998). The genes are operon-like; arranged in different orientations or on opposing strands. The genomic sequences consist of seven exons spread over 6 – 8 kb. Five pig AKRs were also shown to exist in a cluster on chromosome 10 (Nonneman et al., 2006). The intron-exon arrangement of these genes was not investigated. An aldehyde reductase found in red yeast also contained five exons and six introns, with a genomic sequence spanning ~2 kb (Kita et al., 1996).

From all these investigations, a pattern seems to emerge that eukaryotic AKR genes tend to exist in a cluster of operon-like gene arrangements. Within a particular cluster, the genes seem to share similar sequence identities within the exons, introns and the 5′-UTRs, meaning that the genes are similarly regulated. Similar intron-exon structures and gene sizes also exist within the same cluster. In animals, AKR clusters reported to date contain genes consisting of seven, nine and ten exons. The few reports available for plants show genes mostly containing six, seven or nine exons. The plant genes stretch over shorter distances (~3 kb) compared to the animal AKRs which span 6 – 8 kb for the seven exon genes and 14 – 25 kb for the nine and ten exon genes. The high sequence homologies and existence of polymorphic variations among related AKRs make it difficult to discriminate between the closely arranged genes and to decide whether minor sequence variations are a result of sequencing errors or the existence of multiple gene copies.

The clustering is proposed to have arisen from ancient duplication events and subsequent divergence (Penning et al., 1997b). Clustered genes have similar structures and possibly similar functions, and are usually co-regulated as the physical

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close proximity seems to correlate with mRNA expression patterns (Liu and Agarwal, 2005). The analysis of clustered genes was made possible by the availability of chromosomal DNA sequences of humans and mice, resulting from the respective genome sequencing initiatives. Very few genomic sequences from resurrection plants are available in public databases, making a similar study impossible. However, from the Southern blotting results and the sequencing of the genomic clones, it is reasonable to assume that the *XvAld1* exists as part of a gene family in *X. viscosa* and that the genes share substantial sequence similarities and exist in close proximity with each other. Further analyses that utilise the 3′-UTRs to differentiate between closely related sequences may provide a better understanding of the genome organisation.

The genomic organisations of many AKR genes have not been determined. This information would be useful in classification of proteins belonging to the superfamily. When a new nomenclature for AKR proteins was proposed, it was based on the classification of cytochrome P450 proteins (Nelson et al., 1996; Nelson, 1999) except that only amino acid identities were used as not many reports on genomic structures were available (Lou et al., 1994; Jez et al., 1997b). Of the few genomic structures that have been reported, many contain multiple introns and show evidence of gene duplication events (Penning et al., 1997a; Vergnes et al., 2003).

Cytochrome P450 genes also have conserved intron-exon organisations and are often found clustered on a short stretch of chromosomal location (Paquette et al., 2000). There is a good correlation between intron conservation and phylogenetic relationships among cytochrome P450 protein subfamilies, showing that genomic organisation is a viable protein classification method. This second level of gene classification would be important for AKR proteins and the more specific grouping might help in predicting protein functions.

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2.4.4 Promoter sequence analysis

A potential outcome of studies such as this one is the production of crops expressing stress tolerance-related genes under the control of a stress-responsive promoter. In order to understand the functions of *XvAld1* in stress tolerance, it is vital to investigate how the gene is regulated in *X. viscosa*. To accomplish this, the 5′-UTR was amplified and sequenced. Important cis-acting response elements (CAREs) were predicted using several resource databases. Some of the interesting CAREs (Table 2.3) were those associated with hormone signalling. ABREs are of particular interest

as a lot of research has elucidated pathways linking ABA signalling to abiotic stress signalling (Skriver and Mundy, 1990; Bray, 1991). Studies by Shen and Ho (1995) have demonstrated that more than one ABRE in the same sequence is required for ABA-responsiveness. Several ABRE and ABRE-like motifs were identified in the putative promoter sequence, suggesting that *XvAld1* expression is ABA responsive. In addition, several coupling elements, shown to function cooperatively with G-box motifs, have been located on the 5′-UTR of *XvAld1*.

Cis-acting elements for auxin, GA, SA, cytokinin, JA and ethylene responsiveness were also identified, suggesting that the expression of *XvAld1* is partly regulated by hormonal cues. Hormones control many aspects of plant growth and development, as well as being involved in biotic and abiotic stress signalling. Hormones show considerable crosstalk, both in terms of agonistic or antagonistic responses and as demonstrated by sharing of signalling components (Thatcher et al., 2005).

Since *XvAld1* expression is induced under dehydration, identifying stress responsive elements that may be important for this regulatory function was vital. One drought response element (DRE) and several core motifs implicated in dehydration or cold responsiveness were identified. It is interesting to note that only one DRE motif was identified. However, studies by Yamaguchi-Shinozaki and Shinozaki (1994) showed that DRE/CBF motifs do not need to be repeated in order to be functional. Using a core promoter sequence, it was demonstrated that one DRE motif is strong enough to drive gene expression. The binding of DRE motifs is mediated by DRE-binding proteins (DREBs). Although many genes isolated from *X. viscosa* are dehydration responsive, genes coding for the corresponding DRE-binding transcriptional factors (DREBs) remain unidentified.

Several CAREs for tissue specific expression were identified on the *XvAld1* promoter fragment. These include motifs for stem, root, meristems, mesophyll and vascular tissues as well as guard cell and pollen specific expression. This indicates that *XvAld1* may be expressed in many different tissues depending on the prevailing stress conditions or developmental stage of the plant. Of special note are response elements for seed specific expression. These CAREs are found in promoters of genes encoding seed specific storage proteins such as napin, prolamins and glutelins (see Table 2.3). Cis-acting response elements similar to each of the three classes of seed proteins were found on the putative *XvAld1* promoter fragment.

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The presence of CAREs normally found in seed specific proteins is interesting but not unusual. Analysis of *X. viscosa* genes has revealed the expression of gene products that are normally seed specific (Haslekas et al., 1998; Stacy et al., 1999) in vegetative tissues during dehydration (Mowla et al., 2002). A similar finding has been reported of *C. plantigineum* (Bartels, 2005). Illing et al (2005) have proposed that desiccation tolerance in vegetative tissues of *Xerophyta humilis* arose from the appropriation of the developmentally regulated seed-specific program of gene expression into roots and leaves, these having then become environmentally regulated. Microarray analysis of gene expression in dehydrated vegetative tissues and seed of *X. humilis* and comparison with gene expression from seeds of *A. thaliana* lent considerable support to this hypothesis (Illing et al., 2005; Walford pers comm.). The appropriation of the seed-specific strategy of gene expression in vegetative tissues of desiccation tolerant plants may be facilitated by the way stress-responsive genes are regulated. Judging by the CAREs identified on the *XvAld1* putative promoter fragment, it appears that environmental stress and hormonal cues, tissue specific expression and developmental stages of the plant all act cooperatively to regulate the expression of stress responsive genes in *X. viscosa*. In their comparative analysis of *A. thaliana* and *Thellungiella halophyla* transcriptomes, Taji et al. (2004) also hypothesised that the stress tolerance in salt cress may be a result of subtle differences in regulation mechanisms.

The functional dissection of the 870-bp promoter fragment to confirm promoter activity is essential. As discussed above for short sequence motifs, the probability of detecting false positives is high. The signal response specificity of each CARE has to be verified experimentally by fusing the motifs of interest upstream of a minimal heterologous promoter consisting of a TATA box and a transcription initiation site, and testing expression of a reporter gene.

2.4.5 Recombinant protein expression

The recombinant XvAld1 protein was successfully expressed in *E. coli* and was purified using Ni-NTA affinity resin for His-tagged proteins. Polyclonal antibodies raised against the recombinant protein were specific for the aldose reductase protein. The calculated molecular weight of the recombinant protein (40.5 kDa) was different from the apparent molecular weight. The mobility of the recombinant protein on SDS-PAGE was comparable to that of a 45-kDa molecular

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weight marker (Fig. 2.11). Aberrant mobility of a protein on SDS-PAGE is a common phenomenon. The presence of post-translational modifications such as N-glycosylation (Marciani and Papamatheakis, 1978) and phosphorylation (Billings et al., 1979) has been shown to retard protein migration. Other parameters affecting apparent molecular weight on polyacrylamide gels include the conformational properties of the protein (size and shape) and the presence of regions with excessive negative or positive charge (Hu and Ghabrial, 1995). In addition, the oxidised or reduced states of sulfhydryl side chains may also affect protein conformation.

The structural features of XvAld1 protein include two globular domains interspaced with two regions of disorder. Computational analyses predicted PTMs that include both N-glycosylation and phosphorylation. Although β-mercaptoethanol was used during the purification of the recombinant protein, it is likely that some of the cysteine residues in XvAld1 were not completely reduced. Alternatively, the different protein sizes could arise from differential post-translational modification of the recombinant protein in the *E. coli* cells.

Further investigations will be undertaken to explain the anomalous migration evident in Figure 2.11, and the presence of a doublet in Figure 2.12 (lane 1). Interestingly, Bartels et al. (1991) identified a slow migrating, unidentified 36 kDa protein that accumulated as barley embryos matured. The smaller (34 kDa) protein was present in developing embryos younger than 20 days days after pollination, whereas matured embryo accumulated both forms. Roncarati et al. (1995) also detected a faster migrating form (29 kDa) of the barley embryo aldose reductase in some of their immunopurification fractions. They suggested that the protein was a degradation product since it was not present in all the fractions. However, given the similar results from our analyses, it is possible that the faster migrating band may not be a degradation product, but another form of the aldose reductase protein.

2.5 CONCLUSION

Putative posttranslational and functional motifs on the *XvAld1* gene are reported. The upstream non-coding region contains many classes of cis-elements that are putatively associated with stress tolerance. The ectopically expressed protein did not show any enzymatic activity, possibly due to the histidine tag. More work will need to be done to further characterise the putative promoter, the cis-elements therein and the protein.

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CHAPTER 3

Abiotic stress response and localisation of the *X. viscosa* aldose reductase

3.0 SUMMARY

In order to extend our understanding of stress-responsive genes, the expression of *XvAld1* in *X. viscosa* plants exposed to various abiotic stress conditions was determined using quantitative real time PCR. Dehydration to 77% RWC caused a marginal increase in transcript levels. The mRNA levels at 66% RWC were five orders of magnitude higher than under well-watered conditions. The high expression levels of *XvAld1* mRNA were maintained until the leaves had reached an air-dried state (15% RWC). Western blot analysis was used to establish if there was a correlation between mRNA and protein levels with increased desiccation. The XvAld1 protein was undetectable at 90% RWC but was detected at 66% RWC and remained to 15% RWC. The response of *XvAld1* to hormonal cues was determined by separately exposing plants to a single dose of abscisic acid (100 μM), ethylene (1 ppm), jasmonic acid (100 μM) and salicylic acid (100 μM). Leaf samples were collected over a 72-hr period following exposure to the different hormones. The transcription of *XvAld1*, monitored over three days, was marginally induced by exposure all of these hormones. However, there was no significant difference between the transcript levels before the exposure (t=0) and any of the material sampled after the treatment. XvAld1 protein was undetectable by western blot analysis of samples from plants exposed to any of the hormones. To investigate if the low induction was caused by the method of treatment or if it was not induced by exogenous ABA; an additional experiment was performed using total protein that was extracted from samples that had been exposed to ABA once every 24 hr for three days. Western blot analysis of these samples showed increased protein expression at 12 and 48 hr after ABA application, demonstrating that *XvAld1* is responsive to continued exogenous application of ABA. Localisation studies were carried out using both N- and C-terminal fusions to fluorescent proteins and by immunocytochemistry and the XvAld1 protein was found in the cytoplasm as well as in vacuoles.

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3.1 INTRODUCTION

The control of gene expression at transcriptional, posttranscriptional and translational levels has been used to characterise stress response in plants. The up- and down-regulation of genes is important for studying molecular responses to stress. Stress-regulated gene expression has been studied in a few resurrection plant species, that is *Xerophyta humilis, Craterostigma plantagenium, Tortula ruralis and Sporobolus staphianus* (Oliver, 1991; Furini et al., 1997; Wood and Oliver, 1999; Bartels and Salamini, 2001; Ramanjulu and Bartels, 2002; Collett et al., 2004; Le et al., 2007), in addition to the species under study here.

Using RNA dot blots, Mundree et al. (2000) demonstrated that *XvAld1* transcription was induced by dehydration. High light intensity, high and low temperatures, as well as exogenous ABA (10 μM) did not induce increased gene expression. The expression profiles of *XvAld1* in *X. viscosa* under well-watered and various stress conditions are investigated here, using the improved sensitivity of real time PCR. Real time PCR is now widely used for gene quantitation because of its tremendous sensitivity, high sequence specificity and broad detection range. The sensitivity achieved is several orders of magnitude more than end-point assays and probe hybridisation (Schmittgen et al., 2000; Malinen et al., 2003) and can be used to detect a single copy of a particular transcript (Palmer et al., 2003). In order to complement the results on transcript levels, western blotting was performed to investigate any correlation between elevated transcription and protein synthesis.

Computational analysis (section 2.3.2) predicted sorting signals to peroxisomes, microbodies and clathrin-coated endocytic vesicles. Studies using fluorescence microscopy and immunocytochemistry on *X. viscosa* dehydrated sections were performed to determine the subcellular localisation of XvAld1. Green fluorescent protein (GFP), an intrinsically fluorescent protein from *Aequorea victoria*, and its modified spectral variants are a powerful tool for protein localisation in living cells. The cell is dynamic, with components in constant motion. Proteins may move from one subcellular compartment to another through the available intracellular transport systems (Lunn, 2007). The possible function of a protein can be deduced from where it is localised within the cell under specific environmental conditions or developmental stages. Subcellular compartmentation is essential for the regulation of cellular processes by spatial separation. The expression profiles of *XvAld1* under various environmental conditions were investigated. In addition, the subcellular localisation of XvAld1 was determined. Both the spatial and the temporal contexts are important considerations for protein function.

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3.2 MATERIALS AND METHODS

3.2.1 Plant material

X. viscosa plants were collected from Cathedral Peak Nature Reserve in the Drakensberg Mountains (KwaZulu-Natal, South Africa). The plants were potted and grown under green-house conditions as described by Sherwin and Farrant (1996). For stress experiments, plants were transferred to controlled environment growth rooms with light intensity of 150 – 200 $\mu mol.m^{-2}.s^{-1}$ at 22 to 27°C, 50 – 70% humidity and 16 hr photoperiod. The plants were acclimated to the plant growth room conditions for 4 weeks before treatments were carried out. Due to the legal constraints of being able to collect only a few plants per annum (the plants occur in a protected reserve) the number of biological repeats used in this study had to be curtailed.

3.2.2 Dehydration

X. viscosa plants were dehydrated by withholding water from three plants over a 10-day period. The dehydration process was monitored by determining the relative water content (RWC) of leaf samples. Several leaves were excised from the plants, cut into small pieces and the fresh weights (FW) were immediately determined for each plant. For the determination of full turgor, leaf pieces were submerged in sterile distilled water and incubated overnight at 4°C. The water was drained and excess moisture was blotted from the leaf pieces. The weight of the hydrated samples (full turgor weight; FTW) was again determined. The leaf pieces were then dried at 70°C for 48 hr and the dry weight (DW) determined. RWC was calculated using the formula:

$$RWC = [(FW - DW) / (FTW - DW)] \times 100\%$$

Segments from the middle parts of the leaves were used for determining RWC by averaging readings from three replicates. Leaf samples were collected before the stress and on days 2, 4, 6, 8, and 10 of dehydration. Samples not used for RWC determination were quickly frozen in liquid nitrogen and stored at -80°C.

3.2.3 Abscisic acid, jasmonic acid and salicylic acid treatment

To determine if the expression of *XvAld1* was dependent on abscisic acid (ABA), the leaves of three *X. viscosa* plants were sprayed once with 100 µM phytohormone solution. The working solution was prepared fresh by diluting in water an appropriate volume a of 10 mM

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stock solution in 100% ethanol. Jasmonic acid (JA) and salicylic acid (SA) responses were determined by spraying leaves once with freshly prepared 100 μM hormone solution from a 10 mM stock solution. Plants were covered with black plastic bags for 10 min to keep out light immediately after spraying because the hormones are sensitive to light. Leaf samples were collected before each hormone treatment and at time points 3, 6, 12, 24, 30, 36, 48, 54, 60 and 72 hr after treatment. The samples collected before spraying were used as untreated controls.

3.2.4 Ethylene treatment

Whole plants were placed into Scilling plastic bags (Sigma-Aldrich, Germany) designed for holding gases. Ethylene, at a concentration of 1 part per million (ppm), was sprayed once onto the leaves. The plants were removed from the plastic bag 10 min after spraying and kept under growth room conditions. Leaf samples were collected before the treatment and at 3, 6, 12, 24, 30, 36, 48, 54, 60 and 72 hr after treatment.

3.2.5 RNA extraction

Total RNA was isolated using the Trizol reagent (Invitrogen Life Technologies, USA) according to manufacturer's instructions. Solutions used for RNA extraction, where appropriate, were treated with 0.01% diethylpyruvocarbonate (DEPC; Sigma-Aldrich, Germany). In short, leaf samples (~200 mg) were ground in liquid nitrogen and homogenized by vortexing in 0.75 ml of Trizol. After incubation at room temperature (RT) for 5 min, the homogenate was extracted with 0.2 ml of chloroform and incubated at RT for 2 min. Samples were centrifuged at 12 000 x g for 10 min at 4°C. RNA in the aqueous phase was precipitated by adding an equal volume of isopropanol. Pellets were dissolved in DEPC-treated water by incubating the tubes for 10 minutes at 55°C. The RNA was quantified at 260 nm using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The RNA quality was checked on a 1.2% (w/v) ethidium bromide stained agarose gel.

3.2.6 Two-step real time reverse transcription PCR

Total RNA for real time PCR was extracted using the Trizol reagent (Invitrogen Life Technologies, USA) as previously described. Ten units of RNase-free DNase I (New England Biolabs Inc., USA) were added to 50 μl of RNA extract to eliminate residual DNA. The reaction was incubated at 37°C for 15 min. The sample volume was adjusted to 100 μl using

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RNase-free water and RNA was purified using the EZ-10 Spin Column Total RNA Minipreps Super Kit (Bio Basic Inc., Canada), following the manufacturer's protocol. The purified RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). An aliquot was separated on 1.2% agarose gel to check the RNA quality and verify the concentration of the RNA samples.

The purified RNA (2.5 µg) from *X. viscosa* plant material was used for cDNA synthesis. The reaction consisted of; purified total RNA, 1 µM 18-mer oligo-dT or CDSIII primer (Clontech, USA) and RNase-free water to 25 µl. The contents were mixed, spun down and incubated at 72°C for 2 min then immediately chilled on ice. The reverse transcription (RT) master mix, consisting of 200 units M-MuLV RNase H^- Reverse transcriptase (Finnzymes, Finland), 2 µM dNTPs and RNase-free water to a volume of 25 µl per reaction, was prepared in a separate tube. The master mix was aliquoted into the separate RNA samples and the reaction incubated at 42°C for 1 hr. Thereafter, 5 units of RNase H (Finnzymes, Finland) was added to the reaction and incubated at 37°C for 15 min. The cDNA was stored at -20°C until use.

The relative expression of *XvAld1* in *X. viscosa* was determined by real time PCR. The SensiMix (dT) DNA Kit (Quantace, Australia), containing a universal master mix of PCR components, was used. PCR was performed in a reaction volume of 25 µl containing; 0.5 µl of 50 x SYBR Green solution, 12.5 µl of 2 x SensiMix, 1 µl of 10 µM gene specific primers and 2 µl cDNA. Gene specific sense and anti-sense primer sets for *XvAld1* (5′-GGAGGAGTGTGGAGGGAAAT-3′ and 5′-CCGAGAGGTGAATAAGCAGTT-3′), 18S rRNA (*Xv18S*; 5′-CAGGCGCGCAAATTACCCAATCC-3′ and 5′-CCTACCGTCCCGTCCCAAGGTC-3′) were designed (Primer3 programme; Rozen and Skaletsky, 2000) such that PCR products were 150 – 300 bp long and had annealing temperatures of about 60°C. The 18S primers were designed using the *X. humilis* 18S sequence available from the GenBank (Accession number EF418586; Collett et al., 2004). Real time PCR was conducted on a Rotor-Gene RG-3000A PCR machine (Corbett Research, Australia). The thermal cycling conditions were an initial enzyme activation step at 95°C for 10 min followed by 40 cycles of 95°C for 5 sec, 58°C for 8 sec and 72°C for 12 sec. Fluorescence readings were acquired after the extension step. Extra cycles were added where appropriate, depending on the concentration of the target transcript in the reaction. The PCR product was checked for specificity by running a melting curve programme on the Rotor Gene after the 40 cycles. In addition, an aliquot of the end product was separated on an

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ethidium bromide-stained 2% agarose gel and viewed under UV light to confirm that only one amplicon was obtained.

PCR efficiencies were determined from standard curves. Serial dilutions of the *XvAld1* fragment consisting of the full coding sequence were used as template for real time PCR to generate the standard curve. The fragment was prepared by digesting pProExHTa-*XvAld1* (see Appendix D for plasmid map) with *Sal*I and *Xba*I. The dilutions that fell within the range of *XvAld1* and *Xv18S* expression in *X. viscosa* were used for generating the six-point standard curve. This standard curve was imported into every run and used to calculate concentrations of the gene of interest (GOI) and the housekeeping gene (HKG). To account for the run-to-run variations in PCR efficiencies, two of the standard solutions included in every run were used to correct for the differences. The calculated concentration of the GOI was divided by the calculated concentration of the HKG (i.e. GOI/HKG). Thereafter, the values obtained from three biological replicates were averaged and used for relative quantification of transcripts. To evaluate the pattern of *XvAld1* expression under various stress conditions, the transcript levels in non-stressed plants (untreated or time zero) were used as a reference. Relative transcript levels were mean and standard deviation values reported as n-fold relative to the *Xv18S* expression levels (Livak, 1997; Wong and Medrano, 2005).

3.2.7 Protein extraction from plant material

Total protein was extracted from organic fractions of the Trizol reagent (Invitrogen Life Technologies, USA), following RNA extraction, using the manufacturer's protocol. Briefly, proteins were precipitated with 2 volumes of isopropanol. After centrifugation at 12000 x *g* at 4°C, the protein pellet was washed three times with 0.3 M guanidine hydrochloride in 95% ethanol followed by a single wash in 2 ml of absolute ethanol. During each washing step, the pellet was incubated in washing solution for 20 min at room temperature before centrifugation at 7500 x *g* for 5 min. The pellet was air dried for 5 min, resuspended in 1% SDS and stored at -20°C until use.

Alternatively, 0.2 g of whole plant material was ground to a fine powder in liquid nitrogen. One ml of extraction buffer (0.5 M Tris-HCl, 10 mM EDTA, 1% Triton X-100, 2% β-ME) was added and mixed thoroughly by vortexing for 10 min. The homogenate was centrifuged at 12000 x *g* for 5 min at 4°C. The supernatant was transferred to a new tube and extracted twice with an equal volume of Tris-buffered phenol (pH 8). The protein was

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precipitated overnight using 2.5 volumes of 0.1 M ammonium acetate in methanol. The sample was centrifuged at 12000 x *g* for 10 min at 4°C. The pellet was washed with 2 ml of 0.1 M ammonium acetate and again with 2 ml of 80% ice cold acetone. After air-drying, the pellet was resuspended in an appropriate volume of resuspension buffer [125 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol] and kept frozen at -20°C until needed. Protein extractions were done separately for each of the plants analysed. The concentrations of the extracted proteins were determined by the Bradford method as previously described.

3.2.8 SDS-PAGE and chemiluminescent detection

Unless otherwise specified, 15 µg of total soluble protein was loaded per well for separation by SDS-PAGE. The XvAld1 recombinant protein (10 – 25 ng) was used as the positive control. Gel electrophoresis was carried out as previously described, using 12.5% acrylamide. The protein was transferred onto nitrocellulose membrane by electro-blotting as previously described (section 2.2.14). The membrane was incubated for 1 hr in blocking solution (3% BSA in TBS) followed by washing 3 x 10 min in TBSTT. A primary antibody solution (1:5000 dilution of XvAld1 polyclonal antibodies in blocking buffer) was added to the membrane and incubated for 2 hr followed by washing 3 x 10 min in TBSTT buffer. The membrane was then incubated for 1 hr in a secondary antibody [1:20000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich, UK)] in blocking buffer and washed thrice for 10 min in TBSTT buffer. The detection was carried out using the SuperSignal® West Pico Chemiluminescent Substrate Detection System (Pierce, USA) according to the manufacturer's instructions. Fluorescence was captured on CL-XPosure™ Film (Pierce, USA) and the films developed manually.

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3.2.9 Construction of the fluorescent protein fusions

A *GFP-XvAld1* N-terminal fusion was created using the *Sal*I and *Bam*HI sites in the pGDG multiple cloning sites (Goodin et al., 2002; see Appendix D). The *XvAld1* sequence was PCR amplified from the pSK-*XvAld1* plasmid using a forward primer with an incorporated *Sal*I restriction site (5′-GTCGAC**ATG**GCGCATGCACCGT-3′) and a reverse primer containing a *Bam*HI site (5′-CCTAGGTTAGACTTCACCGTCCC-3′). The forward primer contained the translation initiation codon which is in bold. The reverse primer anneals downstream of the XvAld1 termination codon. The PCR amplification was performed as follows; 94ºC for 3 min, 2 cycles at 94ºC for 30 sec, 54ºC for 30 sec, 72ºC for 2 min, 25

cycles at 94ºC for 30 sec, 60ºC for 45 sec, 72ºC for 2 min and 72ºC for 7 min. The pGDG-*XvAld1* plasmid was constructed by ligating the *Sal*I and *Bam*HI digested vector and insert fragments.

The yellow fluorescent protein (YFP) expression vector was constructed as described by Seidel et al. (2005). In brief, the cauliflower mosaic virus (CaMV) 35S promoter was cloned into pEYFP (Clontech, USA) by digestion of both plasmid and insert with *Bam*HI and *Hind*III and subsequent ligation. A PCR-amplified Nos-terminator was digested with *Not*I, incorporated into the forward primer and an endogenous *Eco*RI. The terminator was ligated into YFP vector to generate the p35S-YFP-NosT plasmid (pYFP). C-terminal fusions to the *YFP* gene were constructed by digesting both plasmid and *XvAld1* insert with *Bam*HI and *Kpn*I. The *XvAld1* sequence was obtained by PCR amplification using sense and antisense primers incorporating the *Bam*HI and *Kpn*I sites respectively (YFP forward 5′-GGGATCCAATGGCGCATGCACCGTG-3′ and reverse 5′-AGGTACCTCGCCCTCCCAGAGTTCAG-3′). The PCR amplification was performed as outlined for the N-terminal GFP fusion. The resulting *XvAld1* amplicon did not contain a stop codon, allowing the translation of a read-through chimeric XvAld1-YFP protein. The constructed pGDG-*XvAld1* and pYFP-*XvAld1* plasmids were sequenced to verify that the reading frames were correct. As controls, pGD (vector backbone without GFP), pGDG (an empty GFP-expressing vector) and pYFP (an empty YFP-expressing vector) were used to monitor autofluorescence and the localisation patterns of GFP and YFP respectively. The pYFP::*ABI5* was used as a nuclear-localisation positive control (Lopez-Molina et al., 2003).

Gold particles (1 µm) were sterilised as described by Sanford et al. (1993). Three milligram aliquots of sterile gold were mixed with 5 – 10 µg of the appropriate purified plasmids and were kept shaking on a nutator. One volume of 2.5 mM $CaCl_2$ and 0.4 volumes of 0.1 M spermidine were added. Two volumes of 100% ethanol were used to precipitate the plasmid DNA onto the microcarriers. The tubes were kept shaking in 75 µl absolute ethanol to prevent aggregation. Macrocarriers were inserted into macrocarrier holders, placed into Petri dishes and 9.5 µl of the coated gold was pipetted onto the middle of each macrocarrier. The DNA was allowed to dry for 20-30 minutes.

Onions (*Allium cepa*) were peeled, cut into small rectangular segments and placed into Petri dishes. For each plasmid, six plates were bombarded and the transformation experiments were repeated at least three times. The onion epidermal cells were transformed by particle bombardment as described by Scott et al. (1999). The PDS-1000/He Biolistic Bombardment Delivery System (Bio-Rad Laboratories, Germany) using 1100 psi rupture

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discs and 27 inches Hg. The samples were incubated for 24 hr at 25°C in the dark. The transformed epidermal cells were viewed using an inverted phase contrast epifluorescence ELWD microscope (Nikon, Japan). A DM510 (blue light, BP450-490) filter was used for FITC through a 20 x magnification objective (Ph3 DL; Nikon, Japan). Images of the fluorescent cells were taken using a Carl Zeiss Axiocam colour camera. Alternatively, an Eclipse E600 phase contrast microscope (Nikon, Japan) was used. Images were captured using a Cool Snap Pro Monochrome camera (MediaCybernetics, UK). All micrographs were viewed under 20 x magnification with a 20 x Plan Fluo objective (Ph1 DLL, Nikon, Japan). For identification of nuclei, cells were stained with 10 µg/ml 4,6-diamidino-2-phenylindole (DAPI) in 0.05% Triton X-100 for 5 min. DAPI staining was viewed using a UV filter (BP330-380). GFP and YPF fluorescence were viewed using an FITC filter (BP465-495). Combined fluorescence was visualised with a DFR (DAPI, FITC, Rhodamine) filter.

3.2.10 Immunocytochemistry

Dehydration experiments were performed as outlined in section 3.2.2. For transmission electron microscopy, hydrated (91% RWC) and dehydrated (47%) *X. viscosa* leaf tissues were cut into 5 mm^2 pieces and incubated overnight in fixative [2.5% (w/v) glutaraldehyde, 0.5% (w/v) caffeine in 0.1 M phosphate buffer (PB), pH 7.4]. The specimens were washed 3 x 5 min in 0.1 M PB then dehydrated through a graded alcohol sequence (2 x 5 min in 30% ethanol, 2 x 5 min in 50%, 2 x 5 min in 70%, 2 x 5 min in 90%, 2 x 5 min in 95% and 2 x 10 min in 100% ethanol) and embedded in London Resin White at 60°C overnight. Ultrathin sections were cut using an Ultracut-S ultramicrotome (Leica, Austria). All solutions for immunodetection were prepared using 1 x PBS pH 7.4. The immunogold labelling was carried out by first incubating the sections in 150 mM glycine for 10 min, followed by blocking solution [1% (w/v) BSA, 2% goat serum, 1% (w/v) glycine] for 30 min. The sections were rinsed 3 x 3 min in PBS. The grids were incubated overnight with 1:500 dilution of the primary antibody [polyclonal antibody against XvAld1 in 1% (w/v) BSA]. The control samples were incubated with 1:10 dilution of pre-immune serum or the antibody was omitted. Wash buffer [WB; 0.05% (v/v) Tween-20 in PBS] was used to rinse the section three times for 10 min each. The samples were incubated with a 1:50 dilution of goat anti-rabbit IgG conjugated with 10 µm gold (Sigma-Aldrich, Germany) in 1% BSA for 30 min. Four washing steps of 10 min each were again performed using WB. For high stringency washing, the number of all the washing steps was doubled. The sections were rinsed with

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PBS for 10 min and fixed with 2% (v/v) glutaraldehyde for 4 min and again rinsed in distilled water and dried prior to staining with 0.1% (w/v) uranyl acetate and 2% (w/v) lead citrate. For each of the well-watered and dehydrated samples, two thin sections were each labelled with either polyclonal antibody or pre-immune serum. The samples were examined with a LEO 912 electron microscope (Leo Electron Microscopy Ltd, England). The experiments were repeated three times and all the immunogold-labelled samples were examined.

3.2.11 Stastical analysis

The expression levels, expressed as n-fold relative to untreated controls, were assessed for statistical differences by one-way analysis of variance (ANOVA) with Tukey posttests, using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA, www.graphpad.com).

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3.3 RESULTS

To examine changes in *XvAld1* transcription, mRNA levels were monitored using real time RT-PCR. The expression levels of 18S rRNA, in the same cDNA samples, were used for normalisation to correct for sample-to-sample concentration and PCR efficiency variations. The *XvAld1* expression was expressed as n-fold of 18S rRNA levels (GOI/HKG).

3.3.1 *XvAld1* expression under dehydration

The three individual plants used for this experiment dried at different rates. Although RNA was extracted and real time PCR run for each plant, Figure 3.1 is representative of the trends observed for all three. The levels of *XvAld1* transcript started to increase in leaves once the leaf RWC declined below 77%. At RWC between 66 and 15%, there was approximately five orders of magnitude more transcript than in the hydrated samples.

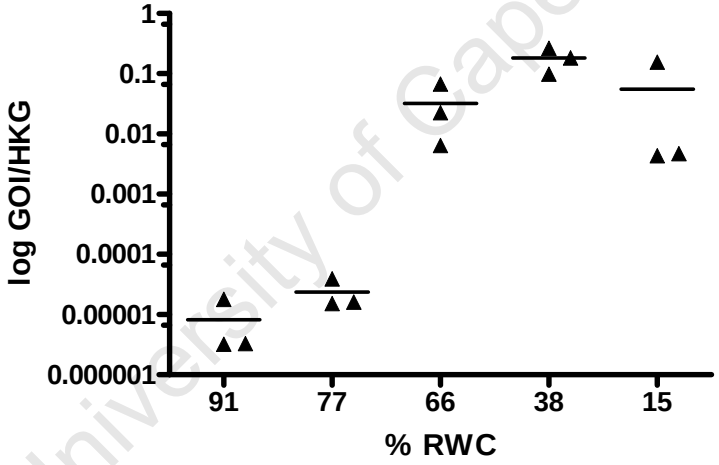

Figure 3.1 *XvAld1* expression profile in leaves under dehydration
Well-watered controls (91% RWC) showed basal transcript levels. The log scale on the y-axis was used to accommodate the wide scale of *XvAld1* transcript levels. While three independent drying curves were analysed, data from only one of the plants is shown due to the variation in rates of drying among plants. However, the data presented here is typical of the trends observed in all three plants.

In order to determine if the induction of the *XvAld1* mRNA resulted in increased protein accumulation, protein samples extracted from leaves at different RWC were separated by SDS-PAGE and western blotting performed using XvAld1 polyclonal antibodies. In well-watered plants, there was no detectable aldose reductase protein (Fig. 3.2; lanes 1-1, 2-1 and 3-1). The protein accumulated once RWC was reduced below 70% (Fig. 3.3) and remained

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high during the course of dehydration, down to 15% RWC. A number of high molecular weight proteins that are not present in the hydrated tissue were detected in the dehydrated samples (lanes 1-4, 2-3, 3-3 and 3-4). These bands are probably multimers of the aldose reductase protein, or complexes with other unrelated proteins. The recombinant AR that was used as a positive control also exhibited multimeric forms in addition to the expected 40.5-kDa protein, the different forms maybe due to the high levels of the protein.

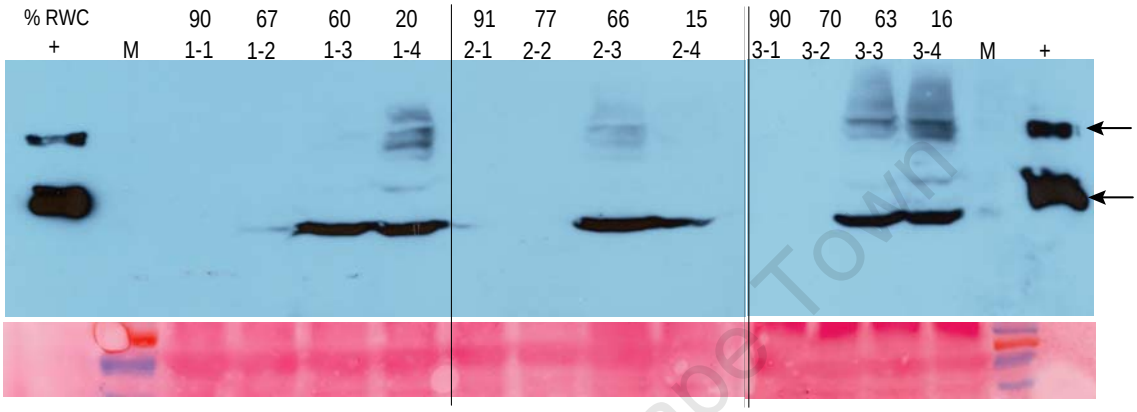

Figure 3.2 Western analysis of aldose reductase accumulation during dehydration of *X. viscosa*. Total soluble protein was extracted from three plants with comparable dehydration rates. Fifteen micrograms of the protein extract was resolved by SDS-PAGE and blotted onto nitrocellulose. Polyclonal antibodies against the recombinant XvAld1 were used to probe for the protein. 1-1 to 1-4: sample from well-watered (90% RWC), day 2 dehydration (67% RWC), day 5 (60% RWC) and day 10 (20% RWC) respectively. The other 2 plants were sampled at the same time points 2-1 to 2-4: 91, 77, 66 and 15% RWC, and 3-1 to 3-4: 90, 70, 63 and 16% RWC respectively. M denotes lanes containing the prestained molecular weight marker. "+" denotes the recombinant protein (100 ng) used as positive control. The Ponceou S stain was used as a loading control.

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3.3.2 Gene expression in response to hormone treatments

mRNA transcript levels from samples treated with the different hormones were analysed by quantitative real time RT-PCR. Exogenous application of the different growth regulators showed variable RNA levels that were not significant upregulation. Because the variation in expression between the different time points was not pronounced, calibration with respect to the transcript levels at time zero was performed to enable a reliable comparative analysis. The expression profiles for each of the hormone treatments failed to show a definitive increase in *XvAld1* transcripts (Fig. 3.3). No aldose reductase protein was detectable by western blotting of samples from all the hormone treated plants (data not shown). Following the low transcriptional and translational induction of *XvAld1* by

phytohormones, a separate ABA experiment was carried out. A repeat experiment where 100 μM ABA was applied at 0, 24 and 48 hr showed some protein expression at 12 and 48 hr (Fig. 3.4). Higher levels of protein were observed at 48 hr (lane 5), compared to that at 12 hr (lane 3).

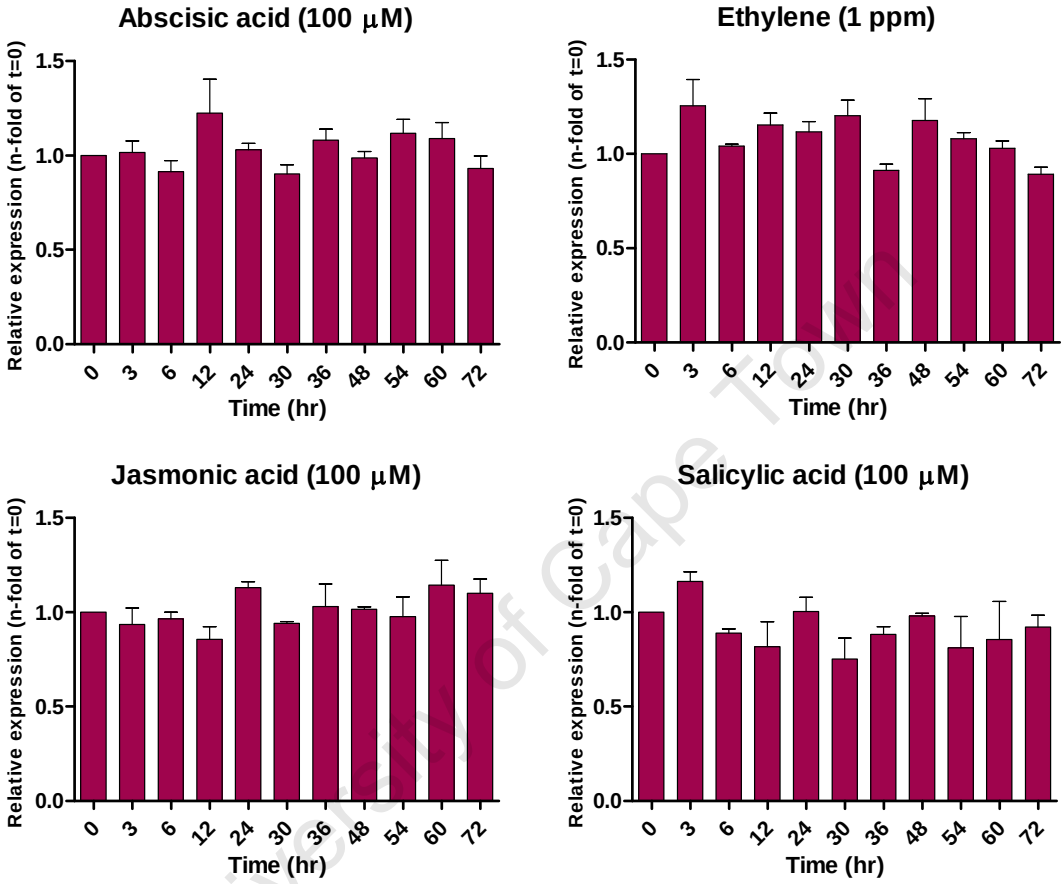

Figure 3.3 *XvAld1* expression after exposure to ABA, ethylene, JA or SA
The indicated hormone concentrations were applied and leaf samples were collected over a 72-hr period. The calculated *XvAld1* transcript levels were normalised using the levels for *Xv18S* RNA (housekeeping gene). One-way ANOVA with Tukey posttests were used to determine any statistically significant differences between the means and standard errors of the transcript levels at the different time points. No differences were observed between the levels of *XvAld1* transcripts at t=0 and any of the sample points after treatment, statistical significance at $p < 0.05$.

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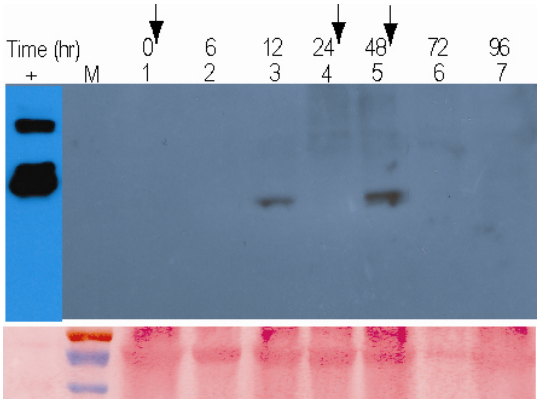

Figure 3.4 Expression profile after exogenous ABA application
Lanes 1 – 7 contain sample from 0, 6, 12, 24, 48, 72 and 96 hr after the first application, respectively. ABA solution (100 µM) was sprayed onto the leaves at the beginning of the experiment, and at 24 and 48 hr after the first application. The panel in red is the Ponceau S stain used as a loading control, showing that the levels of the 72-hr protein sample are lower than the rest. M denotes the pre-stained molecular weight marker and "+", the recombinant protein used as positive control. Arrows indicate the time points of ABA application.

3.3.3 Expression of the XvAld1-fluorescent protein fusions

X. viscosa tissues are hardy and not amenable to manipulation. Analysis of subcellular localisation was therefore performed using onion epidermal cells. The *XvAld1* cDNA was fused to the 3′-end of the gene for GFP. When expressed in onion epidermal cells, the fusion protein largely localised to the periphery of the cytosol (Fig. 3.5 d-f), and the nucleus. Free GFP has a molecular mass of 27 kDa and is unable to cross most subcellular membranes, except the nuclear pore through which it can enter by diffusion (Grebenok et al., 1997). Mature GFP is therefore found throughout the cytoplasm and in the nucleoplasm (Haseloff and Amos, 1995; von Arnim et al., 1998; Haseloff et al., 1999). To obtain detailed information on the localisation, *XvAld1* cDNA was also fused to the 5′-end of the *GFP* gene. The localisation pattern of the resulting N-terminal YFP fusion protein also associated with transvacuolar strands viewed across the cytoplasm and in the nucleus (images g-i).

The GFP-XvAld1 and the XvAld1-YFP fusion proteins have calculated molecular weights of 64 and 65 kDa respectively. This protein size is larger than the reported exclusion limit of the nuclear pores (Grebenok et al., 1997). To investigate if the localisation of the XvAld1-fluorescent protein fusions was indeed both cytosolic and nuclear, cells were counterstained with DAPI to identify the nuclei (Fig. 3.6).

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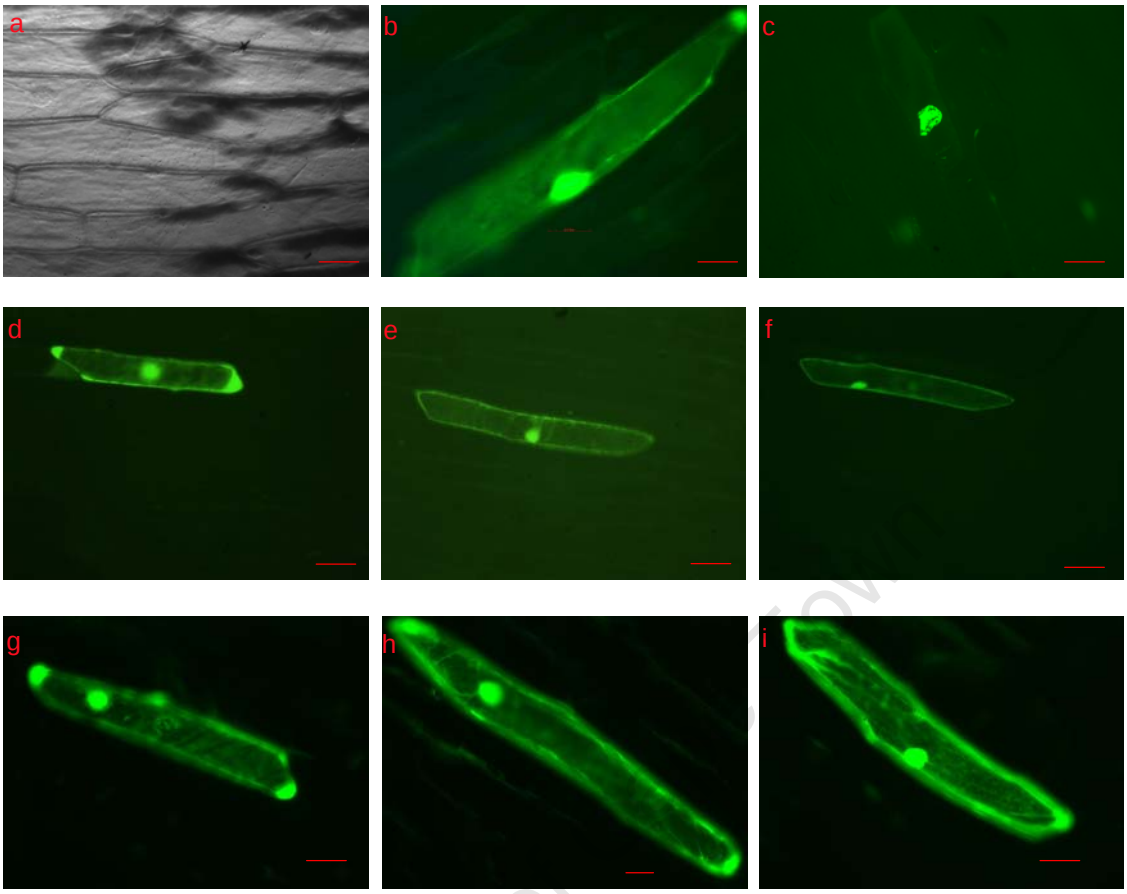


Figure 3.5 Subcellular localisation of GFP-XvAld1 and XvAld1-YFP fusion proteins in onion epidermal cells **a**) A view of epidermal cells under brightfield, **b**) subcellular distribution of unfused GFP, **c**) fusion of YFP with the transcriptional factor ABI5 showing the typical nuclear localisation, **d-f**) subcellular distribution of GFP-XvAld1 and (**g-i**) XvAld1-YFP. Scale bars represent 0.2 mm

Strangely, DAPI did not stain the nuclei of the cells expressing the XvAld1 fusion proteins (marked by asterisks; Fig 3.6, images b, e and h). In fact, other cells close to the fluorescent ones also failed to take up the DAPI stain. Some nuclei, whose nucleic acids failed to stain, were visible on the brightfield images (highlighted by arrowheads, Fig. 3.6c). In contrast, DAPI staining of cells expressing other unrelated fusion proteins was efficient (see image f). Increasing the DAPI concentration from 10 - 30 µg/ml and the incubation time to 10 min improved the staining efficiency (image h). However, the cells expressing the GFP or YFP fused to the aldose reductase still failed to stain with DAPI (cell marked by an asterisk in images h). The XvAld1 overexpressed in onion cells may somehow protect the cell from interacting with DAPI. The dynamics of the XvAld1-fluorescent protein fusions overexpressed in onion cells are shown by fluorescent micrographs taken in sequence (Fig.

3.7). The fluorescent cellular components, which could not be identified with certainty, were in constant motion characteristic of the dynamic nature of living cells.

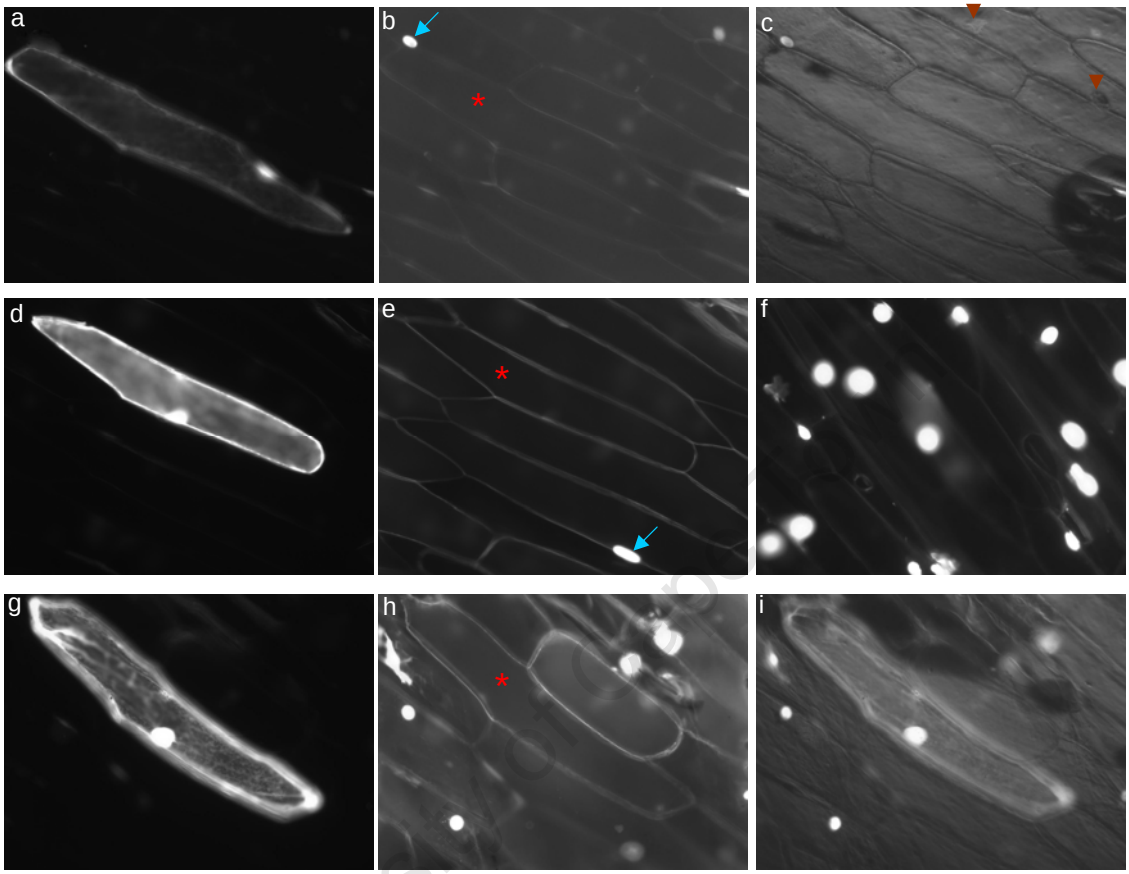
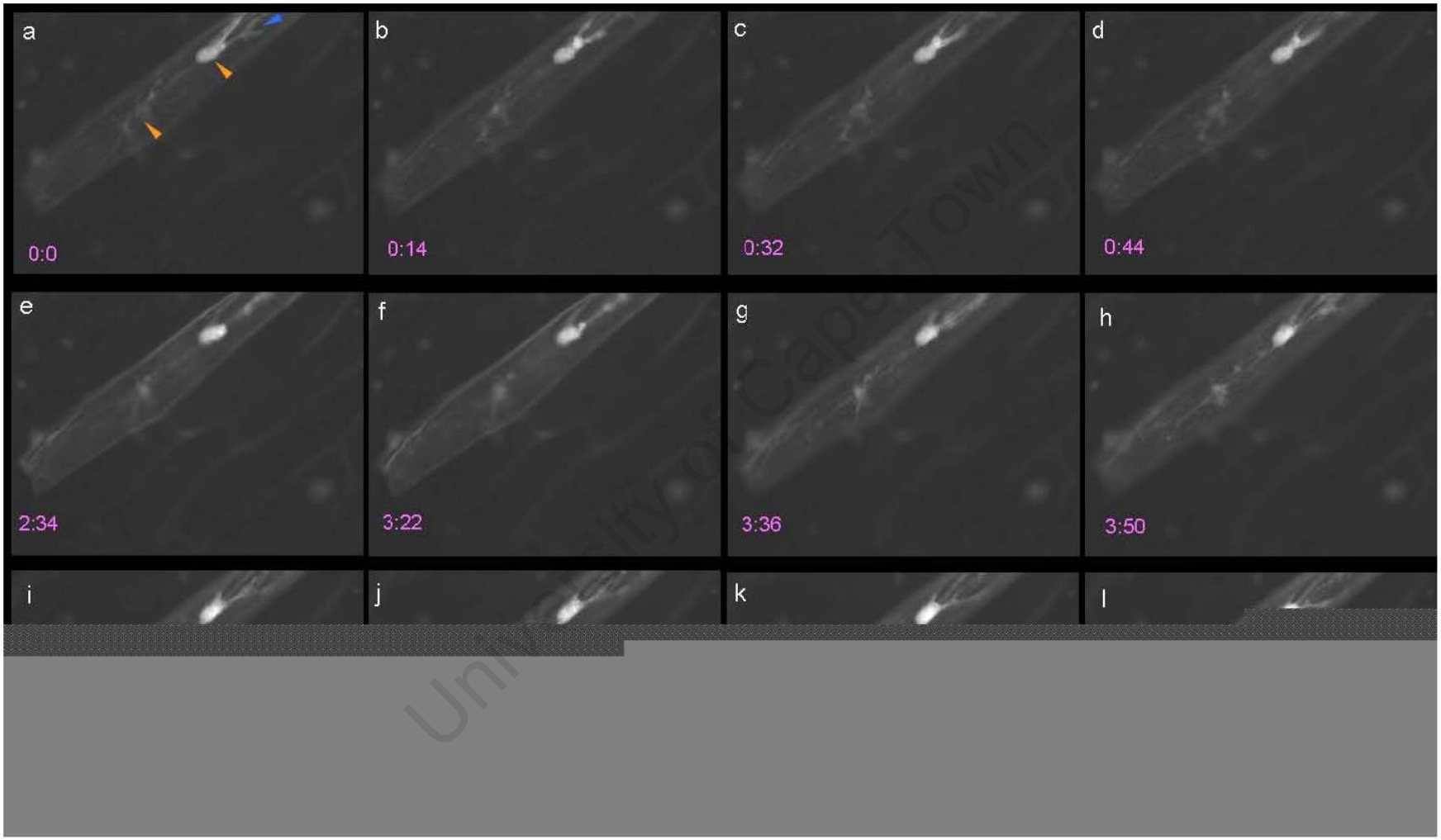


Figure 3.6 The cells expressing the XvAld1-YFP fusion proteins did not stain with DAPI
a, d and **g**) epidermal cells overexpressing the aldose reductase fusion, b, e, h) DAPI staining of cells in images (a), (d) and (g) respectively, **f**) DAPI staining of cells transformed with pYFP-*XvG6*. The cells are viewed with FITC filter only (a, d and g); DAPI filter only, (b, e and h); brightfield and DAPI filters, (c); brightfield, DAPI and FITC filters (i). The arrowheads show positions of visible nuclei, the blue arrows highlight the stained nuclei and the asterisks highlight the fluorescent cells.

Figure 3.7 Analysis of XvAld1 localisation dynamics in onion cells
The localisation of the XvAld1 fusion protein was monitored over time. The protein is associated with a dynamic network resembling the cytoskeleton. The fluorescent components are in constant motion, changing shape and size. Many vesicular organelles darting along the filamentous structures were observed. The arrowheads highlight the segments that show a visible change. The blue arrowhead also highlights the direction of movement on that section. The lapsed time, in minutes and seconds, is shown in pink.



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Because fluorescence microscopy does not achieve the resolution required to identify cell components with certainty, electron microscopy was performed to determine the subcellular localisation of XvAld1 in *X. viscosa* using leaf sections. Since *XvAld1* expression was effectively induced by drying, leaf samples dehydrated to 47% and 29% RWC were analysed by immunocytochemistry. Low immunogold label was observed in the control samples where pre-immune serum was used as primary antibody (Fig. 3.8a), showing low levels of non-specific binding. In hydrated leaf samples, low levels of labelling were detected in the cytoplasm and chloroplasts (image b, green arrows). Unlike the situation in hydrated leaves, in dry leaf tissue there was more intense labelling in the cytoplasm and vacuoles (images c-e). The thylakoids in dehydrated tissues are partially dismantled (Fig. 3.8, image e). In dehydrated tissues, vacuoles become fragmented and fill up with non-aqueous material (image f). The gold labelling in the stroma is light and may be non-specific. In addition, presence of gold label in the space between the cell walls and plasma membrane may also be due to antibody infidelity, or may be an artefact caused by fixation solutions.

Figure 3.8 Transmission electron micrographs of *X. viscosa* mesophyll cells
Leaf sections were incubated with pre-immune serum (**a**) or XvAld1 polyclonal antibody (b-e) and detected using gold-conjugated IgG. Hydrated (91% RWC; **b**), and dehydrated (47% RWC; **c-e**) leaf samples showed heavy gold labelling in cytoplasm, vacuoles and in the space between cell wall and plasmalemma (blue arrows). Image **f** (taken from Mundree and Farrant, 2000) indicates the typical appearance of dehydrated vacuoles, demonstrating the vacuole filling that is observed as speckles in image **a** (black arrows) is not gold labelling. Scale bars represent 500 nm; c, chloroplast; cy, cytoplasm; cw, cell wall; n, nucleus; t, thylakoids; v, vacuole.

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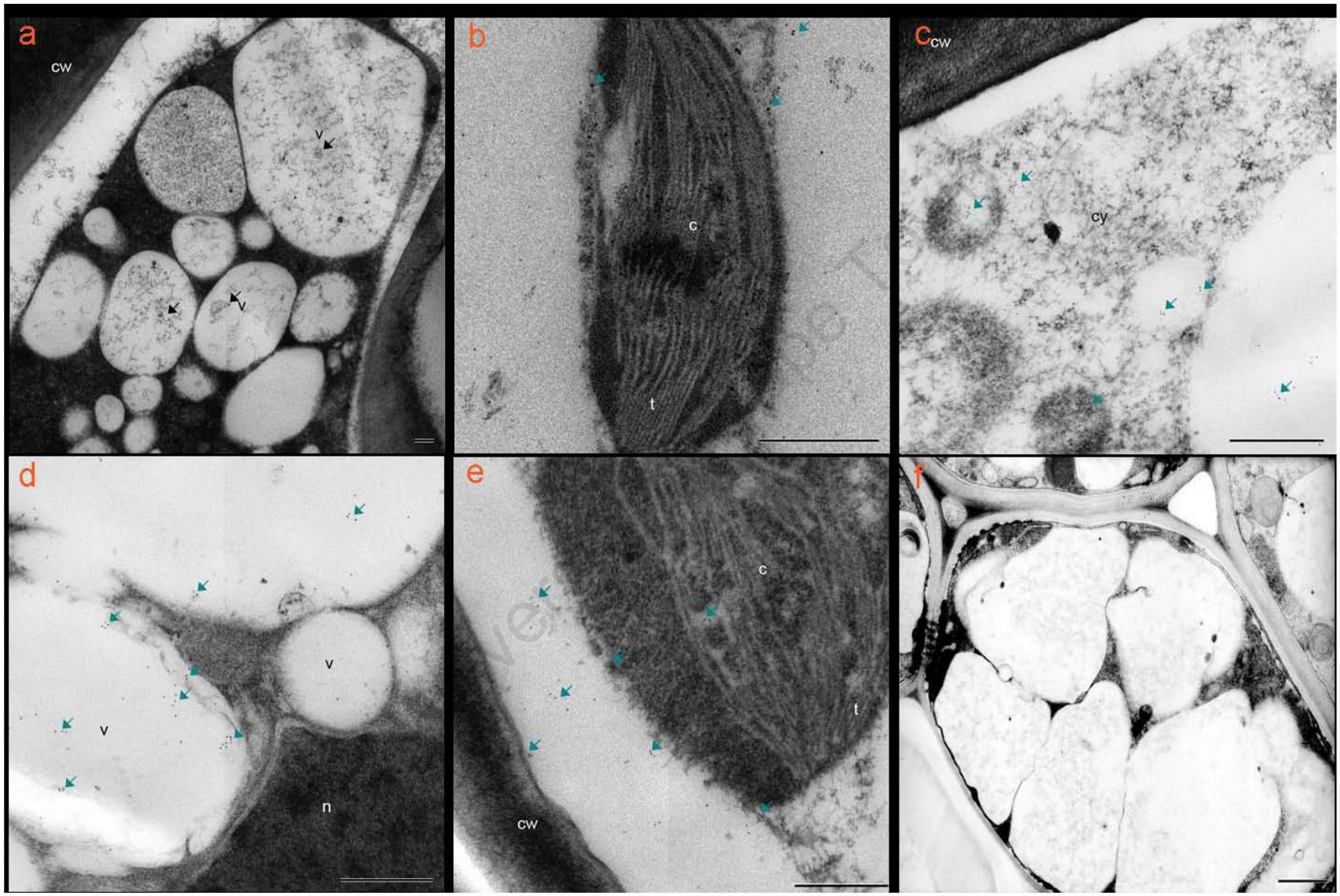
a
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3.4 DISCUSSION

Genomic studies have provided a lot of sequence information and expression patterns associated with the gene products. Many of the ascribed functions of the gene products are deduced from bioinformatics analysis by comparison with sequences of characterised proteins. However, despite all the sequence information, the physiological functions of many gene products remain enigmatic. The biological functions of proteins depend on temporal and spatial localisation, among other factors. For this reason, the regulation of *XvAld1* expression under various abiotic factors and its subcellular localisation were investigated.

3.4.1 The control of *XvAld1* gene expression

Gene transcripts that are elevated during stress are normally involved in effecting stress tolerance (Blomstedt et al., 1998). Quantitative analysis of *XvAld1* transcript revealed that the aldose reductase gene is effectively induced by dehydration. A slight reduction of water levels to ~80% RWC caused a basal increase in mRNA (Fig. 3.1), which in some cases is not supported by a corresponding increase in protein synthesis (Fig. 3.2; lanes 1-2, 2-2, and 3-2). RWC below 70% results in transcript levels that are five orders of magnitude more than the baseline expression levels. Protein synthesis was also increased substantially, and the high protein concentrations were maintained even at 15% RWC. The sustained accumulation of protein suggests that XvAld1 has functional importance during dehydration.

Analysis of *XvAld1* transcript levels after the exposure of different plants to various phytohormones did not show significant induction of gene expression (Fig. 3.3). The levels of accumulated transcript were much lower than the transcript accumulated under dehydration. No proteins were detectable by western blot analysis (data not shown). It is possible that a single dose exposure to any of the hormones was not enough to elicit a response. The poor induction may be due to the nature of the hormone preparations. The hormones are volatile, light-sensitive or unstable, making it difficult to ensure that plants were exposed to the chemicals for prolonged periods. Western blot analysis of protein samples extracted after repeated exposure to ABA showed a phased accumulation of the XvAld1 protein. Such a response is characteristic of circadian-regulated gene expression (Strayer and Kay, 1999;

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McClung, 2006). The protein accumulated at 12 hr after the first ABA application and again at 48 hr. The accumulated protein at 48 hr was substantially more than that at 12 hr, probably indicating that the response is strengthened by prolonged exposure to ABA. However, the fact that there was no detectable protein at 24 hr implies that the protein may be unstable. It is unclear why the accumulation of the aldose reductase was not consistent in ABA treated *X. viscosa*. In the absence of abiotic stress after hormonal application, the protein may be sequestered for subsequent degradation. However, since western blot analyses were only performed on soluble fractions, the possibility of compartmentation remains to be investigated. The sustained accumulation of the aldose reductase under dehydration and its degradation after ABA exposure implies that the protein is probably stable if it is performing some function in the cell. The recombinant protein used as a positive control for western detection appears as a dimer. This may reflect incomplete reduction of disulphide linkages by β-mercaptoethanol.

The steady state transcript and protein levels are dependent on the rates of synthesis and stability of the gene product. The rate of protein turnover is increased under stress conditions (Khedr et al., 2003). The fact that XvAld1 progressively accumulates during dehydration, when most of the cellular processes in resurrection plants are slowed down, probably indicates that it is preferentially translated. RNA turn-over is also affected by circadian effects (Kreps and Simon, 1997; McClung, 2006). Posttranscriptional control of gene expression can also be affected by other RNA species (Sunkar et al., 2007). In the cell, protein-protein interactions are partly controlled by the spatial localisation and the timing of the expression of the interacting partners. Our results suggest that the expression of *XvAld1* is not affected by exposure to a single dose of 100 μM ABA. However, it is also possible that the regulation of *XvAld1* by hormones is an early response and occurs within minutes of the application. The first sample was taken 3 hr after application and the results obtained are probably not representative of the early responses. Experiments using prolonged exposure times and earlier sampling times will be designed to obtain a better understanding of how the different hormones modulate the expression of *XvAld1*.

ABA-regulated gene expression has been widely investigated in plants (Bartels et al., 1990; Shinozaki and Yamaguchi-Shinozaki, 1996; Seki et al., 2002a;

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Yazaki et al., 2003). Seki et al. (2002a) classified ABA-responsive genes into three groups. The first group is upregulated within minutes of ABA application, reaches a maximum within an hour and then the transcript levels drop. The expression levels in the second group reach a maximum within 1 hr and remain constant over 24 hr. The increase in transcript levels in the third group was phased, with expression levels reaching maxima at different points over the 24-hr period. Although the expression of many drought-responsive genes is modulated by ABA, some of these genes may not respond to exogenous application of ABA. Some drought-inducible genes are not regulated by ABA (Gosti et al., 1995; Leung and Giraudat, 1998). The ABA-independent induction of *rd29A* was demonstrated using Arabidopsis mutants and transgenics (Ishitani et al., 1997; Xiong et al., 1999; Nakashima et al., 2006). Many drought-inducible genes contain both the DRE and the ABRE motifs in their promoter sequences (Yamaguchi-Shinozaki and Shinozaki, 2005; 2006). Narusaka et al. (2003) demonstrated that the expression of the *rd29* gene could be induced by ABA, although the induction is weak compared to that of the *rd29B* gene. It is proposed that the initial induction under environmental stress involves DRE elements and a delayed response is mediated by accumulation of endogenous ABA. The *XvAld1* gene may be similarly regulated, being weakly induced by ABA alone. In *X. viscosa*, of the genes investigated to date, the myo-inositol (*XvIno1*; Chopera, 2005) and peroxiredoxin (*XvPrx2*; Govender, 2006) were induced by ABA application in mature plants. The gene for an intrinsic membrane protein (*XvSap1*; Garwe, 2003) was not induced by prolonged exposure to ABA. Other results obtained from treatment of excised leaves could not be compared with experiments performed on whole plants. Comparison with other studies is complicated by differences in application methods, hormone concentrations and plant tissues used.

Given the numerous hormone response elements identified in the *XvAld1* promoter fragment, it was surprising that none of the hormones tested resulted in sustained gene expression. It is possible that the dehydration response elements are strong, and can act individually while the hormone response elements only act cooperatively with other environmental or developmental cues. Several ABRE-like motifs and one DRE were identified on the putative promoter (Chapter 2). These results demonstrate that one DRE is sufficient for a response to dehydration. The responses of *XvAld1* to auxin or cytokinin application were not investigated. It would

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be interesting to investigate the effects of these hormones on gene expression since the putative promoter contains response elements of each of these hormones.

Many gene expression studies investigate only transcriptional regulation or protein accumulation. In order to get a global understanding of the stress response, the gene products must be investigated at all levels. We have reported the expression of *XvAld1* at both the transcriptional and translational levels. However, this analysis must be extended to characterising the activities of the AR under different environmental conditions. The aldose reductase activity during the dehydration of *X. viscosa* was monitored by Mundree et al. (2000) and shown to increase with the reduction in RWC.

A two-step real time reverse transcription (RT) PCR protocol, where the RT and PCR amplifications are performed in separate tubes, was employed. SYBR Green I, a dye that binds to double stranded DNA (dsDNA), was used for fluorescence detection. Relative quantitation methods were used to measure changes in gene expression based on a standard curve, normalisation using 18S ribosomal RNA and calibration with a reference sample (Livak, 1997; Livak and Schmittgen, 2001). Untreated plant material (time 0) was used as controls for a reference of steady-state transcript levels in the absence of stress. This reference was used as a calibrator value for the hormone treatments because the expression profiles did not show a defined trend. Calibration results in a value of one for transcript levels under control conditions. The expression under treatment was considered increased if over one and reduced if less than one. Ribosomal RNA (18S) was used as a housekeeping gene whose expression levels were used to normalise *XvAld1* transcript levels. We have yet to identify a gene whose expression is stable under desiccation. Some researchers use multiple HKGs for normalisation in order to limit the effects of variation caused by using one gene. Few *X. viscosa* sequences are available for designing specific primers suitable for real time PCR. Ubiquitin was the other candidate HKG evaluated. However, the available sequence shared homology with polyubiquitin and several PCR products were obtained. Although the 18S expression profile showed variability, especially under dehydration, it was the least variable housekeeping gene identified. Another problem experienced when using 18S as HKG was that the transcript levels were well above the concentration range of the genes of interest. The calculated concentrations for the GOI under control conditions were vanishingly low (~3 x 10^{-6} ρg/ul) compared to 18S rRNA levels (~0.1 ρg/ul). Because of the low concentrations

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of the GOI, evaluation of expression after hormonal treatment was assessed relative to the transcript levels at time zero. Sample handling errors such as pipetting were not accounted for by normalising since the GOI and HKG detections were performed in separate tubes. However, errors resulting from PCR inhibitors, for example, were minimised by using a two-step protocol since the same pool of cDNA was utilised per time point.

3.4.2 Subcellular localisation of XvAld1

Eukaryotic cells are highly compartmentalised, with each subcellular compartment containing a well defined subset of interacting proteins and substrates. For this reason, protein function is only possible if all the interacting components are available. Thus, insights into protein functions can be gained from the subcellular localisation.

3.4.2.1 Using fluorescent fusions for protein localisation in living cells

Sequence analysis predicted that XvAld1 was localised to the cytoplasm. However, identified functional motifs included targeting to the microbodies (which include peroxisomes and glyoxisomes), Golgi apparatus and recognition sequences for loading into clathrin-coated vesicles (Table 2.1, section 2.3.2). Posttranslational modifications, such as N-glycosylation and N-myristoylation that can affect protein localisation, were also predicted. Because of the possibility for false positive and false negative results of computational predictions, the subcellular localisation of XvAld1 was confirmed by experimentation. The determinination of localisation patterns of proteins fused to the carboxyl- or amino-terminus of the GFP and its spectral variants has been a useful tool for studying cellular dynamics in plants (Haseloff et al., 1997; Haseloff, 1999). Intracellular organisation is dynamic and protein localisation and function is determined by both temporal and spatial contexts. Compartmentation results in spatial separation of cell functions. When a cell is transformed with a GFP-expressing construct, transcription can only occur in the nucleus where the appropriate machinery is located. The resulting mRNA is shunted to the cytoplasm where translation takes place on free ribosomes, or on the rough ER (ER associated with polyribosomes). Targeting of proteins translated on the free ribosomes or rough

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ER to the correct subcellular compartments is facilitated by the ER-Golgi network, or by transport in vesicles.

Using the *XvAld1* cDNA, fused to the 3′- and 5′-ends of the genes for GFP and YFP respectively, the protein was observed in nucleus and cytoplasmic strands (Fig. 3.5). Unfused GFP is ubiquitous in the cytoplasm and can enter the nuclear pore by diffusion (Haseloff and Amos, 1995; Kohler et al., 1997). The fusion protein, however, can only be imported by specific receptors since it is bigger than the size exclusion limit of the nuclear pores (40 - 60 kDa; Grebenok et al., 1997; Hawes et al., 2001). Heterologous expression of the fusion protein may hinder proper sorting because of the large amounts of protein produced by the use of a constitutive promoter (Escobar et al., 2003) leading to abberant localisation. In addition, the recombinant protein may initially localise in the correct compartment and then spill over to other organelles as a result of overloading the cell's transport systems. Intriguingly, counterstaining with DAPI to confirm nuclear localisation failed to identify the nuclei (Fig. 3.6). The transformed cells did not stain with DAPI in all the cells tested. It is conceivable that XvAld1 overexpression in the transformed cells may be protecting nucleic acids from intercalating with DAPI. However, it is also surprising that the cells around the transformed ones failed to stain. Analysis of the fusion protein dynamics in living onion cells (Fig. 3.7) suggests association of the protein with motile structures that may be threads of cytoplasm or the cytoskeleton.

Onion epidermal cells were chosen because they are large, transparent and exist in a monolayer and thus are easier to work with compared to other plant cells (von Arnim et al., 1998; Scott et al., 1999). In addition, these cells are devoid of chlorophyll which may quench GFP fluorescence (Zhou et al., 2005). However, working with the epidermal cells was still complicated by limited knowledge of the subcellular components of onion epidermis. Fluorescent fusions are not easily viewed in the vacuoles due to their high acidity (Scott et al., 1999; Tamura et al., 2003a). However, it is now generally accepted that plant cells may contain two types of vacuoles, a lytic vacuole and a storage vacuole (Paris et al., 1996; Paris et al., 1997; Di Sansebastiano et al., 1998; 2001). The characteristics of the storage vacuoles may differ and are cell type-dependent. All these ultrastructural complexities mean that protein localisation using GFP can be inconclusive. The presence of the fluorescent protein tag may prevent the correct folding and hinder proper localisation (Hanson and Kohler, 2001; Escobar et al., 2003; Dixit et al., 2006). As a result, fluorescence

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imaging is not always straightforward, and requires complementation with other microscopy or biochemical techniques to validate localisation data. The resolution achievable using epifluorescence microscopy is below what is required to clearly define the ultrastructural organisation of the cell.

To address the limitations of epifluorescence microscopy, immunocytochemical localisation of the wild type XvAld1 protein was performed using electron microscopy on dehydrated *X. viscosa* leaf samples. Electron micrographs showed labelling mainly in electron-dense regions of the cytoplasm, and inside vacuoles (Fig. 3.8). These results are in agreement with predictions that XvAld1 is mainly cytosolic, but can potentially be targeted to specific subcellular locations. Taken together, the microscopy results show that the localisation of XvAld1 is complex. No attempt was made to visualise GFP or YFP fluorescence in the vacuoles by bathing with buffer to raise the pH (Scott et al., 1999; Hanson et al., 2002; Tamura et al., 2003a). However, immunolocalisation studies detected XvAld1 in vacuoles where it may perform specific functions associated with either detoxification or osmotic adjustment.

The subcellular localisation of AKR proteins has not been extensively investigated. Numerous studies on plants have focused only on soluble proteins. Bartels et al. (1991) observed that a fraction of the barley AR activity was associated with the microsomes. Mano et al. (2005) showed that a GFP-tagged alkenal reductase localised to the cytosol, vacuoles and nuclei, even though no targeting signals were identified on the protein sequence. In animals, a bovine AR protein was found associated with membranous vesicles despite lacking signal peptides for membrane targeting (Frenette et al., 2003). Another aldose reductase, which also lacks protein targeting sequences, was shown to associate with the mitochondria (Varma et al., 2003). These results reinforce the notion that the understanding of protein sorting is lacking and remains to be fully elucidated.

3.5 CONCLUSION

The expression of *XvAld1* in *X. viscosa* plants exposed to various stresses and hormones was monitored. Exposure to dehydration significantly upregulated the expression of both mRNA and protein. Localisation studies were not conclusive, but XvAld1 was detected in transvaculolar strands, cytoplasm and nuclei.

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CHAPTER 4

Screening and molecular characterisation of transgenic plants expressing the *X. viscosa* aldose reductase

4.0 SUMMARY

Arabidopsis thaliana and *Digitaria sanguinalis* plants were transformed with *XvAld1* cDNA driven by the CaMV 35S and the maize *Ubi-1* promoters using *Agrobacterium tumefaciens*-mediated and biolistics methods respectively. The initial screening for transformants was done on genomic DNA using PCR. Most of the putative transgenic *A. thaliana* and *D. sanguinalis* plants resistant to kanamycin or bialaphos, respectively, also carried the *XvAld1* transgene. Transformants carrying the *XvAld1* and the respective selectable marker genes were selected for further characterisation. The expression of the *XvAld1* mRNA was determined by northern blotting and transcript levels were compared using real time PCR. The majority of the PCR-positive plants also expressed high levels of *XvAld1* mRNA. Western blot analysis showed high protein expression in leaves, stems and siliques of transgenic *A. thaliana* plants. Protein levels in *D. sanguinalis* transgenic plants were comparatively low. All the regenerated *D. sanguinalis* (both the transformed and the wild type plants) were sterile, setting nonviable seeds. In contrast, transgenic *A. thaliana* plants were similar to untransformed controls. Southern blot analysis was used to demonstrate the incorporation of the transgene sequence into the Arabidopsis genome. Simple integration patterns and low copy numbers of transgene DNA were demonstrated. The transformation and molecular characterisation of transgenic plants are described in this chapter.

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4.1 INTRODUCTION

Mundree et al. (2000) showed that the *XvAld1* cDNA complemented the survival of the *E. coli* strain *srl*::Tn10 on 1.25 M sorbitol. Wild type *E. coli* can tolerate osmolarity of up to 0.7 M NaCl (Gowrishankar, 1985). The tolerance is achieved when cells accumulate compatible solutes, enabling them to restore osmotic balance. The *E. coli* strain lacks the ability to take up sorbitol because the *srl* operon responsible for sorbitol transport was disrupted using transposon Tn10 (Csonka and Clark, 1979). The disruption renders the strain susceptible to osmotic stress. The fact that *srl*::Tn10 cells expressing *XvAld1* could grow on high osmoticum showed that the gene product has a role in osmotic stress tolerance.

To extend this hypothesis, transgenic plants were used to confirm the functional role of *XvAld1* in abiotic stress tolerance. *XvAld1* cDNA was cloned into pBI121 (Jefferson, 1987) and pAHC25 vectors (Christensen and Quail, 1996). The vectors were used to transform *A. thaliana* and *D. sanguinalis* respectively. Constitutive expression was driven by the cauliflower mosaic virus (CaMV) 35S promoter in *A. thaliana* and in *D. sanguinalis*, the maize ubiquitin (*Ubi-1*) promoter (Christensen et al., 1992; Christensen and Quail, 1996) was used. *D. sanguinalis*, an African wild grass that was originally cultured for testing genetically engineered resistance to maize streak virus (MSV, Chen et al., 1998b), was used as the monocot model system. Type 1 embryogenic calli, initiated from immature inflorescences, were transformed using biolistics. *A. tumefaciens*-mediated transformation was used to introduce the T-DNA carrying the *XvAld1* construct into *A. thaliana*.

Transgenics have been used to study gene function as well as characterise developmental and environmental stress responses in plants. Stress-responsive gene products may possess regulatory functions or be involved in the protective processes leading to tolerance. Transgenic strategies have been employed to study both these gene classes. The success of transgenic technologies requires successful expression of adequate levels of the foreign gene. Putative transgenic plants were characterised to ascertain the synthesis of the mRNA and protein coded by the transgene. To this end, RT-PCR as well as northern and western blot analyses were performed. In addition, integration of the transgene into the host genome was analysed by Southern blotting.

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4.2 MATERIALS AND METHODS

4.2.1 Plant material

A. thaliana seeds, Columbia ecotype, were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). *D. sanguinalis* inflorescences were collected from the wild. Callus was initiated and cultured as previously described by Chen et al. (1998b).

4.2.2 Plant nutrient media

The full compositions of plant nutrient media used for tissue culture are listed in Appendix B.

4.2.3 Plasmid construction

pAHC25, a pUC19 based vector containing an ubiquitin promoter (*Ubi-I*), is as described by Christensen and Quail (1996). pBI121 is a binary vector driven by a cauliflower mosaic virus (CaMV) 35S promoter. The pAHC25 and pBI121 plasmid maps are shown in Appendix D. The aldose reductase gene was cloned in pAHC25 and pBI121 vectors by S. Walford (1998).

In summary, the GUS gene was removed from the pAHC25 vector by digesting with *Sac*I and *Sma*I. The 5′ overhangs produced by *Sac*I digestion were filled in using T4 DNA polymerase (Promega Corporation, USA). To prevent re-circularisation, the 5′-ends of the linearised vector were dephosphorylated with calf intestinal alkaline phosphatase (Roche Diagnostics, Germany). *XvAld1* cDNA was excised from pSK plasmid using *Xba*I and *Xho*I. The 3′ overhangs were blunted using the 3′-5′ exonuclease activity of T4 DNA polymerase. The *XvAld1* cDNA was blunt end cloned into the pAHC25 vector.

XvAld1 cDNA for cloning into pBI121 was excised from the pSK plasmid by linearising with *Xho*I, removing the 3′ overhangs then digesting with *Xba*I. The pBI121 DNA was digested with *Sac*I, the 5′ overhangs were filled with T4 DNA polymerase and the linearised plasmid was then digested with *Xba*I. The plasmid and insert DNA were

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cloned through blunt-sticky end ligation. The recombinant pAHC25-*XvAld1* and pBI121-*XvAld1* plasmids were selected by mapping with *Sma*I and *Nar*I restriction enzymes.

4.2.4 Transformation of *A. thaliana* by agroinfiltration

The transgenic *A. thaliana*, available as T_1 seeds, were transformed with pBI121-*XvAld1* by S. Walford (1998) using the floral dip method (Clough and Bent, 1998).

4.2.5 Biolistic transformation of *D. sanguinalis*

Embryogenic callus used for particle bombardment was initiated from immature inflorescence and maintained on Murashige and Skoog (MS; Murashige and Skoog, 1962) media (Highveld Biological Pty Ltd, South Africa) as described by Chen et al (1998b). The callus was kept in the dark at room temperature. A day before bombardment, the callus was transferred to MS agar containing 0.2 M mannitol, 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 50 mg/l myo-inositol. Gold particles (0.6 µm diameter) were sterilized in ethanol and resuspended in 50% glycerol (Sanford et al., 1993). Two micrograms of plasmid DNA (pAHC25-*XvAld1* or pAHC25 for the transformation control) was precipitated onto 3 mg gold particles as described by Dunder et al. (1995). Briefly, sterilised 1 µm gold particles were mixed with appropriate volumes of purified pAHC25 or pAHC25-*XvAld1* plasmids and kept shaking on a nutator. One volume of 2.5 mM $CaCl_2$ and 0.4 volumes of 0.1 M spermidine were added. Two volumes of 100% ethanol were used to precipitate the plasmid DNA onto the microcarriers. The tubes were kept shaking in 75 µl absolute ethanol to prevent aggregation. Macrocarriers were inserted into macrocarrier holders, placed into Petri dishes and 9.5 µl of the plasmid DNA-coated gold was pipetted onto the middle of each macrocarrier and allowed to dry for 20-30 min.

The PDS-1000/He Biolistic particle delivery system (Bio-Rad Laboratories, Germany) was used for particle bombardment. Each Petri-dish containing calli was bombarded twice using 900-psi rupture discs at 27 inches Hg. The target Petri-dishes were placed 6 cm from the stopping screen. Calli were protected from direct impact damage by covering with a stainless steel mesh with a 0.2 µm aperture. The bombarded callus was transferred onto MS media and left in the dark at room temperature for 5 days.

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Thereafter, the callus was exposed to 16 hr diffuse light (~40 $\mu molm^{-2}s^{-1}$) and 8 hr darkness for 9 days. The transformed callus was then transferred to MS regeneration medium [MS medium supplemented with 0.1 mg/l naphthylene acetic acid (NAA) and 10 mg/l benzylaminopurine (BA)] containing 3 µg/ml bialaphos (Meiji Seika Kaisha Ltd, Japan). Transformed calli were subcultured fortnightly to remove dead cells and to replenish nutrients. Plant growth room conditions were 16 hr light (100 – 120 $\mu molm^{-2}s^{-1}$) and 8 hr dark at 25°C. Bialaphos resistant putatively transformed *D. sanguinalis* plants were selected on regeneration media until shoots were sufficiently elongated. The regenerated shoots were transferred to rooting media (MS without hormones) until roots were long enough for hardening in soil.

4.2.6 Seed sterilisation

All seeds germinated on agar were surface sterilised before stratification. The seeds were initially washed in 70% ethanol for 5 min. Thereafter, the seeds were sterilised in a solution of 10% commercial bleach [3.5% (m/v) sodium hypochlorite] and 0.02% Triton X-100 for 10 min. The seeds were rinsed with 6 changes of sterile water. All the washing and rinsing steps were performed on a shaker. The seeds were then suspended in 0.1% (w/v) purified tissue culture agar and kept in the dark for 4 days.

4.2.7 Selection of transgenic *A. thaliana* on kanamycin

A. thaliana seeds were stratified on 0.1% agar at 4°C for 3 – 4 days prior to sowing. The seeds were germinated on a plant nutrient solution containing sucrose (PNS; Haughn and Somerville, 1986) solidified with 0.8% (w/v) purified tissue culture agar (Sigma-Aldrich, Germany). Transgenic seeds were germinated on PNS supplemented with 50 µg/ml kanamycin.

4.2.8 Germination tests on *A. thaliana* seeds

Sterilised seeds were plated on PNA without antibiotic selection. About 100 seeds per plate were spotted individually using a pipette tip. Germination was expressed as a percentage of total number of seeds sowed, scored 6 – 7 days after sowing.

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4.2.9 Screening of putative transgenic plants using PCR

Genomic DNA was isolated from putative transgenic plants using Edwards' method (1991). Briefly, 100 mg of leaf tissue were homogenised in 600 ml extraction buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM EDTA, 10 mM β-mercaptoethanol, 1% SDS). The homogenate was incubated at 65°C for 10 min. Thereafter, 210 µl of potassium acetate were added, mixed and incubated on ice for 10 min. Particulate material was precipitated at 12 000 x g for 10 min. The DNA in the supernatant was precipitated by mixing with an equal volume of isopropanol in a clean tube. The DNA pellet was washed with 70% ethanol and dissolved in an appropriate volume of TE (10 mM Tris-HC, 1 mM EDTA) containing 1 mg/ml RNase. A hundred nanograms of DNA were used per PCR reaction. Gene-specific primers (XvAld1-AF; 5′-CGGCACGAGAAGCTACAGAGA-3′ and XvAld1-BR; 5′-CGAAGACCTGGATGTTCTCC-3′) that span the whole aldose reductase coding sequence were used to screen for positive transformants. The PCR reaction consisted of 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 1 x Supertherm buffer, 1 unit per reaction Super-therm Taq DNA polymerase (JMR Holdings, UK) in a 20 µl reaction. Cycling conditions were as follows: 2 min at 94°C; 30 cycles of 94°C for 30 sec, 61°C for 1 min, 72°C for 1 min and finally 72°C for 7 min.

4.2.10 RNA extraction and reverse transcription PCR

RNA was isolated from wild type, and putative transgenic empty vector and *XvAld1* plants using Trizol as previously described (section 3.2.5). RT-PCR was performed using a two-step method. In each case, 1 µg of total RNA was reverse transcribed using the Omniscript™ Reverse Transcriptase kit following the manufacturer's instructions (Qiagen, Germany). The 20 µl reverse transcription reaction consisted of 1 µg RNA template, 1 µM 18-mer oligo-dT primer, 0.5 mM dNTPs, 10 units/ reaction RNase inhibitor (Roche Diagnostics, Germany), 1 x Omniscript buffer and 4 units/ reaction Omniscript Reverse Transcriptase. The reverse transcription reaction was incubated at 37°C for 1 hr. The resulting cDNA (2-4 µl) was used as template for standard PCR consisting of 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.3 µM each of gene-specific forward and reverse primers, 1 x reaction buffer and 1 unit/ reaction Super-therm

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Taq DNA polymerase (JMR Holdings, UK). Screening for the *XvAld1* gene was performed using primers that amplify the complete coding region; XvAld1-AF (5′-CGGCACGAGAAGCTACAGAGA-3′) and XvAld1-BR (5′-CGAAGACCT GGATGTTCTCC-3′). The PCR reactions were performed using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, USA) thermocycler under the following conditions: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 61°C for 30 s and 72°C for 45 s and a final extension step at 72°C for 7 min. PCR screening for the selectable markers was carried out using the *bar* gene primers; Bar-F (5′-CGTCAACCACTACATCGAG) and Bar-R (5′-GAAACCCACGTCATGCCAG-3′) and *NPTII* (neomycin phosphotransferase II) gene primers; NPTII-F (5′-CTGAATGAACTGCAGGACGAGG-3′) and NPTII-R (5′-GCCAACGCTATGTCCTGATAGC-3′). The annealing temperature of the bar and NPTII primers were lowered to 55°C.

4.2.11 Northern and Southern blotting

Five micrograms of total RNA were separated on 1.2% (w/v) agarose gel, stained with ethidium bromide and visualised under UV light. The RNA was transferred to a Hybond-XL nylon membrane (Amersham Biosciences, UK) by the modified downward gravity transfer method (Chomcynski, 1992). Briefly, sponges were soaked in 20 x SSC (3 M NaCl, and 0.3 M tri-sodium citrate) and staked on top of the agarose gel with the membrane underneath. The RNA was allowed to transfer by capillary action and gravity for 2 hr at RT. RNA blotted onto the membrane was cross-linked using a Hoefer^{TM} UVC 500 Crosslinker (Amersham Biosciences, UK). The *XvAld1* probe was labelled as described in section 2.2.5. The probe used for equal loading control was synthesised using primers to an *X. humilis 18S* ribosomal RNA (rRNA) clone (Collett et al., 2004). The primers used to amplify part of the *Xv18S* sequence were: 18S-F; 5′-GGCAGCAGGCGCGCAAATTA-3′ and 18S-R; 5′-GCCATGCACCACCCTAT-3′. PCR amplification to incorporate radioactive [α-^{32}P]-dCTP was carried out as previously outlined for *XvAld1* probe synthesis, except that an annealing temperature of 55°C was used. Hybridisation, washes and autoradiogram development were carried out as previously described. Alternatively, membranes were exposed to a phosphor screen (Kodak, USA) for up to 24 hr in a light proof cassette at RT. Images were developed

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using the Bio-Rad Molecular Imager® FX and analysed using Quantity One® quantitation software (Bio-Rad Laboratories, USA).

Southern blotting was performed as described previously (section 2.2.5). Five micrograms of genomic DNA was digested with *Bcl*I, *Eco*RI and *Xba*I/*Sac*I, separated on 0.8% (w/v) agarose gel and transferred onto nylon membrane (Hybond-N^+, Amersham Biosciences, UK). The membranes were probed with a ^{32}P-labelled 960 bp product from the *XvAld1* coding sequence. Probe synthesis by PCR was performed as previously outlined. Alternatively, the full length *XvAld1* cDNA was labelled with digoxigenin (DIG) using in vitro transcription as previously outlined.

4.2.12 cDNA synthesis and real time PCR

cDNA synthesis and real time PCR were carried out as outlined previously (section 3.2.6**)**. The Arabidopsis β-actin (2-actin) gene expression was used as an internal control (housekeeping gene; HKG). The primers used for the β-actin gene amplification; (5′-CTCGTTTGTGGGAATGGAAG-3′ and 5′-AGGGAAGCAAGAATGGAACC-3′) and the *XvAld1* gene-specific primers (section 3.2.6) were designed using the Primer3 programme (Rozen and Skaletsky, 2000).

4.2.13 Extraction of protein from plant material and western blotting

To extract total soluble protein from plant material, 0.2 g of tissue was ground in 750 µl of protein extraction buffer (PEB; 0.5 M Tris-HCl pH 7.5, 10 mM EDTA, 1% (v/v) Triton X-100, 2% (v/v) β-ME, 1 mM PMSF). The homogenate was clarified by centrifuging for 5 min at 12 000 x *g*. The supernatant was transferred to a clean tube. The protein was extracted with an equal volume of phenol, pH 8 and the phases separated by centrifugation at 12 000 x *g* for 1 min. The aqueous layer was discarded and the protein re-extracted by topping up to the original volume using PEB. The aqueous layer was again discarded. Soluble proteins were precipitated overnight at -20°C using 2.5 volumes of 0.1 M ammonium acetate in methanol. After centrifugation at 12 000 x *g*, the pellet was washed with 0.1 M ammonium acetate in methanol and again in ice-cold 80% (v/v) acetone. The pellet was air dried for 1 hr and dissolved in 1 x PBS. SDS-PAGE and western blot analyses were carried out as outlined (sections 2.2.11 and 2.2.14).

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4.3 RESULTS

4.3.1 Screening of putative transgenic plants on selection media

The putatively transformed *D. sanguinalis* calli were selected on MS agar supplemented with bialaphos. Cell growth was severely inhibited on bialaphos selection, with regeneration taking twelve weeks (Fig. 4.1, Panels A and B) compared to six weeks on non-selective media (Panel D). The putative transformants were bialaphos resistant (Fig. 4.1, Panels A and B), but untransformed *D. sanguinalis* calli did not survive bialaphos selection for longer than one month (Panel C).

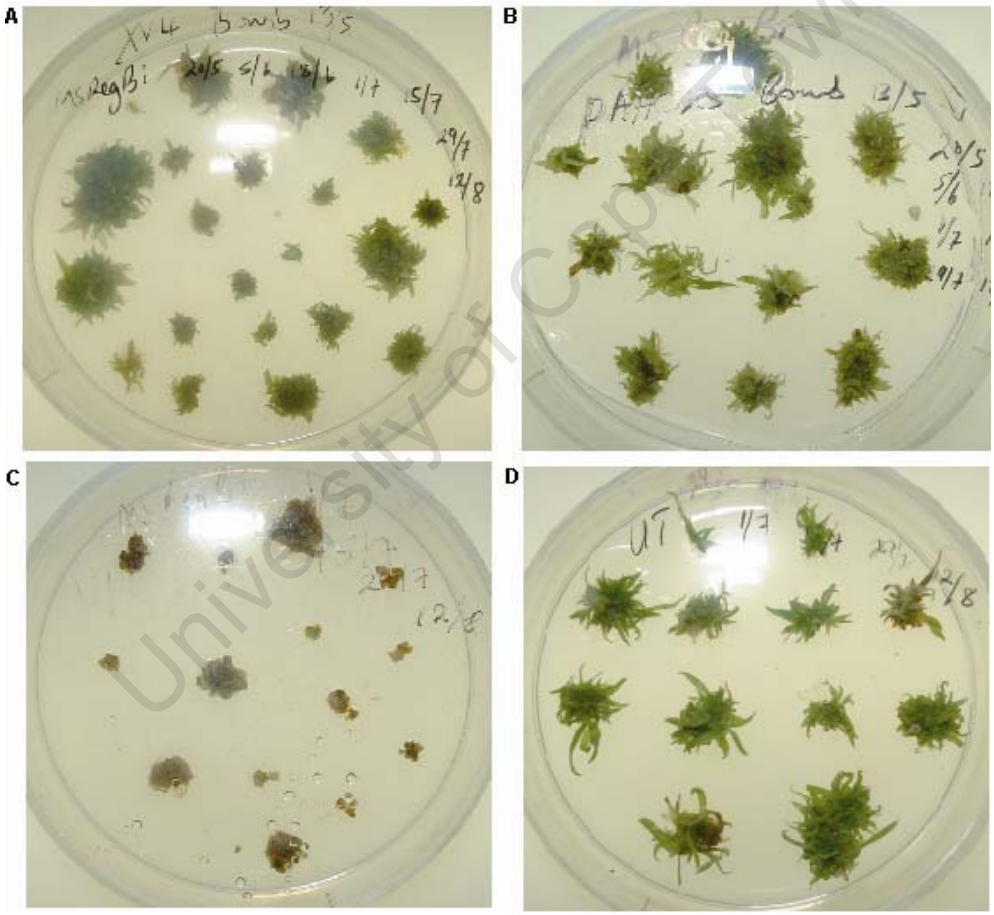

Figure 4.1 Selection of putative transgenic *D. sanguinalis* on bialaphos
D. sanguinalis calli were bombarded with 0.6 μm gold particles coated with the plasmids pAHC25-*XvAld1* (Panel A) or pAHC25 (Panel B). The calli were selected on MS regeneration media containing 3 μg/ml bialaphos until the shoots emerged. The bottom panels demonstrate that wild type (untransformed) *D. sanguinalis* calli could not survive on 3 μg/ml of bialaphos (Panel C) but thrived in the absence of the herbicide (Panel D).

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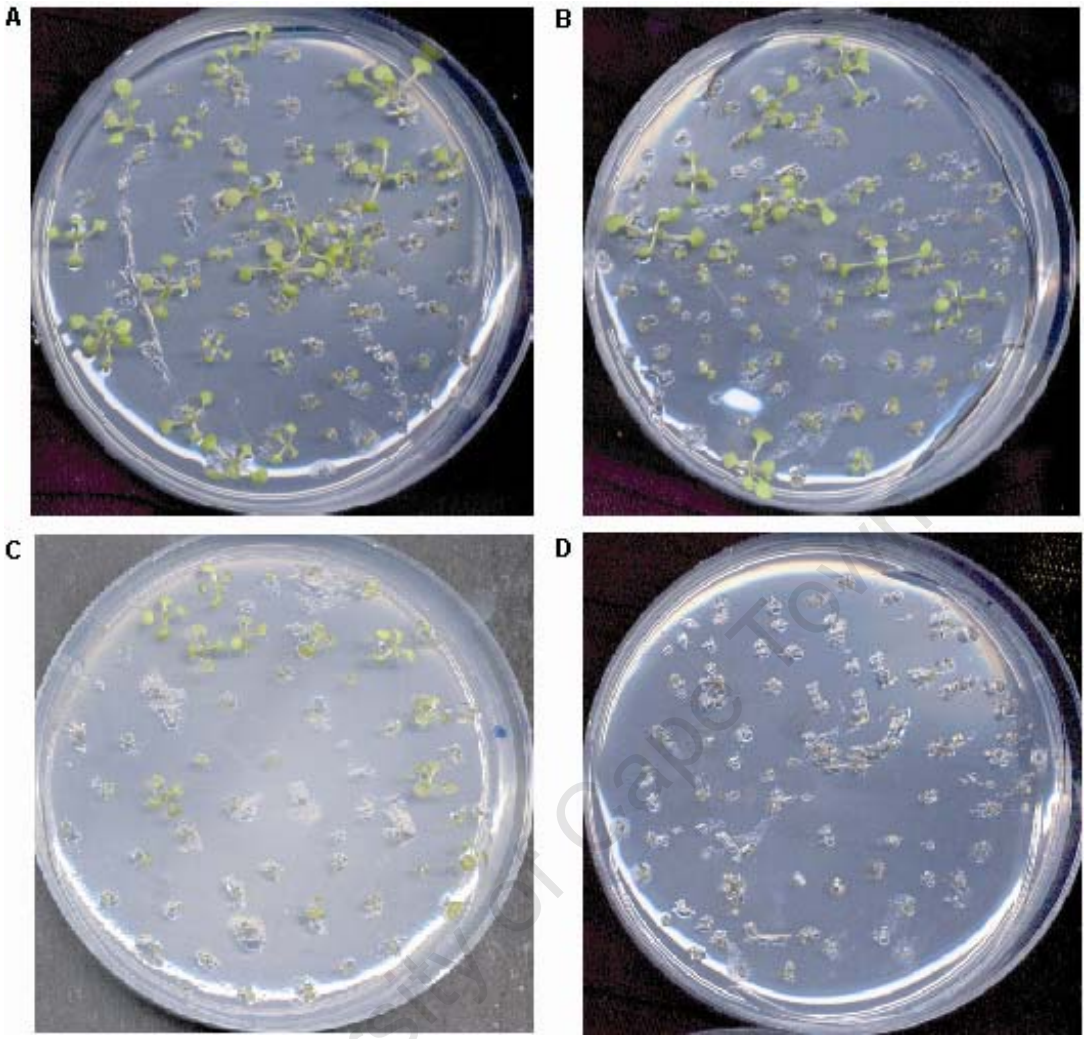

Figure 4.2 Selection of *A. thaliana* T_1 seeds on kanamycin
Sterilised seeds were plated on PNS supplemented with 50 μg/ml kanamycin. The putative transgenic T_2 seedlings that germinated and developed at least four rosette leaves were regarded as resistant. **A**, line 8; **B**, line 12; **C**, "empty" vector transformation control line and **D**, wild type seed.

A. thaliana plants carrying a functional neomycin phosphotransferase II (NPTII) gene also survived on media supplemented with 50 ng/ml kanamycin (Fig. 4.2, Panels A, B and C). None of the wild type plants survived on selective media and most of the seeds failed to germinate. Both the *A. thaliana* and the *D. sanguinalis* transgenic plants were phenotypically similar to the respective wild type control plants. However, the empty vector-transformed Arabidopsis seedlings tended to grow slowly on selection media (Fig. 4.2, Panel C).

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4.3.2 PCR screening for positive transformants

Bialaphos resistant *D. sanguinalis* plantlets were transferred to rooting MS media and transplanted into soil as soon as the roots emerged. Kanamycin resistant *A. thaliana* seedlings were transplanted into soil three weeks after sowing. PCR screening was performed on genomic DNA of both species to confirm the presence of the transgene.

Figure 4.3 PCR screening of putative transgenic *D. sanguinalis* plants
Genomic DNA was used for screening T_1 plantlets with primers specific for the *X. viscosa* aldose reductase gene to produce the expected 960-bp product. M: *Pst*I-digested λ DNA molecular weight marker; lane 1, positive control- *XvAld1* cDNA sequence in pSK plasmid; lane 2, no template negative control; lane 3, untransformed (WT) plants; lane 4, plants transformed with an "empty" pAHC25 vector (VT); lanes 5 to 23, plants transformed with pAHC25-*XvAld1*. The wells were loaded similarly for rows A, B and C such that 3 WT plants, 3 VT plants and 57 putative transgenic plants were screened.

PCR analysis using *XvAld1* gene-specific primers showed that most of the bialaphos resistant plantlets contained the gene of interest (Fig. 4.3). Genomic DNA from wild type and "empty" vector transformed plants (lanes 3 and 4 respectively) did not contain the 960-bp *XvAld1* product. The inheritance of the *XvAld1* transgene in Arabidopsis was evaluated by PCR amplification from genomic DNA isolated from T_2,

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T_3 and T_4 seedlings (Fig. 4.4). Most of the tested sibling lines contained the aldose reductase gene sequence.

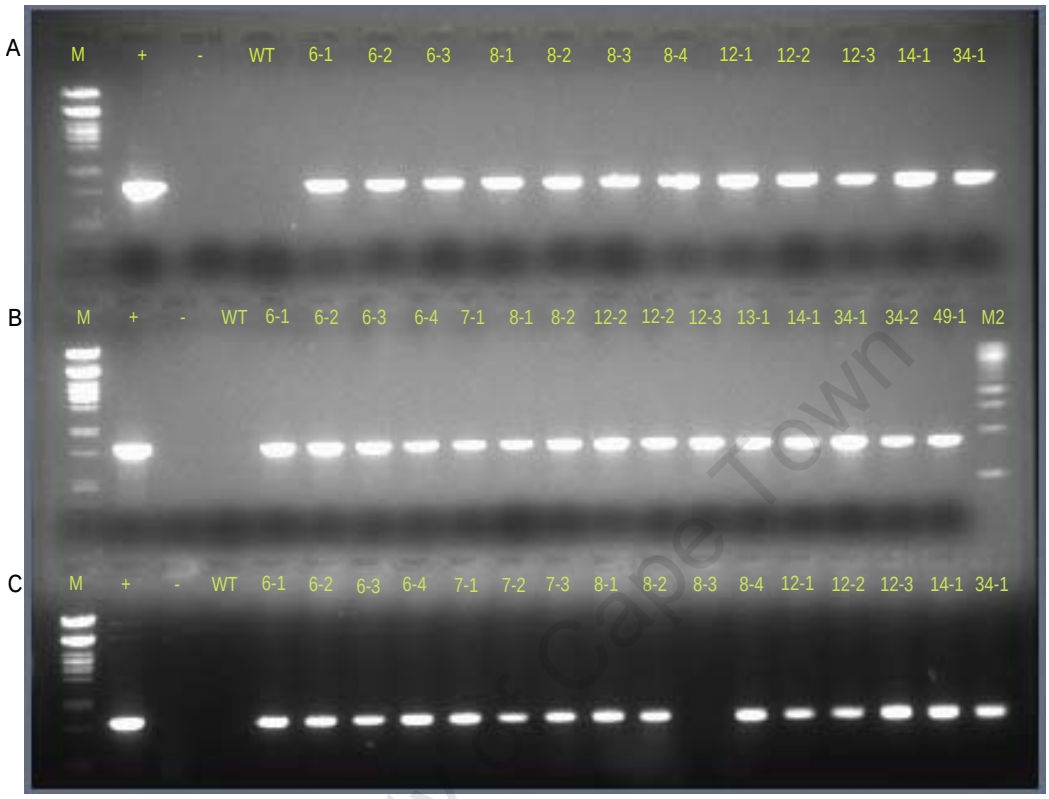

Figure 4.4 PCR screening for putative transgenic *A. thaliana* seedlings
DNA extracted from leaf material was used as template for PCR. A 960-bp product was expected from *XvAld1*gene-specific primers. Rows 1, T_2 plants; 2, T_3 plants; and 3, T_4 plants respectively. The identities of the *A. thaliana* lines and sublines are as indicated by labels under the wells. M and M2 denote *Pst*I digested λ DNA marker and 500 bp ladder respectively.

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4.3.3 Analysis of transgene expression in *A. thaliana* and *D. sanguinalis*

Total RNA was extracted from the PCR positive putative transgenic plants. Analysis of the putative transgenic *D. sanguinalis* using end-point reverse transcriptase PCR showed high levels of expression of the *XvAld1* transcript in some of the lines (Fig. 4.5).

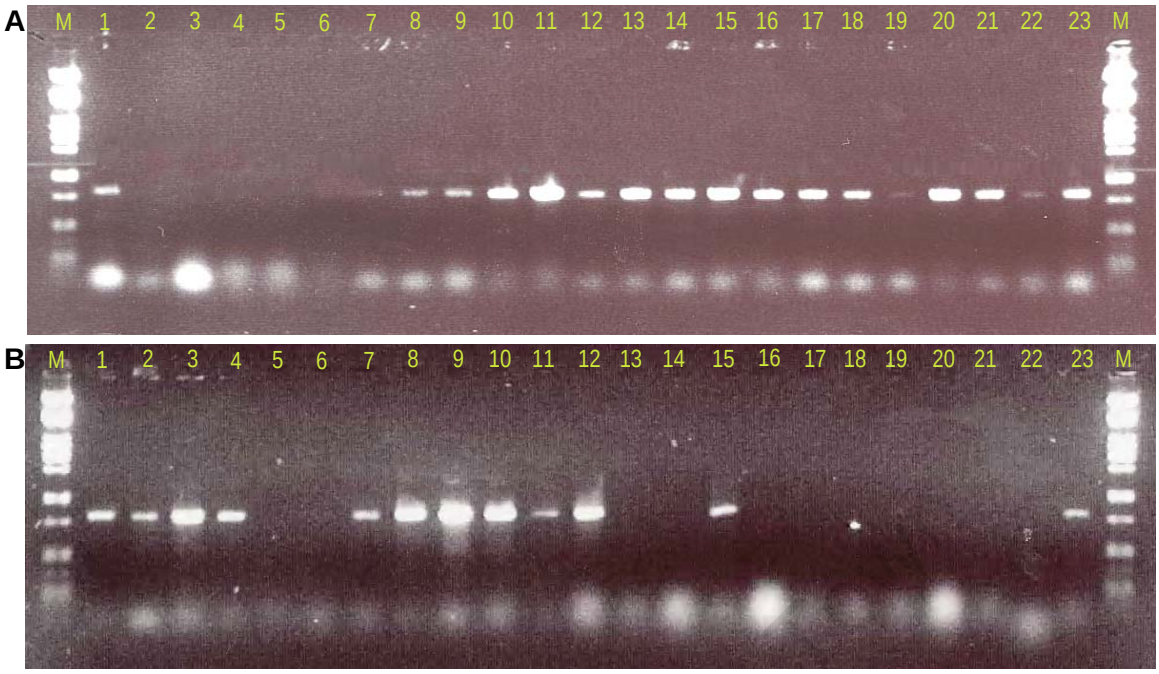

Figure 4.5 RT-PCR for *XvAld1* expression in T_0 putative transgenic *D. sanguinalis*
One microgram of total RNA was reverse-transcribed and the cDNA used as template for PCR with *XvAld1* gene-specific primers. Panel A: Lane 1, positive control using pSK-*XvAld1* as template; lane 2, no template control; lane 3, wild type (untransformed control); lanes 4 – 6, transformation controls (VT); lanes 7 – 23, putative transgenic lines. Panel B: Lanes 1 – 23; putative transgenic lines. M denotes the *Pst*I-digested λ ladder.

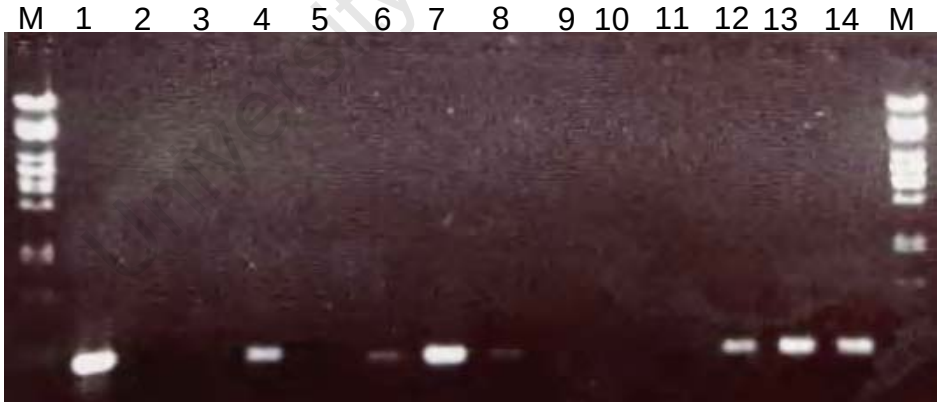

Figure 4.6 RT-PCR analyses for bar gene expression in *D. sanguinalis* T_1 plants.
Lane 1, positive control using pAHC25 plasmid DNA as template; lane 2, WT1; lane 3, VT1; lanes 4 – 14, selected transgenic lines 1, 2, 5, 6, 9, 10, 13, 14, 17, 18 and 21 demonstrated to express *XvAld1*.

The putative transgenic plants that tested positive for the *XvAld1* gene using RT-PCR were selected for further analyses. However, many of the *XvAld1*-containing plants tested negative for the *bar* transcript (Fig. 4.6) but were however resistant to bialaphos.

To support the RT-PCR analysis, and obtain a comparative analysis of transcript levels, northern blots were performed. *D. sanguinalis* plants that showed expression of *XvAld1* transcript using RT-PCR were selected. The detected levels of the *XvAld1* transcript varied among the different transgenic lines (Fig. 4.7). The 18S ribosomal RNA (rRNA) was used as a loading control to enable a comparison of transcript levels. The plants expressed the transgene at lower levels compared to the 18S transcript. Lines 48 and 53 did not contain detectable levels of the transcript while lines 50, 62 and 73 are low expressers (lanes 6 and 8 and lanes 7, 9, 11 and 12 respectively). The transgenic *A. thaliana* plants however, showed relatively high levels of *XvAld1* transcript (Fig. 4.8). Total RNA was extracted from 5 week-old plants selected from lines 8 and 12 (Panels A and B respectively). In both *D. sanguinalis* and *A. thaliana*, no transcript was detected in the wild type or vector transformed controls, showing that these plants were not expressing any related genes.

Real time PCR was used to compare transgene expression in selected *A. thaliana* transgenic lines. Lines 7, 8, 12, and 34 were chosen for analysis because they displayed good germination and high seed yields, important characteristics for downstream physiological analyses. The box plots (Fig. 4.9) represent the spread of expression levels of *XvAld1* transcript in the four independent transgenic lines. The control lines, wild type and transformation control (VT); do not contain detectable levels of *XvAld1* transcript or transcript from any related genes. Transgenic line 7 showed the lowest expression levels, but also demonstrated reproducible relative expression values averaging 0.75 compared to the β-actin levels. Although the values overlap, transgenic lines 8 and 12 show comparable average transcript levels. Line 34 shows the highest expression of close to twice the β-actin transcript levels.

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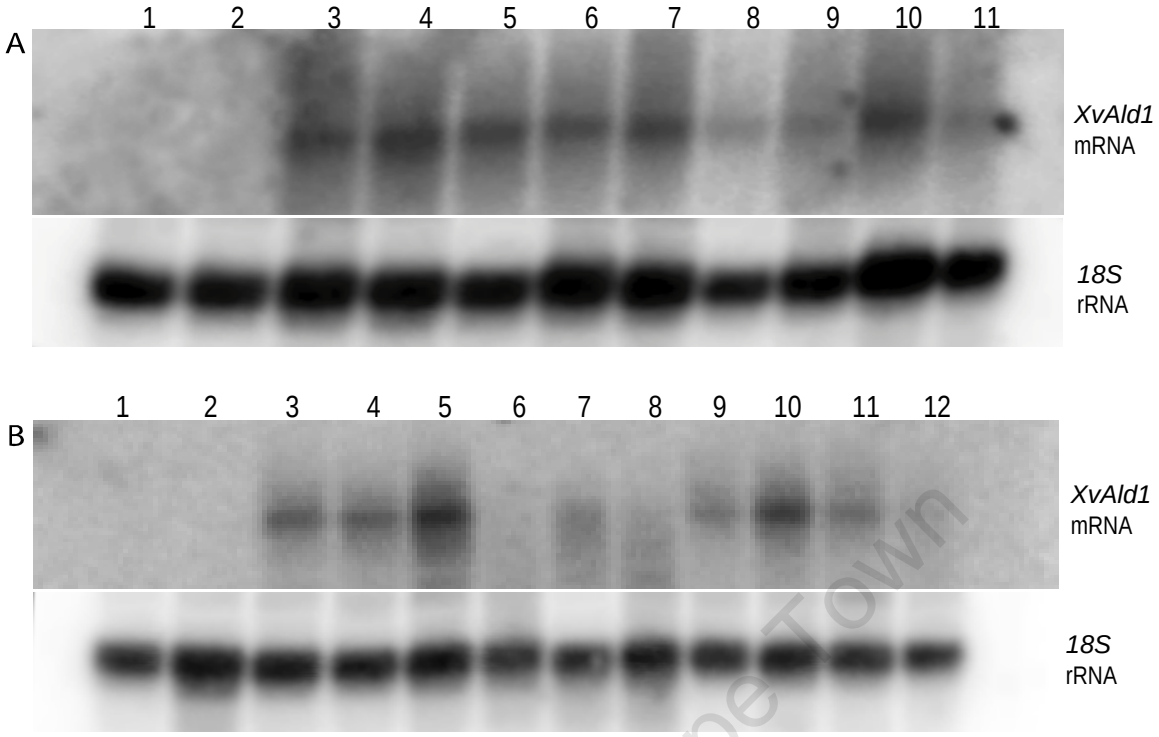

Figure 4.7 Northern blot analysis for levels of *XvAld1* transcript in putative transgenic *D. sanguinalis* plantlets Five micrograms of total RNA from T_1 plants were probed with radioactively labelled *XvAld1* cDNA. Ribosomal RNA (*18S* rRNA) was used as a loading control. Row **A**: Lanes 1 and 2, wild type (WT2) and empty vector transformed (VT2) controls; lanes 3 – 11 putative transgenic *D. sanguinalis* lines 1, 2, 5, 6, 10, 13, 14, 17 and 18 respectively. Row **B**: Lanes 1 and 2, wild type (WT2) and empty vector transformed (VT1) controls; lanes 3 – 12, putative transgenic *D. sanguinalis* lines 21, 22, 45, 48, 50, 53, 62, 64, 72 and 73 respectively.

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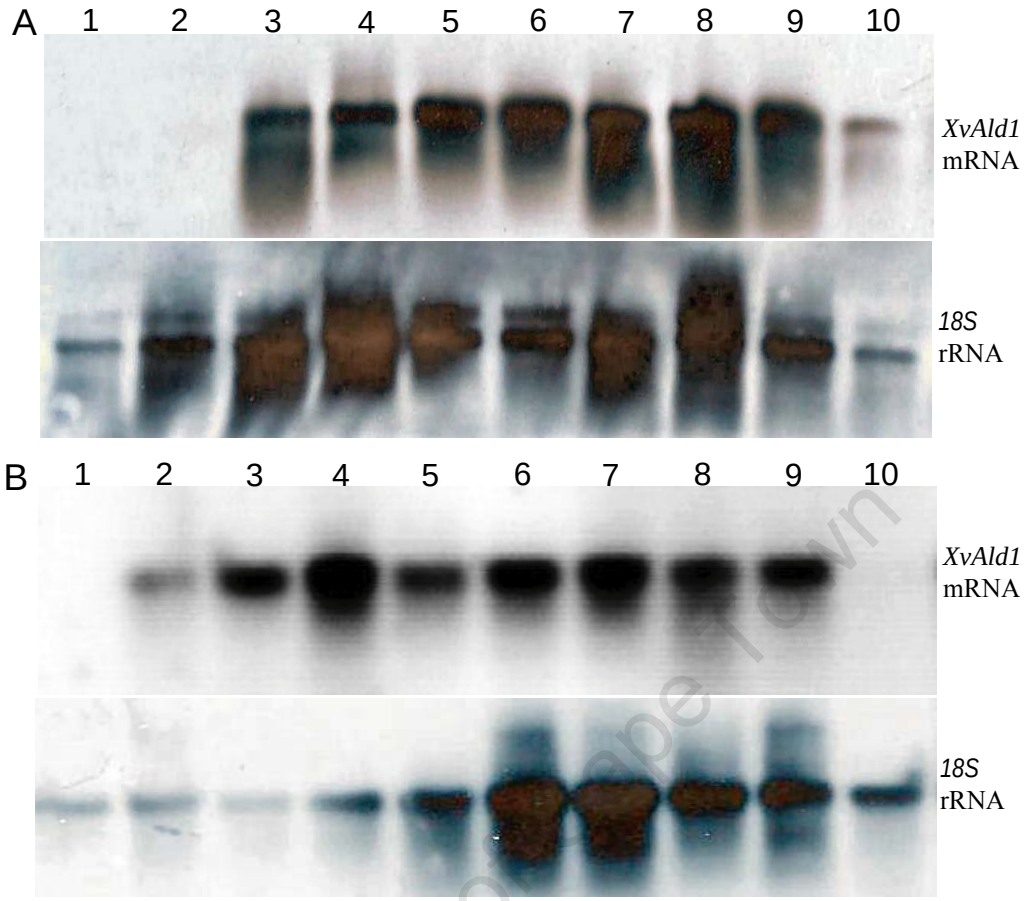

Figure 4.8 Northern blot analysis of *XvAld1* expression in putative transgenic *A. thaliana* Five micrograms of RNA extracted from T_3 plants was separated on agarose gel and blotted onto membranes. The blot in **A** was probed using DIG-labelled *XvAld1*. After the chemiluminescence had ceased, the blot was reprobed with DIG-labelled 18S rRNA. Lanes 1 and 2 , RNA from WT and VT plants; lanes 3 – 10, RNA from transgenic line 8. The blot in **B** was first probed with ^{32}P-labelled *XvAld1* cDNA. After the radioactivity had gone cold, blots were reprobed with DIG-labelled 18S rRNA. Lane 1, WT; lanes 2 – 9, RNA from transgenic line 12; and lane 10, VT plant.

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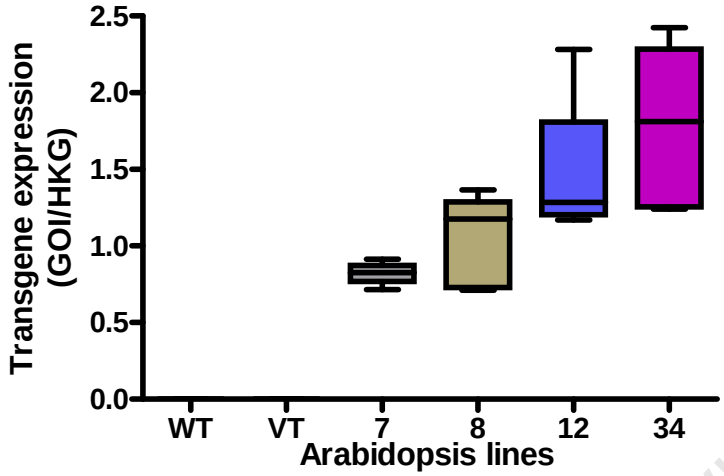

Figure 4.9 Relative expression profile of *XvAld1* transcript in transgenic *A. thaliana*
One microgram of total RNA was reverse transcribed into cDNA. The cDNA was used as template for real time PCR analysis. *XvAld1* expression was calculated relative to the abundance of β-actin transcripts (GOI/HKG). WT, wild type; VT, vector transformed; 7, 8, 12, and 34, transgenic A. thaliana T_5 lines overexpressing the gene.

4.3.4 Accumulation of the XvAld1 protein in transgenic plants

Varying levels of *XvAld1* protein accumulated in the different transgenic Arabidopsis lines (Fig. 4.10). Line 8 expressed the least amount of protein while lines 12 and 34 accumulated the highest levels. However, because no loading control was used to confirm equal loading of protein sample, a quantitative comparison of expression levels may not be accurate. Although line 6 had been shown by PCR to contain the transgene (Fig 4.4), no protein was detectable by western blotting (Fig 4.10, lane 4). The 4 ng of the recombinant protein used as a positive control (top arrowhead; 40.5 kDa) is barely visible compared to the signal for protein in transgenic plants (36 kDa; bottom arrowhead). The *XvAld1* protein expression levels in transgenic *D. sanguinalis* plants were low (Fig. 4.11), in the two generations tested, compared to the levels detected in *A. thaliana* plants.

To investigate the expression of the aldose reductase in different *A. thaliana* plant tissues, total protein was extracted from leaves, stems, immature siliques and roots. Fifteen micrograms of the total protein was probed with polyclonal antibodies against the aldose reductase. The protein was detected in leaves, stems and siliques (Fig. 4.12, lanes 1 – 6) but not in roots (lanes 7 and 8). However, total protein quantities obtained in the

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roots were low as indicated by levels in the loading control (stained pink). Protein levels in siliques were also comparatively low.

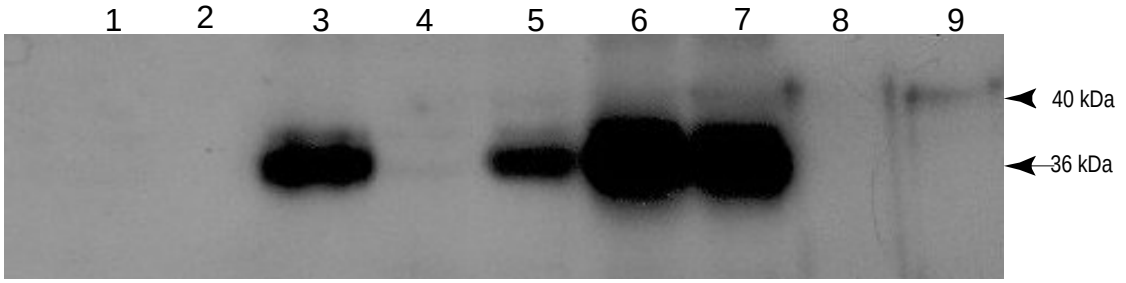

Figure 4.10 Western blot analysis of the expression of *XvAld1* protein in *A. thaliana*
Twenty-five micrograms of total soluble protein were loaded per well, separated by SDS-PAGE and electro-blotted onto nitrocellulose membrane. The membrane was probed with polyclonal antibodies raised against the XvAld1 recombinant protein. Lanes 1 and 2, protein from wild type plants; lanes 3 – 7, protein from T_4 transgenic lines 7, 6, 8, 12 and 34 respectively; lanes 8 and 9, 2 and 4 ng of XvAld1 recombinant protein respectively. The arrowheads show the positions of the 40-kDa His-tagged recombinant protein (top) and the 36-kDa plant aldose reductase.

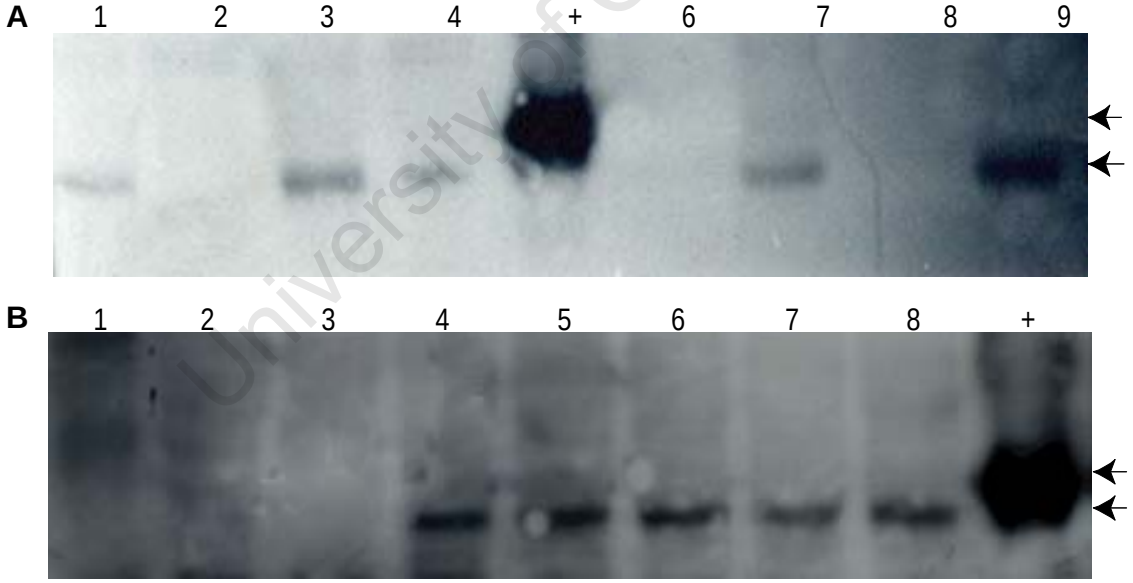

Figure 4.11 Transgenic *D. sanguinalis* plants accumulate the XvAld1 protein
Twenty micrograms of total protein was loaded per well. The membrane was probed with polyclonal antibodies raised against the XvAld1 recombinant protein. **A** Lanes 1 – 9, screening of putative transgenic T_1 lines; **B** Lanes 1, molecular weight marker; lane 2, WT; lane 3, empty vector transformed; lanes 4 – 8, T_2 transgenic lines 1, 2, 5, 6 and 10 respectively; "+", 2 µg of XvAld1 recombinant protein.

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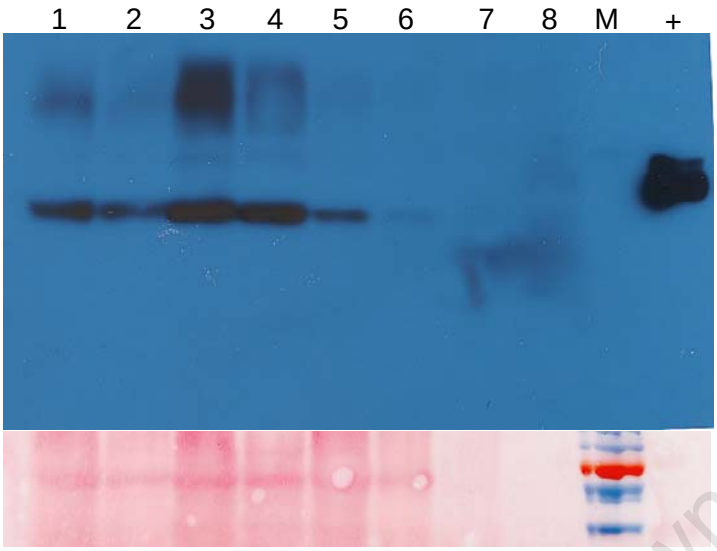

Figure 4.12 Expression of the aldose reductase transgene in different tissues
T_4 generation *A. thaliana* plants at mid-flowering stage were tested for the presence of the XvAld1 protein. Fifteen micrograms of the total soluble protein (extracted from pooled material from four plants) was separated by SDS-PAGE and blotted onto nitrocellulose membrane. Lanes 1 and 2 contain protein from leaf material; lanes 3 and 4, stems; lanes 5 and 6, immature siliques, and lanes 7 and 8, roots. M denotes molecular weight marker and +, 100 ng of the 40.5 kDa XvAld1 recombinant protein used as positive control. Odd numbered lanes contain protein from line 8 and the even ones line 12.

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4.3.5 Southern blot analysis on *A. thaliana* transgenic plants

The integration of the *XvAld1* transgene into the Arabidopsis genome was analysed by Southern blotting. Because no suitable restriction enzymes could be used to release the entire expression cassette, enzymes that cut once or do not cut within the *XvAld1* sequence were selected. Releasing the entire T-DNA could be used to check for rearrangements while enzymes that cut within the cassette will only show integration. A similar banding pattern was observed between the transgenic lines 8 and 12 (Fig. 4.13a). Restriction with *Eco*RI showed two hybridising bands each of approximately 1.2 and 0.8 kb. The *XvAld1* cDNA contains one *Eco*RI site at position 493 of the cDNA while the pBI121 plasmid contains another site beyond the *Nos* terminator (see plasmid map in Appendix D). It is expected that all integration patterns will show at least one common band of ~900 bp. The larger band cannot be accounted for using the restriction sites within the pBI121 T-DNA. It is possible that other *Eco*RI sites exist, besides that one indicated on the plasmid map.

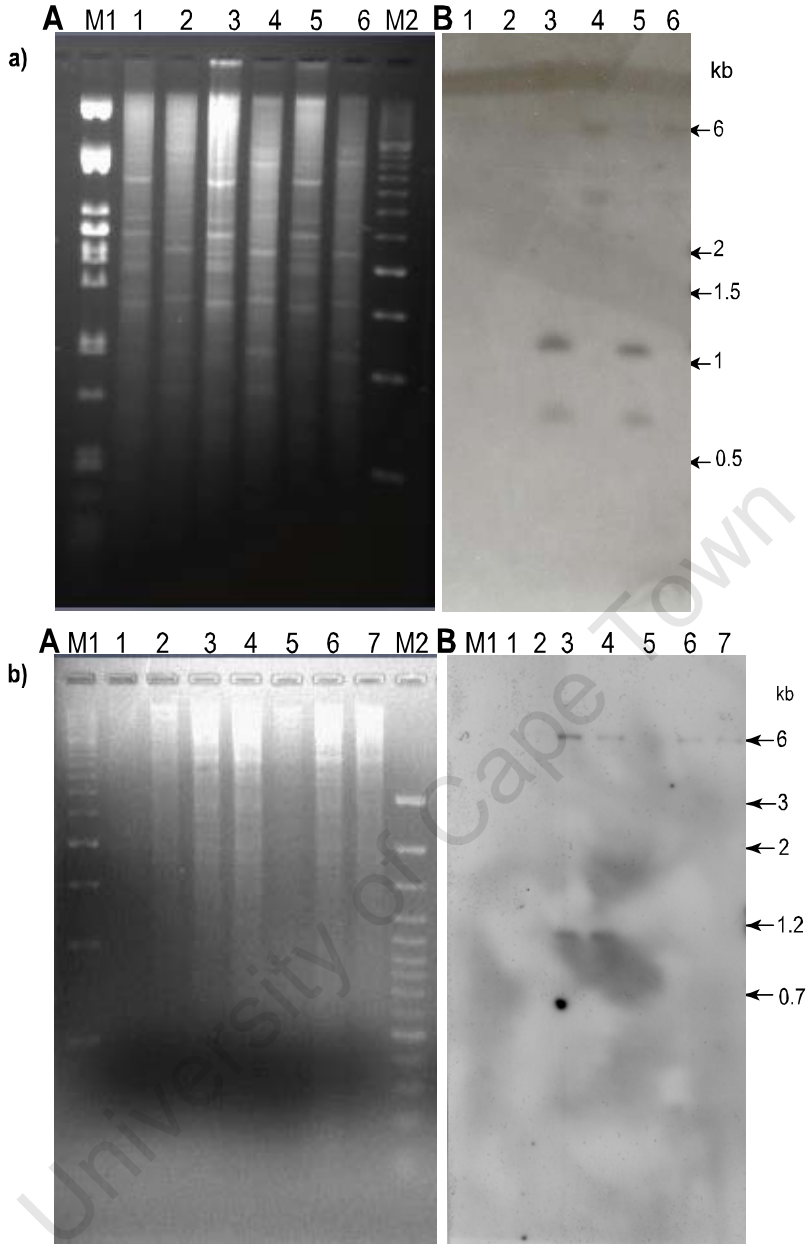

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Figure 4.13 Southern blot analyses on T_4 transgenic *A. thaliana*
a) Genomic DNA from transgenic lines 8 and 12 was analysed using *Eco*RI (lanes 3 and 5) and *Xba*I/*Sac*I (lanes 4 and 6) respectively. Genomic DNA extracted from WT plants was also digested with *Eco*RI and *Xba*I/*Sac*I (lanes 1 and 2 respectively). ^{32}P-labelled *XvAld1* probe was used to identify transgene-hybridising bands (B). M1 and M2 denote *Pst*I-digested λ-DNA marker and the 500-bp ladder (Fermentas, Germany) respectively. **b**) *Bcl*I-digested genomic DNA from transgenic lines 8, 12, 13, 34 and 49, lanes 3-7 respectively, and WT and VT DNA digested with the same enzyme (lanes 1 and 2 respectively) were separated on 0.8 % (w/v) agarose gel. The membrane (B) was probed with DIG-labelled *XvAld1*. M1 and M2 denote the 100-bp and 500-bp ladders (Fermentas, Germany) respectively.

A double digestion with *Sac*I and *Xba*I produced two bands each. The larger bands were ~6 kb and the smaller ones ~3 kb. Neither of the enzymes cuts within the *XvAld1* sequence but *Xba*I and *Sac*I sites flank the insert. Since the two bands detected were larger than the insert size (1.2 kb), it appears that the *Sac*I site was destroyed during cloning. The banding pattern observed for both lines is consistent with the presence of two copies of the transgene. Because the two selected lines showed a similar banding pattern, more transgenic lines were analysed by Southern blotting using a wider variety of enzymes that do not cut within the T-DNA. Many of the enzymes failed to digest the genomic DNA efficiently, making them unsuitable for Southern blotting. The *Bcl*I digests produced two distinguishable groups of transgenic plants (Fig. 4.13b). The first group containing lines 8 and 12 had two hybridising bands, one of 6 kb and the other one slightly smaller than 1.2 kb. The small size of one of the hybridising bands indicates the presence of *Bcl*I restriction site(s) within the T-DNA. The other group consists of lines 34 and 49, both containing one hybridising band at 6 kb. No bands were observed for line 13, probably due to the low levels of DNA (lane 5, Panel A). These results indicate that lines 34 and 49 contain one copy, while lines 8 and 12 contain two copies of the *XvAld1* transgene. As expected, DNA extracted from the control lines (WT and VT) did not hybridise with the *XvAld1* probe.

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4.4 DISCUSSION

The identification of genes that are important in stress response and the understanding of the genetic and physiological basis of stress tolerance are essential for crop improvement. The proposed protective functions of the *X. viscosa* aldose reductase were assessed by making transgenics in two model plants, *A. thaliana* and *D. sanguinalis*.

4.4.1 Screening for transformants and analysis of transgene expression

Putative transgenic *D. sanguinalis* plants were selected for herbicide resistance as well as the presence of the *XvAld1* gene. Using PCR screening, most of the putative transgenic plants tested positive for the presence of *XvAld1* gene (Fig. 4.3). The low rate of false positives can be attributed to the rigorous selection of transformed calli on media supplemented with bialaphos. The bar gene was difficult to amplify using ordinary PCR Taq polymerases because the sequence has high GC content. Screening of genomic DNA resulted in poor amplification of the transgene (data not shown). However, better results were obtained when cDNA was used as template (Fig. 4.6). Other researchers have used polymerases optimised for sequences that are difficult to amplify (Vickers et al., 1996), such as Phusion High Fidelity DNA Polymerase (Finnzymes, Finland) and Expand High Fidelity Enzyme Mix (Roche Diagnostics, Germany). However, these polymerase systems are too expensive for routine amplification such as the screening of putative transgenic plants. PCR screening of putative transgenic *A. thaliana* showed that the transgene was stably inherited stable through the three generations tested (Fig. 4.4).

The presence of a transgene in the host plant genome does not guarantee the expression or the stability of the inserted sequences. Not all PCR-positive transgenic lines expressed detectable levels of the transgene. Northern blot analysis of selected transgenic lines demonstrated the expected variation in expression levels in *D. sanguinalis* (Fig. 4.7). Analysis of transgenic *A. thaliana* was performed on two selected lines to assess the variation of transgene expression between plants from the same parent line. Similar expression levels were observed for line 8 (Panel A). However, transcript levels in line 12 were difficult to compare due to the variable sample loading. Real time PCR showed that *XvAld1* transcript levels in Arabidopsis were high, up to twice as much as the β-actin levels. The plant-to-plant variations

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observed in sibling lines are corroborated by other studies (Goossens et al., 1999; Opsahl et al., 2002). The observed variation in transcript levels may be caused by so-called 'position effects'. Transgene silencing and/or truncation of the expression cassette can abolish expression. The silencing of transgenes is a common phenomenon observed in transgenic plants (Vance and Vaucheret, 2001). Researchers have found little correlation between transgene copy numbers and the levels of expression. It is believed that transgene expression is influenced by the genetic environment into which the sequence is incorporated (Matzke and Matzke, 1995; Iglesias et al., 1997; Gelvin, 1998; Matzke and Matzke, 1998; Vaucheret et al., 1998). Some reports suggest that Agrobacterium-based transformation results in transgene integration into preferred sites such as transcriptionally active or scaffold attachment regions (Koncz et al., 1989; Sawasaki et al., 1998; Shimizu et al., 2001).

Protein accumulation was correspondingly high in transgenic Arabidopsis (Fig. 4.9) while the levels in *D. sanguinalis* were moderate (Fig. 4.10). The tissue specificity of transgene expression under the control of the CaMV 35S promoter may vary depending on plant species or variety (Benfey and Chua, 1989; Benfey et al., 1989). The XvAld1 protein accumulated in all Arabidopsis tissues tested, except the roots (Fig. 4.11). However, the absence may reflect the difficulty of extracting protein from root tissues.

4.4.2 The phenotype of the transformed plants

The regeneration of transformed *D. sanguinalis* calli on selective media took at least 20 weeks (from bombardment to potting), while regenerating wild type plants took six weeks. All the regenerated T_0 plants, including the WT and transformation controls (VT), were sterile. This characteristic was consistent in all the plants generated from the same callus lines. The plants developed inflorescences but failed to set seed. *D. sanguinalis* was originally cultured from the wild for use in testing resistance of transgenic plants to MSV (Chen et al., 1998b). However, the stability of the plants' phenotype under tissue culture has never been investigated. We propose that the loss of fertility observed in the regenerated plants was a result of prolonged propagation in tissue culture. The embryogenic callus lines used for transformation were propagated for months prior to the transformation. Because of the infertility, T_1 plants were regenerated from callus induced from inflorescences of T_0 plants. The

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regenerated *D. sanguinalis* plants were of varying shoot sizes, root lengths and developmental stages. This lack of uniformity made it difficult to assess the growth characteristics and phenotype under environmental stress.

The characterisation of transgenic plants requires the generation of fertile plants. Plants generated from seed would have been developmentally similar, and therefore easier to characterise. Many of the plants regenerated from callus were morphologically different from the *D. sanguinalis* found in the wild. Although the cultured plants were planted as single spikes, they grew into bushy thickets, with short internodes whereas seed-derived plants grow sparsely with long internodes (data not shown). The sterility and phenotypic variations found in callus-derived plants are manifestations of somaclonal variation. This phenomenon has been reported in many transformed plants (Bregitzer et al., 1998; Choi et al., 2001; Dong et al., 2001). Researchers have however demonstrated that phenotypic variations are perpetuated in all plants regenerated from tissue culture, regardless of transformation method or whether or not they have been transformed (Fukuoka et al., 1994). The *in vitro* environment is thought to be mutagenic to cultured tissues. The spontaneous genetic changes occurring in the differentiating cells are heritable and are propagated in subsequent generations (Choi et al., 2001).

Somaclonal variation may be reduced by shortening the time-frame from culture initiation to plant hardening (Chen et al., 1998a; Altpeter et al., 2000; Taylor et al., 2001). Some researchers bombard cells within a week of culture initiation (Goldman et al., 2003). Regeneration of transformed calli takes longer on bialaphos compared to using hygromycin as a selectable marker (Gao et al., 2006). Prolonged tissue culture also lengthens the time it takes to regenerate callus into plantlets following gene transfer (Zhang et al., 2003).

As a result of the morphological abnormalities and sterility of *XvAld1* transgenic plants, subsequent transformations were performed on freshly-induced calli developed from inflorescence collected from the wild. Reducing the time-frame of tissue culture manipulations produced fertile transgenic plants exhibiting normal phenotype (Owor and Bezuidenhout, personal communication). For future experiments, we propose that inflorescences be collected from potted plants and transformation be performed within weeks of calli initiation.

Transgenic *A. thaliana* plants were also compared with the wild type with respect to germination rate and the general phenotype including plant size,

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morphology and days to bolting. No change in phenotype was observed in Arabidopsis plants, except a slight increase in plant size observed in line 8. However, this increase was not investigated by quantifying the differences in biomass between the different lines. All plants showed similar phenotypic, developmental stage and fertility characteristics. Many studies in transgenic plants overexpressing a single stress-responsive gene have reported no changes in the phenotype of the plants (Iturriaga et al., 1992; Garwe, 2003; Sunkar et al., 2003; Zhifang and Loescher, 2003; Deguchi et al., 2006; Garwe et al., 2006). Transgenic Arabidopsis plants were selected for further analysis based on the levels of transgene expression, the stability and heritability of the expression, high yields, and good germination rates. Variable transgene expression levels may be caused by positions into which the foreign DNA is integrated in the host genome.

The analysis of *A. thaliana* transgenic plants was performed at different developmental stages and in various tissues. Constitutive promoters should ideally express the transgene in all tissues at all times. However, it has been demonstrated that the activity of the CaMV 35S promoter is developmentally regulated and also shows varying expression patterns in different tissues and host species (Benfey and Chua, 1989; Benfey et al., 1990; van der Fits and Memelink, 1997). Expression patterns are also dependent on developmental stages and environmental conditions, and may be tissue-specific.

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4.4.3 Transgene integration into the Arabidopsis genome

The integration patterns obtained for Agrobacterium-transformed *A. thaliana* plants were simple, characterised by low copy numbers of one or two (Fig. 4.13). However, interpretation of the banding patterns was complicated by the limited number of unique enzyme sites within the T-DNA. Restriction analysis of the plasmid sequence (AF485783) showed that many common enzymes had multiple sites within the vector. In addition, the plasmid into which *XvAld1* was cloned could have differed somehow from the original clone as a result of repeated propagation since the construct is no longer available from the manufacturer (Clontech, USA).

The prior selection of transgenic plants based on agronomic traits such as germination and seed yield may have caused the similarity in the results obtained from Southern blotting. It is also possible that lines within each of the two separate

groups are not independent transformants but rather seeds from the same transformation event that were separated during selection and then propagated as different lines. However, real time PCR (Fig. 4.9) and western blot analyses (Fig. 4.10 and 4.11) show different transgene levels in the two lines (lines 8 and 12). Other researchers also reported low copy numbers (less than five) for transgenic plants transformed using Agrobacterium-mediated methods (Hobbs et al., 1993; Ingham et al., 2001; Travella et al., 2005). Agrobacterium-mediated transformation methods potentially yield low copy transgenic plants (Frame et al., 2002; Miller et al., 2002) compared to particle bombardment where many copies and complex integration patterns are observed (Altpeter et al., 2000; Dalton et al., 2003; Zhang et al., 2003; Gao et al., 2006). In their comparison of barley plants, Travella et al. (2005) observed that Agrobacterium-derived lines showed stable integration and expression of transgene while plants transformed by biolistics frequently exhibited transgene silencing. Some reports propose that Agrobacterium-mediated transformation preferentially integrates into actively transcribed regions (Szabados et al., 2002). Integration patterns in *D. sanguinalis* were not investigated because all the regenerated transgenic plants were sterile.

There has been a shift towards the use of Agrobacterium-mediated transformation in transgenics programmes. Monocots were traditionally recalcitrant to transformation with Agrobacterium-based methods (Potrykus, 1990; Narasimhulu et al., 1996). However, there are some reports of successful transformation of a range of cereals and grasses using Agrobacterium-mediated methods (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997; Tingey et al., 1997; Patel et al., 2000; Dong et al., 2001; Frame et al., 2002). Despite these successes, the refinement of gene constructs and delivery tools is still crucial. While transformation of dicots can be performed on whole plants, a limited range of monocot cells is amenable to Agroinfection. Transformation is still performed on actively dividing, undifferentiated cells resulting in persistent problems with somaclonal variation (Tingey et al., 1997; Dong et al., 2001).

4.4.4 The use of constitutive promoters for transgenic studies

The mechanisms used to control transgene expression are important in determining the phenotype of the transformed plants. In this study, constitutive

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overexpression was used for both the monocot and dicot model plants. The ubiquitin promoter, used to drive the expression construct transferred into *D. sanguinalis* is a maize endogenous promoter (Christensen et al., 1992; Christensen and Quail, 1996). Virus-derived promoters are another mechanism of achieving constitutive expression. The CaMV 35S promoter was used to drive transgene expression in Arabidopsis plants. Research has demonstrated that constitutive expression does not guarantee the production of the transgene in all cell or tissue types at all times. Several studies have shown that the expression patterns of CaMV 35S-driven constructs can vary depending on plant species or variety, tissue or cell type, and/ or developmental stage (Benfey and Chua, 1989; Benfey and Chua, 1990; Samac et al., 2004). The expression pattern in transgenic Arabidopsis was investigated by probing for the XvAld1 protein in selected tissues. The XvAld1 protein was not detectable in roots. However, the loading control shows that protein levels in the root extracts were low (Fig. 4.11). In Arabidopsis, the roots are fine, web-like and difficult to separate from the soil. The expression patterns in transgenic *D. sanguinalis* were not investigated.

Constitutive overexpression of some gene products may interfere with cellular processes resulting in an aberrant phenotype such as reduced growth or altered development. The use of inducible promoters may overcome such complications. Spatial and temporal regulation of transgene expression can be achieved by employing stress- or chemically-inducible, tissue- or cell-specific, and developmentally-regulated promoters (Gurr and Rushton, 2005). The use of inducible promoters may also give a more representative physiological environment for the study of gene function.

The genomics era has resulted in an explosion in expressed sequence information yet knowledge of the corresponding regulatory mechanisms still lags behind. Further characterisation of the *XvAld1* putative promoter is necessary before it can be used for transgenic studies. The use of an endogenous, stress-inducible promoter may be invaluable for the investigation of stress tolerance in crops.

4.5 CONCLUSION

Putative transgenic plants were screened and characterised to demonstrate transgene expression at nucleic acid and protein levels. Transgenic Arabidopsis lines selected for further experiments yielded many seeds and showed uniform seedling growth.

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CHAPTER 5

Physiological and phenotypic analyses under stress conditions of transgenic Arabidopsis plants overexpressing the *XvAld1* gene

5.0 SUMMARY

Transgenic *A. thaliana* plants, constitutively expressing *XvAld1*, were exposed to various abiotic stresses. The phenotypes, physiological and biochemical properties of the transgenic plants under stress were evaluated and compared to similarly stressed wild type (WT) and vector-transformed (VT) controls. The constitutive expression of *XvAld1* in *A. thaliana* enhanced tolerance to variable levels of salt, osmotic and dehydration stresses. All the transgenic plants performed better than the WT on 75 mM mannitol. However, increasing the osmotic stress to 100 and 150 mM mannitol reduced the vigour of the transgenic plants. The growth of line Xv12 on 14% (w/v) PEG was comparable to that of seedlings on normal tissue culture media. Transgenic line Xv8 exhibited reduced growth but still performed better than the WT. A similar trend was observed for seedlings on salt stress except that growth was markedly reduced after four days on media containing 75 mM NaCl or more. The WT seedlings showed the least growth, while the performance of the transgenic lines was comparatively high, showing that the transgenic seedlings were more tolerant to the range of imposed stresses compared to the WT. Dehydration tolerance in potted plants was assessed based on phenotypic assessment and survival after the resumption of watering. Transgenic plants showed significantly greater tolerance compared to WT and VT. There was little difference in the RWC of transgenic and control plants dehydrated for ten days. In contrast, the water potentials of line Xv8 leaves were more negative compared to the rest of the plants. Despite this, a greater percentage of line Xv8 plants exhibited the tolerance phenotype compared to the WT and the other transgenic lines. Dehydration reduced the efficiency of photosystem II (F_v/F_m) from 0.8 to 0.6 but there was no difference in ratios across all the stressed plants. Assessment of lipid peroxidation products showed that transgenic and well-watered plants accumulated comparable levels of malondialdehyde but levels in WT and VT plants were about six times higher. These results suggest that the overexpression of XvAld1 protects plants from the damaging effects of oxidative stress.

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5.1 INTRODUCTION

Dehydration and salinity are the major stresses affecting agricultural productivity (Boyer, 1982; Holmberg and Bulow, 1998; Flowers, 2004). Breeding for tolerance is hindered by the multigenic nature of many abiotic stresses. Although research has focused on characterising genes that are upregulated during abiotic stress, not all genes that are expressed in response to stress are involved in protective or adaptive responses. On exposure to stress conditions, a subset of genes is induced to counteract the adverse effects on plant growth and development. The gene products either directly protect cells against stress or control the expression of other stress-associated genes (Yamaguchi-Shinozaki and Shinozaki, 2006). Many genes suspected of supporting adaptive responses in plants have been transformed into model plants. Transgenic strategies have been used to overexpress either stress-signalling proteins or gene products possessing protective functions.

Many of the regulatory genes overexpressed in transgenic plants are transcription factors involved in controlling cellular responses to dehydration, salinity and low temperatures (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000; Jaglo et al., 2001). The Arabidopsis DREB1A transcription factor was overexpressed in *A. thaliana* (Kasuga et al., 1999), *Solanum tuberosum* (potato; Behnam et al., 2006; 2007), *Nicotiana tobaccum* (tobacco; Kasuga et al., 2004), *Oryza sativa* (rice; Oh et al., 2005) and *Triticum aestivum* (wheat; Pellegrineschi et al., 2004). Other researchers have also reported stress tolerance in transgenic plants overexpressing kinases (Saijo et al., 2000; Umezawa et al., 2004). The successes of these strategies depended on whether a constitutive or inducible promoter was used and on the plant species under investigation. In general, the use of constitutive promoters resulted in growth and/ or developmental abnormalities. In spite of these aberrations, all researchers reported improved stress tolerance in the transgenic plants.

Of the many functional genes used as targets for engineering abiotic stress tolerance, much work has been devoted to overexpressing genes involved in the biosynthesis of osmoprotectants. Sugar alcohols and other low molecular weight osmolytes accumulate in diverse organisms in response to different environmental

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stresses (Yancey et al., 1982; Sun et al., 1999; Clark et al., 2003). The metabolites are proposed to restore intracellular water balance or to protect macromolecules from damage. The diverse functions of the osmoprotectants make them a favourite tool for genetic manipulation. These efforts have yielded mixed results. Many transgenic plants were reported to accumulate low levels of the polyols; between 0.2 and 1 μmol per gram fresh weight (Bellaloui et al., 1999; Brown et al., 1999; Deguchi et al., 2006). However, Sheveleva et al. (1998) reported sorbitol accumulation of up to 130 μmol per gram fresh weight. High levels of trehalose have also been achieved in transgenic *A. thaliana* (Goddijn et al., 1997; Schluepmann et al., 2003). On the other hand, transgenic plants overexpressing trehalose-6-phosphate synthase failed to accumulate appreciable amounts of the trehalose (Avonce et al., 2004). The accumulation of osmolytes in tissues of plants that do not normally synthesise such metabolites can be detrimental. The ectopic synthesis of osmolyte compounds has sometimes resulted in growth abnormalities, that include stunting, chlorosis and necrosis (Karakas et al., 1997; Sheveleva et al., 1998; Deguchi et al., 2004). The degree of phenotypic aberrations and growth inhibition appeared to correspond with the concentrations of accumulated osmolytes. More recent strategies have involved the use of inducible promoters and engineering of metabolite utilisation pathways (Deguchi et al., 2006). These alternative techniques have yielded plants with normal growth characteristics.

Despite the aberrations observed in many of the transgenic plants, researchers observed improved tolerance to dehydration and salt (Tarczynski et al., 1993; Karakas et al., 1997; Sheveleva et al., 1997; Oberschall et al., 2000; Mohanty et al., 2002; Abebe et al., 2003; Hideg et al., 2003; Sulpice et al., 2003; Zhifang and Loescher, 2003), cold (Hayashi et al., 1997) and nutrient deficiency (Bellaloui et al., 1999; Brown et al., 1999). The marginal levels of metabolite accumulation have caused researchers to reconsider the physiological functions of the transgenes. Similar questions have been raised in studies involving trehalose accumulation in plants. The resulting stress tolerance in the absence of accumulated trehalose also led the investigators to believe that protection was not directly linked to the levels of osmolytes. The physiological roles of many of the osmolytes remain elusive. Evidence of the protective roles is generally correlative and the functions have rarely been demonstrated *in vivo*. The levels of the osmolytes synthesised

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by transgene overexpression are too low to account for osmotic adjustment. Other possible roles include scavenging of ROS (Smirnoff, 1993; Shen et al., 1997b; 1997a) and stabilisation of macromolecules (Chen and Murata, 2002; Hincha and Hagemann, 2004). It is proposed that the enzymes provide protective functions through any one of the intermediate products in the osmolyte biosynthetic pathways. Studies with plants that accumulated low amounts of mannitol also led the researchers to postulate that one of the by-products was responsible for the observed tolerance (Smeekens, 2000; Rolland et al., 2006). It is believed that the biosynthetic intermediates may regulate carbohydrate metabolism in the cell by acting as second messengers that cause downstream changes in gene expression. Sugar metabolites thus control many aspects of plant growth and development (Koch, 1996; Smeekens, 2000; Eastmond et al., 2002).

In this study, the phenotypes of transgenic plants overexpressing *XvAld1* were assessed under various stress conditions. The plants exhibited normal development, and were phenotypically indistinguishable from the WT. As stress tolerance is affected by the developmental stage of the plants, six-day old seedlings or mature plants at the flowering stage were exposed to various stress conditions.

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5.2 MATERIALS AND METHODS

5.2.1 Plant material and growth conditions

A. thaliana plants of Columbia (Col-0) ecotype were used in this study. The WT Col-0 Arabidopsis was obtained from the University of Nottingham Arabidopsis Stock Centre. Transgenic *A. thaliana* plants, generated and screened as outlined in Chapter 4, were used for physiological and biochemical experiments. The selected transgenic lines are from here on referred to with an "Xv" prefix (for example, Xv8 for transgenic line 8). For plate assays, Arabidopsis seeds were germinated on plant nutrient solution (PNS; Haughn and Somerville, 1986), except that the solution lacked sucrose. The PNS was solidified with 0.8% (w/v) purified tissue culture agar (Sigma-Aldrich, Germany) to produce plant nutrient agar (PNA). Prior to plating, all seeds were surface sterilised by washing in 70% ethanol for 5 minutes followed by 10 minutes in 3.5% (w/v) sodium hypochlorite and rinsed six times for 5 min with sterile water. All sterilisation and washing steps were performed on a vortex mixer. Thereafter, seeds were suspended in 0.1% (w/v) tissue culture agar and kept in the dark for 4 days at 4°C to synchronise germination. Six-day old seedlings were transferred to the appropriate stress media. The plants were grown under controlled conditions of 16-hr light, 8-hr darkness, 22°C and 50 - 55% relative humidity unless stated otherwise.

5.2.2 Plant stress treatments on agar plates

Growth of WT, *XvAld1* transgenic and VT Arabidopsis plants was evaluated on PNA (non-stress control), or on PNA supplemented with varying concentrations mannitol or 14% (w/v) polyethylene glycol (PEG) 8000 (results in water potential of -0.35 to -0.4 MPa) to reduce the availability of water to the seedlings. Pre-weighed PEG pellets were dissolved in sterile molten PNA. PEG interferes with the polymerising properties of the agar, making the gel weak. To prevent the gel from collapsing, the depth of the media was poured thinly into the plates. Varying levels of NaCl were used to evaluate tolerance to salinity. The PNA used for stress treatments contained no sucrose. Seedlings of approximately the same growth stage (stage 1.02), as judged by leaf development (Boyes et al., 2001), were selected for the experiments. Seedlings were transferred onto the

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appropriate stress media on square plates. The plates were placed vertically to enable the root to grow along the length of the plates. Growth was monitored over a 10-day period by recording the increase in root lengths of the seedlings. The experiments were repeated at least three times, and each experiment included replicate plates of each stress level. Growth rate, expressed as a percentage increase in root lengths, was calculated using the equation below.

$$\% \text{ Relative growth} = \frac{\text{Final root length} - \text{initial root length}}{\text{Initial root length}} \times 100$$

5.2.3 Water deficit stress in soil

5.2.3.1 Slow drying

Transgenic and WT Arabidopsis seeds were grown in soil containing a 1:1 ratio of Jiffy-7 peat moss (Jiffy Products International AS, Norway) and vermiculite. To ensure uniform water content, 45 ± 1 grams of soil were used for each plant growth in trays. For plants grown in individual pots, 82 ± 1 grams of the soil mixture was used. The sown seeds were stratified at 4°C for four days and then the plants were grown for four weeks. During this time, the plants were watered twice a week on every third or fourth day. Drought stress was imposed on a subset of the plants by withholding water for up to fourteen days. The soil was water saturated a day before stress by filling trays with water. The next day, the remaining water was drained and further watering was withheld to initiate dehydration. The remaining plants were watered as usual and used as controls for normal growth conditions. Stress tolerance was evaluated by counting the number of plants that exhibited the drought-tolerance phenotype after the prolonged dehydration. Drought tolerance was defined as the absence of wilting or drying of leaves. The hydration state of the plants under stress was monitored by determining the relative water content (RWC) and the water activity (a_w) of leaf samples collected during the course of the stress treatment. Fully expanded leaves of the same developmental stage were used for analyses. RWC was determined by weighing leaf material soon after leaves were detached (full turgor weight; FTW). The leaves were subsequently floated in sterile distilled water overnight at 4°C and weighed again after blotting off excess moisture

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(hydrated weight; HW). Thereafter, the samples were dried at 72°C for 48 hr and weighed (dry weight; DW). RWC was calculated using the formula:

$$RWC = [(FTW - DW) / (HW - DW)]*100$$

Midday water potentials were measured between 11 am and noon on 1 cm^2 leaf disks using AquaLab Series 3 (Decagon Devices Inc., USA). Measurements were made on the same leaf samples used for RWC determinations. The experiments were performed at ambient temperature, which varied between 22 and 26°C. The a_w values were converted to water potential using the formula:

$$\psi = RT/M \ln (a_w)$$

where R = 8.318 (the universal gas constant), T is the leaf temperature in Kelvin and M is the molar mass of water.

Plants at the same developmental stage and of similar size were selected for stress experiments. To avoid significant reductions in plant sizes, each plant was sampled once throughout the stress and not more than two rosette leaves were detached for analysis. Sampling was done randomly, but all the sampled plants were marked to avoid oversampling, which leads to marked reductions in plant size. As position was seen to be important, the trays were randomised everyday and trays with well-watered controls were used at boundaries. In addition, at each sampling point leaves were taken from outer, middle and inner positions with the trays. The ability of plants to recover from water deficit stress was ascertained by watering the plants on day 10 and recovery was monitored over seven days.

5.2.3.2 Fast drying

Alternatively, potted *A. thaliana* plants were fast dried by transferring them to a 26 - 30°C plant growth room. The plants were acclimatised for one week. Thereafter,

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watering was withheld for six days. Plants that survived the seven-day dehydration were counted. RWC was determined during the course of dehydration as previously outlined.

5.2.4 Electrolyte leakage

Membrane integrity during drought stress was determined by measuring conductivity using a CM100 Conductivity Meter (Reid and Associates, South Africa). Fully expanded leaves from the same developmental stage were collected at specified time points during the dehydration stress. The leaf samples were placed in 2 ml eppendorf tubes and 1 ml of sterile deionised water was added. The tissue samples were held submerged in the water for 24 hr at 4°C. The initial electrolyte leakage readings (in μS/ml) were determined by calculating the average of at least 10 readings taken at 15 sec intervals. The total leakage was measured after incubating leaf samples in sterile distilled water at 95°C for 30 min and allowing the samples to cool to room temperature. Throughout the experiment, care was taken to avoid mechanical damage to the leaves as this could influence electrolyte leakage readings. It was assumed that leakage after heating the leaf samples represents total leakage. Dehydration damage, expressed as a percentage was calculated using the following equation:

% electrolyte leakage = [(Initial electrolyte leakage) / (Final electrolyte leakage)] *100

The percentage electrolyte leakage was normalised by dividing resulting values by the dry weights calculated for the respective leaf samples.

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5.2.5 Malondialdehyde assay

The assay for malondialdehyde (MDA) was used to determine lipid peroxidation in plant tissues. This assay is used to measure thiobarbituric acid-reactive substances (TBARS). The method of Heath and Packer (1968) was used with modifications. Pre-weighed plant tissue was ground in 1 ml sterile distilled water in an eppendorf tube. The homogenate was mixed with an equal volume of 0.5% 2-thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The solution was heated at 95°C for 30 min and the solids were removed by centrifugation at 16 000 x *g* for 30 min. Absorbance readings of

the supernatant were taken at 532 and 600 nm. The extract was then passed through a Phenomenonex Sep-Pak C18 reversed phase cartridge (Phenomenonex**,** USA) to remove contaminating pigments. Absorbance was again measured at 532 and 600 nm. MDA content was calculated using the equations below.

$$\text{MDA (mM g}^{-1}\text{ FW)} = [((\lambda_{532} - \lambda_{600}) / 155)* \text{ volume of extract}] / \text{g FW}$$

where 155 $mM^{-1}cm^{-1}$ is the extinction coefficient of MDA.

5.2.6 Pigment content after stress

Chlorophylls were extracted from leaves using 100% acetone in the dark and the absorbencies at 470, 644.8 and 661.6 nm were read using a Beckman DU-64 spectrophotometer (Beckman, USA). Total chlorophyll and carotenoid content were calculated using the formulae below (Lichtenthaler, 1987). Pigment contents were expressed as milligrams per millilitre per gram fresh weight.

$$\text{Chl a} = (11.24* \lambda_{661.6}) - (2.04*\lambda_{644.8})$$

$$\text{Chl b} = (20.13* \lambda_{644.8}) - (4.19* \lambda_{661.6})$$

$$\text{Chl a + b} = (7.05* \lambda_{661.6}) + (18.09* \lambda_{644.8})$$

$$\text{Carotenoids} = ((1000* \lambda_{470}) - (1.9* \text{Chl a}) - (63.14* \text{Chl b})) / 214$$

Anthocyanins were extracted in the dark in acidified methanol (79:20:1, methanol: H_2O: HCl) and absorbencies were measured at 530 and 657 nm using a Beckman DU-64 spectrophotometer. The anthocyanin content was calculated using the formula by Sims and Gamon (2002).

$$\text{Anthocyanin content} = \lambda_{530} - \frac{(\lambda_{657})}{3}$$

5.2.7 Photosynthetic efficiency measurements

The quantum efficiency of photosystem II (F_v/F_m) and the rate of photosynthesis were determined using a LI-6400 Portable Photosynthesis System (LI-COR Biosciences

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Inc., USA). Observations were made using the Leaf Chamber Fluorometer and the "Flr Kinetic" auto-program that allowed combining fluorescence and gas exchange measurements over time. The Arabidopsis plants were dark-adapted for 30 min prior to chlorophyll fluorescence measurements using detached leaves.

5.2.8 Seed yields

Following a specified period of dehydration, watering was resumed as before. To assess the effect of stress on the development of surviving plants, the seed yield was measured after the plants had senesced and dried. Plants were harvested in groups of three to six plants. The seeds were collected and weighed after contaminating plant materials had been removed. The yield was expressed as grams seed per plant.

5.2.9 Photography

Photographs of seedlings in tissue culture and soil-grown plants were taken with an Olympus C-770 Ultra zoom (Olympus America Inc., USA) digital camera.

5.2.10 Measurement of the soil moisture content

Soil moisture contents for the plants under progressive dehydration were determined using a ThetaProbe ML2 soil moisture meter (Delta-T Devices Ltd., UK). Since the measurement was destructive to the soil structure, only one measurement was taken for each plant during the course of the experiment.

5.2.11 Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 5.00 for Windows (GraphPad, USA, www.graphpad.com). Two-way analysis of variance (ANOVA) was used to test any significant differences between the different lines on various levels of stress over time. Bonferroni posttests were used to compare the response of each line with that of the WT. One-way ANOVA was used to test any significant differences between parameters where time was not used as a variable. Tukey posttests were utilised for pairwise comparisons between all the lines.

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5.3 RESULTS

5.3.1 Assessing the tolerance of seedlings in tissue culture

To investigate whether constitutive overexpression of *XvAld1* in transgenic plants had an effect on tolerance to abiotic stresses, seedlings were tested in tissue culture. Wu et al. (1996) demonstrated that root elongation can be used as an accurate measure of the growth rate of *A. thaliana* seedlings. Osmotic stress was imposed by growing six-day old seedlings on PNA containing 0, 50, 75, 100, and 150 mM mannitol or on 14% (w/v) PEG 8000. These experiments were repeated at least three times. Higher osmotic stress conditions (200, 300 and 400 mM mannitol) were not repeated because there was little growth over the time course of stress. Seedlings that showed no elongation during the first day or two were considered to be damaged and were not included in the growth rate calculations. A comparative analysis of growth on reduced water availability was made against the growth of well-watered as well as WT and VT controls. However, VT plants were usually smaller than the rest of the seedlings, making it difficult to use them for comparative studies.

The phenotype of WT and transgenic plants under control and osmotic stress conditions after day ten of exposure is shown in Figure 5.1. Both transgenic and WT plants showed vigorous growth under normal conditions (PNA, Panel A) and the roots grew to the bottom of the vertical plates. Under osmotic stress, the roots of transgenic seedlings grew longer than those of the WT (Panels B-E). The shoots of the transgenic plants were also bigger compared to the WT, up to 100 mM mannitol. To gain more insight into the growth patterns of the seedlings under stress, growth curves for root length against time were used as a quantitative representation over the course of the experiments (Fig. 5.2). In addition, growth rates were determined by calculating the slope of the curves using the linear regression tool in GraphPad Prism. The growth rates are indicated as mean and standard deviation below the respective growth curves (Fig. 5.2). Under normal growth conditions, the roots of WT seedlings grew faster than the seedlings from transgenic lines Xv7 and Xv8. The growth rate of WT seedlings grown on PNA containing 50 mM mannitol was greatly reduced (mean 0.32 compared to at least 0.69 for the transgenic plants). The root lengths achieved by the WT plants after ten days on 50 mM mannitol were approximately half those of the transgenic seedlings. In

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addition, the WT shoots were also much smaller compared to all the transgenic seedlings (Panel B; Fig 5.1). The superior shoot and root sizes of the transgenic seedlings were maintained during osmotic stress up to 100 mM mannitol. However, the growth advantage of the transgenic seedlings in terms of shoot and root sizes as well as growth rates was reduced as the mannitol concentration increased. On 150 mM mannitol, there was no difference in shoot size and the differences in root lengths were reduced. However, the growth rates of WT seedlings were lower (mean 0.17) than the transgenic seedlings (0.27 - 0.41; Fig. 5.2). The growth curves show that transgenic line Xv8 performed worse than the other two lines (Xv7 and Xv12). All the transgenic lines exhibited growth rates on stress media that were significantly different from the WT (Fig. 5.2). Significant differences in growth trends between WT and transgenic seedlings were visible from as early as three days on stress media (Fig. 5.2; see Appendix E for detailed posttests results). After four days on PNA supplemented with 14% (w/v) PEG 8000, WT seedlings stopped growing, as judged by changes in root lengths over time (Fig. 5.2) and the slope of the growth curve was almost parallel to the x-axis. The growth of line Xv8 seedlings also slowed down and had almost ceased by day seven. In contrast, line Xv12 continued to grow at a consistent rate. Comparison of the growth rates showed that on average the growth of WT seedlings was half that of Xv8 (mean 0.15 versus 0.29, respectively). On the other hand, the growth rate of Xv12 seedlings (mean 0.65) was more than four times that of the WT.

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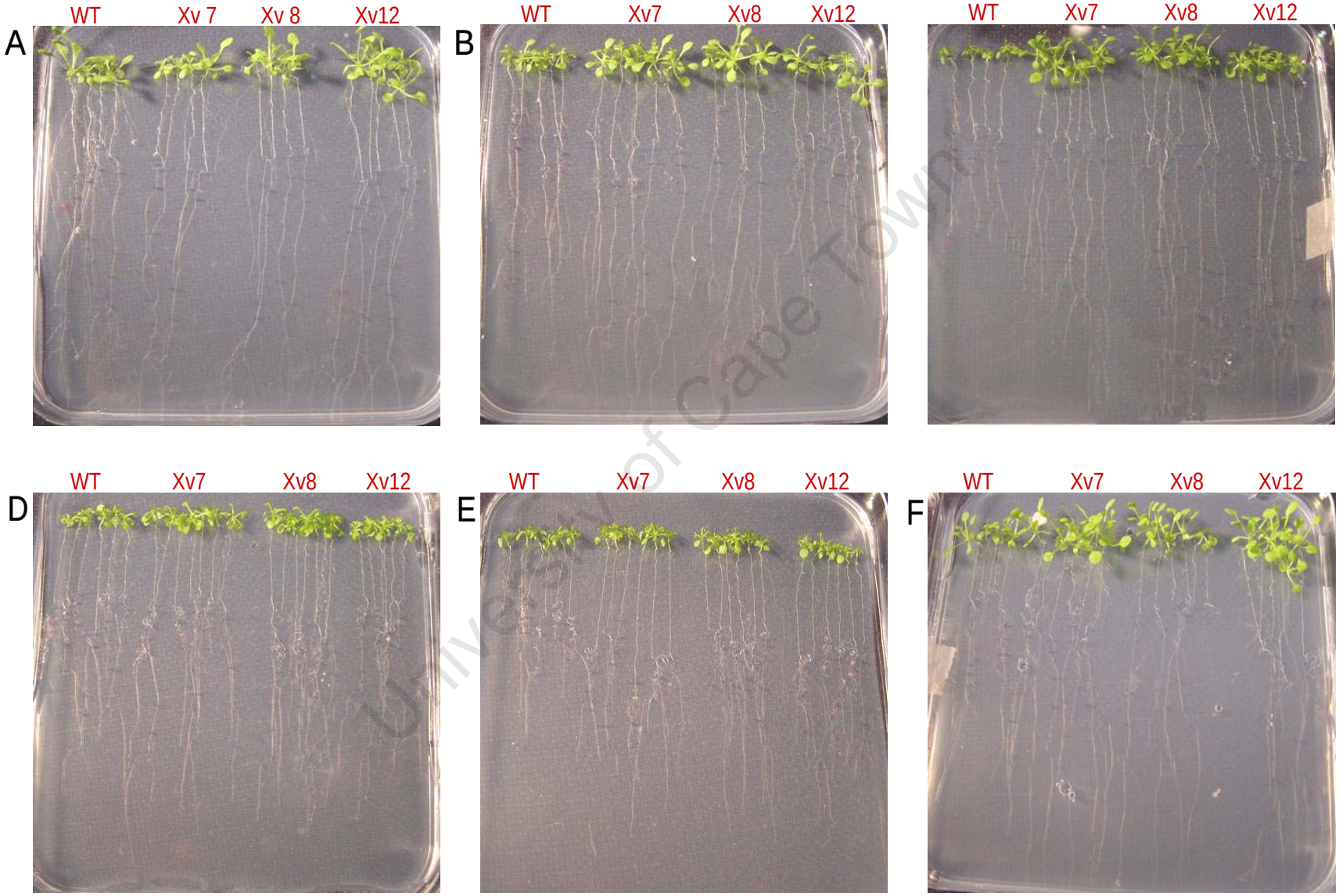
Figure 5.1 The phenotype of *A. thaliana* seedlings under osmotic stress
Six-day old seedlings were transferred onto PNA supplemented with different concentrations of osmoticum. **A**, PNA (normal growth conditions); **B** - **E**, PNA supplemented with 50, 75, 100 and 150 mM mannitol respectively; **F**, PNA supplemented with 14% (w/v) PEG 8000. The plates were photographed after ten days on stress media.



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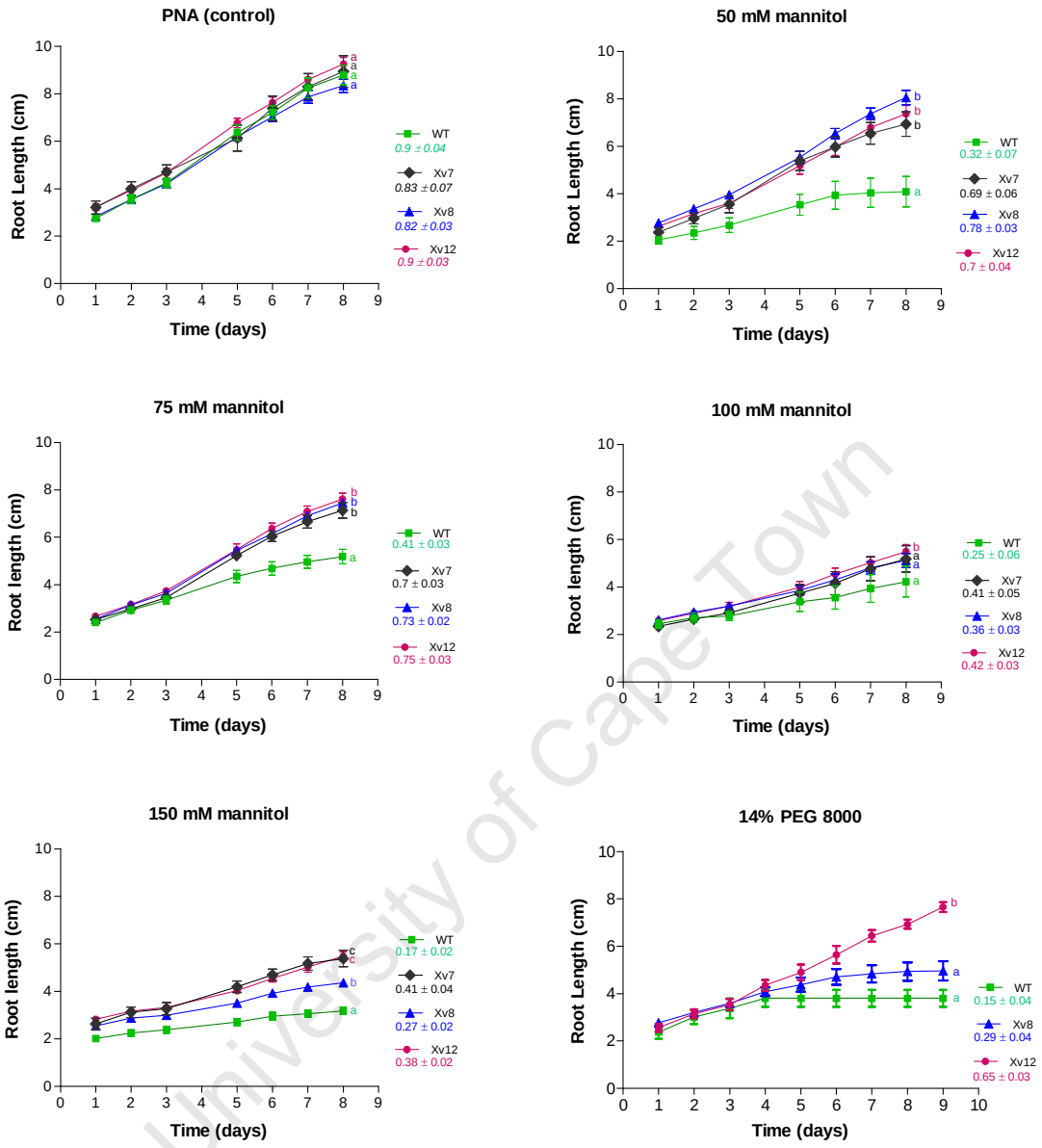

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Figure 5.2 Growth curves for *A. thaliana* seedlings under osmotic stress
The root lengths of seedlings were measured over the course of the experiment. Growth curves were plotted for root length against time in days for the indicated osmoticum concentrations. The growth patterns for transgenic lines Xv7, Xv8, and Xv12 were compared with that of untransformed *A. thaliana* (WT). The plotted values are means and standard deviations of measurements that were pooled from at least three experiments consisting of $4 < n \leq 8$ for the WT seedlings and $6 \leq n \leq 12$ for the transgenic seedlings. Two-way ANOVA with Benferroni posttests was used to assess differences in growth at the end of the experiment. Means with different letters represent significant differences at $P < 0.05$. Detailed statistical analyses over the whole time course are outlined in Appendix E. Growth rates were estimated using the linear regression tool in GraphPad Prism. The calculated values are indicated below the respective legend keys.

Salt stress was imposed by transplanting seedlings onto PNA supplemented with 0, 50, 75 and 100 mM NaCl. When no stress was imposed, there was no difference in growth between the WT and transgenic seedlings (Fig. 5.3A). There was also little difference between the root lengths of seedlings on PNA compared to those on 50 mM NaCl while the effect on shoot growth was discernible (Fig. 5.3, Panels A and B). In addition, the growth curves of WT seedlings were similar to those of the transgenic lines on both PNA and 50 mM NaCl for the first six to seven days (Fig. 5.4). On 75 mM NaCl, the plants started to show signs of reduced growth and chlorosis, possibly due to sodium ion toxicity. Both shoot and root growths were inhibited. Chlorosis was evident on the leaves, anthocyanins accumulated in some of the leaves and new leaves stopped emerging. On 125 mM salt, WT seedlings performed better than transgenic line Xv14, while line Xv8 performed better than the rest (Fig. 5.4). At 150 mM NaCl, root growth for all the plants stopped within the first few days of stress. Growth rates calculated using the slopes of the growth curves also showed that the growth of transgenic seedlings was superior to that of the WT. The exception was line Xv14 on 125 mM NaCl.

Figure 5.3 Phenotype of transgenic seedlings under salt stress
Six-day old seedlings were transplanted from PNA plates to PNA supplemented with 0, 50, 75, 100, 125, 150 mM NaCl; plates **A** - **F** respectively. Pictures were taken after ten days on stress medium.

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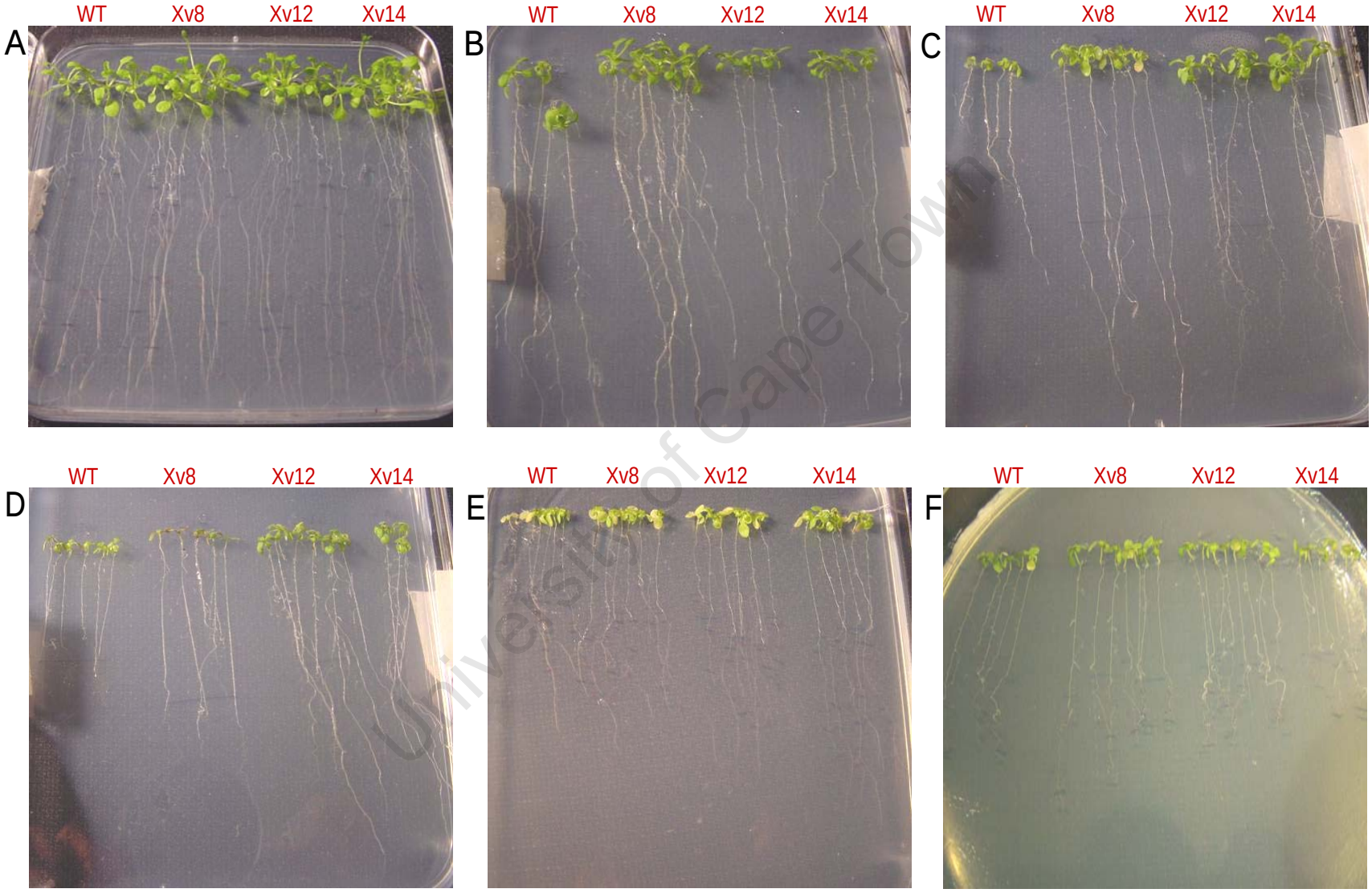

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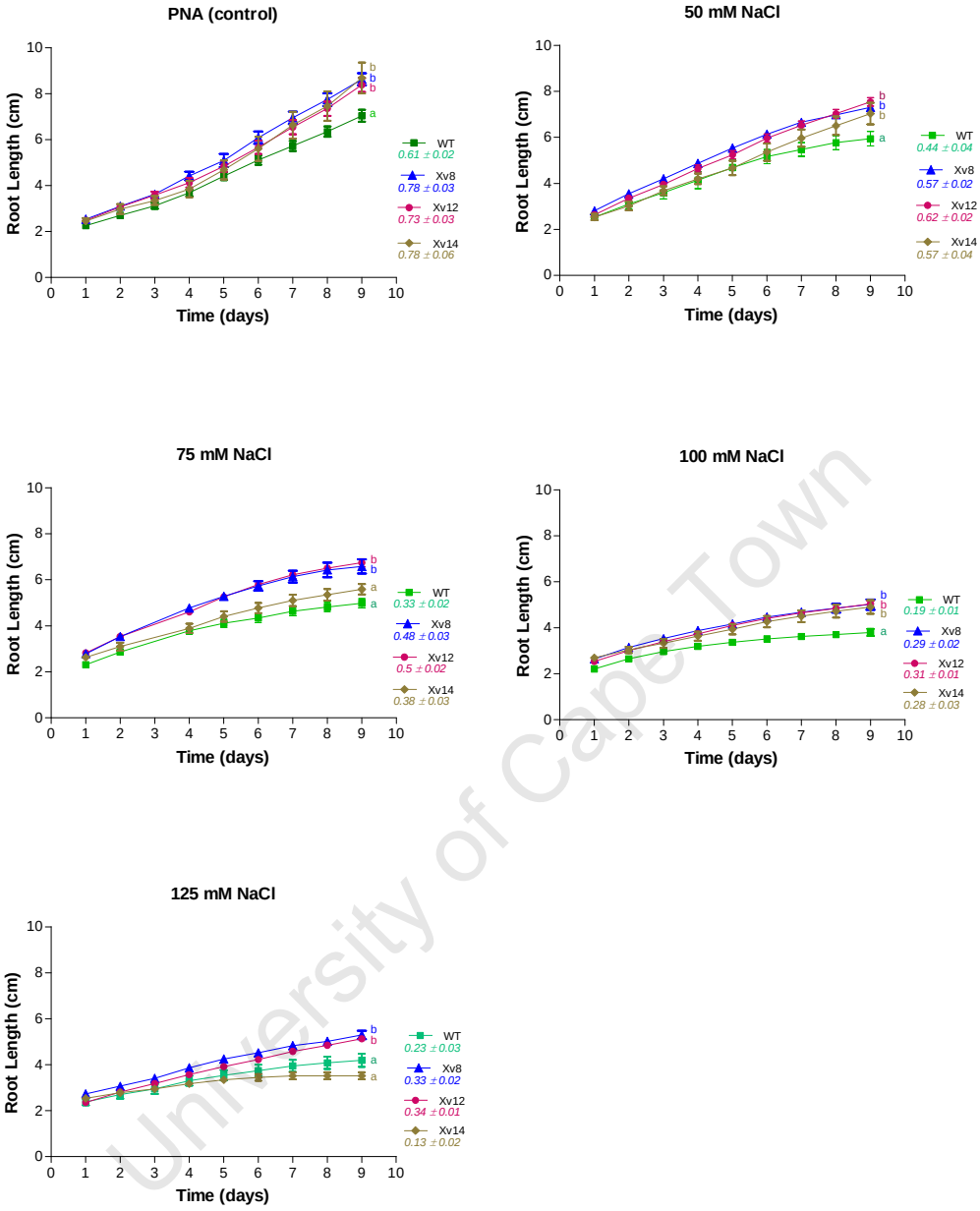

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Figure 5.4 Growth curves for *A. thaliana* seedlings under salt (NaCl) stress
The root lengths of seedlings on media containing salt were monitored daily. The growth curves were plotted for root length against time for the indicated NaCl concentrations. The growth patterns for transgenic lines Xv8, Xv12 and Xv14 were compared with that of untransformed *A. thaliana* (WT). The plotted values were means and standard deviations for $4 < n \leq 12$ for the WT seedlings and $6 \leq n \leq 12$ for the transgenic seedlings. Two-way ANOVA with Benferroni posttests was used to assess differences in growth at the end of the experiment. Means with different letters represent significant differences at $P < 0.05$. Detailed statistical analyses over the whole time course are outlined in Appendix E. Growth rates were estimated using the linear regression tool in GraphPad Prism. The calculated values are indicated below the respective legend keys.

The calculated means and standard deviations of relative root lengths showed variability within the same experiment and between repeated experiments. To enable a comparative analysis of the growth statistics, box plots were used to summarise the spread of the data obtained. Considerable variability in percentage increase of root lengths was observed between sibling seedlings and across the different lines. Variations in percent relative growth were also evident between the repeated experiments (shown by the spread of values represented by the box plots). However, the pooled data showed little difference in root elongation between the WT and transgenic plants grown on PNA (Figs. 5.5 and 5.6). The average values for root growth were between 175 and 200 % for all the lines. On 50 mM mannitol, WT seedlings displayed the lowest growth, averaging 150% (Fig. 5.5). However, the range of root length increase observed for the WT seedlings was wide, ranging from 50 to 225%. More than half of the WT seedlings attained root lengths comparable to those of the transgenic lines. All the transgenic lines tested showed a clear advantage over the WT plants when grown on 75 mM mannitol. More than 75% of transgenic plants performed better than the rest of the WT plants. On 100 and 150 mM mannitol, the growth advantage seen at 75 mM NaCl in the transgenic plants compared to the WT was reduced. The percentage relative growth was also considerably reduced in all the lines. Root growth on 150 mM mannitol ranged between 50 and 75% for the WT seedlings while values for the transgenic plants were largely above 75%, except for line Xv8 which showed values between 50 and 100%. Line Xv7 was the best performer, with most of the growth rates between 75 and 150%. On 14% (w/v) PEG 8000, WT seedlings gained not more than 75% of the initial root lengths and line Xv8 grew by up to 200%. The performance of the WT seedlings was comparable to the growth rates of the lower quartile seedlings in line Xv8. The growth of line Xv12 on PEG was as high as 275%. Most of the line Xv12 seedlings performed better than the upper quartile of line Xv8. In fact, the percentage relative growths of line Xv12 seedlings was comparable to that seen in plants under normal growth conditions (PNA).

On saline media, the transgenic plants also consistently showed less growth inhibition compared to the wild type. On average, similar growth on PNA was observed for all the lines. Wild type seedlings exhibited growth inhibition on 50 mM NaCl, with percentage root elongation falling below 150% compared to 200% average on PNA. The

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growth inhibition observed on transgenic seedlings was less severe. The majority of the roots grew by more than 150% of the initial lengths. The transgenic lines were generally more tolerant to osmotic stress than salinity. Nonetheless, the performance of transgenic lines was significantly better only on 100 mM NaCl when compared to the WT.

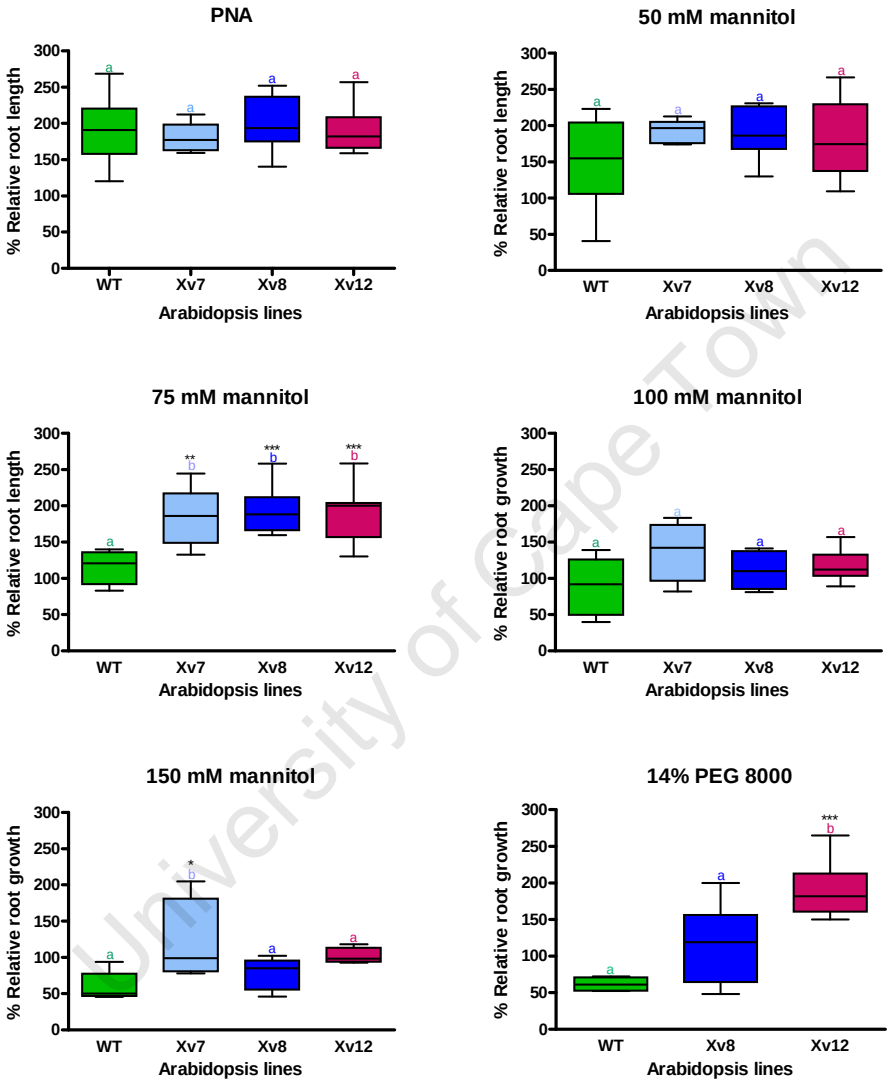

Figure 5.5 Comparison of seedling growth on osmotic stress
The relative root lengths from at least three experiments were used for a comparative analysis of seedling growth on the different levels of osmoticum. For each of the statistics, 50% of the growth rate values lie within the box. The horizontal line inside the box represents the median values. The vertical lines outside the box represent the upper and lower quartile ranges, with the respective horizontal bars showing the highest and lowest values obtained. One-way ANOVA with Tukey posttests was used to assess differences in mean relative growth. Means with different letters represent significant differences at $^{*}P < 0.05$; $^{**}P < 0.01$ or $^{***}P < 0.001$.

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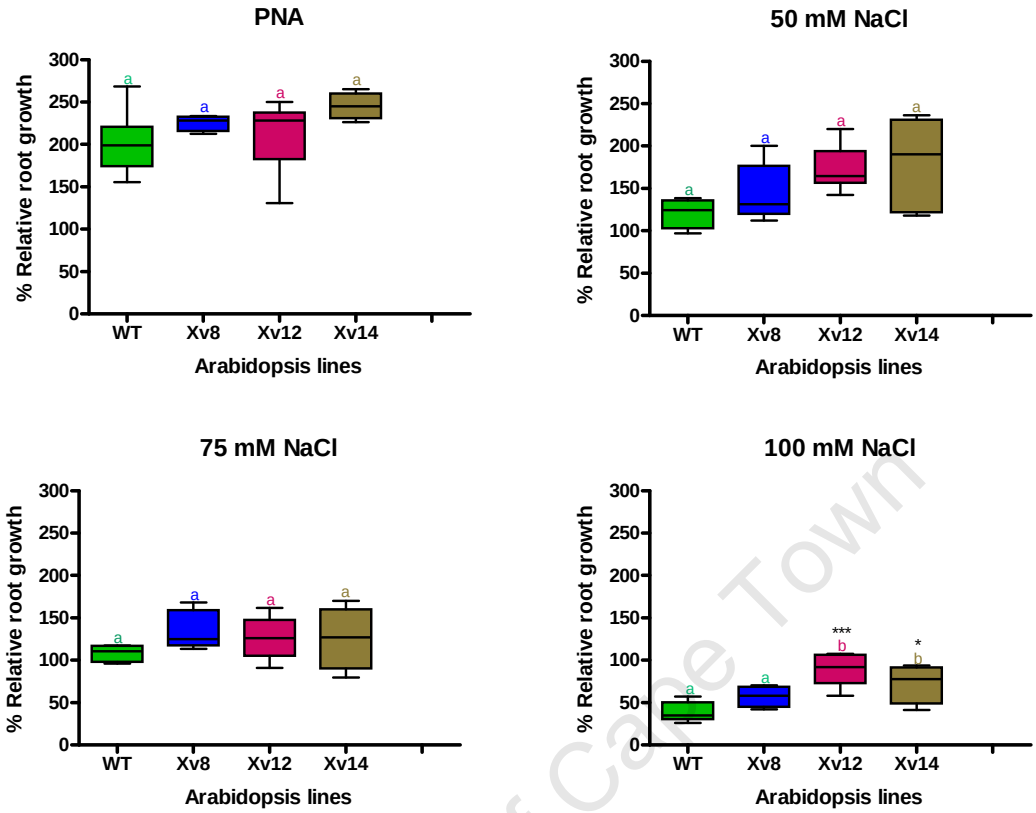

Figure 5.6 Comparison of seedling growth on salt
Box plots were used to represent the range of seedling growth determined from at least three experiments. Fifty-percent of the values are contained within the box. The upper and lower quartile ranges flank the box on either side. One-way ANOVA with Tukey posttests was used to assess differences in mean relative growth. Means with different letters represent significant differences at $^{*}P < 0.05$ or $^{***}P < 0.001$.

Yellow pigmentation was observed in the leaves of some of the seedlings grown on NaCl. To determine if the yellowing of leaves was due to chlorosis or the accumulation of carotenoids and/ or anthocyanins, the pigment content of the shoots was determined. Anthocyanins accumulated only when seedlings were exposed to more than 50 mM NaCl (Fig. 5.7). The anthocyanin levels progressively increased with increasing salt concentration. The levels of the chlorophylls (a + b) were marginally reduced on exposure to 50 mM salt and showed a corresponding decrease with increasing NaCl concentrations. On 100 mM NaCl, the chlorophyll levels had fallen from an average of ~900 mg/ml/g fresh weight, to as low as 350 mg/ml/g. The carotenoids were generally unaffected by the salt stress, demonstrating that yellowing was a result of chlorosis.

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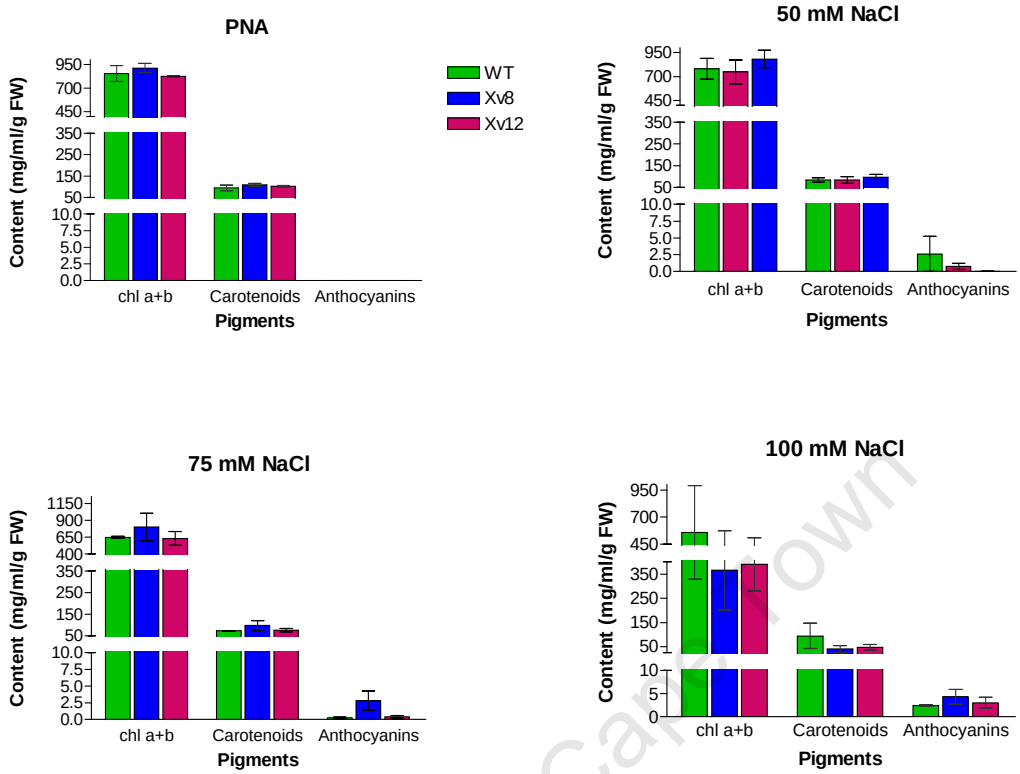

Figure 5.7 Accumulation of pigments in seedlings under salt stress
Photosynthetic pigments were extracted from the shoots of seedling that had been exposed to the indicated salt concentrations for ten days.

5.3.2 Stress tolerance of potted plants under dehydration

To evaluate stress tolerance in mature plants, dehydration stress was imposed on soil-grown plants at the beginning of the reproductive stage. After six days of dehydration, VT plants were severely wilted (Fig. 5.8) and none survived rewatering (not shown). Ten percent of the WT and 30 - 50% of transgenic lines survived the dehydration. Plants that were neither wilted nor dry were regarded as exhibiting a potential drought tolerant/ resistant phenotype. Survival was scored as the percentage of plants showing the tolerance phenotype. To further characterise the indicators of drought tolerance, leaf samples collected during the course of the progressive dehydration were used to determine the drying rates and the membrane integrity. Preliminary studies showed that plants did not recover from RWC below ~45%. As a result, biochemical analyses were not performed on samples that had dried beyond 50% RWC.

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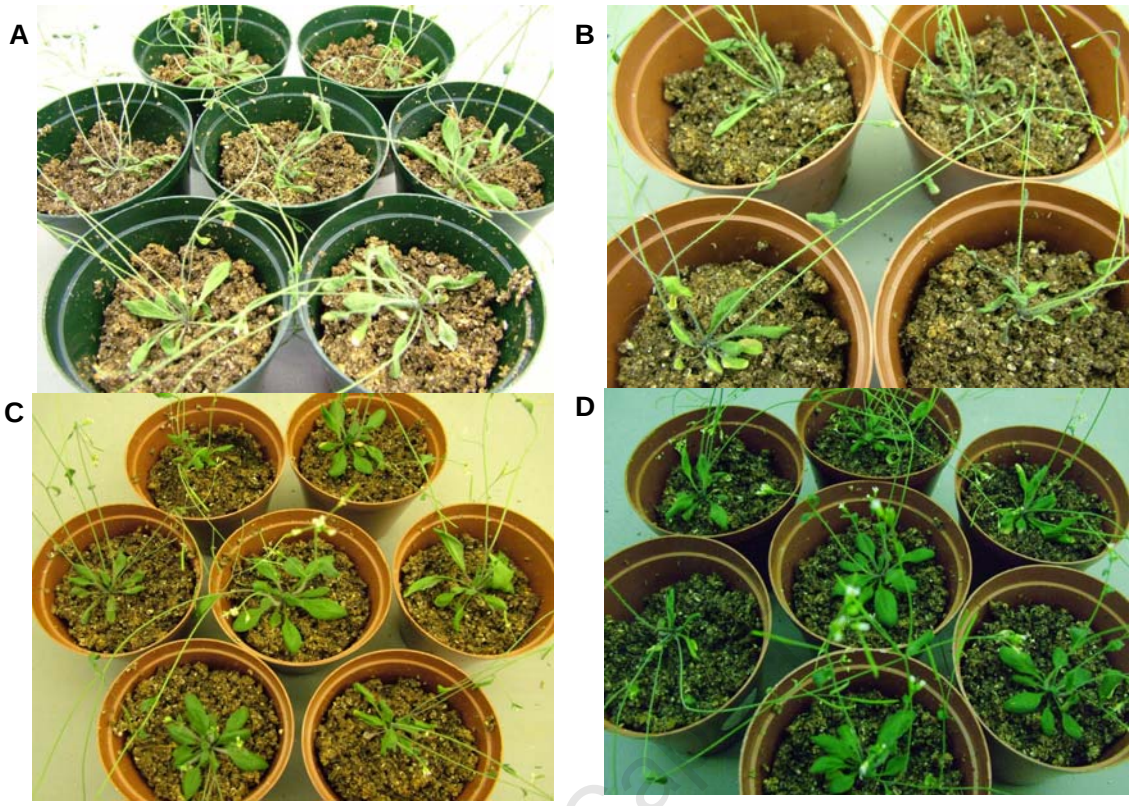

Figure 5.8 Phenotype of potted *A. thaliana* dehydrated for six days
Control (WT and VT) and transgenic *A. thaliana* seeds were stratified at 4°C for four days to synchronise germination. The seeds were sown in a 1:1 mixture of vermiculite and jiffy peat. The plants were kept in a plant growth room at 26 - 28°C and variable relative humidity. The plants were grown under normal watering regime (every third day) for 30 days (stage 6.0; Boyes et al., 2001). Watering was then withheld for six days. One tenth (3/30) of the WT plants (**A**), none (0/15) of the VT plants (**B**), 50% (18/36) of transgenic line Xv8 (**C**) and 30% (11/36) of transgenic line Xv12 (**D**) displayed the tolerance phenotype after six days of stress.

Well-watered plants displayed average RWC of between 70 and 85% (Fig. 5.9A). The *XvAld1* overexpressing transgenic plants and the VT controls had higher water content than WT after two days of dehydration. By day four, the RWC of all the lines had started to drop. The VT line displayed the largest water loss (between 40 and 50% RWC) and the plants had started to wilt. RWC of the WT plants were also comparatively lower than that of the transgenic lines. All the VT plants had wilted by day six of dehydration and the plants had wilted when RWC as low as 35 - 50% were reached. The transgenic lines exhibited the highest water contents, averaging more than 40%, demonstrating that these plants were better able to resist water loss. Under normal growth conditions, wild type

plants displayed half the electrolyte leakage observed in the transgenic plants (Fig. 5.9B). Line Xv8 tended to show high levels of leakage compared to the other transgenic lines at four and six says of dehydration. Line Xv12 showed remarkably low leakage at day four of dehydration. Despite the large variation in the data, the WT plants still displayed the highest electrolyte leakage when exposed to dehydration. A day after the plants were rehydrated, the electrolyte leakage values for the transgenic plants had fallen to the levels seen in control plants before stress imposition but those for the WT were still comparatively high.

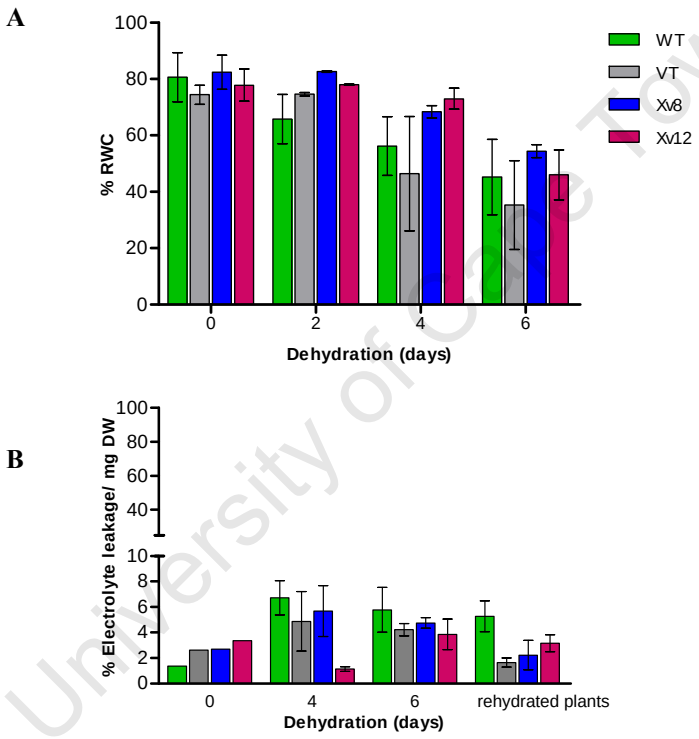

Figure 5.9 The effect of dehydration on leaf water content and membrane integrity
Leaf samples were collected and used to determine RWC (**A**) and electrolyte leakage (**B**). The values were calculated from mean ± standard error (SE) of three experiments. Electrolyte leakages of rehydrated plants were measured a day after watering was resumed (day 7).

To determine whether the stress tolerance could be due to differences in soil drying rates, the soil moisture content was also monitored during the course of the dehydration (Fig. 5.10). The water holding capacities of the soils were comparable, as

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shown by the similar values for wet soils (day 1). As the dehydration progressed, the soil moisture percentages became more variable among sibling plants but showed little difference across the lines. The exception was seen in soil from line VT, which showed the lowest soil water content on day 5. By day six, the readings for more than half of the samples taken were below the detection range of the soil moisture probe (data not shown). Some transgenic lines still exhibited the stress tolerance phenotype under these conditions, demonstrating that these plants could prevent water loss despite the depletion in soil water content.

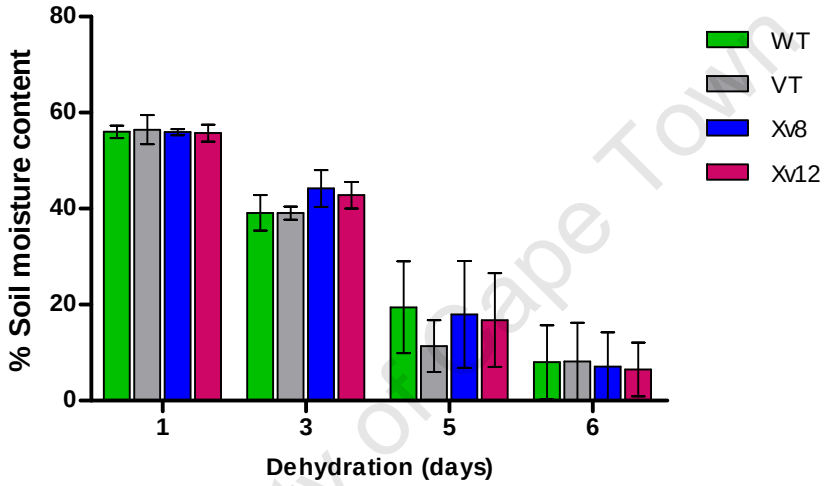

Figure 5.10 The moisture contents of the soil under dehydration
The soil moisture contents for the plants under dehydration were measured using the ThetaProbe ML2 soil moisture meter. Readings for day one were taken after water-saturated soils that had been left to drain for two hours. The values represent mean ± sd where $n = 2$ for wet soils (day 1) and $4 \leq n \leq 6$ for the rest of the data.

Since the temperatures used for fast drying were not the normal growth conditions used for Arabidopsis, another set of dehydration experiments was carried out at 22°C. The duration of the dehydration was also extended to ten days. Prior to the start of stress treatments, there were no phenotypic difference between the WT and the transgenic plants (Fig. 5.11) except that transgenic line Xv8 plants generally had broader leaves compared to the rest (not obvious in the picture). Vector transformed plants were generally smaller in size compared to the rest of the lines. VT plants that appeared similar to the other lines were selected for the dehydration experiments.

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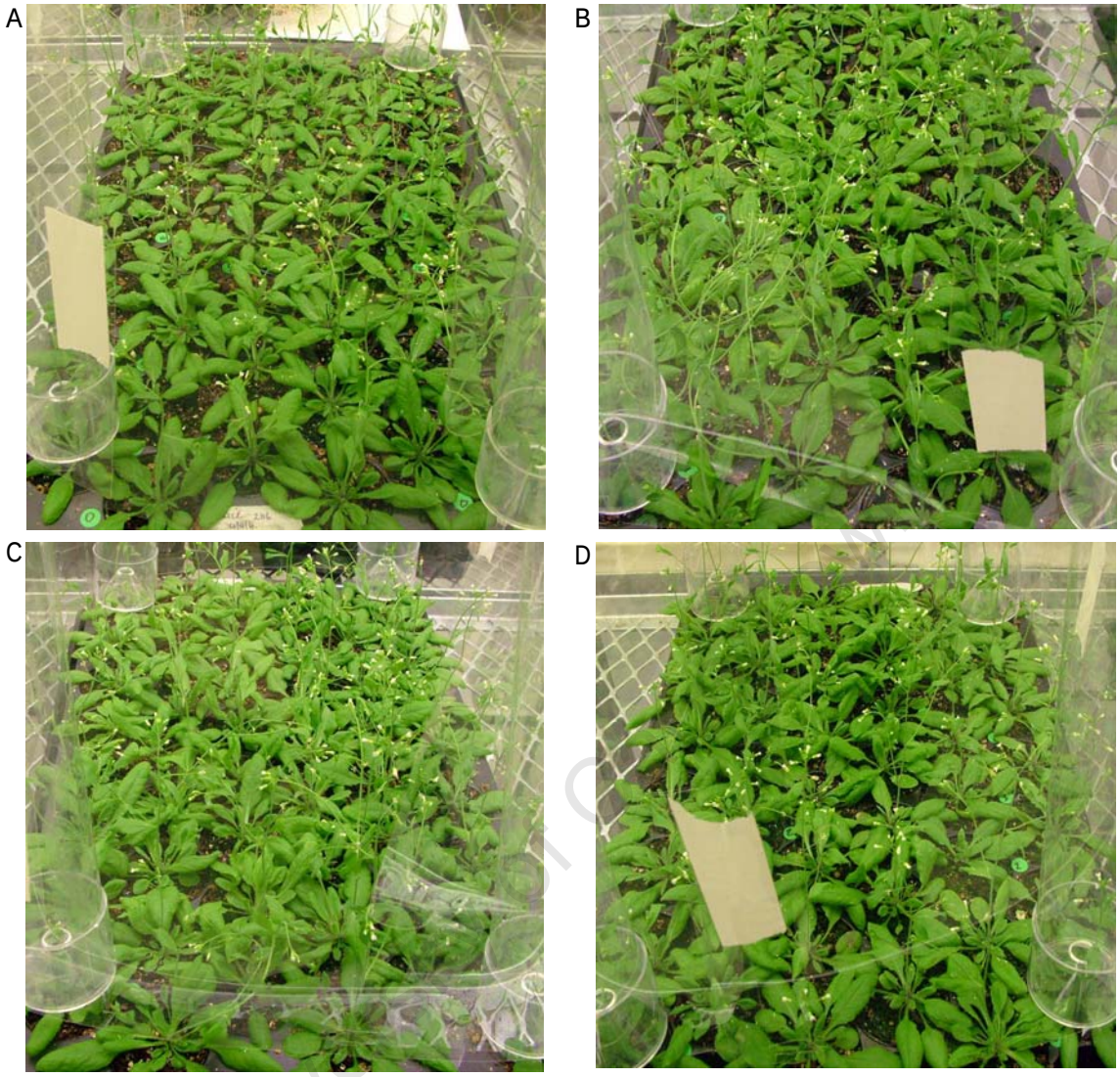

Figure 5.11 Thirty-day old *A. thaliana* plants before exposure to dehydration
The plants were maintained in a plant growth room at 22°C, 50% relative humidity and watered every third day for thirty days. **A**, two rows VT and four rows WT plants; **B**, transgenic line Xv8; **C**, transgenic line Xv12; and **D**, three rows each of transgenic lines Xv7 and Xv34.

After twelve days of dehydration, the stressed plants were photographed (Fig. 5.12) to document phenotypic variations. The percentage of plants exhibiting the drought tolerant/ resistant phenotype was recorded. Most of the WT and VT plants had turned brown by day twelve (Panel A). The WT and VT plants exhibited the least percentage of plants showing the tolerance/ resistance phenotype; 32 and 29%, respectively. In contrast, the majority of the transgenic plants overexpressing *XvAld1* were still green. The percentage

of transgenic lines exhibiting the phenotype varied between forty-six and seventy (Panels B to D).

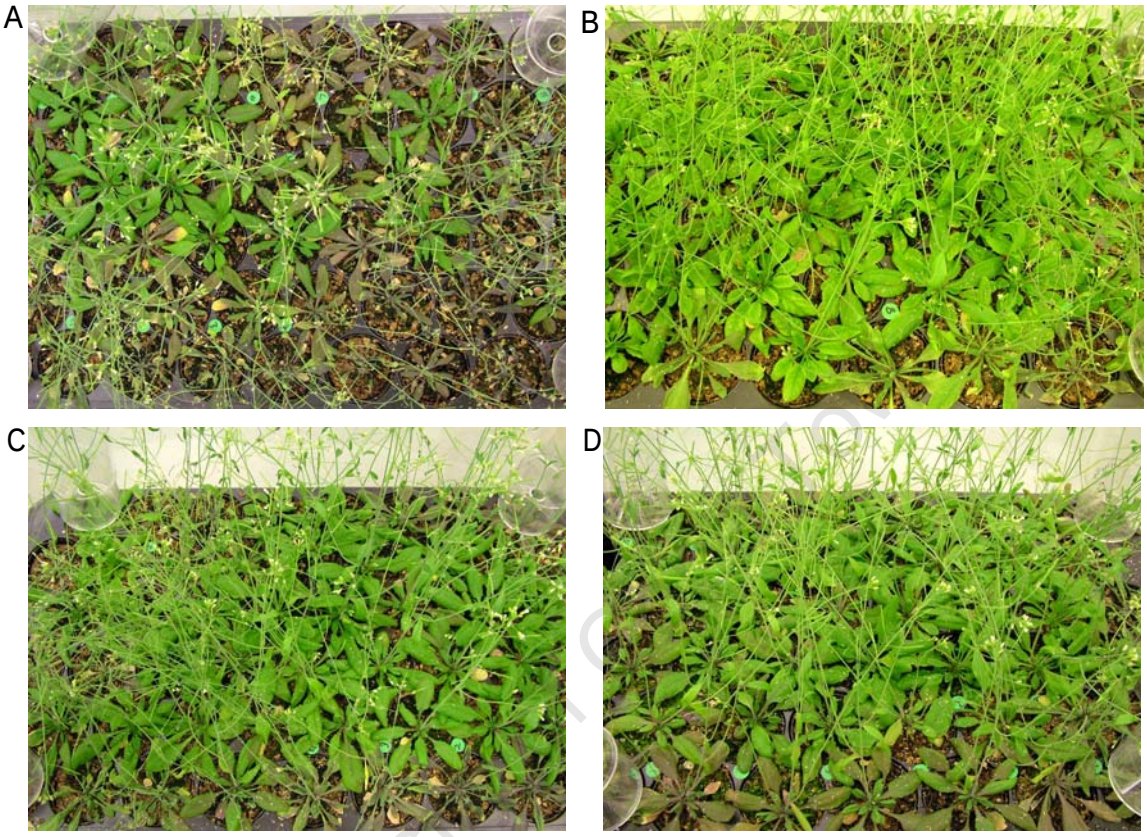

Figure 5.12 Phenotype of control and transgenic *A. thaliana* at day 10 of dehydration
Dehydration stress was imposed on stage 6 plants by withholding water. A day before the stress, the trays were filled with water and the soil imbibed water overnight. The next day (day 30), all trays were emptied of water and turned upside-down. On day ten of stress; 32% of WT plants and 29% of VT (**A**); 61% of transgenic line Xv8 (**B**); 46% of line Xv12 (**C**); 61% and 70% of lines Xv7 and Xv34 (**D**) respectively, exhibited the drought-tolerance phenotype. The rest of the plants had either wilted or dried.

The course of the dehydration was monitored by measuring leaf water potential and RWC of samples collected from stressed plants. In well-watered soils, the RWC of all the plants ranged from ~75 to 90% (Fig. 5.13A). The WT plants showed the lowest range of RWC at full turgor, but this difference was not significant. By day four of stress, the RWC for VT and Xv12 had decreased marginally. However, the water contents in WT and Xv8 leaves had increased to ~90% average, showing that the plants were

resisting water loss. Despite the differences in survival rates, the RWC of samples taken on day ten were similar, averaging ~75% for all the lines. The RWC of the surviving plants, determined three days after resumption of watering (day 13), were also comparable.

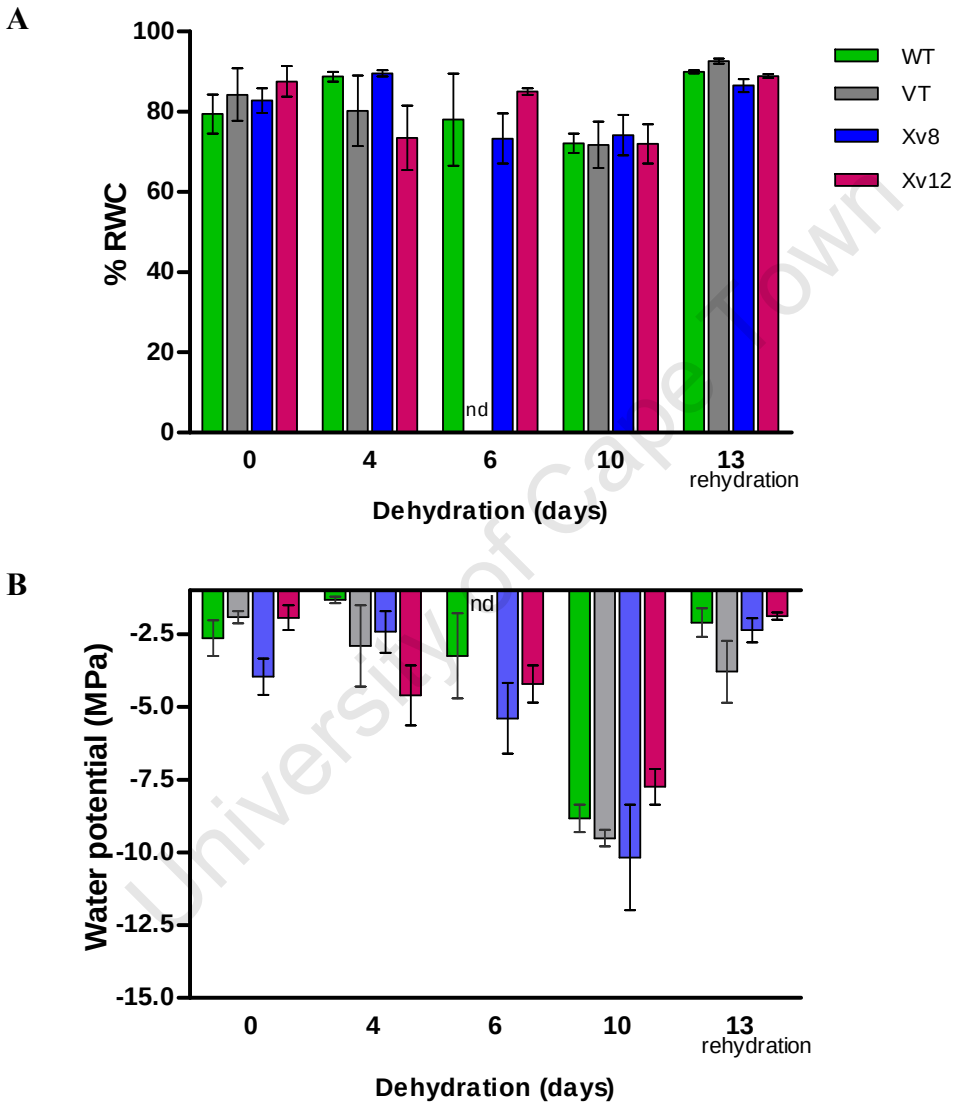

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Figure 5.13 The leaf RWC (**A**) and the corresponding water potentials (**B**) of plants under dehydration. Leaf samples were taken on the indicated days and at every time point, the same leaf samples were used for both determinations. Water potentials were calculated from water activity (a_w) readings. Day thirteen represents the readings made three days after rehydration.

Although little variation was observed for the RWC determinations, the matching leaf water potential calculations were distinctive. Leaf water potentials at full turgor averaged about -2.5 MPa or higher (Fig. 5.13B). However, line Xv8 exhibited lower water potentials, with the lowest calculations almost reaching -5 MPa. As with the RWC results, the water potential values also indicated that the plants were resisting water loss. The water potentials for the WT and Xv8 were increased after mild dehydration (day 4). By day six the water potentials had dropped below -5 MPa and line Xv8 exhibited values below -12 MPa on day ten. Line Xv12 showed relatively lower water potentials at day four of stress. The rest of the values for line Xv12 were higher than those for line Xv8. Resumption of watering raised the water potential values to the levels observed in well-watered plants, except VT plants that seemed to revive slowly.

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) levels in plant tissues. Baseline levels were determined in pooled samples of well-watered plants (untreated; Fig. 5.14). MDA levels in stressed transgenic plants overexpressiong *XvAld1* were low; generally less than double the concentrations in unstressed plants. In contrast, dehydrated WT and VT plants had significantly increased TBA-reactive species (as much as six times the baseline levels).

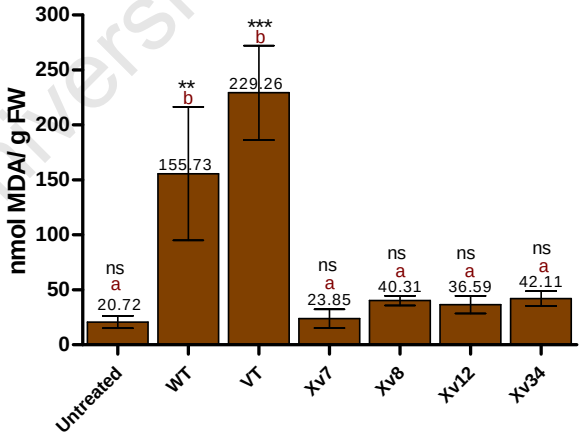

Figure 5.14 MDA accumulation in plants after 10 days of dehydration
The data represents mean and standard deviation values for $3 \leq n \leq 6$. The values over the bars represent the arithmetic means. The experiment was repeated three times. One-way ANOVA with Bonferroni's posttests was used to assess differences in mean MDA levels compared to the untreated samples. Means with different letters represent significant differences at $^{**}P < 0.01$ or $^{***}P < 0.001$; 'ns' denotes 'not significant' ($P > 0.05$).

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Under normal growth conditions, the ratio of chlorophyll fluorescence was within the expected range of healthy chloroplast. The quantum efficiency readings obtained for all the Arabidopsis plants were between 0.75 and 0.85. A gradual decrease in fluorescence readings was observed with progressive drying. On day four, readings for all the plants had fallen to between 0.6 and 0.65. By day seven of dehydration, the F_v/F_m ratio had decreased further to average 0.6 for all the lines. There was no marked difference between the readings for the transgenic plants and the controls (WT and VT).

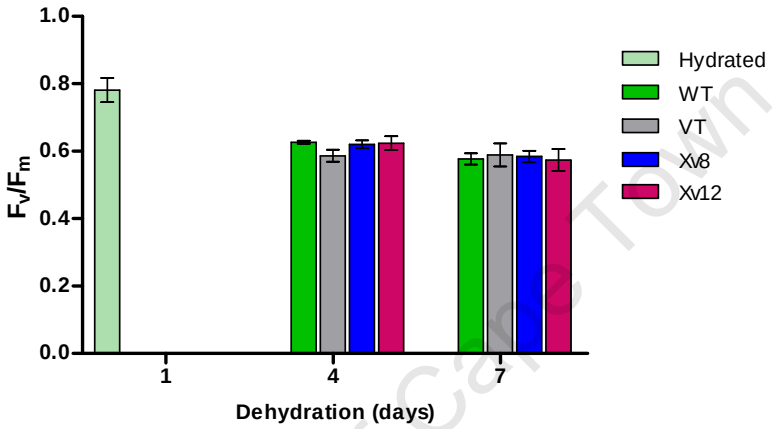

Figure 5.15 Chlorophyll fluorescence
Chlorophyll fluorescence was measured using the Licor 6400 leaf chamber fluorometer on dark adapted leaves. Fluorescence readings for well-watered plants were pooled.

On average, at least fifty percent of the transgenic plants exhibited drought tolerance/ resistance phenotype at day ten of dehydration (Table 5.1). In contrast, 29% of WT and 20% of the VT plants exhibited the same phenotype. Since the ultimate test of survival is the ability to resume healthy growth upon rewatering, survival was used as another measure of stress tolerance. Thus plants were rewatered and seven days after resumption of watering, all the plants that rehydrated or showed any evidence of new growth were scored for the "survival" phenotype. The survival percentages for most of the lines were lower than the percentage of tolerant/ resistant phenotype. These results show that some of the plants that appeared tolerant were actually damaged beyond recovery. There were cases, however, when the percentage of plants that survived was greater than the fraction of plants scored for the drought tolerance/ resistance phenotype (data shown in bold; Table 5.1). This demonstrates that some plants not exhibiting the tolerance phenotype could recover after resumption of watering.

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Table 5.1 Stress tolerance phenotype and survival under drought

Arabidopsis lines	Total number of plants	Phenotype					
		Tolerant/ resistant phenotype			Survival		
		# DTRP	% DTRP	mean ± sd	# survival	% survival	mean ± sd
WT	25	9	36	29.3 ± 0.083	4	16	14.3 ± 0.057
	25	8	32		4	19	
	50	10	20		4	8	
VT	10	2	20	19.7 ± 0.095 **ns**	2	20	18 ± 0.02 **ns**
	17	5	29		3	18	
	50	5	**10**		8	**16**	
Xv7	-	-	-		-	-	
	23	14	61	61 *	10	43	43 *
	-	-	-		-	-	
Xv8	39	24	62	58.7 ± 0.058 **	15	38	46.7 ± 0.081 **
	50	31	62		27	54	
	50	26	52		24	48	
Xv12	38	23	**61**	48.3 ± 0.117 **ns**	24	**64**	47 ± 0.153 **
	50	23	46		22	44	
	50	19	38		17	34	
Xv34	-	-	-	58 ± 0.17 **	-	-	53 ± 0.071 ***
	23	16	70		11	48	
	50	23	**46**		29	**58**	
Xv49	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
	50	19	**38**	38 **ns**	24	**48**	48 *

Watering was withheld from thirty-day old plants for twelve days. DTRP denotes the fraction of plants exhibiting the drought tolerance/ resistance phenotype. On day twelve, watering was resumed. Survival denotes the fraction of plants that revived and showed regrowth seven days after watering was resumed. '-' represents missing data; $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$; 'ns' denotes 'not significant' when compared to the WT.

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Photographs of the recovery phenotype of plants that survived and continued to grow were taken after seven days of the normal watering regime (Fig. 5.16). The surviving WT and VT plants started to accumulate pigments upon rewatering. The plants also showed little evidence of renewed growth, but rather tended to show premature senescence by yellowing and dying sooner than the transgenic plants (data not shown). In contrast, all the transgenic plants displaying the drought tolerance/ resistance phenotype recovered and resumed healthy growth after rehydration. Some of the wilted transgenic plants also revived and continued to grow. The emergence of new leaves and flowers was indicative of continued vegetative growth. These plants showed little evidence of the previous stress such as chlorosis and anthocyanins pigmentation. Transgenic line Xv34 however, accumulated anthocyanins mainly in the midribs of the rosette leaves.

Figure 5.16 The recovery phenotype of plants after resumed watering
The photographs were taken seven days after normal watering regime was resumed. New flowers are visible above the rosette leaves of all the transgenic lines overexpressing *XvAld1*.

To assess the long term effects of stress on reproductive development, the seed yields of plants previously exposed to prolonged dehydration were compared to those of well-watered controls (Fig. 5.17). The best yields under normal growth conditions were obtained for line Xv12 followed by WT. The yields of fast dried transgenic plants only showed a marginal advantage over the WT and VT plants exposed to the same stress. In contrast, when dehydration was imposed slowly, the transgenic plants yielded almost double the seeds harvested from WT plants. The seed yields from VT plants were negligible. When mature plants were irrigated with 100 mM NaCl solution for two weeks, the seed yields were comparable between the WT and VT. Transgenic line Xv12 yielded almost double that of the WT and VT plants. Line Xv8 produced more than four times the seeds harvested from WT and VT. This productivity was similar to that observed for plants grown under the normal watering regime.

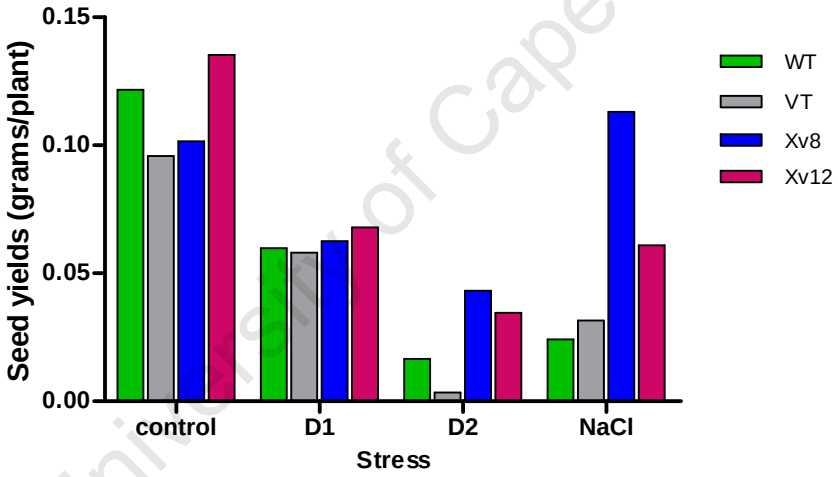

Figure 5.17 Yields of Arabidopsis plants exposed to different stress treatments
Control plants were watered every third day for the whole life cycle. Dehydration stresses were imposed on thirty-day old plants as previously described. D1 and D2 denote plants that were slow dried for six days and ten days, respectively. The salt stressed plants were irrigated every third day with a solution of 100 mM NaCl for two weeks. All the plants were then watered as normal for the rest of the plant life cycle.

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5.4 DISCUSSION

The severity of abiotic stresses is dependent on the plant developmental stage at which the stresses occur. For this reason, abiotic stress tolerance in transgenic plants was assessed on seedlings growing in an in vitro system and on potted mature plants at the reproductive stage. Several methods were used to impose water deficit, and the implications of each method are discussed.

5.4.1 Overexpression of *XvAld1* improves stress tolerance in seedlings

Although the seedlings used were at the same growth stage, root lengths were more variable compared to the shoot sizes. Selecting seedlings of the same root lengths resulted in unnecessary handling of the stems causing bruising and breakage. As a result, expression of growth based on absolute root length could be biased since the rate of root elongation is dependent on plant size. To compensate for the variable root sizes, the growth of each seedling was assessed relative to its length at the beginning of the experiment. Measurement of the growth of root was chosen over that of shoots because the former is favoured under limiting water availability (Hsiao and Xu, 2000). When solidified agar was placed vertically, roots grew on the surface of the medium thus facilitating easy measurement and imaging.

Over the first seven days, the performance of WT lines was similar or better than that of some transgenic lines when grown in the absence of osmotic or salt stresses. Overall, the inhibition of root elongation was more visible in seedlings exposed to salt than to osmotic stress. The differences in growth rates between WT and transgenic plants were statistically significant (Fig. 5.4). However, when relative growth was compared across repeated experiments, statistical differences were observed only on 100 mM NaCl (Fig. 5.6). The inhibition caused by exposure to PEG 8000 was less severe than that on mannitol-containing growth media. However, when considering these differences the fact that 50 mM NaCl has an osmotic pressure similar to that of 100 mM mannitol (Munns 2002) must be taken into account. An interesting phenotype was the cessation of root growth displayed by some seedlings. This phenotype was observed in the WT seedlings at around four days after exposure to 100 mM NaCl or 14% PEG 8000.

These differential responses may be reflective of the specific effects imposed by the various stress agents used. Osmotica such as mannitol and PEG were used to

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impose water deficit on seedlings, mimicking the effects of dehydration. Mannitol is a low molecular weight solute such that it can permeate plant cell walls and accumulate in the apoplasmic space. Permeating solutes may be taken up by the cell, accumulating in the cytoplasm and causing osmotic adjustment that may be beneficial in the short term. However, prolonged exposure to high cytoplasmic levels may be toxic. Such direct contact of membranes with the osmoticum is prevented by using a high molecular weight PEG (Carpita et al., 1979; Verslues et al., 2006). PEG 8000 cannot freely pass through cell walls and therefore can induce water deficit in a manner similar to dry soils without rapidly plasmolysing or accumulating inside the cells. However, this imposition is not gradual, as would happen in drying soils, and transplanted plants are shocked instead of acclimating to the new conditions. The severe growth inhibition observed for plants under mannitol levels of more than 75 mM could be due to specific toxic effects of mannitol accumulation within intercellular regions (Verslues et al., 2006). The inhibition of shoot growth may be an osmotic effect that is specific to mannitol stress and not observed in seedlings cultured on 14% (w/v) PEG 8000. We speculate that the cessation of growth in WT at four days of stress and the considerable growth inhibition in transgenic line Xv8 was a general stress response caused by reduced availability of water. The fact that line Xv12 continued to grow and attain growth rates similar to that achieved in normal growth medium implies acquired osmotic stress tolerance in this transgenic line.

The exposure of seedlings to various concentrations of NaCl most likely resulted in the manifestation of both ionic and osmotic stress effects. Plant responses to salinity are characterised by two phases (Munns, 2002; Verslues et al., 2006). The rapid phase involves osmotic shock and occurs within hours of transfer to stress media. The delayed response is the NaCl poisoning caused by the accumulation of Na^+ ions within the cytoplasm. Our results seem to indicate that the salt specific growth inhibitions were manifested from day four when root elongation in WT seedlings seems to cease. The inhibition of root elongation in the transgenic plants was pronounced when seedlings were exposed to 100 mM NaCl (Fig. 5.4). Low water potentials can cause thinning of roots to facilitate root lengthening (Sharp et al., 1990; van der Weele et al., 2000). Visible root thinning was observed in the presence of high levels of NaCl (75 and 100 mM). However, due to technical limitations, root diameters were not measured. Salt stress also caused chlorosis and accumulation of anthocyanins. However, a quantitative analysis of the pigments showed a similar

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pattern across all the different lines, demonstrating that the change in photosynthetic pigments was a general response to stress (Fig. 5.7). It would be interesting to find out how the accumulation of pigments is affected if the *XvAld1* gene was under the control of its endogenous promoter instead of a constitutive one. Transgenic plants were marginally more tolerant to NaCl exposure compared to the WT. We propose that this tolerance was a result of the protective functions of XvAld1 under osmotic stress. It is unlikely that XvAld1 plays a major role in salinity tolerance because irrigation of *X. viscosa* with 150 mM NaCl (Davis, 2005) or 250 mM NaCl (data not shown**)** did not elicit a response over a 96 hr period. In addition, the *XvAld1* putative promoter did not contain any *cis*-acting response elements for ionic stress. Salt tolerance typically involves three mechanisms; selective exclusion of Na^+ from uptake by roots, extrusion of Na^+ from the cytoplasm by specific pumps and compartmentation of accumulated Na^+ ions in vacuoles (Zhu, 2000; Munns, 2002). None of these mechanisms are in line with the proposed roles of osmolyte molecules or AR enzymes.

Compared to other studies, the overexpression of the *XvAld1* gene appears not to provide substantial improvement to salinity tolerance. Some studies reported survival of Arabidopsis on levels above 200 mM NaCl (Tarczynski et al., 1992; Taji et al., 2004). In this study, tolerance was characterised in seedlings exposed to up to only 100 mM NaCl. Growth was almost completely inhibited in transgenic and control plants grown on media supplemented with 150 mM NaCl. We propose that the protection resulting from *XvAld1* overexpression is not specific to the toxic effects of the Na^+ ions. Rather, the transgenic plants may be protected from the adverse effects of the osmotic environment. As the ion concentrations increase, the osmotic stress tolerance advantage is probably lost. Higher levels of salt (125 and 150 mM NaCl) were not included in the repeated experiments because supplementing PNA with salt interfered with the gelling capacity of the tissue culture agar, making it soft. The agar could be stabilised by increasing the agar concentrations to 0.9%. However, increasing the agar content also altered the matric forces in the media such that growth rate on 0.9% agar was lower than that on 0.8% (data not shown). Roots also tended to coil and get tangled instead of simply growing downwards. As a result of the differences in agar content, comparison of growth could not be made between salt stress levels up to 100 mM and the experiments containing more than 100 mM NaCl. Nutrient media without sucrose were used for the comparison of growth under stress.

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Addition of sucrose in media lowers the osmotic potential of the solution, worsening the effect of the stress. Sucrose levels can also influence ABA responses (Finkelstein et al., 2002; Finkelstein and Gibson, 2002) such that the observed phenotype may result from both hormonal and osmotic effects.

5.4.2 Stress tolerance in mature plants

Dehydration tolerance experiments on mature plants showed that a greater percentage survived when the rate of drying was slow. Between 50 and 70% of the transgenic plants overexpressing *XvAld1* exhibited the drought tolerance phenotype after dehydration for ten days. In contrast, when the plants were fast dried for six days, only 30 to 50% of the transgenic plants survived. The survival rates of the WT and VT plants in both these experiments were significantly lower than those of the transgenic plants. The variation in observed phenotypes between the different experiments indicates that stress tolerance may be affected by the method used to impose the stress. The slow drying allowed time for plants to acclimate to the water deficit. Assuming that all the plants had similar acclimation capabilities, the observed stress tolerance can be attributed to other factors such as the expression of the transgene.

5.4.3 Assessment of the water status in dehydrated plants

The levels of dehydration tolerance observed in soil-grown plants can be affected by various drought avoidance strategies (Verslues et al., 2006). At the onset of stress, the general water conservation characteristics were observed. For plants that were watered every third day, RWC in wet soils ranged between 75 and 85%. By the third day the soil had lost a lot of moisture but RWC increased to more than 90% (data not shown). These results imply that the plants use water conservation strategies such as reducing transpiration rates by regulating the closure of stomata, thus resisting water loss even when the environment was dry. In addition to these general properties, some plants may conserve water better than others. In order to evaluate the effects of these variables, several water relations measurements were carried out. Although the percentages RWC were variable across the different lines, this variation was not statistically different. Measurement of leaf water potential showed more variability, suggesting that it could be a more useful measure of water relations.

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Water potential is also an energy measurement and is more informative if used together with RWC. Transgenic line Xv8 exhibited the lowest (most negative) leaf water potential values. However, this line exhibited survival percentages that were similar to the other transgenic lines. These results may suggest that transgenic plants can withstand more severe dehydration stress than the WT or VT plants. Alternatively, the low water potentials may be caused by accumulation of osmolytes. In wild type and vector transformed controls, leaf water potentials ranged from -1.5 to -2.5 MPa and transgenic plants from -1.5 to -4 MPa (Fig 5.13). These water potential values are more negative compared to values quoted in literature of ~ -0.5 to -1 MPa (Sun et al., 2004; Mane et al., 2007). The reason for the comparatively low water potentials may be the way the values were calculated. Measurements were made as water activity using an AquaLab Series 3. The water activity values were then converted using the equation in section 5.2.3.1.

5.4.4 Transgenic plants accumulate lower levels of toxic lipid aldehydes

Oxidative damage is commonly assessed by measuring the thiobarbituric acid-reactive species (TBARS). MDA is generally used as an indicator for oxidative damage to membranes (Shulaev and Oliver, 2006). A TBARS-based assay for MDA showed that dehydrated transgenic plants overexpressing *XvAld1* accumulated MDA levels similar to well-watered controls (Fig. 5.14). In contrast, the dehydrated control plants (WT and VT) accumulated MDA to levels that were five times more than that of the transgenics. These results imply that the *XvAld1* gene product protects membranes against damage caused by ROS. The protection may be achieved either directly by arresting ROS which can lead to lipid peroxidation or limiting lipid peroxidation by reacting with reactive intermediates such as aldehydes. Estimation of lipid peroxidation products in plant tissues may be valuable measure of plant health and oxidative status, judging by the recovery phenotypes of the transgenic plants compared to the immensely compromised WT and VT (Fig. 5.16). Determination of MDA levels in dry and dead leaf samples could probably have yielded more interesting information that could be used to predict whether or not plants could potentially recover. Other potential biochemical markers of stress could possibly be identified by comparing the status of the plants that recover with those that die after resumption of watering.

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Cells possess an array of detoxification molecules that scavenge ROS (Foyer and Noctor, 2005). Among these enzymes are oxido-reductases. Aldo-keto reductases have been shown to react with a wide array of cytotoxic carbonyl compounds. Lipid peroxidation chain reactions produce chemically reactive compounds such as alkenals, aldehydes, ketones and hydroxyacids (Witz, 1989; Esterbauer et al., 1991). Reactive aldehydes such as 4-hydroxynonenal (HNE; Hartley et al., 1999) and malondialdehyde (HNE; Heath and Packer, 1968; Hodges et al., 1999) are commonly quantified to assess the extent of oxidative stress. An alfalfa AR was shown to reduce HNE to less toxic alcohol derivatives and overexpression of this enzyme in tobacco resulted in reduced lipid peroxidation under stress (Oberschall et al., 2000; Hideg et al., 2003). In the present study, the most striking differences between the transgenic and control plants were in the levels of TBARS. These results indicated that the *XvAld1* transgenic plants possess an effective aldehyde detoxification system that is absent in the control plants. In animals, the AR in kidneys is involved in osmoregulation. Other AR enzymes have been implicated in different pathways involving an array of substrates. Srivastava et al. (2005) reviewed the documented aldose reductase substrates and proposed that AR acts as "a promiscuous aldehyde removase". The wide substrate activity is a property typically required of detoxification enzymes.

The accumulation of ROS may be measured directly, or by detecting the intermediates formed when ROS reacts with other cellular components. Some researchers argue that the accumulation of lipid peroxidation indicators *per se* is not an adequate measure of oxidative stress (Halliwell and Whiteman, 2004; Shulaev and Oliver, 2006). They argue that not all TBA-reactive substances are products of lipid peroxidation. Anthocyanins are among the interfering substances that can cause false positive results in MDA assays (Hodges et al., 1999). In this study, the TBA-reacted solutions were passed through a filter cartridge to remove interfering pigments. In order to validate the MDA results, protein carbonylation was used as another indicator of oxidative stress. Interestingly, carbonyl compounds including aldehydes are proposed to be the in vivo substrates for aldose and aldehyde reductases. Protein oxidation is caused by reactive carbonyl compounds such as aldehydes or ketones. These react with the amino acid side chains; forming adducts with proteins and causing protein denaturation. Spectrophotometric measurement of carbonyl compounds (Reznick and Packer, 1994) was not successful, probably due to the low

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sensitivity of the equipment (data not shown). The expected peak 355 and 390 nm, characteristic of dinitrophenyl hydrazine (DNPH) derivatives, was not consistently detected in all the samples. The detection of oxidatively damaged proteins will be repeated using more sensitive HPLC-based methods (Levine et al., 1994) and/or anti-DNPH monoclonal antibodies.

5.4.5 Osmolyte accumulating transgenic plants

The evidence linking osmolyte accumulation to stress tolerance is mainly correlative. The emerging hypothesis is that the protective effects observed from osmolyte synthesising enzymes is not solely due to the end-product, but the regulatory effects of the biosynthetic intermediates (Avonce et al., 2004; Paul, 2007). Researchers speculate that sugar phosphate metabolic intermediates may be involved in signalling (Prata et al., 1997; Eastmond et al., 2002; Schluepmann et al., 2003; Kolbe et al., 2005) causing changes in carbohydrate metabolism. Altered hexose-sensing may shift the metabolite balance (Koch, 1996; Gibson, 2000; 2005) and contribute to the stress tolerance phenotype observed in transgenic plants. The energy allocation used for accumulating osmolytes may impinge on carbohydrate pools or change the cellular redox balance.

Studies using transgenic plants overexpressing enzymes involved in osmolyte biosynthesis have shown that the levels accumulated are not sufficient for osmotic adjustment. However, protective properties have been observed under stress regardless of these low levels. These observations led to the hypothesis that osmolytes may be involved in other mechanisms besides osmotic adjustment (Hare et al., 1998; Serraj and Sinclair, 2002). Researchers have since demonstrated that some osmolytes are involved in scavenging ROS (Shen et al., 1997b; 1997a; Hong et al., 2000). Aldehydes are the most probable endogenous substrates for many AKR enzymes (Roncarati et al., 1995; Hideg et al., 2003; Srivastava et al., 2004; Ramana et al., 2006).

Metabolomics is an emerging discipline that involves global analysis of different types of metabolic products. Metabolite profiling may help understand the physiological functions of XvAld1 and how sugar metabolism is altered by its overexpression. Other interacting partners also affected by the constitutive presence of the enzyme remain to be identified. The regulation of metabolism is complex and

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poorly understood. However, the overexpression of any gene product will most likely shift the steady state of cellular metabolites. Such analyses on *XvAld1*-overexpressing plants may yield interesting insights into what pathways are affected. The results from the assay for MDA suggest the involvement of XvAld1 in detoxification functions. Metabolite profiling may give different signatures for reactive aldehydes and ketones between transgenic and control plants. In addition, the profiles of sugar metabolites would also indicate if any osmotic adjustment occurs in transgenic plants overexpressing the aldose reductase. In *E. coli*, the His-tagged recombinant protein did not display any reductase activity. It is possible that the histidine tag interfered with the enzymatic functions by interacting with crucial functional groups, or the presence of the additional polypeptide prevented correct folding of the protein. The polypeptide containing the histidine tag could not be cleaved using an enterokinase enzyme. An alternative strategy was the cloning of the *XvAld1* cDNA into pET-SUMO plasmid (Invitrogen, USA). The polypeptide cleavage has been successful using this system. Future work will involve the biochemical characterisation of the AR protein.

5.5 CONCLUSION

Transgenic plants constitutively expressing XvAld1 showed enhanced tolerance to variable levels of NaCl, mannitol, PEG and dehydration when compared to the wild type. Stress tolerance was assessed using RWC, water potential, root growth in seedlings, seed yields, and survival after stipulated stress conditions and durations. Assessment of lipid peroxidation products showed that transgenic plants under stress accumulated levels of MDA that were comparable to well-watered controls. On the other hand, the levels of MDA in similarly stressed WT and VT plants were significantly higher.

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CHAPTER 6

6.0 General discussion and future prospects

Many genes are involved in various adaptive functions that lead to environmental stress tolerance. The transgenic technology has been widely employed to investigate the functions of many genes. However, current genetic engineering strategies are compromised by inadequate understanding of stress tolerance mechanisms. The interacting network of genes involved in the regulation of many stress-protective gene products is poorly characterised.

6.1 Genome organisation and functional motifs

Transcriptional regulation is a major mechanism used by eukaryotic cells to control gene expression. The regulatory molecules involved in controlling the expression of stress-responsive genes in *X. viscosa* are yet to be identified. The cloning and sequencing of a putative promoter is the first step towards identifying control mechanisms governing the expression of *XvAld1*. The functional characterisation of this promoter fragment may lead to the identification of some of the proteins involved in stress-responsive gene expression in *X. viscosa*. The isolation of more sequences upstream of the promoter fragment and downstream of the 3′-UTR will provide more details on the mechanisms involved in the control of gene expression. Characterisation of the interacting promoter-binding elements will probably result in the identification of transcriptional regulators.

The *XvAld1* genomic DNA consists of nine exons interrupted by eight introns. Splicing of the parent mRNA transcript is a posttranscriptional control mechanism. Future work will involve analysis of *XvAld1* transcripts to determine whether posttranscriptional controls, such as differential mRNA splicing, are used to regulate expression.

6.2 The use of inducible promoters compared to constitutive expression

The choice of promoters used for transgenic studies affects the results obtained. In this study, constitutive promoters were used to overexpress the *XvAld1* gene. The advantage of using constitutive promoters is that the protein is expressed in most of the cells over the developmental stages of the plant. However, overexpression

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may compromise the allocation of metabolic resources resulting in phenotypic and/ or developmental aberrations. In this study, *Xvald1* overexpression did not cause visible abnormalities. Given that the constitutive expression of *XvAld1* did not cause any deleterious effects, this strategy may give plants a competitive advantage that may not be possible using inducible expression. Taji et al. (2004) investigated the transcriptomes of *T. halophila* (a salt-tolerant relative of Arabidopsis) and *A. thaliana* under normal growth conditions and when exposed to salt. The results demonstrated that the constitutive expression of stress tolerance genes by *T. halophila* may prime plants to resist damage when stress is imposed. However, constitutive expression may be metabolically expensive, compromising the accumulation of biomass and yield potential. Thus, stress-inducible promoters may be better suited for long term applications in the development of stress tolerant crops. The characterisation of the *XvAld1* putative promoter will provide us with the option of utilising a regulated promoter in future studies. An understanding of the signal-response networks would improve the effectiveness of transgenic strategies. The use of the appropriate regulatory tools, such as endogenous stress-inducible promoters, could improve transgene function.

6.3 The phenotype of transgenic plants under low water potentials

Transgenic Arabidopsis plants overexpressing *XvAld1* were more tolerant to all the stress treatments that caused low water potentials. A larger percentage of transgenic plants survived these treatments compared to controls. The transgenic plants also accumulated lower levels of toxic aldehydes when exposed to stress. Investigating the endogenous *XvAld1*-interacting proteins will help to define the physiological functions of the enzyme. Identifying the *XvAld1*-interacting partners can also generate information on proteins involved in the regulation of gene expression and protein function. The question of how aldose/ aldehyde reductases confer stress tolerance in susceptible plants has not been adequately addressed. These experiments will contribute towards understanding the mechanisms. Identified regulatory partners may be used in transgenic strategies. The overexpression of regulatory genes, such as transcription factors, has been used to control the expression of multiple genes as an alternative to pyramiding (Potenza et al., 2004; Halpin, 2005). The identification of

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similar elements in *X. viscosa* may provide us with several technological options for improving stress tolerance in plants.

6.4 Biochemical characterisations

Biochemical analyses, using the XvAld1 recombinant protein generated by the pProEX expression system, did not show detectable enzymatic activities (data not shown). We propose that the N-terminal polyhistidine tag interfered with the native folding of the protein or blocked some functional amino acid residues. Attempts to cleave the polypeptide tag using an enterokinase enzyme were not successful. Recently, we successfully expressed the recombinant protein using the pET-sumo system and cleaved the polyhistidine tag. The tag-free XvAld1 protein will be used in future studies to identify *in vitro* substrates. Immunoprecipitation of protein complexes will be performed using the XvAld1 recombinant protein and polyclonal antibodies. Characterisation of interacting partners will help define the physiological functions of the protein.

Since the possible physiological roles of XvAld1 are not obvious, further characterisation of the transgenic plants is underway. Biochemical analyses, focusing on a comparison of sugar metabolite profiles between transgenic and WT plants, will shed more light on the mechanisms of protection employed by osmolytes synthase genes.

The work reported in this thesis is an important platform that can be used in future to develop transgenic plants such as maize that is tolerant to various environmental stresses.

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When all is said and done, the questions are only partially answered, and more questions than before still remain…What we know is dwarfed by what we continue to puzzle over. Makasika sei Mwari!? Kubvira kutanga kwemazuva vanhu vachiedza kunzwisisa zvisikwa zwenyu. Asi nanhasi tinenge matununu. Zvinhu zvose zvichingori zvitsva. Mbiri yose ndeyaMusiki! *We honour God for what He conceals; we honour kings for what they can explain (Prov 25 verse 2).*

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APPENDIX A The compositions of tissue culture solutions

a) High osmoticum MS media (1 L)
4.32 g MS salts
10 ml MS vitamins (A.1.1) 30 g Sucrose
2.5 ml 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg/ml)
18.22 g Mannitol
50 mg Myo-inositol
900 ml milli-Q water
Set pH to 5.8 (using 1 M KOH)
8 g Agar (pure) and top-up to 1 L with milli-Q water
Autoclave
Add 1 ml $AgN0_3$

b) MS vitamins
50 mg glycine
125 mg nicotinic acid
250 mg thiamine
250 mg pyridoxine-HCI
2.5 g myo-inositol
500 ml dH_2O
Filter sterilise, aliquot 20 ml volumes into sterilins and store at -20°C

c) 2,4-D (1 mg/ml)

100 mg 2,4-D (poisonous powder) dissolved in 1 M KOH (heat in microwave for 5 s)
Add milli-Q water to 100 ml
Aliquot into 10 ml volumes and store at -20°C

d) $AgN0_3$ (10 mg/L)
200 mg $AgN0_3$
20 ml milli-Q water filter sterilise

e) 50% glycerol
25 ml glycerol (100%)
25 -ml dH_2O
Autoclave

f) 2.5 M $CaC1_2$
36.5 g $CaCl_2$
100 ml dH_2O
Autoclave

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g) 0.1 M Spermidine
Heat the spermidine reagent bottle to 60°C until melted
Pipette 14 µl spermidine and to 1 ml dH_2O
Filter sterilise
(must be made fresh once a month)

h) MS medium for regeneration of transformed *D. sanguinalis* callus (MS regeneration)

MS salts	4.32 g
MS vitamins	10 ml
Sucrose	30 g
NAA (0.1 mg/ml)	1 ml
BA (1 mg/ml)	10 ml
Milli-Q water	900 ml
Set pH to 5.8 using 1 M KOH	
Pure agar (Sigma)	8 g
Make up to 1 L with milli-Q water.	
Autoclave	
Add filter sterilised bialaphos (3 mg/ml)	1 ml

j) MS rooting medium

MS salts	4.32 g
MS vitamins	10 ml
Sucrose	30 g
Milli-Q water	900 ml
Set pH to 5.8 using 1 M KOH	
Pure agar (Sigma)	8 g
Make up to 1 L with milli-Q water.	
Autoclave	
Add filter sterilised bialaphos (3 mg/ml)	1 ml

k) Plant nutrient agar medium (PNA)

Stock	Volume per litre
1 M $KN0_3$	5 ml
1 M $MgSO_4.7H_2O$	2 ml
1 M $Ca(N0_3)_2.4H_2O$	2 ml
20 mM Fe.EDTA (fresh)	2.5 ml
(0.1836 g/ 25 ml, store at 4°C for not more than 2 weeks	
Micronutrients	1 ml
Agar	7.5 g
Sucrose (optional)*	5 g

Autoclave one liter in a 2 liter Erlenmeyer flask for 30 minutes, swirl to mix agar thoroughly and cool to ~50°C.

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After autoclaving, add 2.5 ml of 1 M KPO_4 pH 5.5** per liter.

∗ Media used for stress treatments did not contain any sucrose

∗∗ $Ca(N0_3)_2.4H_2O$ forms a precipitate with $KP0_4$ under high temperatures. To avoid this, filter sterilize the $KP0_4$, and add after autoclaving the PNA.

l) Micronutrients Stock (1000X)

Final concentration	**For 1 L**
70 mM H_3B0_4 (62 g/mol)	4.3 g
14 mM $MnSO_4.H_2O$ (169 g/mol)	2.4 g
0.5 mM $CuSO_4.5H_2O$ (250 g/mol)	0.125 g
1 mM $ZnSO_4.6H_2O$ (288 g/mol)	0.288 g
0.2 mM Na_2MoO_4 (206 g/mol)	0.041 g
10 mM NaCl (58 g/mol)	0.58 g
0.01 mM $CoCl_2.6H_2O$ (238 g/mol)	2.4 mg

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APPENDIX B List of primers

Table B1 Primers used in this study

#	Primer name	Sequence
1	XvAld1-AF	5′-CGGCACGAGAAGCTACAGAGA-3′
2	XvAld1-AR	5′- CTTCACCGTCCCAGAGTTCAG-3′
3	XvAld1-BF	5′-CGCATGCACCGTGTTTTGCTG-3′
4	XvAld1-BR	5′-CGAAGACCTGGATGTTCTCC-3′
5	M13-F	5′-TTCCCAGTCACGACGTTG-3′
6	M13-R	5′-CAGGAAACAGCTATGAC-3′
7	AD1	5′-TG(A/T)GNAG(A/T)ANCA(G/C)AGA-3′
8	AD2	5′-AG(A/T)GNAG(A/T)ANCA(A/T)AGG-3′
9	AD3	5′-CA(A/T)CGICNGAIA(G/C)GAA-3′
10	AD4	5′-TC(G/C)TICGNACIT(A/T)GGA-3′
11	ALD-PR1	5′-GCGTTGCCGGCCTTGTCA-3′
12	ALD-PR2	5′-CCAGACTTCCACGTCCCGAGC-3′
13	ALD-PR3	5′-GGATTGAGTGCCCGCTGAGGA-3′
14	P120	5′-TGAGTGCCCGCTGAGGAGCTTG-3′
15	XvAld1-Gen2F	5′-GATAACCACTATAGTGCTTCATTCATCG-3′
16	XvAld1-Gen2R	5′-GGATATTATTGATTTTCTTCATTTTCTTTCC-3′
17	XvAld1-GenPr2R	5′-CCATTTCCCTCCACACTCCTCCTATGTCG-3′
18	XvAld1-*Sal*I-F	5′-CGTCGACCC**ATG**GCGCATGCACCGTG-3′
19	XvAld1-*Xba*I-R	5′-CTCTAGACCCTAGCATGGCTCTCCAG-3′
20	XvAld1-RTF	5′-GGAGGAGTGTGGAGGGAAAT-3′
21	XvAld1-RTR	5′-CCGAGAGGTGAATAAGCAGTT-3′
22	Xv18S-RTF	5′-CAGGCGCGCAAATTACCCAATCC-3′
23	Xv18S-RTR	5′-CCTACCGTCCCGTCCCAAGGTC-3′
24	18S-F	5′-GGCAGCAGGCGCGCAAATTA-3′
25	18S-R	5′-GCCATGCACCACCCTAT-3′
26	GFP-*Sal*I-F	5′-GTCGAC**ATG**GCGCATGCACCGT-3′
27	GFP- *Bam*HI-R	5′-CCTAGGTTAGACTTCACCGTCCC-3′
28	YFP- *Bam*HI-F	5′-GGGATCCAATGGCGCATGCACCGTG-3′
29	YFP- *Kpn*I-R	5′-AGGTACCTCGCCCTCCCAGAGTTCAG-3′
30	Bar-F	5′-CGTCAACCACTACATCGAG
31	Bar-R	5′-GAAACCCACGTCATGCCAG-3′
32	NPTII-F	5′-CTGAATGAACTGCAGGACGAGG-3′
33	NPTII-R	5′-GCCAACGCTATGTCCTGATAGC-3′
34	β-actin	5′-CTCGTTTGTGGGAATGGAAG-3′
35	β-actin	5′-AGGGAAGCAAGAATGGAACC-3′

I - deoxyinosine
N - any deoxynucleotide
Recognition sequences for restriction enzymes are underlined
Start and stop codons are bold
RT denotes primers used for real time PCR

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APPENDIX C Details of proteins used for multiple sequence alignments

Table C1 Sequences used for multiple alignments

Accession # and abbreviated name	Species name(s)	Enzyme name	AKR classification
P23901_HORVU	*Hordeum vulgare* (Barley)	Aldehyde reductase	AKR4C1
Q39284_BROIN	*Bromus inermis* (Smooth brome grass)	Aldehyde reductase	AKR4C2
Q43320_AVEFA	*Avena fatua* (Wild oats)	Aldehyde reductase	AKR4C3
AF133841_XERVI	*Xerophyta viscosa*	Aldehyde reductase	AKR4C4
CAC32834_DIGPU	*Digitalis purpurea* (Common foxglove)	Aldehyde reductase	AKR4C5
Q9M009_ARATH	*Arabidopsis thaliana* (Mouse-ear cress)	Aldehyde reductase-like	na
AJ223291_SESRO	*Sesbania rostrata* (Prostrate sesbania)	Chalcone polyketide reductase	AKR4B1
AF108435_PAPSO	*Papaver somniferum* (Opium poppy)	NADPH-dependent codeinone reductase	AKR4B2
AAF13736_PAPSO	*Papaver somniferum* (Opium poppy)	Codeinone reductase	AKR4B3
AF039182_FRAAN	*Fragaria ananassa* (Strawberry)	D-galacturonate reductase	AKR4B4
P26690_SOYBN	*Glycine max* (Soybean)	NAD(P)H-dependent 6'-eoxychalcone synthase	AKR4A1
S48851_MEDSA	*Medicago sativa* (Alfalfa)	Chalcone polyketide reductase	AKR4A2
D83718_GLYEC	*Glycyrrhiza echinata* (Hedgehog licorice)	Chalcone polyketide reductase	AKR4A3
BAA13113_GLYGL	*Glycyrrhiza glabra* (Common licorice)	Chalcone polyketide reductase	AKR4A4
P23457_RATNO	*Rattus norvegicus* (Norway rat)	3α- hydroxysteroid dehydragenase (liver)	AKR1C9
Additional sequences used for constructing the homology tree			
NP_006057_HOMSA	*Homo sapiens* (Human)	Aldehyde reductase (liver)	AKR1A1
O80945_ARATH	*Arabidopsis thaliana* (Mouse-ear cress)	Alcohol dehydrogenase-like	na
O82020_MEDSA	*Medicago sativa* (Alfalfa)	Aldehyde reductase	na
CAA54142_RATNO	*Rattus norvegicus* (Norway rat)	Shaker channel β-subunit (Kvb2)	AKR6A2
AAC23646_ARATH	*Arabidopsis thaliana* (Mouse-ear cress)	Alcohol dehydrogenase	na

na - AKR class not assigned (a potential member of the AKR superfamily)

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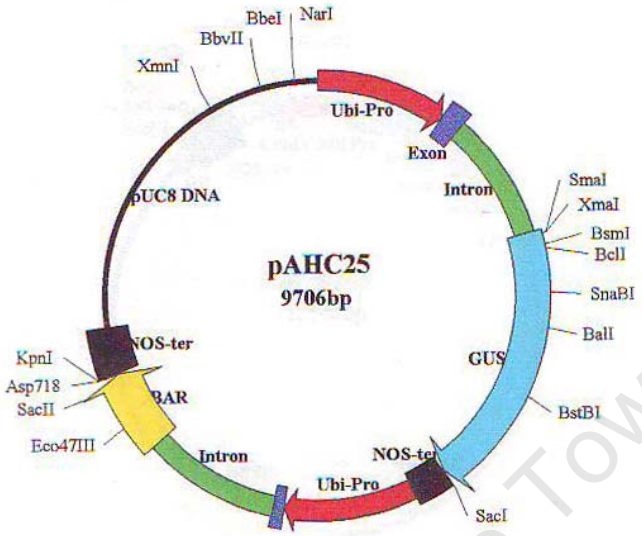
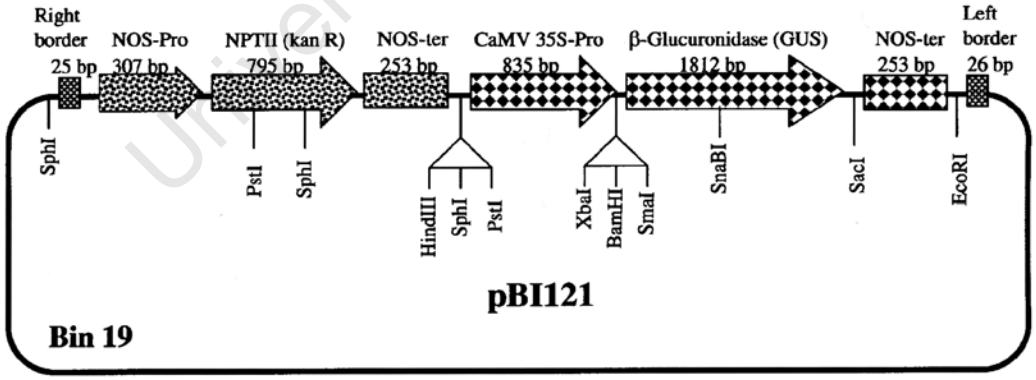
APPENDIX D Plasmid maps

D1 Map of pAHC25



The plasmid pAHC25 contains the selectable marker gene (bar) which codes for the enzyme phosphinothricin acetyl transferase (PAT). The enzyme confers resistance to the herbicide phosphinothricin (bialaphos, basta or glufosate ammonium).

D2 pBI121 map



The *XvAld1* cDNA sequence was cloned pBI121 by replacing the *GUS* gene. *XvAld1* was excised from the pSK plasmid by digesting with *Xho*I, removing the 3′ overhangs then digesting with *Xba*I. The pBI121 DNA was digested with *Sac*I, the 5′ overhangs were filled and the linearised plasmid was then digested with *Xba*I.

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D3 pProEX-HT map

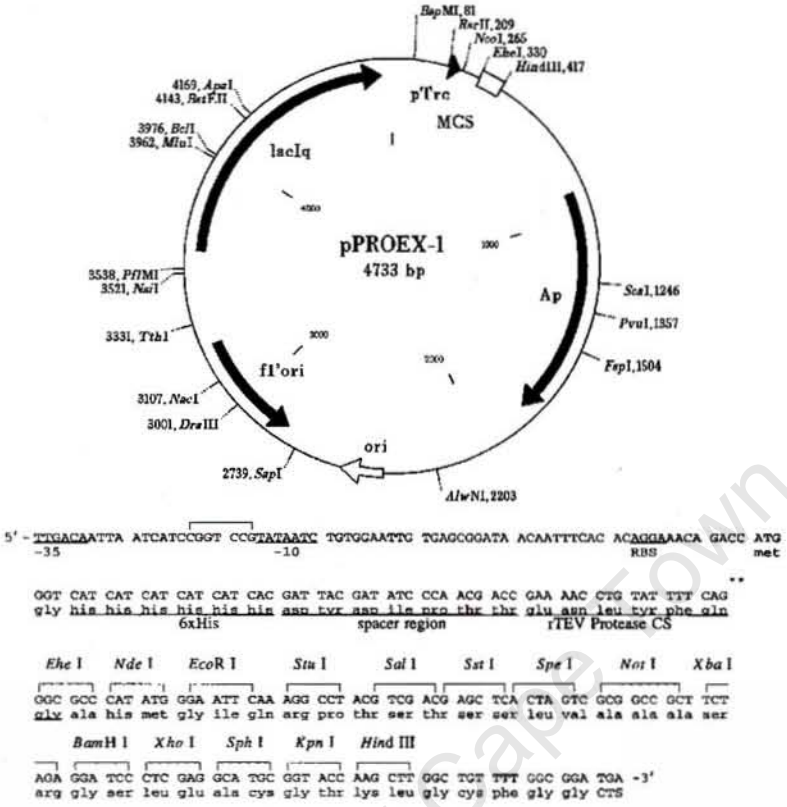

FIGURE 1. Plasmid map of pProEX-1. Selected single cutting restriction endonucleases are presented on the map. The sequence of the promoter and multiple cloning site are shown. The -35 and -10 regions of the pTrc promoter, the ribosome binding site (RBS), the 6xHis, and the spacer region are underlined. The rTEV Protease cleavage site is underlined and bolded. The site of cleavage is indicated with **.

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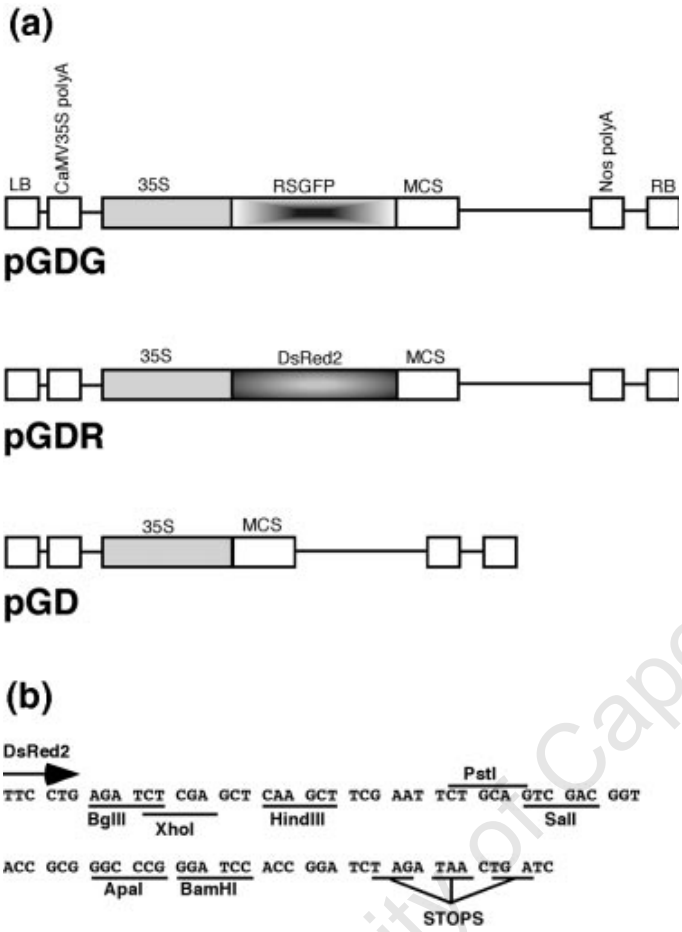
D4 pGD vectors



Maps of the pGD vector series.
All three vectors are derivatives of the binary vector pCAMBIA-1301.
(a) Schematic representations of pGDG, pGDR and pGD. LB and RB denote left and right borders of the T-DNA, respectively. The pGD vectors retain both the CaMV35S polyA and nopaline synthase (Nos polyA) polyadenlylation signals present in pCAMBIA-1301. However, only the Nos polyA signal is utilized in these vectors. (b) Sequence of the multiple cloning site of pGDR. The reading frame is identical in pGDG and pGD and contains the same set of restriction sites. Restriction sites common to all three vectors are shown beneath the multiple cloning site sequence. The PstI site can be used only in pGDG and pGD because the DsRed2 coding sequence contains a PstI site. (Adapted from Goodin et al., 2002).

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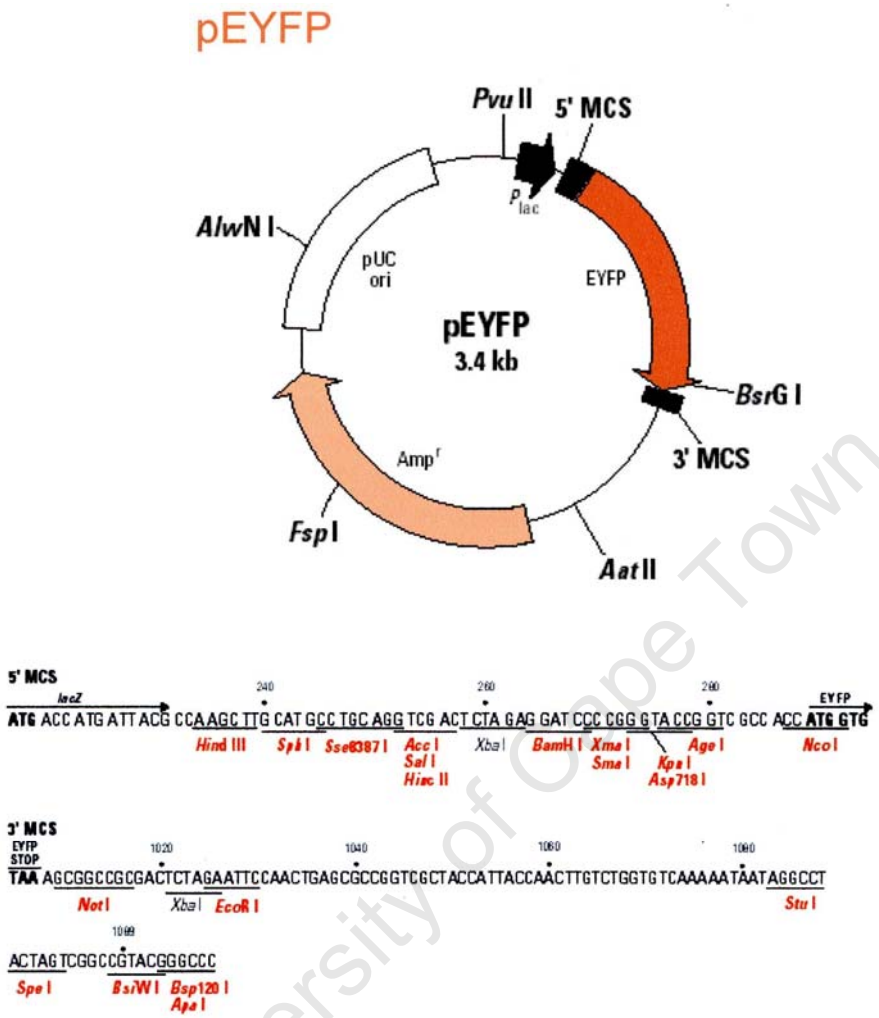
D5 pYFP map



The *XvAld1* open-reading frame was cloned using the *Bam*HI and *Kpn*I restriction sites

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APPENDIX E Bonferroni posttests for growth curves

Table E1 Two-way repeated measures (RM) ANOVA, Bonferroni posttests

PNA- osmotic stress

		WT vs Xv7		WT vs Xv8		WT vs Xv12	
Time (days)		P value	Summary	P value	Summary	P value	Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	5	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	6	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	7	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	8	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns

50 mM mannitol

		WT vs Xv7		WT vs Xv8		WT vs Xv12	
Time (days)		P value	Summary	P value	Summary	P value	Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	5	P<0.01	**	P<0.001	***	P<0.01	**
	6	P<0.01	**	P<0.001	***	P<0.001	***
	7	P<0.001	***	P<0.001	***	P<0.001	***
	8	P<0.001	***	P<0.001	***	P<0.001	***

75 mM mannitol

		WT vs Xv7		WT vs Xv8		WT vs Xv12	
Time (days)		P value	Summary	P value	Summary	P value	Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	5	P > 0.05	ns	P<0.001	***	P<0.001	***
	6	P<0.001	***	P<0.001	***	P<0.001	***
	7	P<0.001	***	P<0.001	***	P<0.001	***
	8	P<0.001	***	P<0.001	***	P<0.001	***

100 mM mannitol

		WT vs Xv7		WT vs Xv8		WT vs Xv12	
Time (days)		P value	Summary	P value	Summary	P value	Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	5	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	6	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	7	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	8	P > 0.05	ns	P > 0.05	ns	P < 0.05	*

150 mM mannitol

		WT vs Xv7		WT vs Xv8		WT vs Xv12	
Time (days)		P value	Summary	P value	Summary	P value	Summary
	1	P > 0.05	ns	P > 0.05	ns	P>0.05	ns
	2	P<0.01	**	P < 0.05	*	P<0.001	***
	3	P<0.01	**	P < 0.05	*	P<0.001	***
	5	P<0.001	***	P<0.001	***	P<0.001	***
	6	P<0.001	***	P<0.001	***	P<0.001	***
	7	P<0.001	***	P<0.001	***	P<0.001	***
	8	P<0.001	***	P<0.001	***	P<0.001	***

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Table E1 continued

PNA salt stress

Time (days)		WT vs Xv8 P value	WT vs Xv8 Summary	WT vs Xv12 P value	WT vs Xv12 Summary	WT vs Xv14 P value	WT vs Xv14 Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	4	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	5	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	6	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	7	P < 0.05	*	P > 0.05	ns	P > 0.05	ns
	8	P < 0.05	*	P > 0.05	ns	P > 0.05	ns
	9	P<0.01	**	P<0.01	**	P<0.01	**

50 mM NaCl

Time (days)		WT vs Xv8 P value	WT vs Xv8 Summary	WT vs Xv12 P value	WT vs Xv12 Summary	WT vs Xv14 P value	WT vs Xv14 Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	4	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	5	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	6	P < 0.05	*	P > 0.05	ns	P > 0.05	ns
	7	P<0.01	**	P<0.01	**	P > 0.05	ns
	8	P<0.01	**	P<0.001	***	P > 0.05	ns
	9	P<0.001	***	P<0.001	***	P < 0.05	*

75 mM NaCl

Time (days)		WT vs Xv8 P value	WT vs Xv8 Summary	WT vs Xv12 P value	WT vs Xv12 Summary	WT vs Xv14 P value	WT vs Xv14 Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P<0.01	**	P<0.01	**	P > 0.05	ns
	4	P<0.001	***	P<0.01	**	P > 0.05	ns
	5	P<0.001	***	P<0.001	***	P > 0.05	ns
	6	P<0.001	***	P<0.001	***	P > 0.05	ns
	7	P<0.001	***	P<0.001	***	P > 0.05	ns
	8	P<0.001	***	P<0.001	***	P > 0.05	ns
	9	P<0.001	***	P<0.001	***	P > 0.05	ns

100 mM NaCl

Time (days)		WT vs Xv8 P value	WT vs Xv8 Summary	WT vs Xv12 P value	WT vs Xv12 Summary	WT vs Xv14 P value	WT vs Xv14 Summary
	1	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns	P > 0.05	ns
	4	P < 0.05	*	P > 0.05	ns	P > 0.05	ns
	5	P<0.01	**	P<0.01	**	P > 0.05	ns
	6	P<0.001	***	P<0.001	***	P<0.01	**
	7	P<0.001	***	P<0.001	***	P<0.001	***
	8	P<0.001	***	P<0.001	***	P<0.001	***
	9	P<0.001	***	P<0.001	***	P<0.001	***

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Table E1 continued

14% PEG

Time (days)		WT vs Xv8 P value	WT vs Xv8 Summary	WT vs Xv12 P value	WT vs Xv12 Summary
	1	P > 0.05	ns	P > 0.05	ns
	2	P > 0.05	ns	P > 0.05	ns
	3	P > 0.05	ns	P > 0.05	ns
	4	P > 0.05	ns	P > 0.05	ns
	5	P > 0.05	ns	P > 0.05	ns
	6	P > 0.05	ns	P<0.001	***
	7	P > 0.05	ns	P<0.001	***
	8	P > 0.05	ns	P<0.001	***
	9	P > 0.05	ns	P<0.001	***

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