The Characterization of *XVSAP1*, a Gene Isolated from the Resurrection Plant *Xerophyta viscosa* Baker, and its Expression in Transgenic Plants

Dahlia Garwe

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“ I was taught that the way of progress is neither swift nor easy”

Marie Curie

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ABBREVIATIONS

ABA	abscisic acid
ABRE	ABA-responsive element
ATP	adenine triphosphate
Bp	base pair(s)
CAP	cold-associated protein
cDNA	copy DNA
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DRE	dehydration responsive element
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
EREBP	ethylene-responsive element binding protein
g	grams
HCl	hydrochloric acid
IP_3	inositol-(1,4,5)triphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase(s)
kDa	kilodalton(s)
λ	lambda
l	litre(s)
LA	Luria-Bertani agar
LB	Luria-Bertani broth
M	molar concentration
µg	microgram(s)
mg	milligram(s)
$MgSO_4$	magnesium sulphate
µl	microlitre(s)
ml	millilitre(s)

µm	micrometer(s)
MES	(2-[N-morpholino]ethanesulfonic acid
mM	millimolar
min	minutes
mRNA	messenger RNA
NaCl	sodium chloride
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometre(s)
ORF	open reading frame
PEG	polyethylene glycol
PMSF	phenylmethanesulfonylflouride
PNA	plant nutrient agar
PTGS	post-transcriptional gene silencing
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RWC	relative water content
SDS	sodium dodecyl sulphate
Spp	species
TBE	tris borate EDTA
TE	tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
u	unit(s) of enzymatic activity
UV	ultraviolet
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

ABSTRACT

The strategy of "complementation by functional sufficiency" was used to isolate a cDNA, designated *XVSAP1,* from a cDNA library constructed from dehydrated *Xerophyta viscosa* Baker leaves. Analysis of the cDNA sequence indicated a highly hydrophobic protein with six transmembrane regions. Southern blot analysis revealed that there are at least two copies of *XVSAP1* in *X.viscosa*. The deduced amino acid sequence showed 49% identity to WCOR413, a low temperature-regulated protein from wheat. The protein also showed between 25% to 56% identity to WCOR413-like proteins from *Arabidopsis thaliana*. Analysis of gene expression using semi-quantitative RT-PCR indicated that *XVSAP1* is induced by dehydration, salt stress (100 mM), both low (4°C) and high temperature (42°C) and high light treatment (1500 μmol $m^{-2} s^{-1}$).

Expression of *XVSAP1* in *Escherichia coli (srl*::Tn10) conferred osmotic stress tolerance when the cells were grown in 1 M sorbitol. Analysis of the expression of *XVSAP1* introduced into *Arabidopsis thaliana* and *Nicotiana tabacum* by Ti plasmid-mediated transformation under the control of a cauliflower mosaic virus 35S promoter and a nos terminator, confirmed constitutive accumulation of the corresponding protein in leaves. The bacterial phosphinothricin acetyl transferase gene (*bar*) was used as a selectable marker. Transgenic plants showed a greater tolerance to osmotic, salt, heat and drought stress. Non-transgenic plants had shorter roots, leaf expansion was inhibited and leaves were more chlorotic than those of the transgenic plants when grown both in tissue culture and hydroponically. Tobacco plants expressing *XVSAP1* attained a significantly higher fresh and dry weight than the untransformed controls. When exposed to water deficit in soil, untransformed tobacco plants were smaller, wilted earlier and were more chlorotic than the transgenic plants. Electrolyte leakage tests demonstrated that

tobacco plants expressing *XVSAP1* had greater membrane permeability to electrolytes. In an attempt to establish the function of *XVSAP1* in *Xerophyta viscosa*, the orthologues of the gene in Arabidopsis, the *A. thaliana* cold associated protein (ATCAP) genes, were silenced using RNA interference. The binary vector pFGC1008 was used to introduce and drive the expression of inverted repeats of at least four of the *ATCAP* genes. Initial results indicated that the transgenic Arabidopsis plants displayed an early flowering phenotype. In addition, the plants were hypersensitive to salt and osmotic stress, respectively. These results indicated that the *ATCAP* genes were associated with the tolerance of Arabidopsis to salt and osmotic stress correlating with the function of *XVSAP1* in *X. viscosa.* The study demonstrated that *XVSAP1* is involved in the response to abiotic stress in *X. viscosa* and confers tolerance to heat, drought, salt and osmotic stresses when expressed in heterologous plant systems.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 OVERVIEW

It is estimated that the world's population will have doubled by the year 2050 (Herrera-Estrella, 2000). In the face of a burgeoning world population, new means of improving crop productivity have to be found in order to provide and maintain adequate food stocks. The productivity and distribution of plants, particularly those of agronomic and horticultural importance, is greatly influenced by environmental stresses such as drought, salinity and extremes of temperature (Boyer, 1982). It has been estimated that two-thirds of the yield potential of major crops are routinely lost due to unfavourable growing environments (Bajaj et al., 1999). As sessile organisms, plants have evolved a wide variety of mechanisms that enable them to grow and reproduce under hostile environmental conditions. Developing an understanding of these strategies could have a significant impact on agricultural productivity besides being of importance to basic research. Traditional plant breeding methods have been utilized for the purpose of increasing plant productivity but with limited success (Flowers and Yeo, 1995; Ingram and Bartels, 1996). This is because conventional breeding methods have less efficient selection techniques, are time consuming and laborious and rely on existing plant genetic variability. Furthermore, it is often difficult to modify single traits using these methods (Holmberg and Bulow, 1998). It has also been observed that quantitative trait loci (QTLs) that are linked to tolerance, are dependent on the development stage of the plant (Foolad, 1999).

In recent years, genetic engineering has been used to improve stress tolerance in plants and much work has been done in trying to understand the molecular basis for stress tolerance (Bray, 1997). The main advantage of using transgenic approaches for crop improvement is that genes can be sourced from any organism whereas with conventional plant breeding the genes can only be transferred among closely related plant species. Further only the gene or genes of interest are transferred to a crop thus rendering extensive back-crossing unnecessary. Most strategies rely on the transfer of genes that encode either biochemical pathways or endpoints of signalling pathways that are controlled by a constitutively active promoter. These gene products protect either directly or indirectly against environmental stresses. However, a lack understanding of the complexity and interplay of osmotic, desiccation and temperature tolerance mechanisms and their corresponding signalling pathways has generally limited the success of these approaches (Cushman and Bohnert, 2000). Despite this, transgenic approaches have resulted in the development of plants with improved stress tolerance (Bray, 1997; Holmstrom et al., 1996; Mendoza et al., 1996; Murata et al., 1992; Sokamoto et al, 1998; Tarczynski et al. 1993; Thomashow, 1999; Xu et al, 1996).

A potential rich source of genes that could confer tolerance to abiotic stresses is a small group of around 200 species of angiosperms known as the resurrection plants (Gaff, 1971, Bartels and Salamini, 2001). These plants have developed mechanisms that allow them to withstand severe water deficit and are unique in their ability to tolerate drying of their vegetative tissues. Most flowering plants are unable to survive being subjected to a water deficit equivalent to less than 85% to 98% (v/v) relative humidity (Bartels and Salamini, 2001). However certain species and specific structures are able to withstand severe desiccation. These include mosses and ferns, (Oliver and Bewley, 1996), the resurrection plants (Gaff, 1971, Ingram and Bartels, 1996; Sherwin and Farrant,

1996) and seeds (McCarty, 1995). In orthodox seeds as much as 90% of the original water is removed in attaining a state of almost complete dormancy (McCarty, 1995). This desiccated state allows survival under extreme environmental conditions and favours wide seed dispersal. Resurrection plants on the other hand, have evolved an array of morphological and physiological mechanisms that allow the plants to survive desiccation. It is thought that they developed strategies that limit the damage incurred during dehydration to a repairable level, maintain physiological integrity in the dried state and mobilize repair mechanisms on rehydration (Oliver et al., 1998). Resurrection plants will be described in more detail in section 1.5.

Genes, which are postulated to play a role in desiccation tolerance have been isolated from *Craterostigma plantagenium* (Bartels and Salamini, 2001; Furini et al., 1997; Ituriaga et al., 1992). *Tortula ruralis* (Chen et al., 2002; Oliver et al., 1998) and *Xerophyta viscosa* (Garwe et al., 2003; Mowla et al., 2002; Mundree et al., 2000; Ndima et al., 2001). It is hoped that some of these genes could be used to improve the tolerance of crop plants to abiotic stresses and consequently improve their yield. The characterization of *XVSAP1* and transgenic plants expressing this gene form the basis of this dissertation.

1.2 WATER DEFICIT AND DESICCATION

An understanding of the responses of plants to environmental stresses such as drought, salinity and low temperatures and consequent improvement of the productivity of crop plants must begin with an appreciation of the processes that occur when plants are exposed to these stresses. Biochemical activities by all known forms of life require water. The water molecules in their liquid state form a

large hydrogen-bonded network that ensures the structural stability of biological molecules such as proteins, nucleic acids and the fluid-bilayer structure of membranes. Water is also needed as a solvent, a transport medium, an evaporative coolant and an electron donor in the Hill reaction (Bohnert et al., 1995). Abiotic stresses such as those mentioned above result in the depletion of cellular water or induce a water deficit in the plant. Water deficit, which occurs when the rate of transpiration is exceeded by water uptake, causes a flux of water from the cells or dehydration of the cytoplasm which leads to a loss of turgor, an increase in the concentration of intracellular solutes and changes in cell volume and membrane shape. Further, cellular water deficit can result in denaturation of proteins and disruption of membrane integrity (Bray, 1997). More severe water loss from the cells is known as desiccation. Desiccation is when most of the protoplasmic water is lost and a low amount of tightly bound water remains in the cell. However, as already mentioned in section 1.1, such deficit is a normal component of some processes such as seed development (McCarty, 1995).

Abiotic stresses induce the expression of a number of genes (Hasegawa et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 1996; Swire-Clark and Marcotte, 1999). The products of the expression of these genes can be classified into two groups, namely those that regulate gene expression and signal transduction, and those that directly protect against environmental stresses (Fig.1) (Shinozaki and Yamaguchi- Shinozaki, 1997). The first group includes molecules such as calcium-dependent kinases, mitogen-activated kinases which are induced by drought, cold and high salt (Knight et al., 1996; Mizoguchi et al., 1997), signalling molecules such as phopholipase C and transcription factors such as basic leucine zipper (bZIP) proteins. The second group encompasses proteins that are likely to function by protecting cells from dehydration, such as the enzymes

required for the biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes (Bray 1997; Ingram and Bartels, 1996). Numerous stress-inducible proteins have been identified in both groups and their corresponding genes isolated (Bray, 1997; Garwe et al., 2003; Holmstrom et al., 1996; Mendoza et al., 1996; Mowla et al., 2002; Mundree et al., 2000; Murata et al., 1992; Tarczynski et al., 1993; Thomashow, 1999; Zhu et al., 1997).

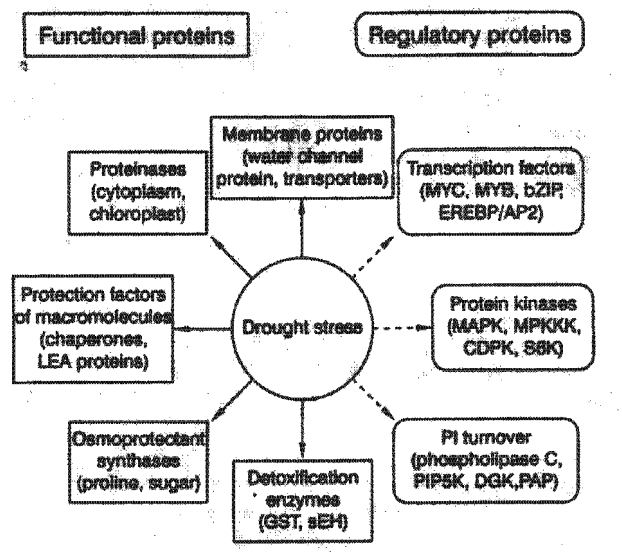

Figure 1.1 Drought stress-inducible genes and their possible functions in stress tolerance and response. The products of gene expression can be divided into functional proteins and proteins with a regulatory role. Functional proteins probably function in stress tolerance and the regulatory proteins group contains protein factors involved in further regulation of signal transduction and gene expression. (adapted from Shinozaki et al., 1999).

1.3. REGULATION OF GENE EXPRESSION AND SIGNAL TRANSDUCTION

Expression of genes in response to different environmental stimuli results from a complex signal transduction cascade that commences with perception of the stimulus followed by the processing, amplification and integration of the signal. The final step is a response reaction in the form of *de novo* gene expression (Ingram and Bartels, 1996). A primary stress condition may activate multiple signalling pathways and these pathways may connect or interact with one another using shared components (Fig 1.2).

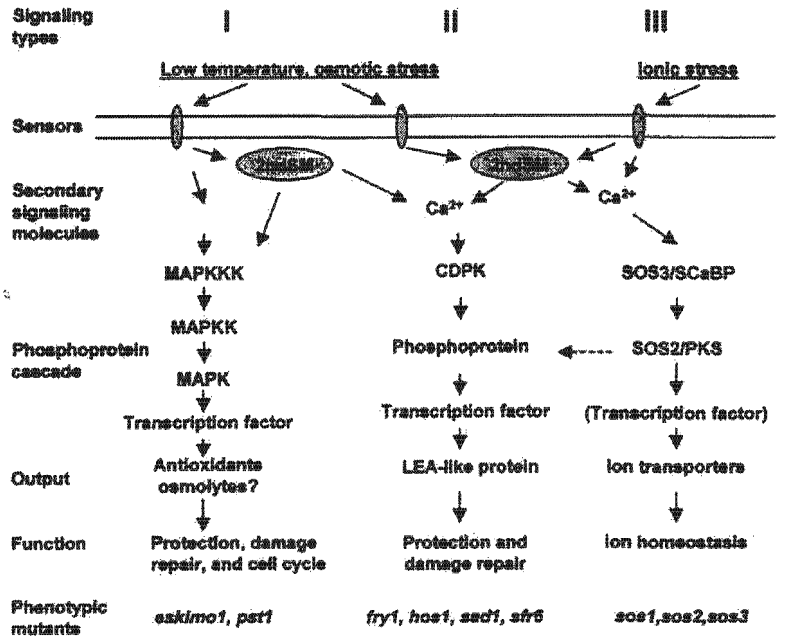

Figure 1.2 Major types of signalling for plants during cold, drought and salt stress. Type I signalling involves the generation of ROS scavenging enzymes, antioxidants and osmolytes. Type II signalling involves the production of stress-responsive proteins mostly of undefined functions. Type III involves the SOS3 pathway, which is specific to ionic stress. (adapted from Xiong et al., 2002).

1.3.1 Signal Perception

All organisms, from bacteria, yeast and plants to mammalian cells respond to signals from the extracellular environment. These signals are then transduced from the cell surface to the interior of the cell. Despite great effort, knowledge of how plants sense and transduce low temperature, drought and salinity signals is still very limited. One major constraint has been the slow pace of application of molecular genetics due to the scarcity of reliable phenotypes specific to the various environment stresses (Xiong and Zhu, 2001). The advent of microarray analysis of expression at the genome level should assist in the dissection of the complex network of osmotic stress signalling. Current thinking is that the decrease in turgor pressure due to water deficit, leads to a change in the osmotic potential across the plasma membrane, which in turn triggers the water-stress response at the molecular level (Ingram and Bartels, 1996). Other proposed signals to the cell of water deficit include loss in membrane flexibility, change in cell volume or membrane area, or a change in solute concentrations (Bray, 1997). It has also been suggested that the reduction in membrane fluidity that occurs at low temperature, may also be the primary sensor for low temperature. Orvar et al. (2000) used dimethyl sulfoxide to reduce the membrane fluidity of alfalfa cell cultures cells at 25°C. Using measurements of cell viability and *cas3* gene expression as reporters of cold signalling in the cells, they were able to demonstrate a modest increase in *cas30* transcript levels and a concomitant increase in the freezing tolerance of cells.

Environmental signals are thought to be first perceived by specific receptors that, upon activation, initiate (or suppress) a cascade that transmits the signal intracellularly and in many cases, activates nuclear transcription factors to induce the expression of specific sets of genes. In animal systems,

many of the extracellular signals are perceived by membrane associated receptor kinases. Although none of the receptors for cold, drought, salinity or ABA in plants are known with certainty, indications are that receptor-like protein kinases, two-component histidine kinases as well as G-protein-associated receptors may represent the sensors of these signals. It should be noted that the existence of plant G-protein–associated receptors has not been proved conclusively although G-proteins have been implicated in environmental stress signalling.

Receptor-like kinases consist of three domains: an extracellular domain that may function in ligand binding or protein-protein interactions, a transmembrane domain and an intracellular kinase domain. Unlike animal receptor-like kinases that usually have tyrosine serine/threonine signature sequences, plant receptor-like kinases have serine/threonine signature sequences (Xiong and Zhu, 2001). In Arabidopsis, for example, RPK1 is a receptor-like kinase with extracellular luecine-rich repeats that may be involved in multiple- stress signal transduction. Hong et al. (1977) found that dehydration, high salt, low temperature and ABA induce the gene coding for the kinase within a period of one hour.

Osmosensors are another type of cellular sensor involved in the recognition of water-deficit stress. These types of sensors were first identified in prokaryotes and subsequently well characterised in yeast and bacterial mutants with altered perception of osmotic stress. Osmosensors are two-component systems that contain a cytoplasmic histidine kinase sensor, which is autophosphorylated on perception of a signal and an aspartate receiver in the response regulator that relays the phosphorylation signal (Wurgler-Murphy and Saito, 1997). One of the best-characterised two-component histidine kinases is the *Saccharomyces cerevisae* osmosensor Sln1. This signal unit is

made up of three proteins, Sln1p (a transmembrane histidine kinase), Ssk1p and Ypd1p (Fig. 1.3). Sln1p is thought to act as a sensor protein, phosphorylating response regulator proteins Ypd1p and Ssk1p under conditions of high osmolarity. The Sln 1 phospho-relay system is initiated by the autophoshorylation of Sln 1 at His576. This phosphate is then transferred sequentially to Sln 1-Asp1144, then Ypd 1-His64, and finally to Ssk 1-Asp554. Several two-component histidine kinases have been identified in the Arabidopsis genome (Urao et al., 2000), although there is currently no evidence confirming their role as thermosensors. In earlier work Urao et al. (1999) isolated a histidine kinase gene, *AtHK1,* from Arabidopsis by PCR which is structurally related to *Sln1*. The gene product was able to rescue the salt sensitivity of yeast mutants with deletions of Sln1 and Sho1 indicating that *AtHK1* may function as an osmosensor in plants. However, its exact role has not yet been confirmed.

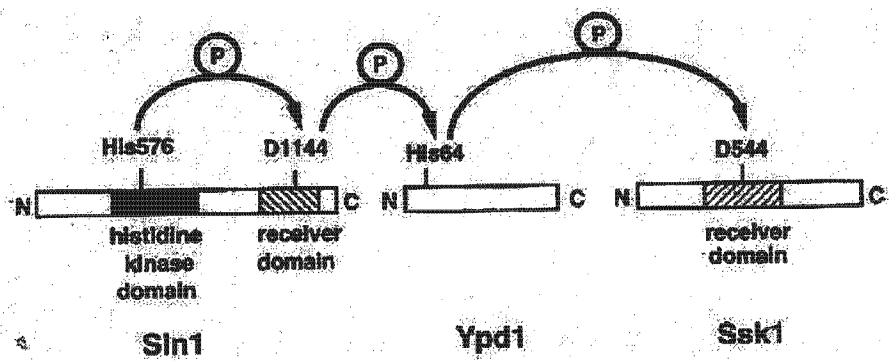

Figure 1.3 Phospho-relay system of the HOG pathway. A phospho-relay system consisting of three proteins, Sln1, Ypd1 and Ssk1 activates the HOG pathway. Sln1, the osmosensor, contains both a histidine kinase domain (black rectangle) and a receiver domain at its C terminus (hatched triangle). Upon signal sensing Sln1 autophosphorylates His576. Ssk1 contains a receiver domain (hatched rectangle) and is the activator of the MAPKKKs Ssk2 and Ssk22. Arrows indicated direction of phospho-transfer. Mutation of the His or Asp residues blocks phospho-transfer and results in constitutive activation of the pathway, suggesting that phosphorylation prevents activation of the HOG MAPK cascade (Banuett, 1998).

A transmembrane protein, Sho,1 was reported by Maede et al. (1995) to be an osmosensor activated under conditions of high osmolarity. The protein consists of four closely packed hydrophobic transmembrane peptides. The carboxyl-terminal region contains an Src homology 3 (SH3) domain that modulates various signal transduction pathways. Sho1p activates the Pbs2 kinase. *PBS2* is a gene that confers resistance to polymyxin B when over-expressed and its product is a member of protein kinase genes that include a MAP kinase kinase (MAPKK; Pbs2p). The details of the activation mechanisms are unknown (Brewster et al., 1993; Shinozaki and Yamaguchi-Shinozaki, 1997).

Calcium is known to be involved in the response to environmental stresses. Cold, drought and salinity have been shown to induce transient Ca^{2+} influx into the cell cytoplasm (Knight et al., 2000; Sanders et al., 1999). It has been suggested that the channels responsible for this influx of Ca^{2+} may represent a type of sensor for these stress signals.

1.3.2 Signal Transduction

There appears to be a complex signaling mechanism involving a range of molecules. The plasma membrane, as the selective barrier between the cells and the external environment, plays a key role in the perception and transmission of environmental changes. Activated G-box-binding proteins signal a variety of plasma membrane-bound phospholipases and channels that in turn regulate second-messenger production. The G-box is a ubiquitous regulatory element with the sequence CACGTG. Other second messengers including, glutathione, ascorbic acid and hydrogen peroxide have been identified (Trewavas and Malho, 1995).

Reversible protein phosphorylation is one of the most widely used mechanisms to regulate biological processes, including intracellular signal transduction (Wurgler-Murphy and Saito, 1997). Protein kinases, which catalyze protein phosphorylation, can be classified according to the amino acid species they phosphorylate namely protein serine/threonine kinase and histidine kinases (PHKs). In prokaryotes PHKs are invariably part of the two-component signal transducing system. Many variations exist among the two systems in prokaryotes but they all share a common theme – a phospho-relay reaction between a histidine residue in the sensor and an aspartate residue in the response regulator. A serine/ threonine protein kinase from wheat PKABA1, was among the first plant protein kinase genes to be found that is up-regulated by drought, low temperature and NaCl (Holappa and Walker-Simmons, 1995).

Ssk 1 is an activator of the ubiquitous signaling module, the mitogen-activated protein kinase (MAPK) cascade. This family of homologous protein kinases is found only in eukaryotes. In yeast phosphorylated Ssk1p is an activator of the MAPK cascade referred to as the HOG (high osmolarity glycerol response) MAPK cascade. This cascade is composed of a pair of redundant MAPKKs (Ssk2 and Ssk22), the MAPKK Pbs2 and the MAPK Hog 1. Activated Hog1 is believed to translocate to the nucleus, where it phosphorylates one or more transcription factors that are responsible for inducing *GPD1* and other genes. The *GPD1* gene encodes NAD^+ - dependent glycerol-3- phosphate dehydrogenase, a key enzyme for glycerol biosynthesis. Glycerol is a compatible osmolyte that helps the cells to adapt to conditions of high osmolarity (Shinozaki and Yamaguchi-Shinozaki, 1997; Wurgler-Murphy and Saito, 1997). There is evidence that MAP kinase cascades are actively involved in the response of Arabidopsis to water-deficit stress

(Mizoguchi et al., 1997). For example, the Arabidopsis MAP kinases, ATMPK4 and ATMPK6, are activated by various abiotic stresses.

Calcium dependent protein kinases (CDPKs) are a family of protein kinases, unique to plants, that are is also involved in stress signal transduction in plant cells. There are at least 34 putative CPDKs in the Arabidopsis genome (Harmon et al., 2000). CPDKs consist of a serine/ threonine protein kinase domain and a C-terminal calmodulin-like domain with up to four EF hand motifs. Most CPDKs have an N-terminal motif for myristoylation that potentially facilitates membrane association. Urao et al. (1994) demonstrated that expression of the Arabidopsis *ATCDPK1* and *ATCDPK2* was rapidly induced by drought and salt treatments but not by cold, heat shock or ABA. This result indicated that the expression of *ATCPDK* is ABA-independent. Sheen (1996) transiently expressed a chimeric gene consisting of a green fluorescent protein (GFP) reporter driven by a cold-, salt-, dark- and ABA-responsive promoter (HVA1) in maize leaf protoplasts. The reporter gene was activated by co-expression with a constitutively active ATCDPK1 while a non-active CPDK mutant alone failed to do the same, thus proving the functional role of CDPKs in signal transduction. Similarly, a membrane associated CPDK in rice was activated by cold and salt treatment and overexpression of the *OsCPDK7* gene resulted in increased cold and osmotic stress tolerance in rice (Saijo et al., 2000).

Calcium ions increase rapidly in response to environmental stresses and are generally considered to function as a second messenger in plants (Knight et al., 1991). It has been demonstrated that calcium is released from internal stores and the apoplastic spaces and that the release is controlled by ligand-sensitive Ca^{2+} channels (Knight, 2000; Monroy et al., 1998; Sanders et al., 1999). Using

different Ca^{2+} channel blockers, Knight et al. (1997) demonstrated that the expression of drought-responsive genes was inhibited during mannitol treatment in Arabidopsis, denoting the importance of calcium signalling during stress. The role of calcium ions in signalling is further illustrated by the function of calcineurin B-like proteins (calcium binding proteins). These proteins are implicated in a variety of signalling pathways in animals and also in adaptation to salt stress in yeast and plants, and require calcium for their function (Liu and Zhu, 1998; Pardo et al., 1998). It was shown that in Arabidopsis, for example, a calcineurin B-like protein, AtCBL1was induced in response to drought, wounding and cold stress (Kudla et al., 1999).

The SOS3 family of proteins is yet another group of Ca^{2+} -binding proteins. SOS3 is homologous to the regulatory subunit of yeast calcineurin (CNB) and animal neuronal calcium sensors (Liu and Zhu, 1998) and is composed of three EF- hand motifs. SOS3 binds Ca2+ with low affinity compared to calmodulin (Ishitani et al., 2000) suggesting that the function of SOS3 salt tolerance is only evident at locations where the cellular transient increase in Ca^{2+} is very large (Xiong et al., 2002).

The phosphoinositide complex has been implicated to function in the transduction of environmental stimuli such as gravity, light, pathogen and osmotic stress. Analogous to animal cells, phopholipase C in plants is believed to hydrolyze phosphatidylinisitol-4,5-biphosphate to generate two second messengers, diacylglycerol and inositol 1,4,5-triphosphate (IP_3) (Munnik and Meijer, 2001) then activate protein kinase C and induce the release of Ca^{2+} from intracellular stores, respectively.

Increasing evidence suggests that phosholipase D (PLD) is also involved in signal transduction. PLD hydrolyses phosholipids to generate phosphatidic acid (PA). PA is believed to act as a second messenger in plants (Wang, 1999). The enzyme appears to be activated by drought and osmotic stress through a G-protein and leads to transient increases in PA levels in plants (Frank et al., 2000; Munnik et al., 2000). PLD activity also mediates ABA-induced stomatal closure.

Reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anions and hydrogen peroxide are believed to act as signals inducing the ROS scavengers and other protective mechanisms in plants (Gong et al., 1998). Although not much is known yet about the connection between ROS signal transduction and osmotic stress signalling, the involvement of ROS in pathogenesis signal transduction is well known (Lamb and Dixon, 1997). ROS have been shown to activate Ca^{2+} channels in guard cells and subsequent stomatal closure (Pei et al., 1998). ROS are also involved in thermotolerance (Gong et al., 1998) and ABA synthesis (Zhao et al., 2001). There is also evidence to suggest that a histidine kinase (Desikan et al., 2001) and the transcription factor DREB2A (Xiong and Zhu, 2001) are up-regulated by oxidative stress.

It is thought that there are at least four independent signal pathways that function in the activation of stress-inducible genes (Fig. 1.4) (Shinozaki and Yamaguchi–Shinozaki, 1997). Two of the pathways are ABA-dependent (I and II) and two are ABA-independent (III and IV). There is now increasing evidence that there are two other ABA-independent pathways that are involved in low-temperature-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki, 2000). One of these pathways involves *hos5*, which functions in DREB2A signalling (Xiong et al., 1999) and the other pathway involves *esk1* which functions in response to cold via DRE-independent pathway (Xin and

Browse, 1998). These signalling pathways do not act in a parallel manner but instead "cross-talk" and converge to activate stress-responsive gene expression (Ishitani et al., 1997). Yamaguchi-Shinozaki and Shinozaki (1994) studied the expression of the *rd29*A gene and found that at least two separate regulatory systems function in gene expression during cold or drought stress. One of these systems is ABA-dependent and the other is ABA-independent.

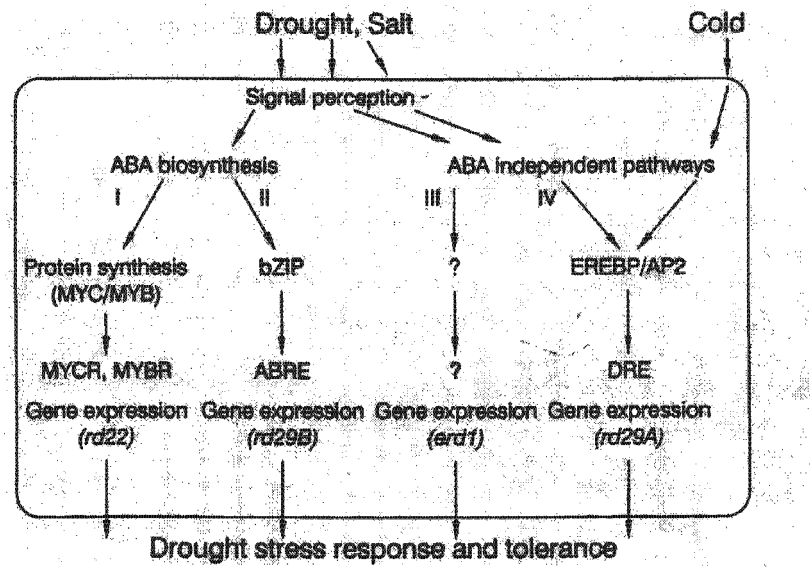

Figure 1.4. Signal transduction pathways between the perception of a water-stress signal and gene expression in Arabidopsis. At least four signal transduction pathways exist (I-IV): two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein biosynthesis is required in one of the ABA-dependent pathway (I). In another ABA-dependent pathway, ABA-responsive element (ABRE) functions as an ABA-responsive element and does not require protein biosynthesis (II). In one of the ABA-independent pathways, a drought responsive element (DRE) is involved in the regulation of genes not only by drought and salt but also by cold stress (IV). Drought and salt control another ABA-independent pathway, but not by cold (III) (Shinozaki et al., 1999).

1.3.3 ABA-dependent Gene Expression

One of the major signals operating during drought stress is the phytohormone abscisic acid (ABA). Both genetic and biochemical studies have established that the phytohormone ABA is crucial in the response of plants to desiccation, salt, and cold (Bray, 1997). Many water-stress inducible genes are up-regulated by exogenous ABA treatment and the levels of endogenous ABA increase significantly in many plants under stress conditions (Bartels et al., 1990; Bray, 1997; Busk and Pages, 1998). Furthermore, ABA is necessary for the regulation of several events during late seed development (McCarty, 1995).

In pathway II (Fig.1.4), ABA- responsive elements (ABREs, PyACGTGGC) are thought to exist in the promoter regions of the dehydration-induced genes. ABREs were first identified in wheat *Em* (Litts et al., 1987) and rice *rab* genes and function as *cis*-acting DNA elements involved in ABA-regulated gene expression (Mundy et al., 1990). These elements contain the palindromic motif CACGTG with the G-box ACGT core element. ACGT elements have been observed in many plant genes regulated by environmental and physiological factors such as UV light, anaerobiosis and wounding (Shinozaki and Yamaguchi-Shinozaki, 1996). ABRE and G-box binding proteins have a basic region adjacent to a leucine zipper motif (bZIP). DNA-binding studies have shown that nucleotides flanking the ACGT core specify the DNA-protein interactions and subsequent gene activation (Ingram and Bartels, 1996; Iwaza et al., 1993; Shinozaki and Yamaguchi–Shinozaki, 1997). It has emerged in recent years that coupling elements are required for the regulation of genes activated by ABREs. For example, the wheat *HVA22* gene is regulated by an ABA-responsive complex consisting of the CE1 (TGCCACCG) coupling element as well as an ABRE element

(Bray, 1997). However, it is not clear how ABA activates bZIP proteins to bind to ABRE and initiate transcription of the ABA-inducible genes.

Protein biosynthesis is required for one of the ABA-dependent pathways (Fig.1.4, pathway I) (Yamaguchi-Shinozaki and Shinozaki, 1993). In Arabidopsis, ABA mediates a drought inducible gene *rd*22. A 67-bp region of the *rd*22 promoter contains several conserved motifs of DNA-binding proteins, such as MYC (a family of transcription factors with basic-helix loop and Leu-zipper motif) and MYB (transcription factors with the Trp cluster motif) but no ABREs. This suggests that a drought inducible MYC homologue may function in the ABA-inducible expression of *rd*22 (Abe et al., 1997). Abiotic stress induces genes for several transcription factors including MYB-related and bZIP–related factors in higher plants. High salt conditions and exogenous ABA rapidly induce *Atmyb2,* a gene encoding an MYB-related protein. However, *Atmyb2* is not induced by cold or heat stress. (Urao et al., 1993). A cold-inducible gene encoding a bZIP transcription factor has been isolated from rice (*lip29)* (Nakagawa et al., 1996) and maize (*mlip15*) (Kusano et al., 1995). These genes also respond to high salt and exogenous ABA treatment.

1.3.4 ABA-independent Gene Expression

Analysis of drought-inducible genes using ABA- deficient mutants revealed that although many genes respond to ABA, there are some that do not. Several genes including *rd29A, cor6.6* and *cor47* (*rd17)* were induced by exogenous ABA treatment but were also induced by salt, cold or drought in *aba* (ABA-deficient) and *abi* (ABA-insensitive) Arabidopsis mutants (Shinozaki and

Yamaguchi-Shinozaki, 1997). This suggests that these genes do not require ABA for their expression under stress conditions although they do respond to ABA.

Examination of cold-responsive genes in Arabidopsis led to the identification of a novel *cis*-acting element containing the 9-bp sequence, TACCGACAT, which is involved in the ABA-independent response of *rd29A* to conditions of drought, cold or high salinity. This element termed the C-repeat (CRT) drought responsive element (DRE) is not involved in the ABA-responsive, slow expression of *rd29A* but is essential for the regulation of dehydration–responsive gene expression. DRE-related motifs have been reported in the promoter regions of many cold and drought inducible genes (Baker et al., 1994; Nordin et al., 1993; Shinozaki and Yamaguchi-Shinozaki, 1996; Wang et al., 1994). Investigations by Liu et al., (1998) revealed that there are two DRE binding proteins (DREB1A and DREB2A) in Arabidopsis that specifically interact with the DRE sequence in the promoter region of the *rd29A* gene under stress conditions. In a separate study, Gilmour et al., (1998) identified similar transcriptional activators that bind to the CRT/DRE which they designated CBF1, CBF2 and CBF3. These activators correspond to DREB1B, DREB1C and DREB1A respectively (Liu et al., 1998). The proteins which all contain the AP2/EREBP (ethylene-responsive element binding protein) -DNA-binding domain, have nearly identical amino acid sequences.

Liu et al., (1998) suggested that DREB1 and DREB2 function as *trans*-acting factors in two separate signal transduction pathways. DREB1 functions in the response to low-temperature and DREB2 to dehydration. In subsequent work, Kasuga et al. (1999) reported that over-expression of the cDNA encoding DREB1A in transgenic plants activated the expression of many stress

tolerance genes under normal growing conditions resulting in improved tolerance to drought, freezing and high salt. They also discovered that expression of DREB1A under the control of the constitutive 35S CaMV promoter in transgenic plants resulted in severe growth retardation under normal growing conditions. However, substituting the 35S promoter with the stress-inducible *rd29A* promoter not only gave much improved growth, but also provided even greater tolerance to stresses with a water deficit component. More recently, Jaglo et al. (2001) reported that the CRT/DRE binding factor cold-response pathway is conserved in *Brassica napus*, wheat, rye and tomato. They concluded that the components of the CBF cold-response pathway are highly conserved in flowering plants and not limited to those that cold acclimate. Hsieh et al. (2002) confirmed that overexpression of *CBF1* improved the chilling and oxidative stress tolerance in transgenic tomatoes. The DRE from Arabidopsis mediates transcription in response to desiccation, cold or high salt but not in response to ABA. Intriguingly, the DRE appears to have a different role in maize than in Arabidopsis in that the *rab17* promoter from maize contains two DRE-like elements that participate in induction by ABA (Busk et al., 1997).

There are several drought-inducible genes that do not respond to either cold or ABA treatment suggesting that there is a fourth pathway (Fig 1.4, pathway III) in the dehydration-stress response. These genes include *ERDI,* which encodes a Clp protease regulatory subunit (Nakashima et al., 1997), and *rd19* and *rd21,* which encode cysteine proteases (Shinozaki and Yamaguchi-Shinozaki, 1997). Not much is known about this pathway.

The ability of the whole plant to respond and to survive cellular water deficit depends on whole-plant mechanisms that can integrate the signalling mechanisms and subsequently the cellular

responses. However, the question is how do plants survive water deficit? What mechanisms are in place in order to cope with the multitude of abiotic stresses that a plant may encounter in its life cycle?

1.4 MOLECULAR RESPONSES AND TOLERANCE TO WATER DEFICIT

Plants may withstand imposed environmental stresses by employing mechanisms that promote avoidance of water loss, protect the cellular machinery and repair damage. The severity of the water loss, the developmental stage and physiological condition of the plant determines the response to cellular water deficit (Bray, 1997). A slow rate may allow acclimation to the water deficit and limit the extent of injury while a rapid loss may suppress acclimation. As higher plants are immobile, they are limited in their response to conditions inducing water deficit. Consequently, they have evolved two major mechanisms to cope with water stress: stress avoidance and tolerance. At the cellular level, for example, avoidance is achieved by lowering the osmotic potential of the cell in order to allow the uptake of water and the maintenance of turgor. The physiological responses of plants to water deficit arise out of changes in cellular gene expression. In trying to understand the response to stresses involving a water deficit component, interest has centered on differentially expressed genes because it has been postulated that induction of new genes will permit adaptation to stresses. However, the simple accumulation of mRNA does not equate with an adaptive function for any specific gene as additional mechanisms, such as translational regulation and post-translational modification, may still be required for a fully functional gene product.

1.4.1 LEA Proteins

The synthesis of globular and extremely hydrophilic proteins known as late embryogenesis abundant (LEA) proteins is one of the well-documented responses to abiotic stress. These proteins are glycine-rich and remain soluble even when boiled. LEA proteins were first observed to accumulate during the maturation phase in seeds developing desiccation tolerance (Dure et al., 1981). Many groups of LEA proteins have now been defined by their sequence homologies in different plant species. It has been suggested that they have a role to play in the maintenance of protein or membrane structure and sequestration of ions. In addition, their randomly coiled moieties are consistent with structures involved in the binding of water. They are also believed to operate as molecular chaperones (Skriver and Mundy, 1990; Bray, 1997). A LEA protein gene, *HV1A*, isolated from barley, was found to confer tolerance to water deficit and high salinity stress in transgenic rice (Xu et al., 1996). Dehydrins, a subgroup (D-II) of the LEA proteins, have also been observed to accumulate in response to drought, cold, high salinity and exogenous application of ABA (Close, 1997). The proteins associate with macromolecules ranging from nucleoprotein complexes to endo-membrane sheaths in the cytoplasm. It has been hypothesized that dehydrins are surfactants capable of inhibiting the coagulation of a range of macromolecules, thereby preserving structural integrity (Close, 1996; Gee et al.,1994). NDong et al. (2002) confirmed that WCS19 (Chauvin et al., 1993) is a stromatal protein belonging to a new class of organelle-targeted group 3 LEA proteins. Constitutive expression of the WCS19 protein protected cold-acclimated leaves from freeze-induced damage.

Danyluk et al., (1994) identified a low temperature responsive dehydrin-like gene from wheat, which they named w*cor410*. The gene belongs to a family of homologous members, w*cor410, wcor410b and wcor410c.* They found a positive correlation between the accumulation of the *wcor410* transcripts and the capacity of wheat to develop freezing tolerance. Members of the *wcor410* family were also found to be up-regulated extensively by water stress, polyethylene glycol, ABA and to a lesser extent by salt and wounding. In a subsequent publication (1998), these authors showed that the WCOR410 protein accumulates in the vicinity of the plasma membrane of cells in the vascular transition area where freeze-induced dehydration is likely to be more severe. They proposed that the abundance, properties and localization of the protein suggested that it was involved in the cryo-protection of the plasma membrane against freezing or dehydration stress.

1.4.2 Osmolytes

The accumulation of osmolytes is considered to be an important strategy that plants use to overcome abiotic stresses. Osmolytes are compatible solutes that occur in all organisms from archeabacteria to higher plants and animals. Three types of osmolytes namely: quarternary ammonium compounds such as glycine betaine; polyols and sugars such as mannitol and trehalose and; amino acids such as proline, are known (Ingram and Bartels, 1996). Osmolytes are highly soluble compounds that carry no net charge at physiological pH and are non-toxic at high concentrations. They are thought to function by raising the osmotic potential of the cell and also by stabilizing proteins and membranes when a water deficit occurs (Holmberg and Bulow, 1998; McNeil et al., 1999).

An improvement in osmotic and salinity stress tolerance has been obtained mainly by the introduction of transgenes leading to the synthesis of osmolytes such as glycine betaine, mannitol and proline in tobacco (Hayashi et al., 1997; Holmstrom et al., 1996; Kishor et al., 1995; Tarczynski et al., 1993). Glycine betaine is an important osmolyte distributed widely amongst plants. In many plants, it is synthesized through a two-step oxidation of choline by choline monooxygenase and betaine aldehyde dehydrogenase (Burnet et al., 1995). Attempts to introduce or improve the generation of glycine betaine in plants by the separate introduction of the genes coding for these enzymes have not been successful because insufficient choline substrate and the intermediate betaine aldehyde were available within the cell (Nuccio et al., 1998). It has been suggested that betaine probably functions as an osmotic adjustment agent and a stabilizer of protein structure and function (Sokamoto et al., 1998). However, transgenic rice containing the betaine aldehyde dehydrogenase gene supplied with exogenous betaine aldehyde to generate sufficient glycinebetaine, showed improved tolerance against both salt and low temperature (Kishitani et al., 2000). In addition, exogenous application of betaine improved freezing tolerance by more than 5°C in both acclimated and non-acclimated wheat plants (Allard et al., 1998).

Much work has gone into studying the amino acid-derived osmoprotectant proline. Proline biosynthesis from the amino acid glutamate was achieved by using the pyrroline-5-carboxylate synthetase (*p5cs)* gene from the mothbean *Vigna aconitfolia.* Transgenic tobacco plants with increased proline content were shown be more tolerant to osmotic stress than the control plants (Kishor, et al. 1995) and transgenic rice plants accumulating proline showed an enhanced biomass production (Zhu et al., 1998).

The accumulation of sugar related compounds such as sorbitol, fructans, trehalose and raffinose is another part of the mechanisms that plants use to cope with environmental stresses. An osmolyte of interest is the sugar alcohol sorbitol that accumulates under conditions of environmental stress (Bartels and Ingram, 1996). It is thought that the sugar, in common with other osmolytes, acts as a membrane stabilizer by substituting for water. Through hydrogen bonding with polar groups on the membrane surface, sorbitol is believed to assist in maintaining the structural integrity of the cell membranes. It has been suggested that the other method by which sugars, including sorbitol, protect the cell during desiccation is by the formation of glass. Koster (1991) explained this by proposing that rather than solutes crystallizing, a supersaturated liquid is produced due to the presence of the sugars. This liquid has the mechanical properties of a solid and prevents cellular collapse. Gilmour et al., (2000) noted that transgenic plants expressing CBF3 (C-repeat binding factor) not only had elevated levels of COR proteins, but also had increased levels of total sugars and proline which presumably assisted in increasing the tolerance of the plants to cold and other stresses.

Mannitol, a sugar alcohol, has been overproduced in both Arabidopsis and tobacco by expressing the bacterial *mltd* gene, encoding mannitol-1-phosphate dehydrogenase (Tarczynski and Bohnert, 1993). Enhanced tolerance was observed under conditions of high salinity stress.

1.4.3 Detoxification

It has been established that the negative effect of stress on plants may be in part a consequence of the oxidative damage to important molecules by ROS such as hydroxyl radicals, superoxide anions and hydrogen peroxide (Gong et al., 1998). ROS are usually generated as a consequence of the normal cellular activities of the plant such as photorespiration. Plants are endowed with numerous antioxidant defense mechanisms and cope very well with oxidative stress when conditions are normal. However, under severe stress conditions, damage in the form of photo-bleaching of chlorophyll, base mutations, destruction of proteins, breakage of DNA strands and lipid peroxidation of membranes may occur (Bowler et al., 1992; Smirnoff, 1993). There is, therefore, an increase in the synthesis of antioxidants such as tocopherols, ascorbate and tripeptide glutathione (GSH) and enzymes such as superoxide dismutase (SOD), gluthatione peroxidase, glutathione reductase and catalases to scavenge excess ROS when stress conditions occur.

It has been shown that overexpression of various genes involved in the ROS scavenging systems can have a protective effect. SODs react with superoxide radicals to produce hydrogen peroxide and water. Overexpression of SOD can lead to protection against high light intensity and low temperature. For example, Van Camp et al. (1996) showed that the expression of an Fe superoxide dismutase from Arabidopsis protected both the plasmalemma and photosystem II against superoxides generated during illumination of tobacco leaf discs impregnated with methyl viologen. Roxas et al. (1997) showed that the over-expression of an enzyme with the combined activities of glutathione S-transferase and glutathione peroxidase in transgenic tobacco resulted in significantly faster growth than in the wild-type during chilling and salt stress.

1.4.4 Heat Shock Proteins

Another important response of plants to a variety of biotic and abiotic stresses is the production of heat- shock proteins (Hsps) (Vierling, 1991). Prolonged exposure to conditions of extreme stress can result in cell and tissue death. Hsps appear to be involved in maintaining cell function and survival during stress and in facilitating recovery from stress. For example, the Hsp70 proteins are believed to act as molecular chaperones and are found in the cytosol and most organelles, assisting in protein folding and translocation. During heat stress, Hsp 70s may be involved in the refolding of denatured proteins or protecting them from stress-induced damage (Lindquist and Craig, 1988). Chloroplast and mitochondrion localized Hsps appear to protect electron transport when plants are exposed to heat and oxidative stress (Downs et al., 1988).

1.4.5 Lipid Modifications

Low temperature is one of the major environmental factors limiting plant growth. When tropical and subtropical plants are exposed to low temperature, they suffer chilling injuries and growth is stunted (Bartels and Nelson, 1994; Gomes et al., 2000,). Freezing injury in most plant tissues results largely from the severe dehydration that occurs upon ice formation, and the cellular membrane systems are a primary sites of freeze-induced injury (Thomashow, 2001). Several cold-induced genes and their products have been isolated and characterized in a number of dicotyledonous and monocotyledonous species (Guy et al., 1985; Hughes and Dunn, 1996, Thomashow, 1999). Normal functioning of integral membrane proteins such as transporters and

receptor proteins depends on the fluidity of the membrane which in turn is strongly influenced at a given temperature by its lipid composition (Hazel, 1995). One of the best-documented responses of plants to chilling stress is the increase in polyunsaturated acyl chains or membrane phospholipids, which allows membrane fluidity to be maintained (Nishida and Murata, 1996). An alternative method to modify the level of unsaturation in membrane lipids is to express fatty acid desaturase genes in plants. Kodoma et al. (1994) found that tobacco plants over-expressing the Arabidopsis *FAD7* gene, which codes for the chloroplast ω-3 desaturase, had enhanced chilling tolerance.

1.4.6 Proteinase inhibitors

High proteolytic activity, cysteine proteases, coinciding with programmed cell death is one the consequences of severe abiotic stress (Fukuda, 1996). It is thought that these enzymes may be involved in the recovery of amino acids by digesting unwanted or damaged proteins for the synthesis of more proteins (Ingram and Bartels, 1996). However, plants also possess many protease inhibitors and it has been suggested that some of these inhibitors play a vital role by inhibiting the activity of proteases during conditions of water deficit (Solomon et al., 1999).

1.5 RESURRECTION PLANTS

The extremophiles known as resurrection plants have developed mechanisms that allow them to withstand severe water deficit. These plants can lose over 90% of their relative water content, survive in their dried state for prolonged periods and then resume active life when water becomes available again (Bartels et al., 1990; Gaff, 1971; Sherwin and Farrant, 1996). Not surprisingly, most resurrection plant species inhabit the arid areas of the world mainly in southern Africa,

southern America and western Australia (Gaff, 1971: 1987). Resurrection plants often grow in shallow soil on rocky outcrops (Fig. 1.5) where the water supply is limited and where there is little shelter from inclement weather (Sherwin and Farrant, 1996). The growth and reproduction of these plants occurs when water is available but upon drying, resurrection species can remain dormant for lengthy periods of time.

Figure 1.5 *Xerophyta viscosa* growing in a rocky crevice, one of its natural habitats. Note that both the hydrated and dehydrated states of the grass are represented (arrow).

Desiccation-tolerant species are represented in most taxonomic groups, ranging from pteridophytes to dicotyledons. Based on current knowledge, resurrection plants have been divided into two categories: fully-desiccation tolerant plants and modified desiccation-tolerant plants. (Oliver et al., 1998). The first group is comprised of plants that can withstand the total loss of free protoplasmic

water without being affected by the rate of water loss. Fully-desiccation tolerant plants do not have well developed water-retaining morphologies and thus the internal water content of these plants rapidly equilibrates to the water potential of the environment. Fully-desiccation tolerant plants such as lichens, algae and bryophytes experience drying rates that are extreme and they can reach air dryness within an hour or less (Oliver et al., 1998). The rate of water loss and the events before dehydration appear to be critical for the survival the modified desiccation-tolerant plants. This group comprises members of more complex plant families such as the ferns and angiosperms. Tolerance is only achieved after several hours or days. If the speed of dehydration is too fast, these plants do not acquire tolerance to desiccation. This observation suggests that the acquisition of desiccation tolerance is an active process and requires specific biochemical changes and the synthesis of desiccation-related molecules. The monocotyledonous *X. viscosa* belongs to this latter sub-group of resurrection angiosperms. The plant dehydrates slowly to a relative water content of about 4% and then rehydrates completely within 80 hours (Sherwin and Farrant, 1996).

Modified desiccation-tolerant angiosperms have been further classified into the poikilochlorophyllous type, such as the *Xerophyta spp.* that lose their chlorophyll on drying and the homoiochlorophyllous type, such as *Craterostigma wilmsi,* that do not lose their chlorophyll (Sherwin and Farrant, 1996). Poikilochlorophyllous desiccation-tolerant plants occur only among modified-desiccation tolerant plants and apparently only among the monocotyledonous plants (Gaff, 1977, 1989; Oliver et al., 2000). On drying, the leaves of *X. viscosa* lose chlorophyll and turn yellow. They also fold in half along the mid-rib (Sherwin and Farrant, 1996). As light-chlorophyll interactions can cause damage to the photosynthetic apparatus during dehydration, the plant has to be able to repair the damage or regenerate damaged organelles. Sherwin and Farrant

(1997) suggested that the loss of chlorophyll and the dismantling of thylakoid membranes during desiccation reduce photooxidative damage in poikilochlorophyllous plants.

1.5.1 Desiccation Tolerance in Resurrection Plants

The acquisition of desiccation tolerance in resurrection plants is complex. Desiccation tolerance requires biomembranes to withstand the chemical and mechanical stresses that accompany shrinkage of cells and tissues during water loss, and cellular expansion on re-absorption of water. Moreover, the plant has to maintain functional integrity of macromolecules such as proteins and nucleic acids and minimize the accumulation of toxins and free radical damage. The initiation of repair mechanisms upon rehydration is equally important.

It is thought that two basic mechanisms exist which allow desiccation tolerant plants to survive water deprivation. The first involves the protection of cellular integrity through inducible and constitutive mechanisms while the second involves the repair of desiccation- or rehydration-induced damage. However, both mechanisms are probably employed for desiccation tolerance with different plants utilizing one strategy more than the other (Oliver and Bewley, 1997). In dehydration-tolerant leaves and seeds of angiosperms, the protective mechanism activates the synthesis of specific transcripts and proteins in response to dehydration, whilst in the mosses desiccation tolerance is based on repair mechanisms involving components that are constitutively present in the cells (Oliver et al., 1998).

The molecular basis of desiccation tolerance has been studied in only a few species representing different groups: the moss *Tortula ruralis* (Oliver and Bewley, 1997), the monocotyledonous *Sporolobolous. stapfianus* (Neale et al., 2000) and the dicotyledonous *Craterostigma plantagineum* (Bartels et al., 1990). Theories on the mechanisms by which resurrection plants tolerate dehydration have mostly been derived from observations of the cellular processes that occur during the drying of the plant (Ingram and Bartels, 1996). It has been demonstrated that several genes are differentially expressed in response to dehydration (Blomstedt et al., 1998; Bockel et al., 1998; Itturiaga et al., 1992; Schneider et al., 1993). These include genes encoding regulatory molecules such as transcription factors and genes encoding membrane proteins involved in transport processes, genes encoding proteins with protective properties and genes encoding enzymes related to carbohydrate metabolism.

1.5.1.1 Regulation of Gene Expression in Desiccation-Tolerant Plants

Desiccation tolerance is a multigenetic trait and several signalling pathways exist in desiccation-tolerant species like *C. plantagenium*. Not much is known about the regulatory circuits in resurrection plants, but a number of individual factors contributing to various pathways have been characterised. The plant hormone, ABA, just as in desiccation-sensitive plants, appears to play a central role in the response to dehydration. Exogenous application of ABA to *C. plantagineum* plants and callus tissue induces dehydration-responsive genes. In fact, ABA is essential for the acquisition of desiccation tolerance by callus tissue (Bartels et al., 1990).

Most of the information currently available is derived from promoter analyses and from differential screening. Furini et al., (1996) showed that the promoter of the gene *CdeT6-19* isolated from *C.*

plantagineum was inducible by dehydration or ABA in the tissues of tobacco and Arabidopsis. In the same vein, two cDNA clones, *CpPLD-1* and *CpPLD-2* encoding phopholipase D (PLD) have been isolated. PLD is believed to be involved in the formation of phospholipid-based signaling molecules, which may be among the primary events in the signaling cascade that leads from the perception of water stress to stress-adaptation. In *C. plantagineum,* PLD is induced within minutes by dehydration but not by ABA (Frank et al., 2000).

Promoters for ABA-responsive LEA-like genes *pcC6-19*, *pcC27-45* and *pcC11-24*, were found to be responsive to dehydration although there were no common sequence motifs amongst them (Michel et al. 1993; Velasco et al., 1998). All the promoters were active in seeds and pollen but only *pcC6-19* was inducible by dehydration or ABA in the vegetative tissues of tobacco and Arabidopsis. *PcC6-19* also does not require protein synthesis for ABA-mediated transcription.

Other molecules with putative transcriptional or signalling activities have also been isolated mainly from *C. plantagineum.* These include members of the homeodomain Leu zipper (HDZIP) family which are thought to regulate developmental processes and respond to pathogen-induced and abiotic stress (Frank et al., 2000), heat shock transcription factors (Bockel et al., 1998) and a MYB transcription factor (Itturiaga et al., 1996).

1.5.1.2 Protective Molecules

Blomstedt et al., (1998) found that a number of genes activated in the early stages of dehydration in resurrection plants are similar to those expressed in the desiccating seeds of most plants. The synthesis of the globular LEA proteins is one of the well-documented responses to dehydration,

salinity and cold stress. A number of genes which possess homology to LEA proteins have been isolated (Ingram and Bartels, 1996; Schneider et al., 1993). It has been proposed that LEA proteins in resurrection plants also have the same protective role of stabilizing internal structures as they do in non-tolerant plants. *CdeT27-45*, a LEA-like gene from *C. plantagineum*, is strongly expressed in vegetative tissue in response to dehydration or ABA treatment (Piatwoski et al., 1990). It has been established that specific nuclear proteins are necessary for the ABA-induced expression of *CdeT27-45* (Hillbricht et al., 2002). A region of 29 bp, pr3.75, was identified to be essential for protein interaction. Hillbricht et al., (2002) confirmed that CpR18 was a transcription factor that binds to pr3.75 for the ABA-mediated expression of the *CdeT27-45* gene. CpR18 is a novel protein containing an SAP-domain and a zinc finger at the carboxy-terminus. The protein shares the same pattern motifs with expressed sequence tag clones from tomato, barley and rice. The SAP-domain is essential in plants for association of chromatin *in vivo* and mediates the formation of stable dimers that bind nucleic acids (Babiychuk et al., 2001).

The accumulation of non-reducing sugars is also a common response in resurrection plants to abiotic stresses. In *C. plantagineum* the unusual C8 sugar octulose represents 90% of the soluble sugars in photosynthetically active leaves. During dehydration, octulose declines and sucrose accumulates perhaps as a result of the activation of aldolases and tansketolases. The reverse happens on rehydration (Bartels and Salamini, 2001). Although the overall sugar content is similar in both hydrated and dehydrated leaves, roots contain only a small amount of octulose. It has been suggested that octulose results from photosynthesis and accumulates in the leaves during the light period. Some octulose is then probably transported to the roots via the phloem (Norwood et al., 2000). Interestingly, several modified desiccation-tolerant species accumulate sucrose and other

derivatives during dehydration. Sucrose and trehalose increase in *Myrothamnus flabellifolia* (Bianchi et al., 1993) and sucrose accumulates in *S. stapfianus* (Oliver, 1996) during dehydration.

Other proteins with protective functions include some proteins with enzymatic function such as the aldehyde dehydrogenase described by Kirch et al., (2001) and small heat shock proteins (Alamillo et al., 1995). The evidence gathered so far indicates that desiccation tolerance in resurrection plants, particularly *C. plantagenium,* is probably not due to unique structural genes present only in these plants. Most of the genes are also present in non-tolerant plants. The differences in tolerance between the two groups probably arises from differences in expression patterns (Bartels and Salamini, 2001).

1.5.2 Recovery from desiccation

In all desiccation-tolerant vegetative plants and tissues, cellular damage is caused either by the in-rush of water on rehydration or a consequence of the drying process or both (Bewley, 1979). The processes associated with tissue recovery on rehydration in resurrection plants have been less extensively studied with only a few rehydration associated proteins identified (Oliver, 1991). Most of the information available is on the fully desiccation-tolerant moss *T. ruralis.* It has been postulated that the moss relies more on the activation of pre-existing repair mechanisms for desiccation tolerance than it does on either pre-established or activated protection systems (Oliver, 1991). During dehydration there is a rapid decline in protein synthesis and there is no new protein synthesis even if the rate of dehydration is slow. It was suggested that the moss responds to desiccation by an alteration in protein synthesis upon rehydration, which is primarily a measure of the result of a change in translational controls. It was demonstrated that during the first two hours

of rehydration the synthesis of 74 proteins termed rehydrins is initiated or significantly increased and the synthesis of 25 proteins termed hydrins is terminated or significantly reduced. Rehydrin synthesis was initiated only when the moss has lost between 50 and 20% of its water. This indicates that a certain amount of water loss is required before the protein-based recovery mechanism is activated. Protein analysis using a purified antibody raised against the common carboxy-terminus of maize seedling dehydrins (Close, 1993) indicated that *T. ruralis* produces two major dehydrins, 35 kDa and 80-90 kDa, that are present in the hydrated state. They do not appear to increase during dehydration whether the water loss occurs slowly or rapidly

The assertion that *T. ruralis* has constitutive protective mechanisms in place is supported by its sugar metabolism. The only sugar available for cellular protection in *T. ruralis* is sucrose (Smirnoff, 1992), which constitutes 10% of the dry mass of the gametophytic cells of the moss. This amount of sucrose is sufficient for membrane protection. The concentration of the sugar. however, does not change either during drying or rehydration. This lack of change in the concentration of sucrose appears to be a common feature of desiccation-tolerant mosses (Smirnoff, 1992). Also, the photosynthetic apparatus in *T. ruralis* is well protected during dehydration. This is evidenced by the fact that photosynthesis recovers very rapidly on rehydration. Tuba et al., (1996) found that it takes only 20 minutes for a positive carbon balance to be re-established.

In desiccation-tolerant angiosperms, recovery is more complex. All desiccation-tolerant vegetative plants and tissues exhibit cellular damage immediately upon dehydration. The membranes are particularly affected as is evidenced by ion leakage. Studies by Tuba *et al.*, (1993) showed that in poikilochlorophyllous plants such as *Xerophyta spp.*, the internal structures of the chloroplasts

including chlorophyll and caretonoids were broken down completely after a period of desiccation. The rebuilding of chloroplasts and the photosynthetic apparatus only occurred after full turgour and maximum leaf water content had been re-established. This response probably requires rehydration-induced changes in gene expression. However, it is thought that protective mechanisms during dehydration rather than repair on rehydration appear to dominate in modified desiccation-tolerant resurrection plants (Sherwin and Farrant, 1996).

1.6 IMPROVING STRESS TOLERANCE BY GENE TRANFER

The transfer of genes encoding protective proteins or enzymes from other organisms can improve tolerance to the environmental stresses that plants may encounter. However, there are many obstacles to foreign gene expression in higher plants. Techniques available for transferring foreign genes into genomes of plants include *Agrobacterium*-mediated gene transfer, electroporation, particle bombardment and vacuum-infiltration (Walden and Schell, 1990). However, certain techniques work better in some cases than in others and sometimes the best method has to be determined empirically. Obtaining adequate expression of a particular gene especially at the right time may not be easily achieved. In addition, expression of a particular gene in specific tissues and organelles is often essential for achieving the desired result. With most proteins, correct processing and folding are often pre-requisites for function. This may not occur if the protein is expressed in a foreign system.

An unsuitable or inhibitory environment could result in sub-optimal enzyme activity thus failing to mimic the reactions occurring in the original environment. Another problem is that it is not possible

to foresee all the metabolic changes that might occur when a foreign gene is expressed in a plant. Endogenous enzyme activity may deplete the product or form toxic compounds thus making it impossible to assess the function of a gene in a transgenic plant (Holmberg and Bulow, 1998).

Abiotic stresses elicit multigenetic responses within the plant cells. The response to abiotic stress, therefore, involves a range of different biochemical and physiological mechanisms. The expression of foreign genes in various crop plants has resulted in plants with an altered response to environmental stresses. The expression of the transcription factors CBF1 and DREB1A has shown that is possible to alter the expression levels of several genes at the same time by the transfer a single transcriptional factor. Numerous examples of single genes improving the tolerance of abiotic stresses have also been mentioned in the preceding discussion.

Genes that could potentially improve the drought tolerance of agriculturally important plants such as maize and wheat have been isolated from *C. plantagenium* (Itturiaga et al., 1992) and *X. viscosa* (Garwe et al., 2003; Mowla et al., 2002; Mundree et al., 2000; Ndima et al., 2001). An aldolase reductase homologue (*ALDXV4)* cloned from the resurrection plant, *X. viscosa* Baker, was found to confer tolerance to osmotic stress in *Escherichia coli* cells. Northern blot analysis showed that *ALDXV4* was expressed only under conditions of dehydration stress in *X.viscosa.* The gene showed significant similarity to known aldolase reductases. The highest identities were with *Hordeum vulgare* (66%), *Bromegrass* (65%) and *Avena* (66%) aldose reductase-like proteins (Mundree et al., 2000).

1.7 OBJECTIVES OF THIS STUDY

XVSAP1 was isolated using the strategy of "complementation by functional sufficiency" from a cDNA library constructed from dehydrated *X. viscosa* leaves as described by Mundree et al. (2000). In summary, *XVSAP1* conferred tolerance to osmotically stressed *E. coli* (*srl*::Tn10) cells. The gene codes for a protein that is homologous to a cold tolerance protein, WCOR413, from *Triticum aestivum* (Danyluk and Sarhan, 1996). The gene also bears close identity (56%) to other uncharacterized proteins from *A. thaliana.* The Arabidopsis proteins have 64% identity to WCOR413.

The objectives of this work were two-fold:

(i) The initial aim was to conduct a molecular characterization of the *XVSAP1* cDNA using such techniques as:

(a) Sequence analysis

(b) Southern blot analysis to determine the copy number of the gene in the *X. viscosa* genome

(c) Northern blot analysis to determine the expression patterns of the gene in response to abiotic stresses such as cold, heat, salt, high light intensity and to the phytohormone ABA

(d) Western blot analysis to determine expression patterns of the XVSAP1 protein.

(ii) The second aim was to transform *A. thaliana* and *N. tabacum* with *XVSAP1* and to determine if the gene conferred abiotic stress tolerance to these model plants.

CHAPTER TWO

MOLECULAR CHARACTERIZATION OF *XVSAP1* FROM *X. VISCOSA*

SUMMARY

The strategy of "complementation by functional sufficiency" was used to isolate a cDNA designated *XVSAP1* from a cDNA library constructed from RNA isolated from dehydrated *X.viscosa* leaves. Analysis of the cDNA sequence indicated a highly hydrophobic protein with six transmembrane regions. The deduced amino acid sequence showed 49% identity to WCOR413, a low temperature-regulated protein from wheat and 53% identity with another cold-associated protein in rice. The protein also showed between 25% to 56% identity to WCOR413-like proteins from Arabidopsis. Southern blot analysis revealed that there are multiple copies of *XVSAP1* in *X. viscosa. XVSAP1* is, therefore, likely to code for an integral membrane protein which is expressed in response to low temperature

2.1 INTRODUCTION

In an attempt to gain some insight into the mechanisms utilized by *X. viscosa* in desiccation tolerance, Mundree et al. (2000), using an approach called complementation by functional sufficiency (Fig. 2.1), isolated genes from *X. viscosa* that were expressed during dehydration. *X. viscosa* was dehydrated to 85%, 37% and 5% relative water content.

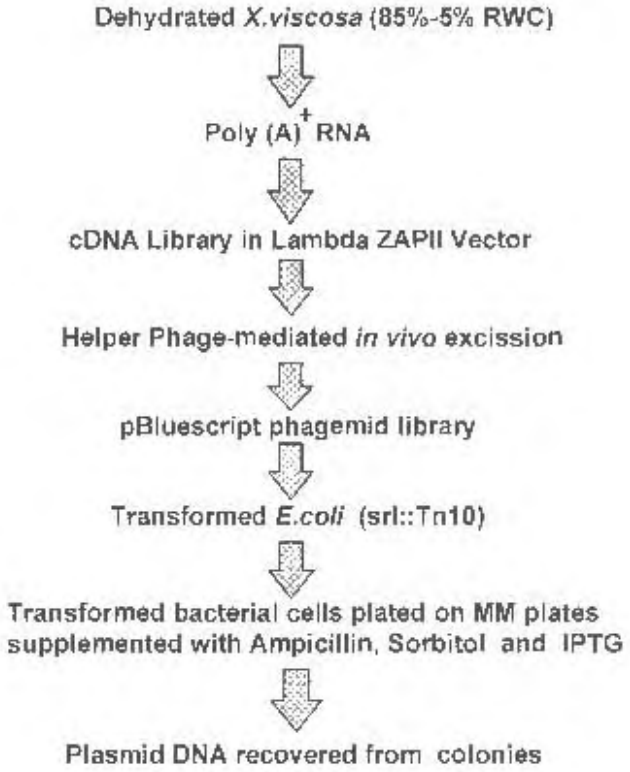

Figure 2.1 Illustration of the strategy of Complementation by Functional Sufficiency (Mundree et al., 2000)

A cDNA library constructed from the dehydrated leaf material was then used to transform *E. coli* (*srl*::Tn10) cells. Nine clones that were able to confer tolerance to osmotically stressed *E. coli* (*srl*::Tn10) cells were selected from M9 minimal medium plates containing 1.25 M sorbitol and their respective plasmids isolated. It was predicted that all cDNAs selected in this manner would have a role to play in abiotic stresses inducing osmotic stress or dehydration. The cDNA designated *XVSAP1* was one of these clones. This chapter describes the isolation and characterization of *XVSAP1* based on the gene sequence as well as the determination of the gene copy number in *X. viscosa*.

2.2 MATERIALS AND METHODS

2.2.1 Plant Material and Growth Conditions

X. viscosa plants were collected from the Buffelskloof Nature Reserve (Mpumalanga Province, South Africa) and 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). Experimental plants were watered to ensure full hydration prior to the stress experiments. Relative water content (RWC) was determined according to the formula:

RWC =[(Fresh Weight-Dry Weight)/Dry Weight)]/[(Hydrated Weight-Dry Weight)/Dry Weight)] X 100.

Leaves were weighed immediately after excision to determine the fresh weight and hydrated weight was determined after floating the leaves in water for 1 hour. Dry weight was obtained after drying the leaves at 70°C for 24 hours.

2.2.2 *XVSAP1* cDNA Isolation

Construction and screening of the cDNA library was as previously described (Fig.2.1, Mundree et al., 2000). Briefly, a cDNA library was constructed from *X. viscosa* leaves that had been to dehydrated to 85%, 37%, and 5% RWC. The library was converted into an infective phagemid library and used to transform *E. coli* (*srl*::Tn10). Nine clones that conferred tolerance to osmotically stressed *E. coli* cells growing on minimal medium supplemented with 1.25 M sorbitol,

100 μg /ml ampicillin and 1 mM isopropyl β-thiogalactopyranoside (IPTG) were selected. A cDNA insert in pBluescriptSK+ (Stratagene, La Jolla, CA) named *XVSAP1* was used for the experiments described in this project.

2.2.3 *XVSAP1* Sequence Analysis

The nucleotide sequence of *XVSAP1* was determined on both strands using the MegaBACE 500 (Molecular Dynamics, USA). Sequencing reactions were carried out using the DYEnamic ET Dye terminator sequencing kit (Molecular Dynamics, USA) according to the manufacturer's instructions. The BLAST programme of the National Centre for Biotechnology Information (Altschul et al., 1990) was used to search databases for sequence similarities. Nucleotide and amino acid sequence comparisons were done using CLUSTAL W (1.5) on the BCM server. The ProfileScan tool on the ISREC bioinformatics server was used to scan *XVSAP1* for conserved motifs. DNAMAN (Version 4.3, Lynnon BioSoft) was used to construct the homology tree.

2.2.4 Southern blot analysis

Genomic DNA from *X. viscosa* was isolated using the Nucleon Phytopure DNA extraction and purification kit (Amersham, Buckinghamshire, England) according to the manufacturer's instructions. Alternatively the DNA was isolated using a method based on the plant DNA preparation procedure described by Dellaporta et al. (1983). In all cases approximately 1 g of leaf tissue was ground to a fine powder in liquid nitrogen before extraction. DNA was precipitated using isopropanol, resuspended in TE buffer and quantitated spectrophotometrically.

Aliquots of 15 μg DNA were restricted with *Eco*RI/ *XhoI, Bgl*II, *Hind*III and *Eco*RV restriction endonucleases at 37°C and electrophoresed on 1% agarose gels at 20V overnight. Electrophoresed DNA was blotted onto nylon membranes (Hybond $-N^+$, Amersham) by capillary transfer (Sambrook et al., 1989). DNA was fixed using a cross-linker (Stratalinker 1800, Stratagene).

The complete *XVSAP1* cDNA was labelled with digoxigenin (DIG) using PCR labelling according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). The plasmid pSK-*XVSAP1* (100 pg) was used as template. A ratio of DIG-dUTP:dTTP of 1:6 and *XVSAP1*-specific primers (forward primer, 5' – GCACGAGGCAGATTTGAA TTG –3'; reverse primer, 5'-ATATGGACACGCATGACCCA-3') were used. Reactions were conducted using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, CA, USA) thermocycler under the following conditions: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 61°C for 40 s and 72°C for 45 s and a final extension step for 6 min. Labelling efficiency was tested by running the labelled product on a 0.8% agarose gel and staining with ethidium bromide. Blots were hybridized with the labelled *XVSAP1* probe for 16 hours at 42°C. The blots were subsequently washed with 2 X SSC (sodium citrate buffer, 0.1% SDS) at room temperature and more stringently with 0.5 X SSC, 0.1% SDS at 68°C. The chemiluminescent alkaline substrate disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3.1.13.7]decan}-4-yl) phenyl phosphate (CSPD, Roche Diagnostics GmbH, Germany), was used for detection according to the manufacturer's instructions.

2.3 RESULTS

2.3.1 Sequence analysis of XVSAP1

The nucleotide sequence of *XVSAP1* was found to be 942 bp with an open reading frame of 867 bp (Fig. 2.2). The deduced amino acid sequence encodes a basic protein of 264 amino acids with a molecular weight of 29.6 kDa and a predicted pI of 9.12. A Prosite motif search revealed that the protein has two prokaryotic membrane lipoprotein lipid attachment sites between amino acid residues 149 – 159 and 239 - 249. Five possible N-myristoylation sites were also found and these are indicated on the *XVSAP1* sequence at positions 42 - 47, 146 – 151, 170 – 175, 181 – 186, and 247 – 253.

A hydropathic plot [based on the method of Kyte and Doolittle, 1982 (window of 19 amino acid residues)] (Fig.2.3) predicted a protein rich in hydrophobic residues with an average hydrophobicity index of 0.81. The sequence consists of at least six transmembrane helices suggesting that XVSAP1 is likely to be an integral membrane protein.

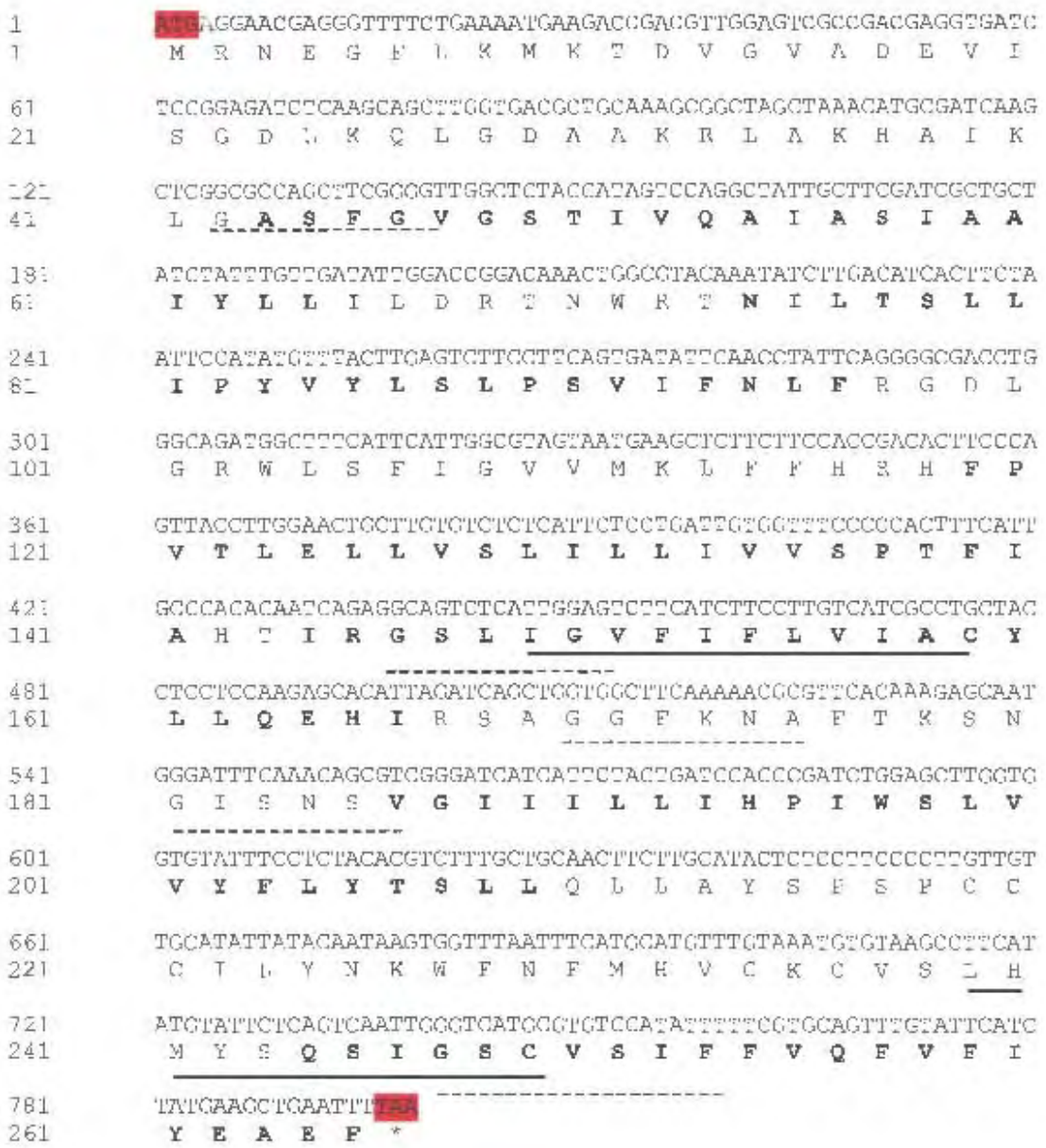

Figure 2.2 The nucleotide and deduced amino acid sequences of *XVSAP1*. The start and stop codons are indicated by shading. The N-myristoylation sites are indicated by broken lines and the prokaryotic membrane lipoprotein lipid attachment sites are underlined by bold lines. The regions of the six transmembrane helices are indicated in bold letters.

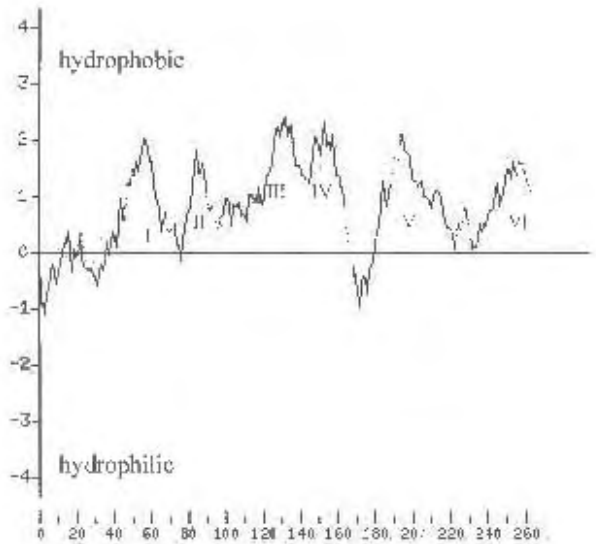

Figure 2.3 A hydrophobicity profile of XVSAP1 protein as determined by the method of Kyte and Doolittle (1982) using a window of 19 amino acid residues. The six putative transmembrane domains are indicated by Roman numerals.

A computer search of protein sequence databanks revealed that XVSAP1 showed 49% identity to WCOR413, a cold-responsive protein isolated from wheat and between 25 - 56% identity to cold associated proteins identified in *A. thaliana* (Fig.2.4). The protein also has 53% identity to a cold-associated protein from rice. Results from the BLAST programme indicate that the region extending from the lysine residue at position 36 to the phenylalanine residue at 119 (Fig. 2.2) bears 12 % identity with K^+ transporter family that is conserved across phyla (Qiuntero and Blatt, 1997). A homology tree based on the amino acid sequences of *XVSAP1* and its homologues (Fig 2.5) indicates that the first three ATCAPs from Arabidopsis are the most closely related with over 70% identity. XVSAP1 is closest to these three homologues, The rice-cold associated protein (RCAP) and WCOR413 are closely related with nearly 70% identity.

Figure 2.4 Comparison of the deduced amino acid sequence of XVSAP1 with related proteins. The proteins are WCOR413 (A# T06810), a cold regulated protein from wheat, cold associated proteins (ATCAP1- 4: A# T08404, AAD41971, CAB16776, T02423 respectively) from Arabidopsis and an *O. sativa* cold acclimation WCOR413-like protein (RCAP: A# AAG13395). Only Arabidopsis proteins showing the highest homology are shown. Percentages following the sequences indicate the percent identity to XVSAP1. Identical amino acid residues are indicated by asterisks. Similar amino acids are shown by points. A# refers to the GenBank accession numbers.

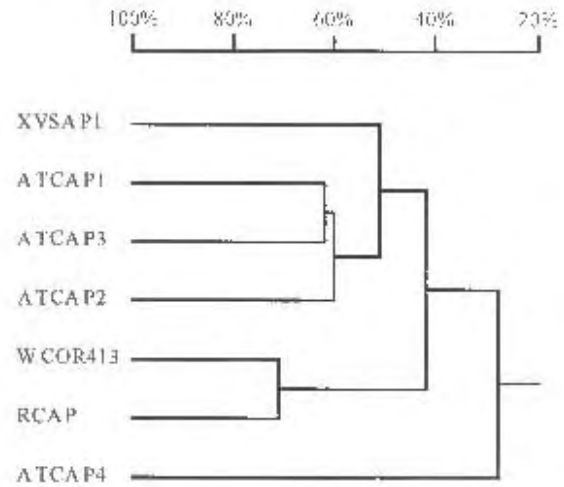

Figure 2.5 Homology tree for *XVSAP1* and related proteins.

2.3.2 Southern blot analysis

Southern blot analyses of *X. viscosa* genomic DNA probed with *XVSAP1* cDNA were carried out to confirm that *XVSAP1* was present in the *X. viscosa* genome and also to determine the gene copy number. A gel run to determine the efficiency of labelling using the DIG Probe synthesis kit confirmed successful labelling (Fig.2.6). As expected, the labelled probe migrated slower than the unlabelled control due to the presence of the DIG label. Of the restriction enzymes used, only *Bgl*II has a predicted restriction site within *XVSAP1*. At least seven hybridization bands were detected with this enzyme (Fig. 2.7). A double-digestion with *Eco*RI and *Xho*I and restriction with *Hind*III and *EcoR*V, each resulted in at least four hybridization fragments of varying intensities. These results indicate that there are multiple copies of *XVSAP1* in *X. viscosa* and taken with the results obtained from the homology searches, suggest that *XVSAP1* belongs to a small gene family.

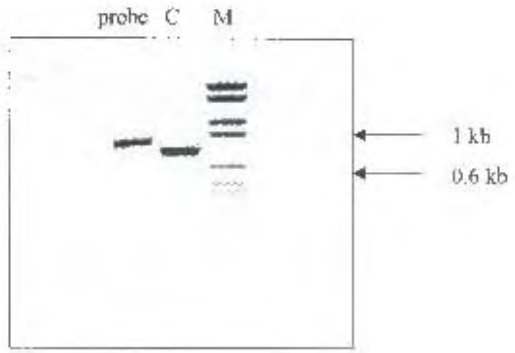

Figure 2.6 Probe synthesis using the PCR DIG Probe Synthesis Kit. Probe refers to the DIG-labelled XVSAP1 probe, C is the unlabelled control probe and M is marker II (Roche Diagnostics, Germany). Note that the labelled probe migrates slower than the control due to the presence of the DIG label.

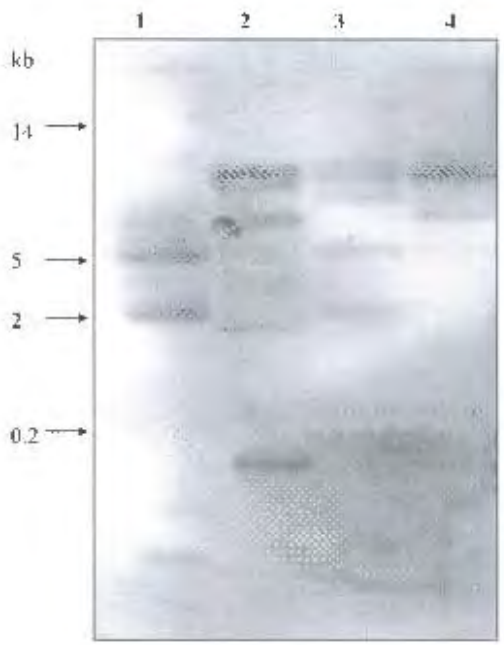

Figure 2.7 Southern blot analysis of genomic DNA from *X. viscosa*. 15 µg of DNA was cut with *Eco*RI/*Xho*I (lane 1), *Bgl*II (lane 2), *Hind*III (lane 3) and *Eco*RV (lane 4), electrophoresed on a 1% agarose gel, transferred to a nylon membrane and probed with DIG-labeled *XVSAP1* cDNA.

2.4 DISCUSSION

The cDNA designated *XVSAP1* was isolated from dehydrated *X.viscosa* leaves using "complementation by functional sufficiency" as described by Mundree et al. (2000).
The predicted XVSAP1 protein contains 264 amino acids and the hydropathic plot showed that XVSAP1 is a highly hydrophobic protein that is probably anchored in the plasma membrane. Immunolocalization studies would be useful in confirming the location of the protein. XVSAP1 showed significant identity to proteins identified in wheat, rice and Arabidopsis. Only the wheat protein, WCOR413, has been partially characterised in this group. The wheat WCOR series of stress-inducible proteins from wheat resembles soluble hydrophilic dehydrins in contrast to XVSAP1 and WCOR413, which are predicted to be integral membrane proteins. Data supplied with the protein sequence in the GenBank (Accession number T06810) indicates that the WCOR413 is a cold regulated protein. The proteins from rice and Arabidopsis are classified as cold associated proteins on the basis of their similarity to WCOR413 at the amino acid level. As is evident from the results of the homology analysis, XVSAP1 is more closely related to the first three ATCAPs from Arabidopsis than to WCOR413. The level of identity between XVSAP1 and its homologues suggests that the protein may also be a cold-regulated protein and may have a conserved function in stress adaptation.

Several low-temperature (LT)-regulated genes have been isolated and characterized in many species including Arabidopsis, barley, *Brassica napus,* wheat and alfalfa (Breton, et al., 2000; Thomashow, 1999). These genes have been classified into three groups based on their presumed function. The first group is comprised of structural proteins that play a protective role during cold

stress. The second group consists of proteins involved in the regulation of gene expression and signal transduction. The group includes enzymes that are responsible for the biosynthesis of osmoprotectants, membrane lipids and those with a role in the antioxidant response (Breton, 2000).

Some of these LT-regulated genes have been shown to play a role in the tolerance of various plant species to low temperature. The Arabidopsis *COR* genes [also designated *LTI* (low temperature-induced), *KIN* (cold-inducible), and *RD* (responsive to desiccation)] are induced in response to low temperature or other conditions that are associated with water deficit such as high salinity and drought. Steponkus et al. (1998) demonstrated that COR15a, a 15-kDa polypeptide targeted to the chloroplasts, increases freezing tolerance by stabilizing membranes against freezing-associated dehydration. Kaye et al. (1998) showed that tobacco plants expressing the two cold acclimation proteins (CAPs) from spinach, CAP160 and CAP85, had slightly improved tolerance to freezing stress as determined by electrolyte leakage tests. In wheat, the expression of several LT-induced genes positively correlated with the potential to develop LT-tolerance. Among these is the *wsc*120 gene that encodes a 39-kDa polypeptide, WCS120, a member of the D-11 or dehydrin class of LEA proteins (Danyluk et al., 1998) and *wcs*19 which encodes chloroplastic proteins related to group 3 LEA proteins (NDong et al., 2002). Danyluk et al. (1994) identified a LT- responsive acidic dehydrin, *wcor410* that enhances the freezing tolerance of wheat. Additionally, betaine, salinity and drought stress also induce *wcor*410 and another cold-regulated gene, *wcor*413 (Allard et al., 1998). *wcor*413, in contrast to most of the characterized COR genes in wheat, is a hydrophobic protein (Danyluk and Sarhan, 1996). Sequence homology revealed that XVSAP1 had 49% identity with WCOR413 as well as a number of other uncharacterized proteins in Arabidopsis and rice. These uncharacterized proteins have some homology to WCOR413 and they all possess at least three

transmembrane regions. The Arabidopsis homologues, in common with XVSAP1, also have a prokaryotic membrane lipoprotein lipid attachment site and are predicted to have a role to play in stress response.

An analysis of the genomic organization of *XVSAP1* by Southern blotting confirmed that the gene is indeed present in the *X. viscosa* genome. As with the Arabidopsis homologues, there is more than one copy of the gene in the nuclear genome suggesting that *XVSAP1* belongs to a small gene family.

An examination of the sequence of XVSAP1 revealed very few clues to the possible functions of the protein in conferring stress tolerance. It is possible that XVSAP1 may be involved in the transport of substances or ions across the plasma membrane as a region stretching from amino acid residue 36 to 119 bears 12% identity with a K^+ potassium transporter family. The family is conserved across phyla, having plant (AtKT), yeast (HAK) and bacterial (KUP) sequences as members (Qiuntero and Blatt, 1997). However, no data are currently available to support this possibility.

It is conceivable that XVSAP1 may be modified for full function as is indicated by the presence of two prokaryotic membrane lipoprotein lipid attachment sites. Modification of proteins by the covalent attachment of lipids appears to be widespread in living systems (Hayashi and Wu, 1990). The presence of these sites supports the concept that XVSAP1 associates closely with the cell membrane. It is interesting to note that the Arabidopsis homologues also have prokaryotic membrane lipoprotein lipid attachment sites. XVSAP1 is possibly further processed for full

function by the attachment of lipids as is implied by the existence of five putative N-myristoylation sites. The process of N-myristoylation is a co-translational modification that involves the covalent reaction of myristate and the amino-terminal glycine residue of a growing polypeptide. Proteins so modified have diverse functions and the myristate appears to be critical for mediating protein-protein and/or protein-membrane interactions (Ishitani et al., 2000; Johnson et al., 1994). However there is currently no evidence that myristoylation is required for the function of XVSAP1 or indeed that it occurs at all.

CHAPTER THREE

ANALYSIS OF *XVSAP1* EXPRESSION IN *X. VISCOSA*

SUMMARY

The *XVSAP1* cDNA was isolated from dehydrated *X. viscosa* leaves on the basis of its ability to confer osmotic stress tolerance to *E.coli* (*srl*::Tn10) cells. It was postulated that *XVSAP1* was probably induced in response to water deficit. Analysis of gene expression using semi-quantitative RT-PCR confirmed that this was indeed the case. It was shown that *XVSAP1* is induced by dehydration, salt stress (100 mM), both low (4°C) and high temperature (42°C) and high light treatment (1500 μmol m^{-2} s^{-1}). Western blot analysis revealed that the gene expression correlated with the accumulation of XVSAP1 protein in the stressed plants. These results suggested that *XVSAP1* might have a substantial role to play in the response of *X. viscosa* to abiotic stresses.

3.1 INTRODUCTION

The expression of genes in response to various stimuli is usually detected using classic methods such as Northern blotting or Northern hybridization, *in situ* hybridization or ribonuclease (RNase) protection assays. The techniques are invaluable for the analysis of gene expression at the transcript (mRNA) level and allow the steady state mRNA level of a gene to be assessed in a particular tissue or organ. However, these techniques are limited by their sensitivity, particularly in small tissue samples. Even under optimum conditions, a minimum of about $4x10^3$ copies of an RNA transcript is required for detection of a specific mRNA by the RNase protection assay, which is the most sensitive non-PCR based mRNA detection and quantitation procedure (Lee et al., 1996). The combined use of reverse transcription followed by the polymerase chain reaction (RT-PCR)

revolutionised the study of gene expression despite an ongoing debate about its accuracy. The polymerase chain reaction originally described by Saiki et al. (1991), is a highly sensitive and specific method for detection of nucleic acids and is a useful tool for quantitation of specific nucleic acids present in a sample. Using RT-PCR, it is possible to characterize the expression profile of any gene, regardless of the quantity of the starting material or the relative abundance of the mRNA transcript. RT-PCR has been shown to be 1000 to 10000-fold more sensitive than the traditional RNA blot techniques (Wang et al, 1989). Even the detection of the expression of multiple mRNAs or mRNAs expressed in mixed cell populations is made much simpler using RT-PCR. Additionally, the RT-PCR procedure can be completed within a few hours.

Although RT-PCR has many advantages over RNA blots, it can be difficult to obtain quantitative information. This is due to the exponential nature of the PCR amplification, where small variations in amplification result in dramatic changes in product yields. In addition, the amount of product plateaus during the later stages of the reaction due to consumption of necessary components and generation of inhibitors. These characteristics of PCR can conceal differences in the initial amount of target sequences during the course of amplification.

RT-PCR can be aimed at absolute or relative quantification. Relative quantification is also called semi-quantitative RT-PCR. Absolute quantitation by RT-PCR aims to state the number of copies of a specific RNA per cell or unit mass of tissue. However, for most purposes, semi-quantitative RT-PCR is adequate. The method involves amplification of RT products in the exponential phase. Since the PCR cycle numbers in the exponential phase are highly dependent on the abundance of the target transcript, the exponential phase must be determined such that all given amounts of target

mRNA display linear accumulation. Another feature of semi-quantitative RT-PCR is the use of internal controls, which help in overcoming the inherent problems associated with PCR (Zimmerman and Mannhalter, 1996).

One form of internal control is a second set of primers designed to amplify a ubiquitously and constitutively expressed mRNA in the same PCR tube. "House-keeping" genes such as those coding for the enzymes dihydrofolate reductase and glyceraldehyde phosphate dehydrogenase have been used for this purpose. Unfortunately, mRNA levels of these genes do not always remain constant (Siebert and Larrick, 1992). The use of RNA competitors as internal standards in all steps of the reaction including the RT step has generally become accepted as a reliable and sensitive method for the quantification of mRNAs (Raeymaekers, 1995). The assay is based on the competitive co-amplification of a specific target sequence and a known concentration of an internal standard in one reaction tube. For the assay to work, the internal standard has to share primer recognition sites with the target sequence, both the target sequence and the internal standard must be PCR-amplified with the same efficiency and it must be possible to analyze the PCR-amplified products of target sequence and internal standard separately (Zimmerman and Mannhalter, 1996). The easiest way to distinguish between the target and internal standard is by differences in the size of the two products. This can be achieved, for example, by constructing standards having the same sequence as the specific target but containing a deletion or an insertion. Differences between the target and standard can also be obtained by incorporation of restriction sites. Following PCR, the standards containing such restriction sites are digested with the relevant restriction enzymes. Since the digested products are smaller in size, they can easily be distinguished from the target. In this

work, the target sequence and the internal standard were distinguished by a difference in size between the PCR products.

The most widely used technique for analysing protein mixtures is probably SDS polyacrylamide gel electrophoresis (SDS-PAGE). In this technique the protein sample to be analysed is denatured and coated with the anionic detergent sodium dodecylsulfate (SDS) by heating in the presence of SDS and a reducing agent. The SDS forms a complex with the protein that has a high net negative charge proportional to the length of the protein or polypeptide chain. Samples are separated on sieve-like polyacrylamide gel with the protein components migrating towards the anode. Since all of the proteins have a net negative charge that is in proportion to their size, the proteins are separated on the basis of differences in charge and molecular mass. A discontinuous gel system composed of a stacking and separating gel with different salt concentrations and/or pH gives SDS-PAGE excellent resolution powers (Hames, 1990, Laemmli, 1970). Following gel electrophoresis, resolved proteins can be visualized by staining with either Coomassie Blue or silver stain. However, to confirm the expression of a particular protein, immuno-blotting or western blotting is done.

Western blotting is a widely-used and powerful technique used to detect and identify proteins using antibodies. After separation of sample proteins by SDS-PAGE, the proteins are transferred onto a thin support membrane composed of either nitrocellulose or more durable materials such as polyvinylidene diflouride using an electric current. The membrane binds and immobilizes the proteins preserving the same pattern as on the original gel. The protein blot is then exposed to an antibody solution containing antibodies that recognize and bind to the specific protein of interest.

Bound antibodies are detected using a variety of techniques usually involving use of a secondary antibody which binds to the primary antibody. This is known as an indirect detection. Secondary antibodies are commonly conjugated to detectable markers including enzymes such as horseradish peroxidase or alkaline phosphatase and flourescent markers. Detection methods most often used involve the use of chemiluminescent subtrates which produce light at the target site and can be detected by exposing the membrane to x-ray film. The use of chromogenic substrates which produce a coloured precipitate on reacting with enzymes bound to secondary antibodies is also another popular detection method.

This chapter describes the analysis of the expression of *XVSAP1* in *X. viscosa* in response to various artificially imposed abiotic stresses. The accumulation of the XVSAP1 protein in response to dehydration, heat and salt stress was also assessed.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material and Growth Conditions

X. viscosa plants were collected and maintained as described in section 2.2.1

3.2.2 Stress induction

X. viscosa plants were subjected to a variety of treatments simulating abiotic stresses. In all cases, leaf samples (5-6 leaves) were taken from the experimental plants just before commencing

treatments (time 0). Samples of 5-6 leaves were collected from each experimental plant every 24 h thereafter except for the cold treatment, where samples were collected every 6 h. In the case of ABA, samples were taken every 3 h after treatment of the leaves. All leaf samples collected were frozen in liquid nitrogen and stored at -70°C until further use.

Dehydration

X. viscosa plants were dehydrated by withholding water from two plants for a period of two weeks. Leaves were detached from the plants at 90%, 78%, 63%, 51%, 44% and 4% RWC. Leaf samples were also collected at 4%, 32%, 42%, 85% and 92% RWC on re-hydrating. Leaf samples used for western blot analysis were dehydrated to 60%, 50%, 30%, 10% and 4% RWC.

Heat Treatment

For the heat treatment, fully hydrated two mature plants were kept in a phytotron at 42°C (humidity 50-70%, 16 h light and 8 h dark cycle) for a week. The plants were watered regularly to maintain them at full hydration.

Cold Treatment

To determine the effect of cold stress, two mature plants were kept at 4°C in a cold room with a light intensity of ~300 $\mu mol.m^{-1}.s^{-1}$ (humidity 60-70%, 16 h light and 8 h dark cycle). The plants were kept hydrated throughout the duration of the experiment. Leaf samples were taken every 6 h for 60 h.

Salt Treatment

To test the response of *X. viscosa* to high salinity, two mature plants in the greenhouse were irrigated with 100 mM NaCl daily for 7 days. Plants were allowed to drain freely after each irrigation.

High light intensity treatment

The high light treatment was carried out by exposing two mature plants to light at 1500 μmol m^{-2} s^{-1} for 4 days in a phytotron (25°C, humidity 50-70%). Plants were irrigated with water daily to keep them fully hydrated.

ABA treatment

To determine if ABA had an effect on the expression of *XVSAP1*, the leaves of two *X. viscosa* plants were sprayed with the phytohormone at a concentration of 100 μM in water once every 24 h.

3.2.3 RNA isolation

Solutions used for RNA extractions, where appropriate, were treated with 0.01% diethylpyruvocarbonate (DEPC) and all Eppendorf tubes and micropippette tips were autoclaved twice before use. Total RNA was isolated using the Trizol reagent (Gibco-BRL, Germany). *X. viscosa* leaves (200 mg) were ground in liquid nitrogen and homogenized in 0.75 ml of the reagent. Following incubation for 5 min at room temperature, 0.2 ml chloroform was added followed by a further incubation at room temperature for 10 min. Samples were centrifuged at 12 000 x g for 10 min at 4°C and the RNA was precipitated using isopropanol. RNA was quantitated

spectrophotometrically, separated on a 1.2% agarose formaldehyde gel and stained with ethidium bromide to verify quantitation.

3.2.3.1 Semi-quantitative RT-PCR

Creation of Internal Standard

XVSAP1 in pBluescriptSK+ was restricted at 37°C using *Nde1* to remove a 473-bp fragment. The remaining construct with *Nde1* cohesive ends was ligated using ligase enzyme to create a truncated *XVSAP1*. A 5-µl aliquot of the ligation mix was mixed with 100 µl competent JM109 *E. coli* cells. The mixture was retained on ice for 10 minutes and then transferred to a heating block at 37°C. The *E.coli* cells were heat-shocked for 5 min and then immediately returned to ice for a further 5 min. Transformed cells were selected on Luria Agar (LA) plates (1% tryptone, 0.5% yeast extract, 5% NaCl, 15% agar) containing ampicillin (100 ug/ml). Single colonies were picked and grown in Luria Broth (1% tryptone, 0.5% yeast extract, 5% NaCl) overnight. Plasmid DNA was purified from the cultures using a plasmid isolation kit (Roche Diagnostics, Germany). The structure of the construct was confirmed by sequencing.

A 2-µg aliquot of the plasmid construct was linearised by restriction using the *Apa1* site on pBluescriptSK. A sense competitor was transcribed *in vitro* using the T3 promoter site of the plasmid to create the internal standard. The linearized plasmid DNA (1 µg) was mixed with RNase-free distilled water to a final volume of 18 µl and 2 µl 10x transcription buffer added. RNA polymerase (2 µl) was added and the mixture incubated for 2 h at 37^0C. Template DNA was removed by adding 2 µl DNase I (RNase free) and incubating for 15 min at 37^0C. The mixture was then placed on ice and 2 µl 0.2 M EDTA (pH 8.0) added to stop the reaction. The transcripts were

precipitated by adding 2.5 µl 4M LiCl and 75 µl cold ethanol to the reaction. After storing the mixture at –70°C for 30 min, the nucleic acids were centrifuged at 12 000 g and the supernatant discarded. The precipitates were washed with 75% ethanol and the pellets dissolved in RNase-free water. The RNA transcripts were quantitated spectrophotometrically.

Determining the Linear Range for PCR

To determine the linear range of the PCR reaction, RNA isolated from the 44% RWC sample was reverse transcribed as described below. A PCR master mix was prepared using *XVSAP1* specific primers. The mix was split into separate 10 PCR tubes and an aliquot removed every two cycles of the PCR reaction. The PCR products were run on a 0.8% agarose gel and the results quantified by determining the optical density of each PCR band (Gel Trak programme) on gel pictures (Gel documentation system GDS 2000, UVP Ltd, Cambridge, U.K). The results were plotted on a graph and a cycle number in the linear range of the graph chosen for subsequent PCR reactions.

Reverse Transcription and PCR of RNA Isolated from Stress Treatments

All RNA samples were treated with DNase I (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions to eliminate DNA contamination. In each case, 2 µg RNA was used for the reverse transcription reaction. The internal control RNA was prepared as described above. Two picograms of the truncated *XVSAP1* were used in all the RT reactions except for the ABA RT-PCR where 0.5 pg of the internal standard was used. The reverse transcription reactions were performed using the Omniscript reverse transcriptase kit according to the manufacturer's directions (Qiagen GmbH, Germany). The RNase inhibitor was obtained from Roche Diagnostics GmbH, Germany. Undiluted cDNA (5 µl) from the RT step was used in 50-µl PCR reactions. The primer pair described in section 2.2.5 produced an 829-bp product from *XVSAP1* and a 342-bp product

from the truncated clone. Reactions were conducted using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, CA, USA) thermocycler under the following conditions: 95°C for 2 min followed by 23 cycles of 95°C for 30 s, 61°C for 40 s and 72°C for 45 s and a final extension step for 6 min. The linear portion of the reaction was determined to be between 18 and 25 cycles and 23 cycles were used for all subsequent experiments.

After PCR, the samples were resolved by electrophoresis on a 0.8% agarose gel and stained with ethidium bromide. Gel pictures were obtained using the gel documentation system GDS 2000 (UVP Ltd, Cambridge, and U.K).

3.2.5 Protein Extraction and Western Blot Analysis

Microsomal membrane fractions were prepared from leaf tissue of *X. viscosa*, tobacco and Arabidopsis principally as described by Schumacher et al. (1999). Approximately 1 g of leaf tissue was homogenized with 6 ml of homogenization buffer [0.35 M sucrose, 70 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 3 mM EDTA, 0.15% (w/v) BSA, 1.5% (v/v) PVP-40, 4 mM DTT, 1 mM PMSF]. The homogenate was filtered through cheesecloth and the filtrate centrifuged at 15 000 x g for 15 min at 4°C in a JA-20 rotor (Beckman J2-21 M/E, UK). The supernatant was centrifuged at 100 000 x g for 2 h at 4°C in a SW50.I rotor (Beckman L7-55, UK). The microsomal membrane protein pellet was washed once with homogenization buffer and then resuspended in 100 µl resuspension buffer [0.35 mM sucrose, 10 mM Tris-MES (pH 7.0), 2 mM DTT, 1 mM PMSF] for 30 min on ice.

The protein was quantified using a Micro-BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). The separation of proteins by SDS-PAGE was performed as described by Laemmli (1970). A 15-µg aliquot of each protein was loaded for SDS-PAGE. Gels were stained with Coomassie blue (A.3.9) or used for western blotting. Proteins were transferred electrophoretically to nitrocellulose membranes (Osmonics, 0.45 µm pore size) using Towbin buffer (25 mM Tris, 192 mM Glycine, 20% methanol) and briefly stained with Ponceau S [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] to confirm transfer and equal loading. The blots were blocked with 5% Blotto [5% wt/v nonfat dry milk, 0.05% Tween20, in Tris buffered saline (20 mM Tris pH 7.4, 0.15 mM NaCl)] and incubated with antibodies that were raised in rabbits against a synthetic peptide corresponding to a hydrophilic portion of XVSAP1 (LLQEHIRSAGGFKNAFTKSNGI). The polyclonal serum was first purified by precipitating with 14 % polyethylene glycol (PEG) and then used at a 1: 500 dilution. After three washes in Blotto, the blots were incubated with goat anti-rabbit IgG coupled to HRP diluted at 1:5000 (Sigma-Aldrich, UK) for 1 h. This was followed by three washes in Tris–buffered saline. The blots were visualized using the ECL detection system (Amersham) according to the manufacturer's instructions.

3.3 RESULTS

3.3.1 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to compare the relative transcript levels after various stress treatments. The competitor RNA prepared was used as an internal control for variations in the RT and PCR reactions. *XVSAP1* was induced by dehydration with the transcript appearing only at 51%

and 44% RWC (Fig. 3.1A). There was no evidence of the transcript at 4% RWC. *XVSAP1* was not detected during rehydration of *X. viscosa* (Fig. 3.1B). The dehydration-hydration curve (Fig. 3.1C) for the above treatment revealed that the plant took 12 d to dehydrate to 4% RWC and then completely rehydrated within 4 d after watering.

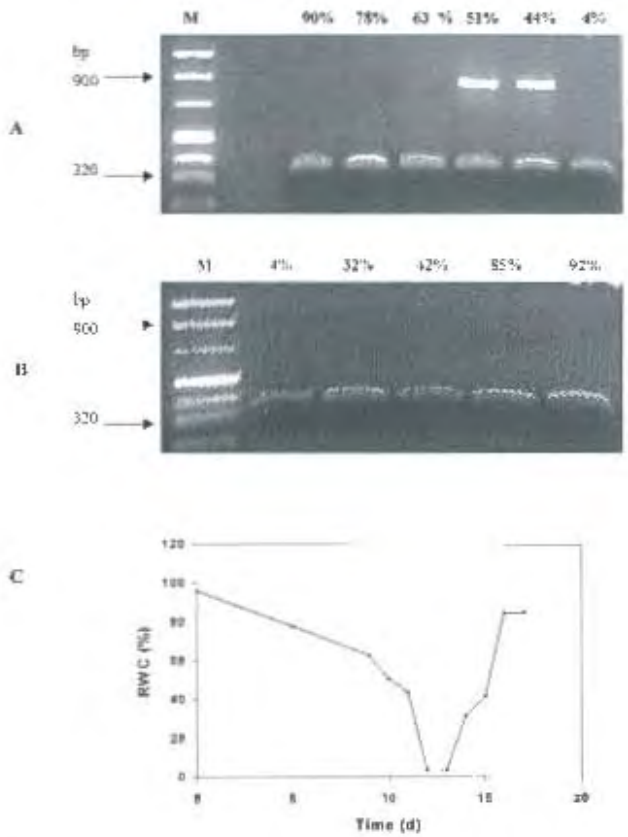

Figure 3.1 Expression of the *XVSAP1* transcript during dehydration and rehydration of *X. viscosa*. Expression of the *XVSAP1* transcript was determined using semi-quantitative RT-PCR. PCR products were visualized on agarose gels stained with ethidium bromide. The native *XVSAP1* product was 830 bp and the competitor 345 bp. M refers to Marker VIII (Roche Diagnostics, Germany). Percentages refer to the relative water content (RWC). (A) *X. viscosa* plants were dehydrated from 90% RWC to 4% RWC by withholding water for a period of 2 weeks. (B) The same *X.viscosa* plants were rehydrated over 5 d by watering. (C) The RWC variation during the dehydration-hydration of *X. viscosa*.

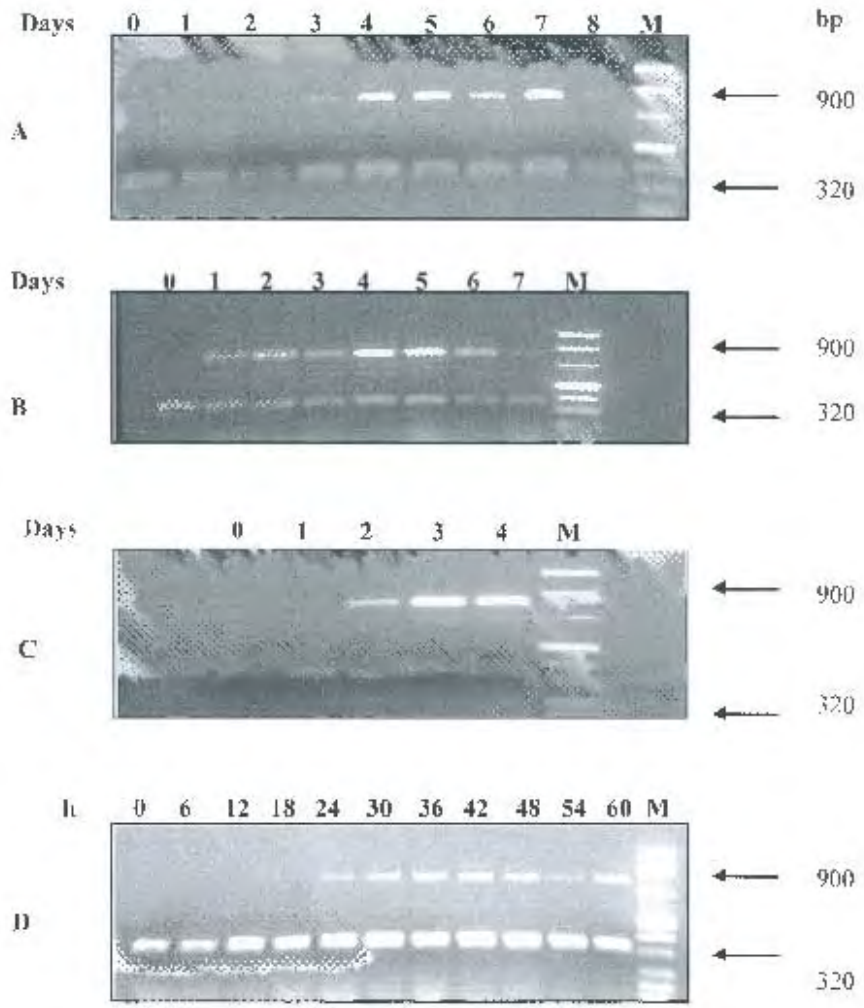

Figure 3.2 Induction of *XVSAP1* by heat, NaCl, high light intensity and low temperature treatments in *X. viscosa*. Induction was compared using semi-quantitative RT-PCR. PCR products were visualized on agarose gels stained with ethidium bromide. The native *XVSAP1* product was 830 bp and the competitor 345 bp. M refers to Marker VIII (Roche Diagnostics, Germany). (A) Plants were kept in a 42°C incubator for 7 d to induce heat stress. (B) Salt shock was induced by irrigating potted plants with 100 mM NaCl. (C) Plants were exposed to high light at 1500 µmol $m^{-2} s^{-1}$ for 4 d in a phytotron (25°C, humidity 50-70%) for the high light treatment. (D) Cold treatment was achieved by keeping plants at 4°C for 60 h.

Heat (Fig. 3.2A), salt (Fig. 3.2B) and high light (Fig. 3.2C) resulted in a significant induction of *XVSAP1*. The transcripts took three days to appear with heat shock and had declined by day 8 of the treatment. Salt shock resulted in the induction of *XVSAP1* expression within 24 h. During the treatment, the transcripts were evident for 7 d but began to decline on the sixth day of the treatment. The transcripts appeared within 48 h with high light treatment whereas with the cold treatment (Fig. 3.2D), the transcripts were evident within the first 24 h. Levels of *XVSAP1* transcription reached a peak after about 42 h during cold treatment.

3.3.2 Western Blotting

The accumulation of XVSAP1 protein in response to dehydration, salt and heat stress was analysed by western blotting. Protein was extracted from leaf material detached from dehydrated *X. viscosa* plants at selected RWCs. XVSAP1 protein began to accumulate in the leaves at 60% RWC (Fig. 3.3A) and had reached high levels by the time the plant had dried down to 50% RWC. Protein levels remained high even when the plant had reached 4% RWC.

There was no evidence of XVSAP1 protein when the salt treatment commenced but by the end of 24 hours, the protein had begun to accumulate (Fig. 3.3B). The maximum amount of protein was reached on day 3. By day 4, XVSAP1 had started to decline although the protein was still in evidence on day 5 when the last leaf sample was collected. In contrast, XVSAP1 protein accumulated to a maximal level within 24 h in response to heat. The amount of protein appeared to remain at a constant level after the initial increase for duration of the experiment (Fig.3.3C).

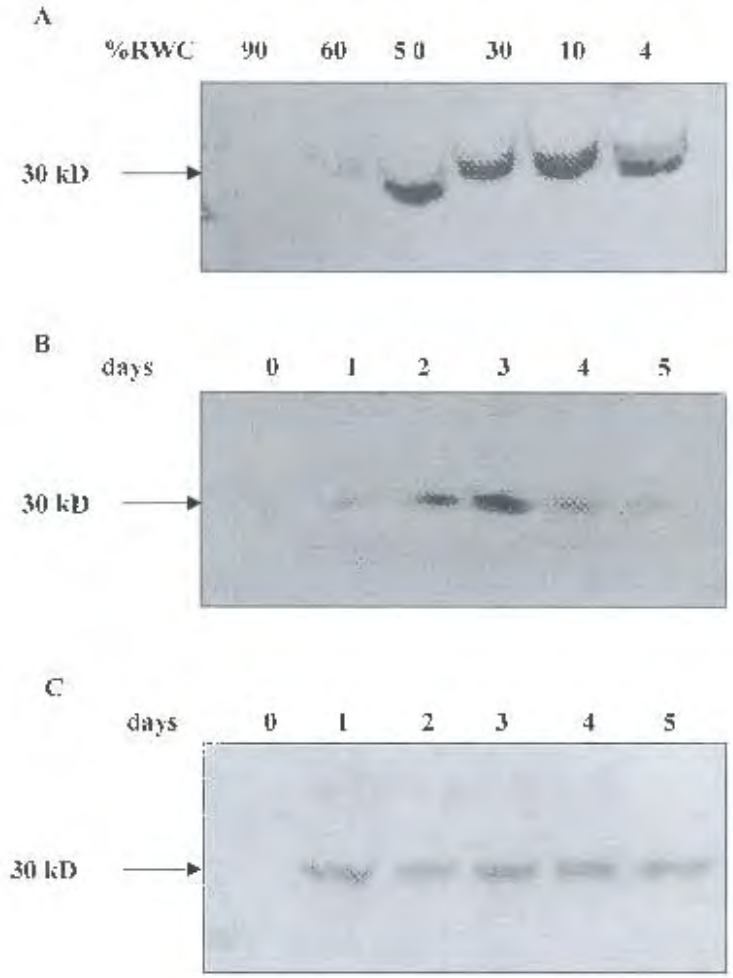

Figure 3.3 Immunoblot detection of XVSAP1 protein accumulation in *X. viscosa* during dehydration. Equal amounts of microsomal membrane proteins (15 µg) were separated on 12% SDS-PAGE and subjected to western blot analysis as described in Materials and Methods. The 30-kD XVSAP1 protein is indicated by arrows. (A) Plants were dehydrated until RWC had reached 4%. Protein was extracted from leaf material collected at selected RWC as indicated. (B) Salt shock was induced by irrigating potted plants with 100 mM NaCl and protein extracted from leaf material collected on the days indicated. (C) Plants were kept in a 42°C incubator for 5 d to induce heat stress.

3.4 DISCUSSION

Semi-quantitative RT-PCR was used to assess the response of *XVSAP1* to various simulated environmental stresses. It has been shown that many genes induced by drought are also induced by salt and cold stresses (Zhu et al., 1997). All these factors result in osmotic stress or water deficit in plant cells. As *XVSAP1* was isolated on the basis of its ability to confer tolerance to osmotically stressed *E. coli* (*srl*::Tn10), it was necessary to establish if *XVSAP1* was involved in the response to these abiotic stresses. An examination of the expression of *XVSAP1* during dehydration and rehydration of *X. viscosa* showed that only dehydration and not rehydration induces the expression of the gene. Interestingly, *XVSAP1* expression was strongly induced at 51% and 44% RWC and not at any other stage. This indicates that *XVSAP1* is not required in the initial stages of dehydration but is only expressed when dehydration becomes severe and the plant has dried down to approximately 51% RWC. Protein accumulation data correlated well with the RT-PCR results. The XVSAP1 protein only began to accumulate at about 60% RWC. No expression of the gene was evident at 4% RWC. Since XVSAP1 is likely to be an integral membrane protein, one of the roles it could play is the stabilization of membranes during the drying process. As the plant dries further, its metabolic processes decline and eventually stop. This could explain why the expression of *XVSAP1* is not observed at 4% RWC. However, the protein persisted at 4% RWC. No expression of *XVSAP1* was observed during rehydration indicating that *XVSAP1* has no role to play during this process. However, the absence of the transcript does not imply absence of the protein product. It has been observed that most of the components required for recovery from desiccation are already present in the plant during dehydration in *X. humilis* (Dace et al., 1998). In

X. viscosa, XVSAP1 could be one of those components involved in the repair of membrane damage that results from severe water deficit.

Heat and high light stresses both strongly induced the accumulation of *XVSAP1* mRNA. In both cases, the transcript only started to accumulate at least 48 h after imposition of the stress. The results obtained suggest that *XVSAP1* is not involved in the initial stages of the response to heat or high light intensity. It is expected that *X. viscosa* would have mechanisms in place to deal with such stresses in the short term as the extremophile grows in environments where it is regularly exposed to high temperature and high light intensity. However, when the duration of these stresses increases, other mechanisms that would have a protective effect come into play. It is known that heat stress affects most cellular processes and causes denaturation of proteins, cellular enzymes, and damage to membranes. The damage is due to the temperature change itself as well as heat-induced oxidative stresses (Karim et al., 1999; Munro and Pelham, 1985). Survival after heat stress requires an ability to tolerate or repair oxidative damage as well other kinds of heat-induced damage. It is expected that XVSAP1 would have a role to play in the protection of membranes against heat damage. The data obtained from the western blot analysis indicates that the protein starts accumulating only 24 h after the imposition of stress. This seems to contradict the information obtained from the RT-PCR analysis. However, the difference could be attributed to the individual plants used. It is possible that the *X. viscosa* plants do not react in exactly the same manner to the imposition of a stress condition. Nonetheless, the arguments raised above would still apply. The appearance of the protein does not occur until at least 24 h after the commencement of the heat stress and therefore the reaction could still be viewed as being adaptive. The RT-PCR and

western blotting data agree in that there appeared to be no change in the relative abundance of either the mRNA nor the protein after the peak has been reached.

In a similar manner, high light intensity can result in the formation of ROS. If the free radicals are not quenched, damage in the form of photo-bleaching and lipid peroxidation occurs (Smirnoff, 1993). *X. viscosa* appears to withstand damage caused by light by a combination of protective and avoidance mechanisms. The poikilochlorophyllous resurrection plant loses its chlorophyll and dismantles its photosynthetic apparatus while the levels of anthocyanins and antioxidant enzymes increase affording the plant a degree of protection (Sherwin and Farrant, 1997). As with heat stress, it is proposed that XVSAP1 is involved in the protection of membranes possibly by maintaining structural integrity.

High exogenous salt concentrations cause an imbalance of cellular ions resulting in ion toxicity, osmotic stress and production of ROS (Hasegawa et al., 2000). Various molecules including proteins that protect membrane integrity, control ion homeostasis and play a role in ROS scavenging have been reported to attenuate salt stress effects (Hasegawa et al., 2000; Ingram and Bartels, 1996; Ishitani et al., 1997). Studies in both wheat and barley showed that the induction of genes by salt occurs within 2 h and that many transcripts decrease in abundance within 24 h (Robinson et al., 1990). In the case of *XVSAP1,* the transcript appeared within 24 hours after salt shock and persisted for 7 days. This result correlates well with the western blot analysis data. The accumulation of the protein was observed to begin some 24 h after imposition of the salt stress and the protein persisted throughout the duration of the experiment. The response of *XVSAP1* to salt

was delayed compared to other salt-responsive genes and it also lasted for a longer period. This supports the earlier theory that the gene is expressed on persistence of a particular abiotic stress.

XVSAP1 has a relatively high identity to cold responsive WCOR413 and the uncharacterised Arabidopsis homologues (Fig. 2.4). It was, therefore, reasonable to consider that the gene could be induced by cold. This proved to be the case as *XVSAP1* was detected within 24 h after the commencement of the treatment. The results correlate well with those obtained with other *COR* genes. Cold-induced mRNAs generally begin to accumulate within a few hours at low temperature and remain at high levels until removal of the stress. The *CBF* genes are induced within 15 min of Arabidopsis plants being exposed to low temperatures followed at about 2 h by induction of cold-regulated genes that contain the CRT/DRE regulatory element (Gilmour et al., 1998; Thomashow, 1998). It is expected that in the natural habitats of *X. viscosa,* temperatures at night could decrease to below zero degrees on occasion. *XVSAP1* would therefore form part of the mechanism that assists *X. viscosa* to cope with the stress particularly since chilling injury is mainly a consequence of destablization of cell membranes.

It has been established that many genes that respond to drought and /or cold stress are also induced by exogenous applications of ABA (Bray 1997; Chandler and Robertson, 1994). However, in the case of *X.viscosa,* ABA treatment *in planta* failed to induce *XVSAP1*. Moreover, placing of excised leaves in a 100 μM solution of ABA did not have an effect on the expression of *XVSAP1* despite the fact that less competitor RNA was used in the RT-PCR reaction. Shinozaki and Yamaguchi-Shinozaki (1997) suggested that there are at least four independent signal pathways that function in the activation of stress-inducible genes. Two of these are ABA- dependent (pathways I and II) and

two are ABA-independent (pathways III and IV). The fact that *XVSAP1* was not induced by exogenous applications of ABA suggests that *XVSAP1* responds to environmental stresses through an ABA-independent pathway. It is also possible that the response to ABA is transient and was not detectable under the experimental conditions used.

CHAPTER FOUR

EXPRESSION OF *XVSAP1* IN *E. COLI, S. CEREVISIAE, N. TABACUM* AND *A. THALIANA*

SUMMARY

To confirm the functional role of *XVSAP1* in osmotic stress tolerance, use was made of the *E.coli* (*srl*::Tn10) mutant strain. The cDNA was cloned into pProEX HT, a prokaryotic expression vector and used to transform the mutant *E. coli* cells. *XVSAP1* was also cloned into the yeast shuttle vector pHVX2 and transformed into a salt-sensitive phenotype of a calcineurin-deficient *S. cerevisiae* mutant (Δ*cnb1*). The same construct was used to transform an osmotic stress-sensitive yeast mutant strain (Δ*hog1*). Expression of *XVSAP1* in *E. coli (srl*::Tn10) conferred osmotic stress tolerance when the cells were grown in 1 M sorbitol. However, expression of the cDNA in yeast did not complement the mutations in the strains used.

XVSAP1 was introduced into Arabidopsis and *N. tabacum* by Ti plasmid-mediated transformation under the control of a cauliflower mosaic virus 35S promoter and a nos terminator. Expression of *XVSAP1* in both Arabidopsis and tobacco plants led to the constitutive accumulation of the corresponding protein in the leaves. Transgenic Arabidopsis grown on plant nutrient agar in petri dishes and tobacco grown hydroponically were more tolerant to salt and osmotic stress. Non-transgenic plants had shorter roots, leaf expansion was inhibited and leaves were more chlorotic than those of the transgenic plants were. In addition, transgenic tobacco plants attained a higher fresh and dry weight than the untransformed controls. Transgenic tobacco also showed a significantly greater tolerance to drought stress when grown in soil. Untransformed plants were

smaller, wilted earlier and were more chlorotic in response to drought stress. The transgenic plants expressing *XVSAP1* had greater membrane permeability to electrolytes (measured by electrolyte leakage) which resulted in less negative water potential and thus the ability to continue normal growth. The expression of *XVSAP1* had no effect on the efficiency of photosystem II under normal growth conditions or under drought stress. These experiments confirmed that expression of *XVSAP1* confers tolerance to NaCl and osmotic stress.

4.1 INTRODUCTION

To confirm the functional role of *XVSAP1* in osmotic stress tolerance, use was made of the *E. coli* (*srl*::Tn10) mutant strain. The cDNA was cloned into pProEX HT, a prokaryotic expression vector and used to transform the mutant *E. coli* cells. The pProEX HT bacterial expression system allows the high-level production of recombinant proteins in *E.coli*. The gene of interest is cloned into an appropriate vector and expression of protein from the *Trc* promoter and *lac*I^q gene, induced by IPTG. There are three different vectors available in the pProEX HT expression system, which allow the in-frame cloning of any genes in the multiple cloning site. Further, the system incorporates a tag composed of six histidine residues at the N-terminus of the recombinant protein that allows purification of the protein using a technique called immobilized metal chelate affinity chromatography (pProEX HT Prokaryotic Expression System product pamphlet).

In IMCAC, multivalent metal ions such as Ni^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} are bound to a metal chelating adsorbent such as tetradentate nitrilotriacetic acid (NTA), immobilised on a chromatographic medium. Some ligand binding sites remain available on the metal chelator for selective interaction

with proteins. The histidine tag, fused to the recombinant protein, specifically interacts with the chelated ions, thereby immobilizing the protein on the medium. Elution is achieved by lowering the pH to between 4.5 to 5.3 or by increasing the levels of a competitive eluting agent, such as imidazole. Low pH causes the histidine residues to become protonated and to dissociate from the resin whilst increasing the levels of a competitive agent deprives the proteins of binding sites (Janknecht et al., 1991; Petty, 1996; QIAexpress manual, Qiagen). After purification, the histidine tag can be removed by a protease, which recognises a Tobacco Etch virus cleavage site located just before the multiple cloning site (pProEX HT Prokaryotic Expression System product pamphlet)

It was expected that *E.coli* (*srl*::Tn10) cells expressing the XVSAP1 protein via the pProEX HT system would be able to withstand osmotic stress. *E.coli* usually tolerates media with an osmolarity corresponding to about 0.7 M NaCl by accumulating compatible osmolytes to restore osmotic balance. However, studies by Csonka and Clark (1979) showed that the *srl*::Tn10 strain lacked the ability to take up and catabolise sorbitol due to the disruption of the *srl* operon. *E. coli* (*srl*::Tn10) cells are, therefore, unable to grow in high concentrations of the osmoticum.

Yeast is considered an ideal model for the large-scale functional analysis of genes as well as a molecular genetic model for abiotic stress, particularly salt tolerance, in plants. The biggest advantage that yeast offers is the ability to create mutations through homologous recombination in which precise gene mutation can be incorporated into the yeast genome for rapid phenotype evaluation. It is evident that many transcriptional stress responses are shared between many diverse species including yeast, fungi, the ice plant *Mesembryanthemum crystallinum,* and Arabidopsis (Bohnert et al., 2001). Heterologous plant gene expression in yeast is a powerful tool for the

identification and isolation of plant genes significant in physiological processes. It has been found that there is substantial functional conservation of cellular processes in plants and yeast and numerous plant genes have been identified by their ability to suppress or complement mutant phenotypes in yeast (Frommer and Ninneman, 1995). These genes include those that encode biosynthetic enzymes, transcription factors, transporters and ion channels.

Functional complementation of a salt-sensitive calcineurin (CaN)-deficient mutant (Δ*cnb1*) and an osmotic stress-sensitive yeast mutant strain (Δ*hog1*) using *XVSAP1* was attempted. *XVSAP1*was cloned into the yeast shuttle vector pHVX2 to create the vector pHVX2-*XVSAP1*, which was then used to transform the yeast mutants. The pHVX2 vector contains the phosphoglycerate kinase (PGK) constitutive promoter, the yeast LEU2 selectable marker and the 2 μm yeast origin of replication (Gietz and Sugino, 1988).

In this chapter the characterization of *E.coli* (*srl*::Tn10), yeast (Δ*cnb1*, Δ*hog1*), Arabidopsis and tobacco transformed with *XVSAP1* is described. The expression of *XVSAP1* in tobacco and Arabidopsis enhanced tolerance to salt and osmotic stress and also to water deficit. The results presented here indicate that the gene may be a useful molecular tool to improve crop productivity.

4.2 MATERIALS AND METHODS

4.2.1 XVSAP1 Expression in Osmotically Stressed Escherichia coli

XVSAP1 was cloned into the pPROEX HT Prokaryotic Expression Vector System (Life Technologies, Inc, USA) using the *Eco*RI/*Xho*I restriction sites. *E. coli* (*srl*::Tn10) cells were transformed with the pPROEX HT-*XVSAP1* construct and grown in M9 minimal medium(A.1.3) supplemented with 1 mM $MgSO_4.7H_2O$, 0.2% glycerol, 0.1% vitamin B and 100 µg /ml ampicillin. Cell cultures were induced in duplicate by adding 0.2 mM IPTG after the OD_{600} of the cells had reached approximately 0.5. The cells were allowed to grow for a further 2 h before an osmotic stress was imposed by adding 4 M sorbitol to a final concentration of 1 M. The growth of the cells was monitored by taking absorbance readings at 600 nm over a 48 h period. The experiment was repeated three times.

4.2.2. Yeast Strains, Growth Conditions and Complementation Analysis

The entire open reading frame of *XVSAP1* was cloned into the *Eco*RI – *Xho*I site of pHVX2 (a shuttle vector derived from Yeplac 181, with a 2 µm ori, a PGK promoter and a LEU selection marker) to create pHVX2-*XVSAP1*. Both this construct and the vector alone were used to transform the *S. cerevisiae* mutant strains Δ*cnb1* – W3031A (Mat a his3-11, leu 2-112, trp 1-1, ade 2-1, ura 3-1 cnb1::HIS3) and Δ*hog1*- W303A (Mat a, his3-11, leu 2-112, trp1-1, ade 2-1, ura 3-1 hog 1::TRP1). W3031A (wild type) Mat a, his3-11, leu 2-112, trp 1-1, ade 2-1, ura3-1) was used as the

positive control. Standard procedures for yeast culture and manipulation were used (Kaiser, 1994). Yeast cells were grown on SD medium (0.7% Difco YNB-AA, 2% dextrose, the appropriate amino acid supplements and 2% bacteriological agar for plate assays.) or on YPD medium (1% Difco yeast extract, 2% peptone, 2% dextrose and 2% bacteriological agar for plate assays). Sodium chloride (0.8 M and 1 M), lithium chloride (0.2 M) and sorbitol (2 M) were added to SD and YPD as indicated. Spot assays were conducted as described by Matsumoto et al., (2001).

4.2.3 Plant Material and Growth Conditions

A. thaliana ecotype Columbia and *N. tabacum* L cv. Kutsaga E1 were used. Seeds were stratified in 0.1% agar at 4°C prior to sowing to promote even germination either in soil or on plates. Where appropriate, Arabidopsis seeds were germinated on plant nutrient agar (PNA, Haughn and Somerville, 1986) without sucrose. Tobacco seeds were germinated on half-strength Murashige and Skoog (MS-2) medium supplemented with 5% sucrose and solidified with 0.8% (w/v) agar. For plate assays, both Arabidopsis and tobacco seeds were surface sterilized by washing in 75% ethanol followed by 10% commercial bleach (active ingredient 3.5% m/v sodium hypochlorite) and rinsed five times in sterile water before sowing. All plants were grown in a growth room (16-h day, 8-h night, 22°C, and 65% humidity) except where stated.

Wu et al.(1996) have shown that root elongation is an accurate and convenient indicator of Arabidopsis seedling growth. Using this parameter, the growth of transgenic and non-transformed control Arabidopsis was evaluated *in vitro* on plant nutrient agar (PNA; Haughn and Somerville, 1986) except that the media contained no sucrose, and on PNA supplemented with either NaCl or

mannitol. Root growth was expressed as a percentage relative to growth on unsupplemented PNA. The experiments were repeated at least three times and similar trends were observed. Seedlings of approximately the same root length were selected at the beginning of each experiment.

4.2.4 Plant Transformation

XVSAP1, a cDNA insert in pBluescript(SK+) (Mundree et al., 2000) was excised from the vector using *Eco*RI and *Xho*I restriction enzymes and directionally cloned into the binary plant transformation vector, pSMB, (Mylne and Botella, 1998) placing *XVSAP1* under the control of the 35S CaMV promoter and a *nos* terminator. The construct pSMB-*XVSAP1* was introduced into *A. tumefaciens* (GV3103) by electroporation (Bio-rad Gene Pulser, Hercules, California). Arabidopsis was transformed using the floral dip method (Clough and Bent, 1998). Tobacco leaf disc transformation was carried out using standard protocols (Horsch et al., 1985). Bialaphos (Meiji Seika Kaisha Ltd, Japan) and a concentration of 8 mg/L was used to select for transformants and where appropriate, Arabidopsis seedlings were selected on PNA without sucrose containing bialaphos at a concentration of 5 mg/L. Arabidopsis plate assays were carried out on PNA without sucrose supplementation and tobacco assays on one-half-strength MS (MS-2). Transgenic tobacco and Arabidopsis plants were verified by PCR using a primer pair (forward primer, 5' – GCACGAGGCAGATTTGAA TTG –3'; reverse primer, 5'- ATATGGACACGCATGACCCA-3') specific for *XVSAP1*. T2 seeds from independent transformation events were used for further experiments.

4.2.5 Southern Blot and RT-PCR Analysis of Transgenic Plants

Genomic DNA was isolated from Arabidopsis and tobacco plants using the plant DNA preparation procedure described by Dellaporta et al., (1983), except that in all cases approximately 1 g of leaf tissue was used and DNA was precipitated with isopropanol. Ten micrograms of genomic DNA from transgenic plants were restricted with *Bgl*II and *Hind*III, blotted onto a nylon membrane (Amersham, 045 μM) and probed with the full-length digoxigenin (DIG) labelled *XVSAP1* cDNA as described in section 2.2.5. RNA from the transgenic plants was isolated using the Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. All RNA samples were treated with DNase I (Roche Diagnostics GmbH, Germany), according to the manufacturer's instructions to eliminate DNA contamination. Two micrograms of RNA were reverse-transcribed using the Omniscript kit (Qiagen GmbH, Germany) as indicated by the manufacturer. The cDNA (5 μl) from the RT step was used in 50-μl PCR reactions undiluted. The primer pair described in section 2.2.5 produced an 829-bp product from *XVSAP1.* Reactions were conducted using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, CA, USA) thermocycler under the following conditions: 95°C for 2 min followed by 25 cycles of 95°C for 30 s, 61°C for 40 s and 72°C for 45 s and a final extension step for 7 min.

4.2.6. Protein Extraction and Western Blot Analysis

Protein was isolated from microsomal membrane fractions prepared from the leaves of tobacco and Arabidopsis as described in section 3.2.5. Separation of the protein by SDS-PAGE and detection was also as described in section 3.2.5.

4.2.7. Plant Stress Treatments

Tobacco or Arabidopsis test seeds were placed in single rows and allowed to germinate with the plates placed vertically to permit easier observation of growth patterns. Osmotic stress was imposed by growing seedlings on PNA containing 0, 50, 100, 200, 300 and 400 mM mannitol. Salt treatment was carried out on PNA containing 0, 50, 75, 100 and 150 mM NaCl. Observations were made over a period of 7 days. Tobacco plants were also grown in hydroponic culture. Tobacco seedlings germinated and grown on plates for two weeks were transferred to tubs containing one-fourth strength MS (MS-4) and allowed to adapt to the new growing conditions for four weeks. Plants were then transferred to MS-4 containing either 200 mM NaCl or 9% (w/v) polyethylene glycol (PEG) of molecular weight 3500. Control plants were transferred to fresh MS-4. Plants were treated for 7 days and then returned to fresh MS-4. The fresh weight, dry weight and root lengths of the transgenic and control plants were determined after a seven-day recovery period. Percentage relative root growth was determined by calculating percent root growth compared to growth of untransformed Arabidopsis on non-stress media.

4.2.8 Water Deficit Stress and Evaluation

Transgenic and control tobacco seedlings from plates were grown in soil until they were six weeks old. Drought stress was imposed on half of the plants by withholding water for 12 days. The remaining plants were watered as normal. To determine pigment content after drought stress, pigments were extracted from tobacco leaves with 100% acetone and the absorbance at 470, 644.8 and 661.6 nm read (Beckman DU-64 spectrophotometer, Fullerton, California). Total chlorophyll ($a + b$) and carotenoid ($x + c$) were calculated as indicated by Lichtenthaler (1987) and expressed on a dry weight basis. An OS-500 Modulated Flourometer (Opsi-Sciences, Haverhill, USA) was used to determine quantum efficiency of photosystem II (Fv/Fm) on previously dark adapted leaves. Membrane integrity following drought stress was determined by measuring conductivity after freeze-fracturing leaf samples in liquid nitrogen. Mid-day water potential of the tobacco plants was determined using a Scholander pressure bomb (Scholander et al., 1965) on excised leaves (fourth true leaf).

4.2.9 Statistics

Repeated Measures Analysis of Variance (Von Ende, 1993) using the Statistical Analysis System (SAS) was used for assessing the performance of the different Arabidopsis lines during NaCl and mannitol treatments. One-way analysis of variance (ANOVA) followed by Scheffe's multiple range tests at $p<0.05$ were conducted to compare different tobacco lines in each treatment (drought-stressed or well-watered) using the Statgraphics Plus computer program (Statistical Graphic Corporation, 1993, New York, USA).

4.3 RESULTS

4.3.1 *XVSAP1* Expression in Osmotically Stressed *E. coli*

E. coli (*srl*::Tn10) (Csonka and Clark, 1979) cannot grow on minimal media containing high concentrations of sorbitol. To confirm the osmo-protection function of *XVSAP1*, the cDNA was cloned into a prokaryotic protein expression vector to yield pPROEXHT-*XVSAP1*. *E. coli* (*srl*::Tn10) cells transformed with this plasmid exhibited significantly better growth in the presence of 1 M sorbitol over a period of 48 h, compared to *E. coli* (*srl*::Tn10) transformed with the vector only, after induction with IPTG (Fig. 4.1). Although the imposition of osmotic stress by the addition of sorbitol caused an initial decrease in the growth rate of both cultures, 2 h after the stress was imposed there was a steady increase in the growth rate of the experimental cultures.

4.3.2 *XVSAP1* Expression in *S. cerevisiae*

Functional complementation of a salt-sensitive Δ*cnb1* yeast mutant was unsuccessful, as was the complementation of the osmotic-stress sensitive Δ*hog1* mutant. There were no differences in the growth of the mutant strains transformed with the pHVX2-*XVSAP1* construct and the mutant strains transformed with the pHVX2 vector alone either on salt or osmotic stress media when the strains were grown in spot assays (Fig 4.2Y). However, sequencing and restriction analysis of the pHVX2-*XVSAP1* constructs (Fig 4.2X) confirmed successful cloning of *XVSAP1* into the multiple cloning site of the pHVX2 vector and colony PCR was used to confirm the presence of the pHVX2-*XVSAP1* in the yeast cells.

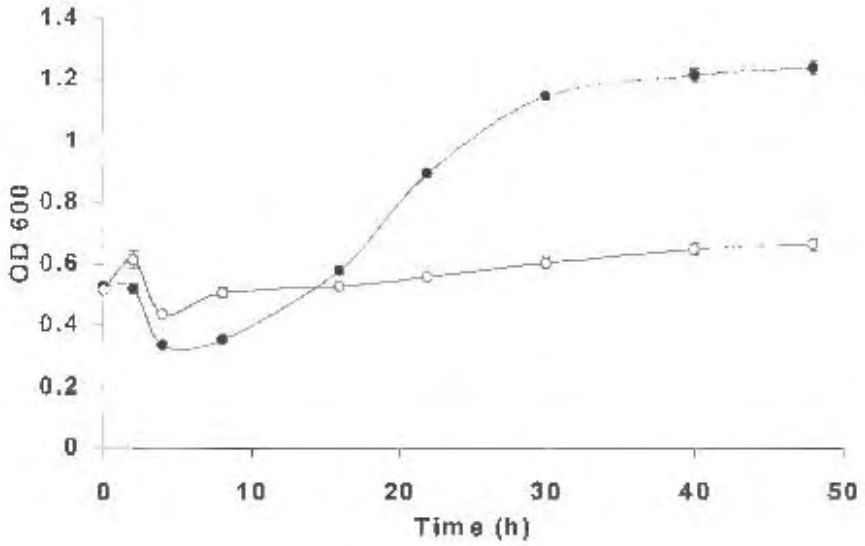

Figure 4.1 Growth analysis of *E. coli (srl*::Tn10) cells transformed with the prokaryotic protein expression vector PROEX HT (open circles) and cells transformed with pPROEXHT-*XVSAP1* (closed circles) in minimal media. The expression of XVSAP1 was induced with IPTG at time zero. The cells were allowed to grow for a further 2 h before sorbitol was added to a final concentration of 1 M. Samples were taken at intervals and the absorbance at 600 nm determined. Error bars represent standard deviation based on the average of triplicate samples.

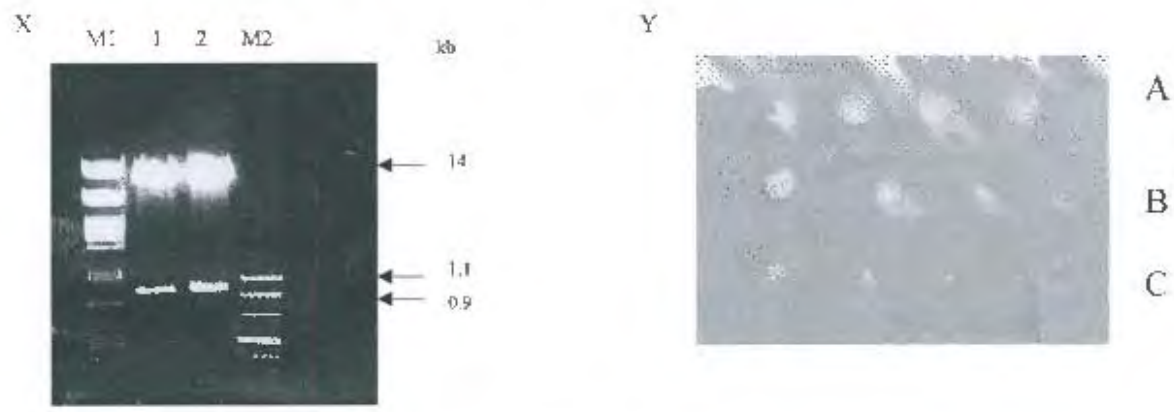

Figure 4.2 Restriction analysis of pHVX2-*XVSAP1* to confirm correct ligation (X) and complementation of yeast mutant with *XVSAP1*(Y). Digestion of the vector construct with *Eco*R1 and *Xho*1 (lanes 1 and 2) resulted in the release of the *XVSAP1* gene. M1 refers to the λ *Pst* I DNA marker and M2 is Marker VIII (Roche, Germany). A is the wild type yeast, B is the Δ*hog1* mutant transformed with the pHVX2-*XVSAP1* construct and C is the Δ*hog1* mutant transformed with the vector alone.

4.3.3. Transformation of Arabidopsis and Tobacco with *XVSAP1* and Analysis of Transgenic Plants

XVSAP1 was cloned into the binary plant transformation vector pSMB and the construct pMSB-*XVSAP1* (Fig. 4.3A) was introduced into Arabidopsis and tobacco by Ti plasmid-mediated transformation. Primary transformants were selected in tissue culture on the basis of their resistance to bialaphos (bar gene). Twenty-one and 15 transgenic lines of Arabidopsis and tobacco were obtained, respectively. Further, PCR analysis of genomic DNA was used to used to show the incorporation of *XVSAP1*.. Arabidopsis and tobacco plants transformed with *XVSAP1* were phenotypically similar to the wild type control. Three lines from Arabidopsis and tobacco respectively were chosen for further analysis and a homozygous recessive line (azygous) from tissue culture for both Arabidopsis and tobacco was selected as a negative control. The integration and gene copy number of *XVSAP1* were determined by Southern blot analysis of genomic DNA using the full-length *XVSAP1* cDNA as a probe (Fig. 4.3B and C). The hybridization patterns of the chosen transgenic plants confirmed integration of the transgene into the plant genome of both Arabidopsis and tobacco. Of the enzymes chosen, *Bgl*II cuts once within the *XVSAP1* gene sequence. The results obtained with Arabidopsis indicate that line 6K contains more than one copy of the gene whereas the other two lines (10C and 21G) each have one copy. The tobacco lines chosen (A2, A5 and A7) had very similar banding patterns although the plants were from different transformation experiments. The results confirmed integration of one copy of the transgene in each of the tobacco lines.

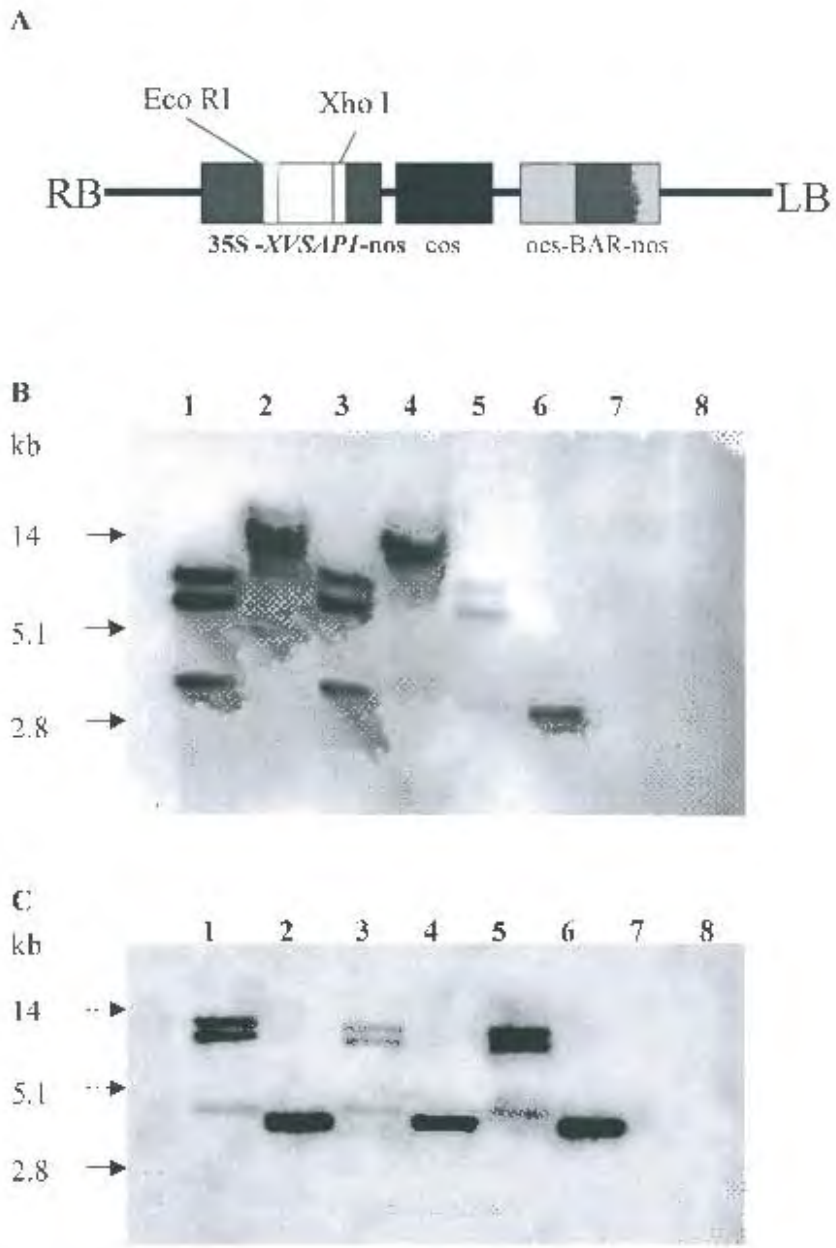

Figure 4.3 (A) Schematic diagram of the pSMB-*XVSAP1* construct transformed into Arabidopsis and tobacco. The bacterial phosphinothricin acetyl transferase gene (*bar*) was used as the selectable marker. The right (RB) and left (LB) borders of *A. tumefaciens* T-DNA flank all elements. Southern blot analysis of transgenic Arabidopsis (B) and tobacco (C) lines transformed with *XVSAP1* and the untransformed controls. Ten micrograms of genomic DNA were digested with either *Hind*III or *Bgl*II, separated on a 1% agarose gel and blotted onto a nylon membrane. Blots were probed with full-length DIG-labelled *XVSAP1*. 6K (lanes 1 and 2), 10C (lanes 3 and 4), 21G (lanes 5 and 6) are transgenic lines, and WT (wild-type control, lanes 7 and 8) refers to the. Odd number lanes – *Bgl*II digestions; even number lanes – *Hind*III digestions. The DNA size markers are shown in kb on the left.

The expression of *XVSAP1* in the transgenic plant leaves was assessed by RT-PCR using primers specific for *XVSAP1*. The results showed that a transcript of approximately 900 bp, the expected size, accumulated in both the transgenic tobacco and Arabidopsis lines but not in the wild-type and the azygous controls (Fig. 4.4).

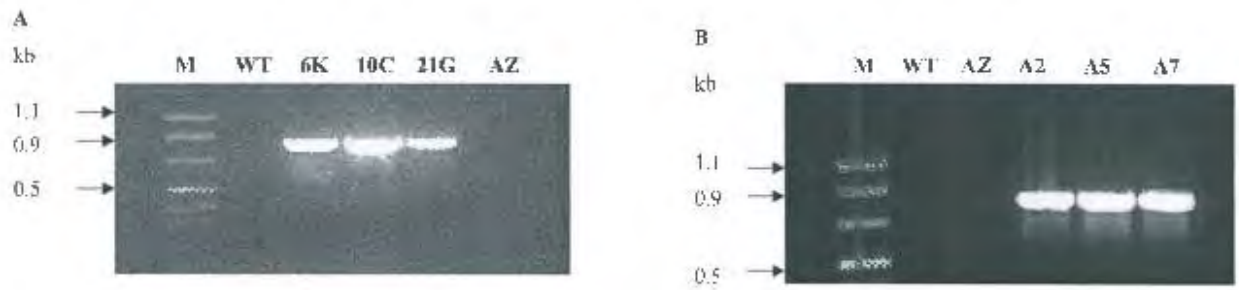

Figure 4.4 RT-PCR analysis of *XVSAP1* expression in transgenic and untransformed Arabidopsis (A) and tobacco (B) lines. Total RNA was isolated and treated with DNase I. Two micrograms of RNA were reverse-transcribed and the cDNAs generated were used as template for PCR using *XVSAP1* specific primers. 6K, 10C and 21G are Arabidopsis transgenic lines. A2, A5 and AZ are tobacco transgenic lines. WT and AZ are the appropriate wild type and azygous lines respectively, for each plant species. M refers to Marker VIII (Roche, Germany).

4.3.4 Western Blot Analysis of Transgenic Arabidopsis and Tobacco

The expression of XVSAP1 protein in the transgenic lines was analyzed using SDS-PAGE. Microsomal membrane protein extracts were prepared from leaf tissue and the XVSAP1 protein detected by polyclonal antibodies raised against a synthetic peptide corresponding to a portion of the protein determined by sequence inspection to be the most antigenic. Protein from *X. viscosa* was used as the positive control in the detection of XVSAP1 in the transgenic lines. A single band of approximately 30 kD was detected in *X. viscosa* protein although the deduced size was 29 kD (section 3.3.2). However, in both Arabidopsis (Fig. 4.5A) and tobacco (Fig. 4.5B), at least three

bands were observed. In each case, the 30-kD protein was detected but there was cross-reactivity in both Arabidopsis and tobacco with proteins estimated to be approximately 25 kD and 50 kD.

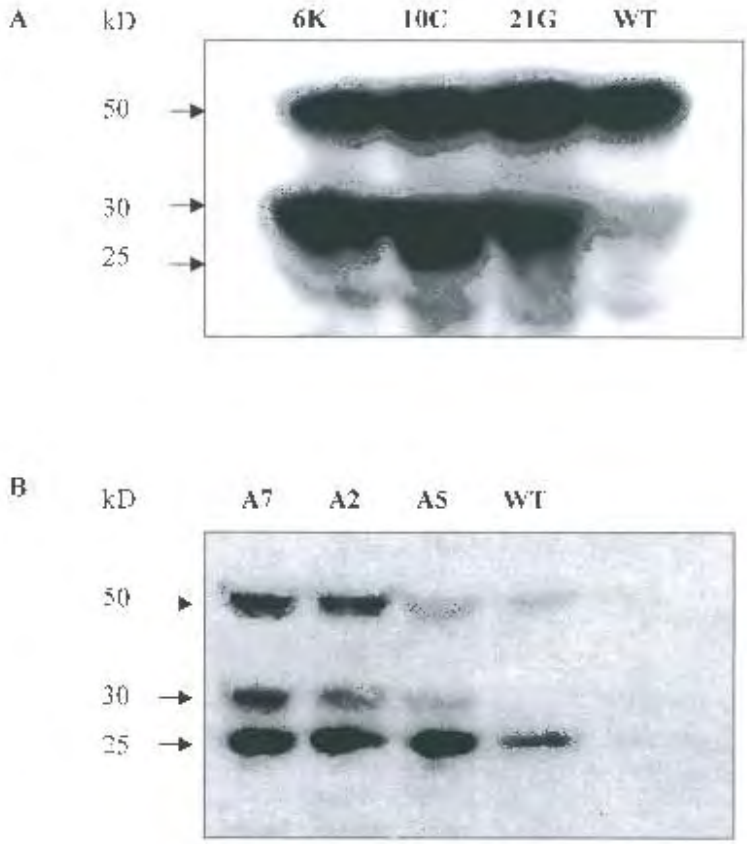

Figure 4.5 Western blot analysis of XVSAP1 protein in Arabidopsis (A) and tobacco (B). Microsomal membrane protein (15 µg) was separated by SDS-PAGE and blotted onto nitrocellulose membranes. The blots were incubated with polyclonal antibodies raised against a synthetic peptide as described in Materials and Methods.

4.3.5 Transgenic Arabidopsis Plants Expressing XVSAP1 are More Tolerant to Salt, Osmotic and Heat stress.

All lines tested grew well on PNA without supplementation although transgenic lines, 6K and 21G grew significantly faster (Figs. 4.6 and 4.7). These lines always germinated earlier than the wild type and azygous controls on PNA or stress media.

When NaCl was added to PNA at a concentration of 50 mM, there were generally no apparent differences in the growth rates of the transgenics and the controls except that in the example shown, transgenic line 21G appeared to be growing faster than the other lines. However, at 75 mM (Figs. 4.6 and 4.7), the transgenic lines exhibited a higher rate of root elongation. After 7 days of growth on 75 mM NaCl line 6K showed 90% relative root growth compared with only 41% for the wild type control. Interestingly, for this particular experiment, analysis indicated that there was significant interaction between the concentration and the line ($p=<0.0001$). For example line 21G grew faster on both 50 and 100 mM NaCl, whereas line 6K grew faster on 0 mM and 75 mM NaCl. Although it is true that the transgenic lines always grew faster than the untransformed control lines, it was not always the same transgenic line showing the most vigorous growth on the various salt concentrations.

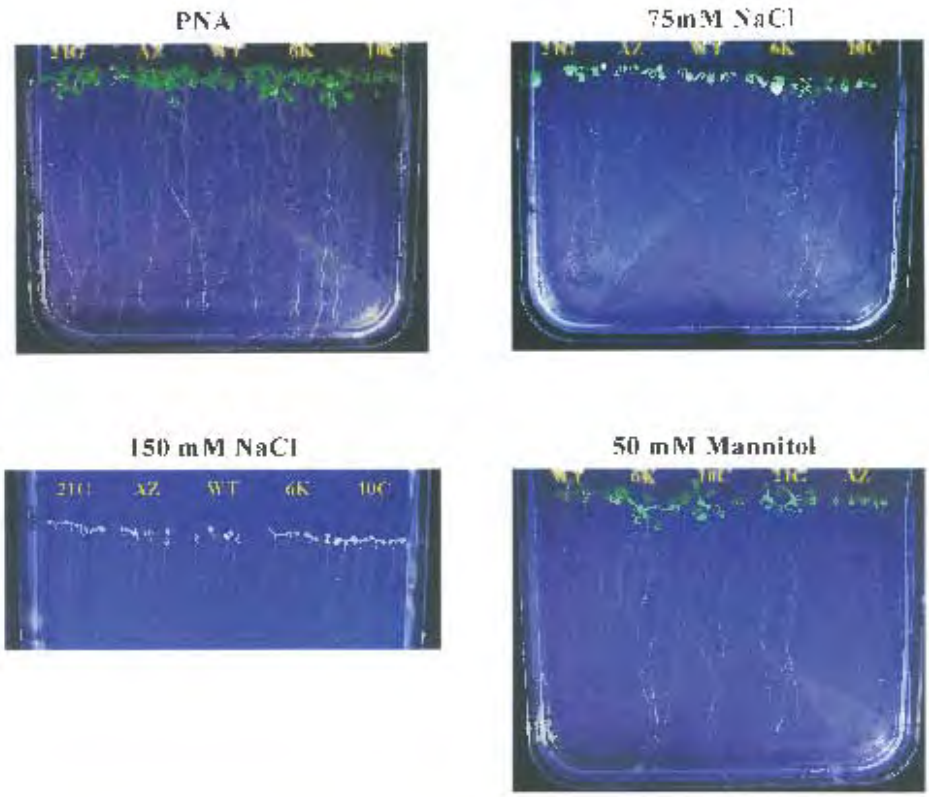

Figure 4.6 Phenotype of Arabidopsis plants transformed with *XVSAP1* (6K, 10C, 21G), the azygous control (AZ) and the untransformed control (WT) on salt and osmotic stress media. Five-day-old seedlings were transferred to plant nutrient agar without sucrose (PNA) or to PNA supplemented with the indicated concentrations of NaCl and mannitol. The pictures were taken 7 days after commencement of the treatment.

Nevertheless, the two transgenic lines, 21G and 6K, consistently displayed a higher relative root growth on sodium chloride concentrations. There was marked growth inhibition at 100 mM NaCl in the wild type and azygous seedlings whereas the transgenic lines continued to grow well with a 43% difference in relative growth between line 21G and the wild type. However, at 150 mM NaCl, all seedlings failed to thrive and were dead after 7 days (Fig.4.6).

At the end of 7 days on salt stress media, there were signs of NaCl damage in all the lines including chlorosis and bleaching of the leaves. However, these symptoms were less severe in the transgenic lines. Some of the plants in the 6K line remained green even at 100 mM NaCl although leaf expansion was retarded.

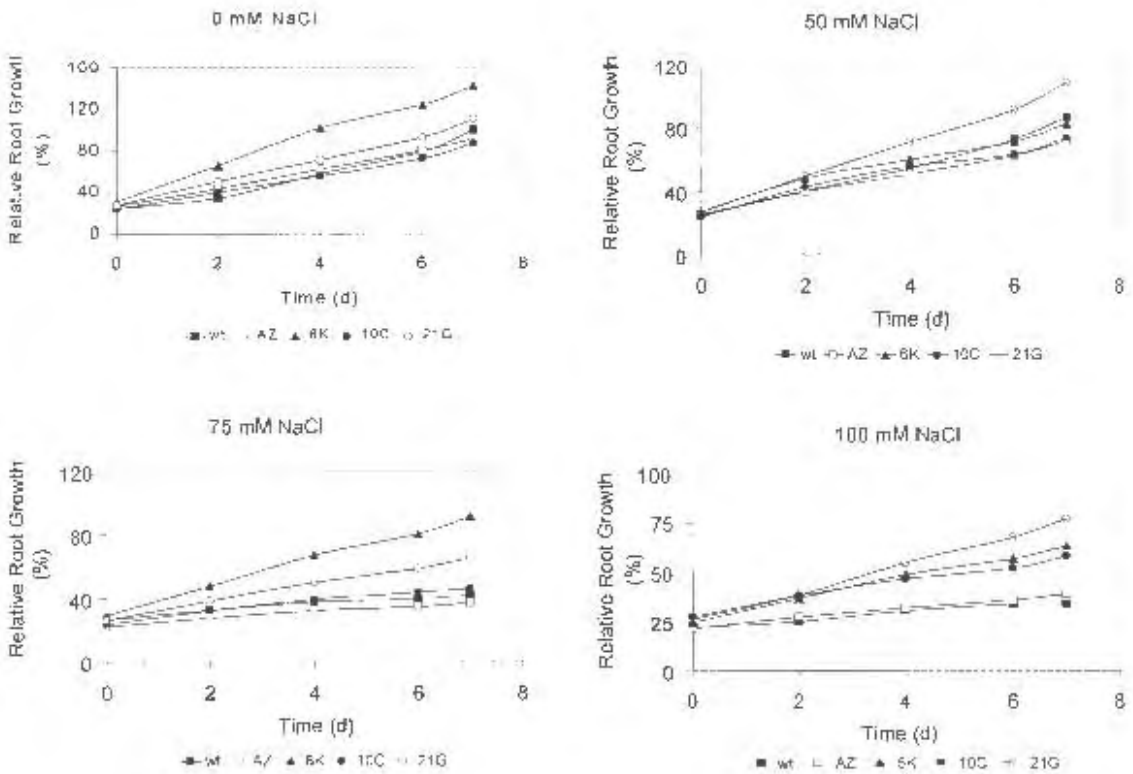

Figure 4.7 Comparison of relative root growth of Arabidopsis plants transformed with *XVSAP1* (6K, 10C, 21G) the azygous control (AZ) and the untransformed control (WT) on salt stress media. Five-day-old seedlings were transferred to plant nutrient agar without sucrose (PNA) or to PNA supplemented with the indicated concentrations of NaCl. Root length was determined from eight plants in each treatment.

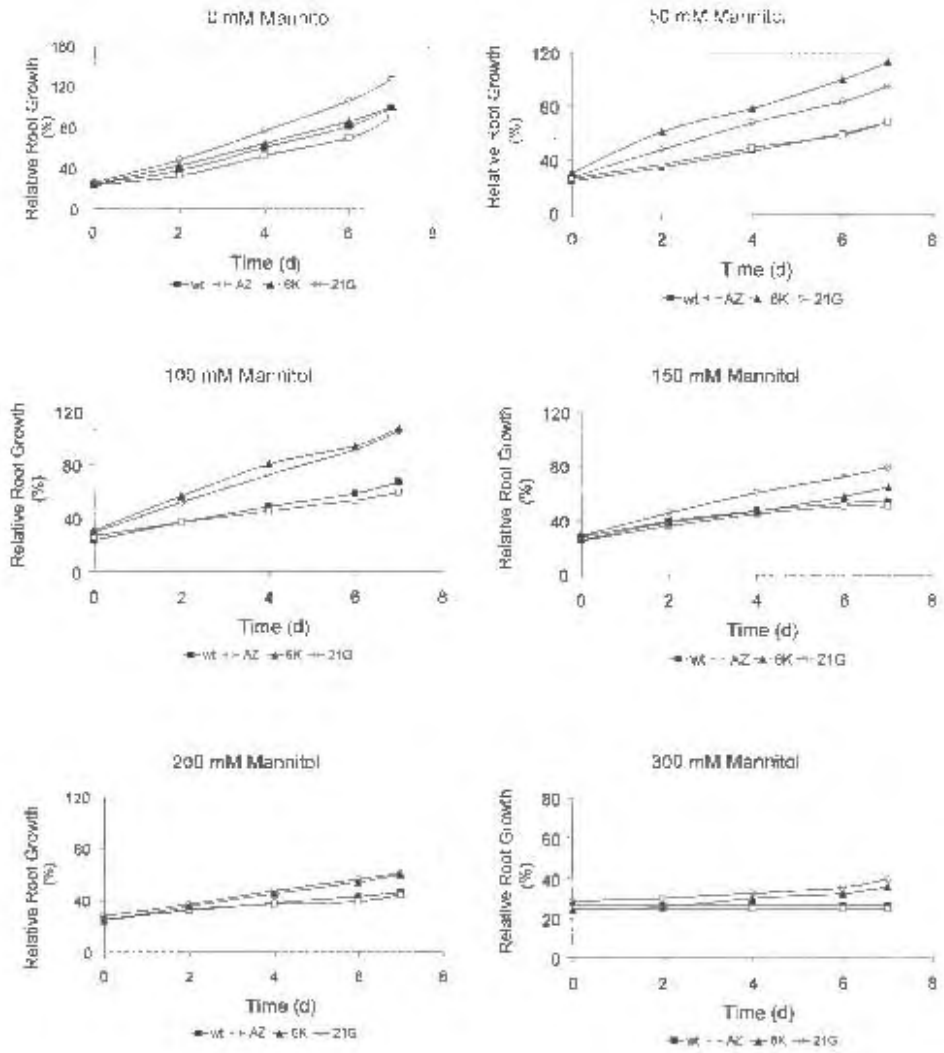

Figure 4.8 Comparison of the relative root growth of Arabidopsis plants transformed with *XVSAP1* (6K, 10C, 21G), the azygous control (AZ) and the untransformed control (WT) on osmotic stress media. Five-day-old seedlings were transferred to plant nutrient agar without sucrose (PNA) or PNA supplemented with the indicated concentrations of mannitol. Root length was determined from eight plants.

Tolerance to osmotic stress was determined by growing Arabidopsis seedlings on PNA media supplemented with mannitol. Only two transgenic lines, 21G and 6K, were used in this experiment. The growth patterns observed were similar to those on salt in that the transgenic lines generally grew better than the untransformed controls with line 21 G showing the highest growth rate at 0 mM mannitol (Fig. 4.8). At 50 mM mannitol both transgenic lines were clearly coping better with the osmotic stress imposed. There was a 47% difference in relative growth on day 7 between the transgenic line 6K and the wild type control (Figs.4.6 and 4.8). At 150 mM mannitol line 21G began to exhibit a greater tolerance to osmotic stress. Even on media containing mannitol at a concentration as high as 300 mM, there was a significant difference between the relative root growth of line 21G and the wild type control ($p = 0.0001$). However, at this concentration, the growth rate was greatly reduced even for the transgenic lines, with line 21G only achieving a relative root growth of 39% by day 7.

Transgenic (6I, 10C, 21G) and wild type (WT) Arabidopsis plants were exposed to a high temperature of 42 °C for 2 hours and then allowed to recover in the growth room. After the heat treatment, all the plants began to wilt and exhibited a slightly chlorotic appearance. After a week the wild type plants had wilted and the leaves were almost completely bleached. The transgenic plants on the other hand had begun to recover from the heat shock although some of the leaves were damaged beyond recovery and progressively became more bleached. At the end of two weeks, the transgenic plants had new leaves and were beginning to flower whereas the wild type Arabidopsis plants were completely bleached and dead (Fig 4.9).

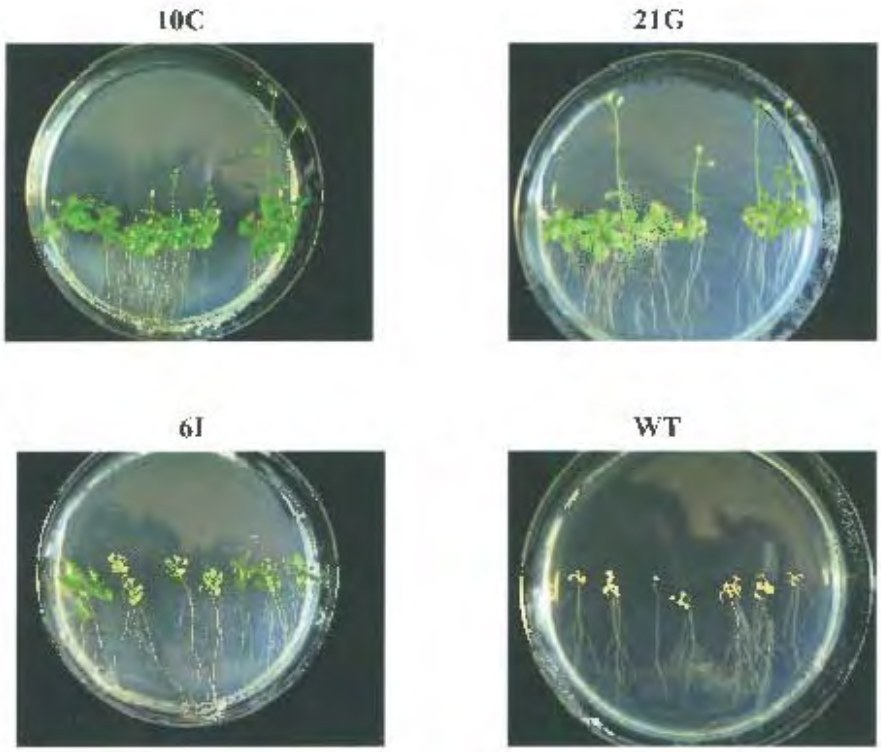

Figure 4.9 Phenotype of Arabidopsis plants transformed with XVSAP1 (6I, 10C, 21G) and untransformed control plants (WT) 2 weeks after heat shock. 10-day-old seedlings were transferred to plant nutrient agar (PNA) without sucrose and allowed to grow until they were 3 weeks old. Transferring the plants to an incubator at 42 °C for 2 hours induced heat shock. Plants were then returned to the growth room. The pictures were taken two weeks after the heat treatment.

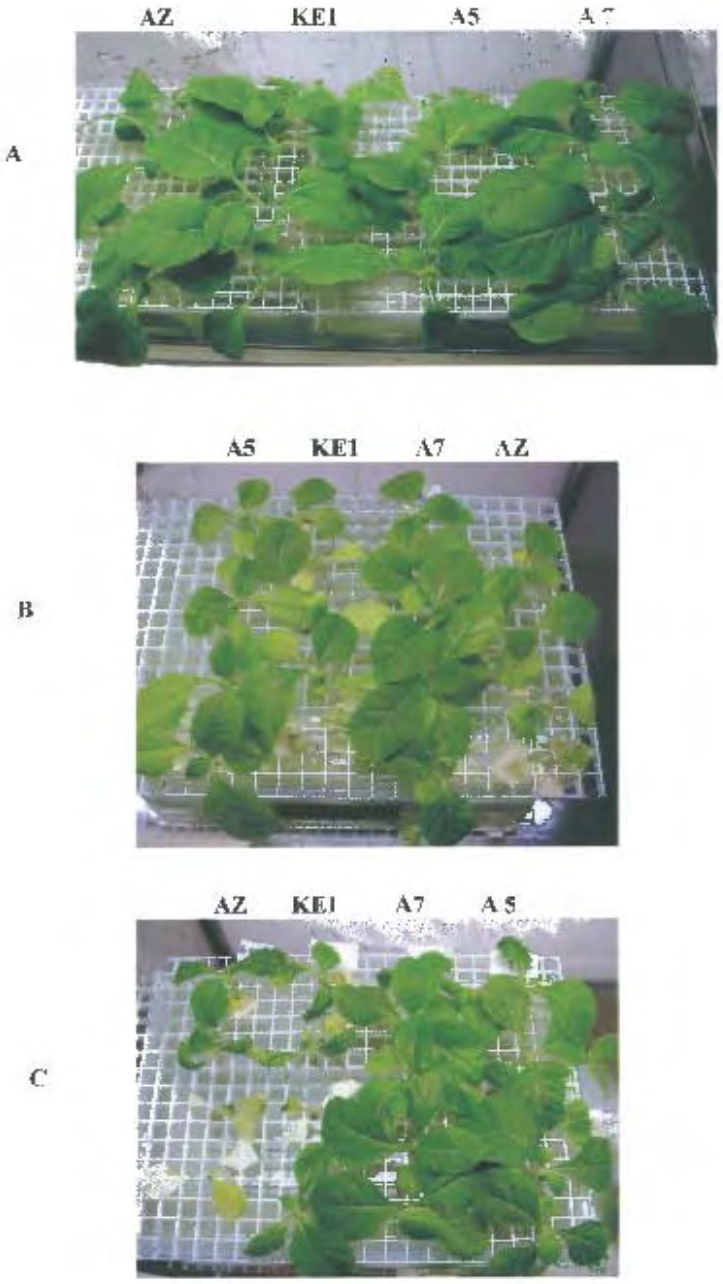

Figure 4.10 Improved stress tolerance of tobacco plants expressing *XVSAP1*. Six-week old tobacco seedlings grown in one-fourth-strength MS (MS-4) solution were transferred either to fresh MS-4 medium (A) or MS-4 supplemented with 200 mM NaCl (B) or MS-4 supplemented with 9% polyethylene glycol (avg. mol. wt. 3.350) (C) for 7 d. Plants were then returned to fresh MS-4 solution. Photographs were taken 7 d after recovery in fresh MS-4 solution. A5 and A7 are transgenic lines expressing *XVSAP1*, KE1 is the untransformed control and AZ is the azygous control.

4.3.6 Transgenic Tobacco Plants Expressing *XVSAP1* are More Tolerant to Salt and Osmotic Stress.

Although transgenic and untransformed controls plants were of similar size at the beginning of the experiment, transgenic plants, by visual inspection, grew better than the untransformed plants under the test conditions (Fig.4.10). However, compared to growth in unsupplemented MS (Fig. 4.10A), growth and leaf expansion were retarded in all lines when the plants were exposed to salt (Fig. 4.10B) and osmotic (Fig. 4.10C) stress. This reduction in vigour was more severe in the untransformed controls than in the transgenic lines. Both transgenic and untransformed control lines displayed a level of chlorosis when grown in 200 mM NaCl. More pale or yellow leaves were observed in the untransformed plants and in some cases, plant leaves were completely bleached. Plants grown on PEG initially developed a dark green colour but the untransformed plants quickly began to exhibit symptoms of stress. Leaf expansion was inhibited, leaves curled and became progressively bleached during stress treatment. After a week-long recovery period, transgenic lines A7 and A5 had recovered whilst the control lines KE1 and AZ did not fully recover. By the time the experiment was terminated some whole plants in the control lines had died.

Tolerance was also evaluated on the basis of total fresh weight (FW) and dry weight (DW) and the root length after allowing the plants to recover for a week. Before treatment, transgenic and control plants were similar in morphology and the FW was also found to be comparable (results not shown). The results obtained from this evaluation supported those obtained by visual inspection. NaCl and PEG both caused a significant reduction in growth in all lines as can be seen in Fig. 4.11A.

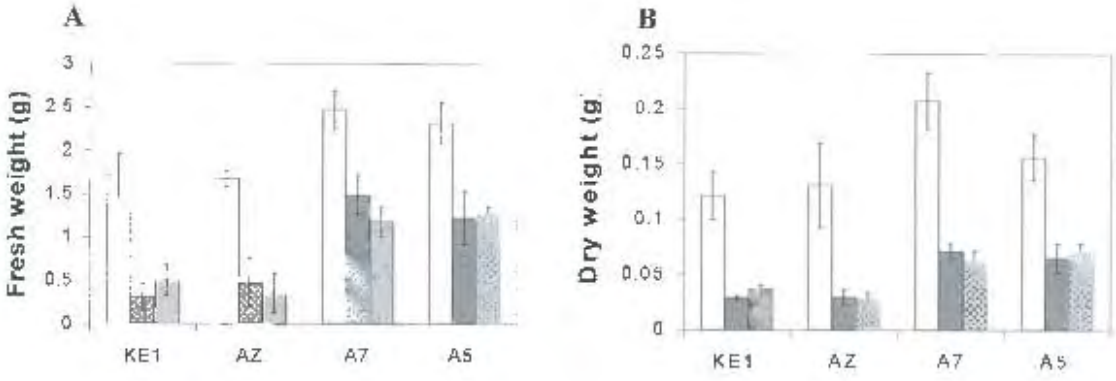

Figure 4.11 Comparison on the basis of fresh (A) and dry weight (B) of tobacco plants transformed with *XVSAP1* (A5, A7), the azygous control (AZ) and the untransformed control (KE1) after salt and osmotic stress. Six-week old tobacco plants grown hydroponically in one-fourth-strength MS (MS-4) solution were transferred either to fresh MS-4 medium (MS, white bars) or MS-4 supplemented with 200 mM NaCl (dark grey bars) or MS-4 supplemented with 9% polyethylene glycol (PEG, avg mol. wt. 3,350, light grey bars) for one week. Measurements were taken one week after recovery in fresh MS-4 solution. Error bars represent standard deviation based on the mass of eight plants per treatment.

For example, the fresh weight of the untransformed line KE1 when grown on salt was only 18% of the fresh weight of the same line growing in unsupplemented MS media. In comparison, line A7, a transgenic line was able to attain 65% of its fresh weight in plain MS media and line A5 attained 43%. This trend was also observed when plants were grown in MS media supplemented with PEG. The azygous control line grew to only 20% of the determined fresh weight on MS-4 compared to 51% for line A7. The transgenic lines showed greater tolerance to both salt and PEG and were able to accumulate more biomass than the untransformed lines. For example, the control line KE1, accumulated 24% and 23% biomass on NaCl and PEG respectively compared to growth in MS-4, whilst transgenic line A7 accumulated 45% and 40% biomass.

There were no differences in the root length of both transgenic and non-transgenic lines grown in MS-4 medium (Fig. 4.12). However, there were differences between these lines when the root length was determined after NaCl or PEG stress. The roots of line A7 plants grown in NaCl were approximately half the length of the control plants in MS-4 whilst the roots of both untransformed lines were 3-fold shorter than those of the same lines but grown in MS-4. In general NaCl appeared to have a more severe effect than the osmotic stress on the transgenic lines.

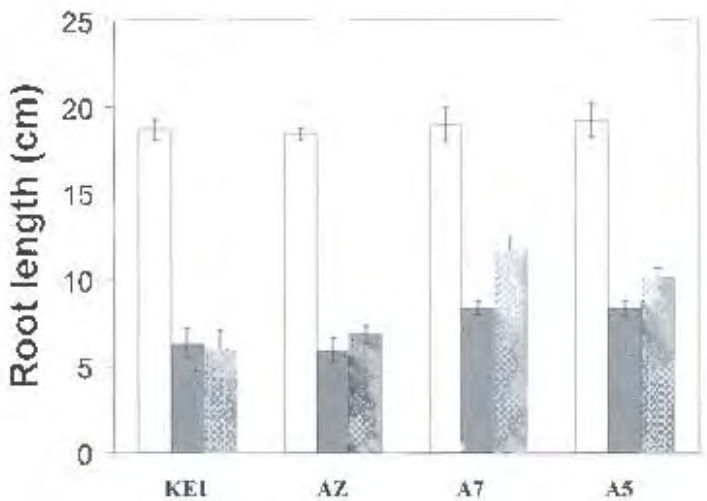

Figure 4.12 Comparison of the root length of hydroponically grown tobacco plants after salt and osmotic stress. Six-week old tobacco plants grown in one-fourth-strength MS (MS-4) solution were transferred either to fresh MS-4 medium (MS, white bars) or MS-4 supplemented with 200 mM NaCl (dark grey bars) or MS-4 supplemented with 9% polyethylene glycol (PEG, avg mol. wt 3,350, light grey bars) for one week. Plants were then returned to fresh MS-4 solution. Measurements were taken one week after recovery in fresh MS-4 solution. A5 and A7 are transgenic lines expressing *XVSAP1*, KE1 is the untransformed control and AZ is the azygous control. Error bars represent standard deviation based on the root length of eight plants per treatment.

4.3.7 Tobacco Plants Expressing *XVSAP1* Show Enhanced Tolerance to Water Deficit

Tobacco plants were germinated on half-strength MS (MS-2) media and transferred to soil when they were two weeks old. The plants were grown on soil until they were six weeks old and then watering was withdrawn. A control group of plants was watered as normal. Five days after the withdrawal of water, the untransformed control plants had started to show wilting symptoms which gradually increased in severity. Figure 4.13 shows the phenotype of one transgenic line, A7, and the wild type (KE1) ten days after withdrawal of water. Well-watered plants from the control group are also shown. The first true leaves in both the transgenic line and the untransformed control had wilted although the wilting was more advanced in the latter. The untransformed plants were also smaller in appearance indicating that growth had slowed to a greater extent in response to the water stress.

Figure 4.13 Phenotype of drought-stressed tobacco. A representative transgenic (A7) and wild type (KE1) line are shown. Drought stress was imposed by withholding water. The control plants were watered as normal. Photographs were taken 10 days after the imposition of the stress.

The untransformed plants became progressively chlorotic whereas the transgenic plants remained green for a longer period. Because of the increasing chlorosis of the leaves of the drought-stressed plants, the chlorophyll content was determined. The results (Fig. 4.14) confirmed that there were significant differences in the sum of chlorophyll *a* and *b* between the transgenic lines and the non-transgenic controls. The transgenic lines contained more chlorophyll in the unstressed condition as well as after stress although total chlorophyll was reduced in both the transgenic and non-transgenic lines after 12 days of drought-stress. For example transgenic line A2 contained 1583 mg ml^{-1} g^{-1} dw chlorophyll compared with 704 mg ml^{-1} g^{-1} chlorophyll for line KE1.

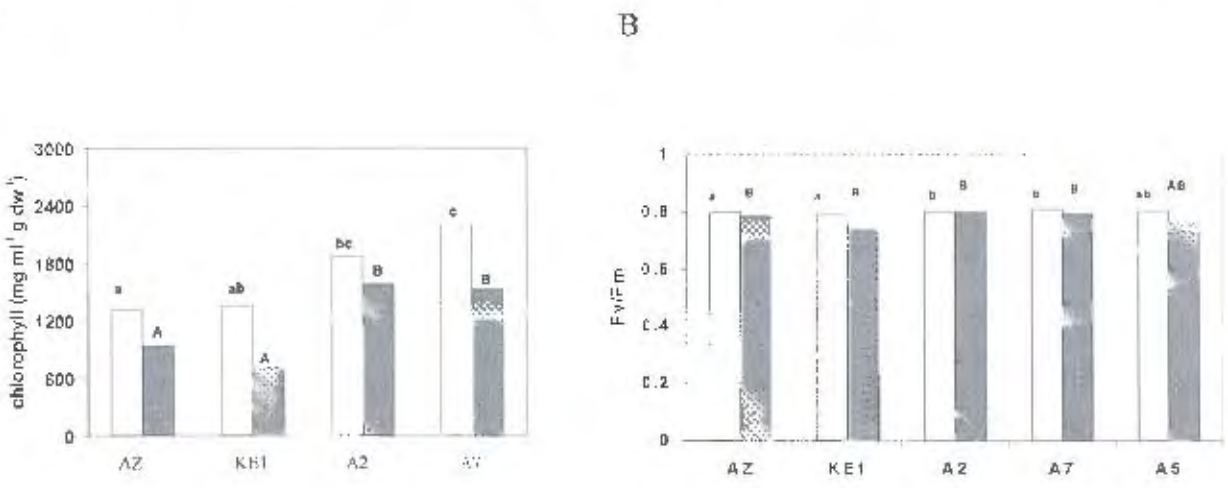

Figure 4.14 Determination of total chlorophyll (A) and the quantum efficiency of photosystem II (B) in tobacco plants transformed with *XVSAP1* (A2, A7) and untransformed controls (AZ and KE1) during water deficit. Plants were drought-stressed by withholding watering for 12 d. Total chlorophyll was extracted using acetone and the amount determined spectrophotometrically. Quantum efficiency (Fv/Fm) was measured on dark-adapted leaves using a flourometer. White bars represent unstressed plants and grey bars stressed plants. Different letters represent significant differences according to the LSD test (P [0.05).

The differences in the chlorophyll content led to the speculation that the photosynthetic efficiency of the drought-stressed plants may have been affected. The ratio of variable to maximal fluorescence (Fv/Fm) is indicative of the potential photoefficiency of photosystem II (Krause and Weis, 1991). Neither wild type nor transgenic plants showed any photoinhibition by water stress (Fig. 4.14B).

The water status of the transgenic and untransformed tobacco plants was assessed by determining the mid-day water potential using a Scholander pressure bomb. The water potential of the untransformed plants after a 12-day drought period was significantly more negative than that of the transgenic plants (Fig. 4.15). The water potential for the three transgenic lines used in this study was about -4.0 Mpa whereas that of the controls had dropped to approximately –8.0 Mpa.

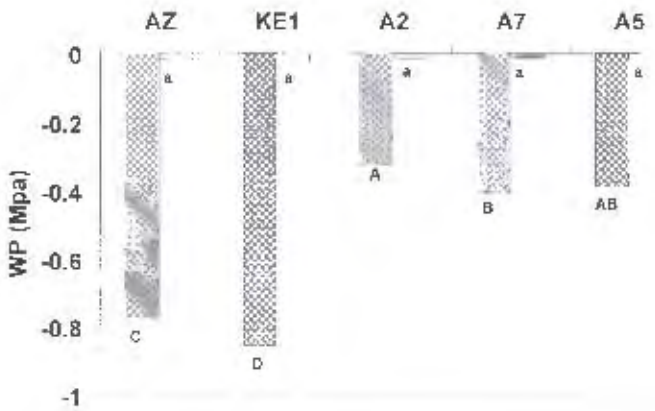

Figure 4.15 Comparison of the water potential of tobacco plants expressing *AVSAP1* (A2, A5, A7), the azygous control (AZ) and the untransformed control (KE1) 12 d after imposition of drought stress by withholding watering. Mid-day water potential of the tobacco leaves was determined using a Scholander pressure bomb. White bars represent unstressed control plants and grey bars stressed plants. Different letters represent significant differences according to the LSD test (P [0.05).

XVSAP1 is highly hydrophobic and is most likely located in the plasma membrane. In order to determine if the protein was playing a role in maintaining membrane integrity during drought stress, the electrolyte leakage of drought-stressed plants was determined. Contrary to expectations, the transgenic plants had a higher initial conductivity than the non-transgenic plants 12 d after the imposition of drought-stress (Fig. 4.16). The differences between the transgenics and the control lines were, however, small.

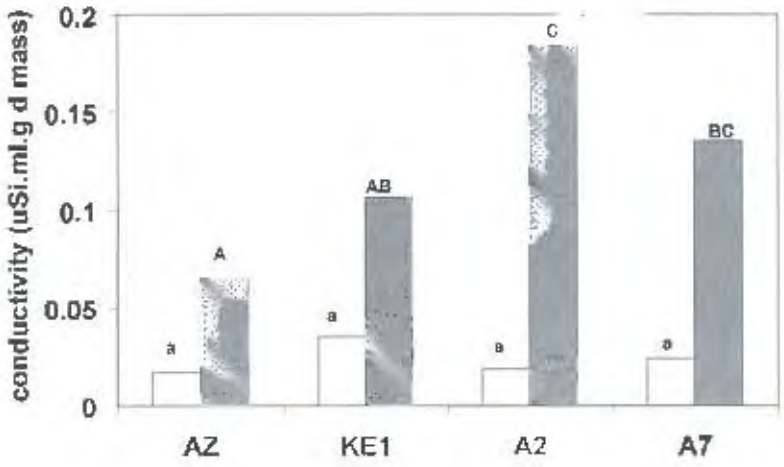

Figure 4.16 Electrolyte leakage of drought-stressed tobacco plants 12 d after imposition of drought stress. A2 and A7 are transgenic plants expressing *XVSAP1*, AZ the azygous line and KE1, the untransformed control. White bars represent unstressed control plants and grey bars stressed plants. Different letters represent significant differences according to the LSD test (P [0.05).

4.4 DISCUSSION

The *E. coli (srl*:: Tn10) mutant strain used in this study is unable to catabolize sorbitol. The cells are, therefore, unable to grow in minimal media in which sorbitol is the sole carbon and energy source (Csonka and Clark, 1979). *XVSAP1* cloned into a prokaryotic expression vector was able to rescue *E. coli (srl*:: Tn10) cells growing in media containing a high concentration of sorbitol confirming the hypothesis that the gene is associated with osmotic stress tolerance.

Ion homeostasis in a salt stress environment is principally regulated by a signal transduction pathway that involves Ca^{2+} and the Ca^{2+}/calmodulin-dependent PP2B phosphatase, calcineurin (Nakamura et al., 1993). This pathway transcriptionally regulates Na^{+}/Li^{+} uptake across the plasma membrane. The high osmolarity (HOG) cascade is responsible for mediating glycerol biosynthesis in response to osmotic stress (see Chapter 1). Since *XVSAP1* is involved in the response of *X. viscosa* to osmotic and salt stress, and since it is also an integral membrane protein, it was speculated that the protein could complement the *S. cerevisae* mutant strains, Δ*cnb1* and Δ*hog1*. However, attempts to complement yeast mutant strains were not successful. Assuming that an *XVSAP1* functional homologue exists in yeast, it is possible that *XVSAP1* complements a gene downstream of the mutations and, therefore, had no role to play in the yeast experiments conducted. It is also a possibility that *XVSAP1* has no yeast functional homologue in the respective pathways and had no effect when the mutant strains were exposed to osmotic and salt stress. Another possibility could be that the XVSAP1 protein was not expressed because of vector construct failure. These aspects require further investigation.

Transgenic Arabidopsis and tobacco plants constitutively expressing *XVSAP1* from *X. viscosa* were obtained and evaluated for tolerance to salt, osmotic stress and water-deficit stress. It is generally accepted that tolerance to abiotic stresses is a multi-genetic trait mediated by a number of biochemical and physiological processes (Zhu, 2000). It has been suggested that meaningful tolerance to abiotic stresses such as salt and drought can only be achieved by pyramiding several stress-responsive genes in a single genotype (Cushman and Bohnert, 2000). However, the expression of single genes has been shown to improve tolerance to various stresses. McKersie et al. (1996) showed that expression of a superoxide dismutase gene from *Nicotiana plumbaginifolia* in transgenic alfalfa resulted in increased drought tolerance under field conditions. Transformation of tobacco with genes or cDNAs coding for enzymes leading to the accumulation of osmolytes such as mannitol and proline has resulted in plants that are more tolerant to salt and water stress (Tarczynski et al., 1993; Kishor et al., 1995). Successes in achieving tolerance to abiotic stresses have also been registered through genetic engineering for the accumulation of sugars. The *IMTI* gene isolated from the halophyte *Mesembryanthemum crystallinum* increased tolerance to drought and salt stress in transgenic tobacco plants as a result of the accumulation of the methylated sugar alcohol D-onitol (Sheveleva et al., 1997). One of the more striking examples of a single gene conferring tolerance to abiotic stresses was the overexpression of AtNHX1 in Arabidospis (Apse et al., 1999) and tomato (Zhang and Blumwald, 2001) which improved salt tolerance substantially.

Although a number of genes have been shown to be up-regulated by abiotic stresses in resurrection plants (Furini et al., 1997; Bockel et al., 1998; Oliver et al., 1998; Mundree et al., 2000; Neale et al., 2000; Mowla et al., 2002), very little has been reported on the expression of these stress-associated genes in heterologous plant systems. Individual expression of three desiccation-related

proteins from *C. plantagenium* dsp21, 16 and 15 in transgenic tobacco did not alter the response of the plants to water stress (Itturiaga et al., 1992). In this study it has been demonstrated that the expression of one gene, *XVSAP1,* resulted in increased tolerance to, heat, salt and osmotic stress. Transgenic Arabidopsis exhibited greater relative root growth under these stress conditions whilst transgenic tobacco plants accumulated more biomass and had longer root systems under similar conditions. It could be argued that the gene was merely promoting the growth of transgenic plants rather than actually conferring tolerance. However the magnitude of differences between the transgenic plants and the control plants was much greater after stress treatment than when the plants were grown on normal MS or PNA media.

As stated in section 2.3.1, searches using the BLAST programme showed that there are at least four orthologues of *XVSAP1* in Arabidopsis. This was confirmed by the results obtained from the western analysis. The synthetic peptide used for immunizing rabbits was prepared from the sequence of one of the few antigenic sequences of the translated protein. This region is present in at least three of the Arabidopsis orthologues revealed by computer searches. It was expected that the resultant antibodies would cross-react with the Arabidopsis homologues. The apparent molecular mass of XVSAP1 was a little higher than that predicted by the amino acid sequence of the open reading frame. This may reflect anomalous migration in the SDS-PAGE gel although the difference of 1 kDa may not significant.

It is noteworthy that there was also cross- reactivity with tobacco membrane proteins indicating that there are XVSAP1 orthologues in tobacco. The banding pattern was similar to that obtained with Arabidospis suggesting that there could be more than two homologues. It would appear that

these orthologues do not play a part in improving tolerance to abiotic stresses in either the transgenic plants created nor the wild type and azygous control plants. This could be explained by taking into consideration that although there was a significant amount of identity between XVSAP1 and the Arabidopsis orthologues (maximum of 56%), perhaps the active part of the protein could be the area that bears no identity to any characterized proteins to date. Another explanation could be that an activation signal could be missing from these orthologues which is present in the *X. viscosa* gene. In fact, it has been suggested that some of the stress-related genes present in resurrection plants are also present in normal plants. Molecules involved in desiccation tolerance in the seeds of normal plants are to a large extent found in the vegetative tissues of resurrection plants such as *C. plantagenium* (Bartels and Salamini, 2001). However, the regulatory mechanisms that govern the expression of some of these genes which lead to variations in tolerance to various stresses may differ (Zhu, 2001). It is also possible that XVSAP1 has a completely different role to that of the identified homologues in Arabidospis.

Expression of transgenes in transgenic plants is often correlated with copy number and the so-called positional effect (Hobbs et al., 1993; Peach and Velten, 1991). Having more than one copy of the transgene did not appear to confer an increased tolerance to abiotic stresses in Arabidopsis transformed with *XVSAP1*. Transgenic Arabidopsis lines 6K and 21G had comparable tolerance to both mannitol and NaCl although line 6K had at least two copies of the gene. Line 10C on the other hand only had one copy of the gene but did not significantly outperform the wild-type and azygous controls ($p=0.0663$ and $p=0.1991$ respectively) on salt stress media except on 100 mM NaCl. Western blot data confirmed that the gene was indeed expressed. The behaviour exhibited by this line could be the result of the position at which the gene was inserted within the Arabidopsis

genome. It must be noted, however, that having more than one copy of a transgene can also result in the silencing of the endogenous gene (Flavel, 1994).

Environmental stresses such as drought, salinity and temperature cause a reduction in water availability resulting in water deficit conditions in plant cells. These conditions induce osmotic stress. Indeed, in a recent publication Munns (2002) argued that in the short term, salt shock induces an osmotic stress rather than salt-toxic effect. *XVSAP1* was isolated on the basis of its ability to provide functional sufficiency to osmotically stressed *E. coli* (*srl*::Tn 10) cells and therefore its function in stresses having a dehydrative component was expected (Garwe et al., 2003). However, the exact function of *XVSAP1* in *X. viscosa* remains obscure. Because of the highly hydrophobic nature of XVSAP1 it is likely that the protein is localized in the plasma membrane. As plant responses to dehydration may result from protein structure and membrane damage (Shinozaki and Yamaguchi-Shinozaki, 1997), it is possible that XVSAP1 may be playing a protective role in the transgenic plants by stabilizing specific protein complexes within the membrane or the membranes themselves.

During the dehydration experiment, the water-stressed plants became increasingly chlorotic and this was particularly obvious in the untransformed controls. The presence of light during dehydration in photosynthetically active leaves can lead to the formation of ROS. These can cause photo bleaching of chlorophyll and lipid peroxidation of membranes (Smirnoff, 1993). Results obtained by measuring photosystem II efficiency indicated that *XVSAP1* is not associated with the photosynthetic system in tobacco although transgenic plants remained green for a longer duration during dehydration stress. It is suggested that because transgenic tobacco plants were able to retain

water for a longer period as evidenced by the water potential data, stress symptoms such as the loss of chlorophyll were delayed.

The electrolyte leakage data implies that the XVSAP1 protein plays a role in maintaining homeostasis. The transgenic tobacco membranes appeared more leaky or more permeable to ions. This result is particularly significant when it is considered that the region extending from the lysine residue at position 36 to the phenylalanine residue at 119 in the deduced protein sequence of XVSAP1 and bears 12 % identity with the K^+ potassium transporter family. It could be that this putative transporter region might play a role in the leakage or active transport of electrolytes to maintain homeostasis. The information obtained from the conductivity experiments is not conclusive and data obtained from experiments using as a patch-clamp, for example, would assist in elucidating the possible role of *XVSAP1* in ion transport. Nonetheless, taken together, the data presented shows that XVSAP1 confers tolerance to stresses with a water deficit component.

CHAPTER FIVE

SILENCING OF *XVSAP1* ORTHOLOGUES IN *A. THALIANA*

SUMMARY

There are initiatives to identify and ascertain the biological function of every gene in various organisms. Considerable progress has been made in such organisms as nematodes, Arabidopsis and humans. Some of the techniques used to determine gene function include reverse genetics and RNA interference (RNAi). In an attempt to establish the function of *XVSAP1* in *X. viscosa*, the orthologues of the gene in Arabidopsis, the *A. thaliana* cold-associated protein (ATCAP) genes, were silenced using RNAi. The binary vector pFGC1008 was used to introduce and drive the expression of inverted repeats of at least four of the *ATCAP* genes. Initial results indicated that the transgenic Arabidopsis plants displayed an early flowering phenotype. Additionally, the plants exhibited a hypersensitivity to salt and osmotic stress. These results indicated that the *ATCAP* genes were associated with the tolerance of Arabidopsis to salt and osmotic stress correlating with the function of *XVSAP1* in *X. viscosa.*

5.1 INTRODUCTION

Several projects are currently underway to sequence the genomes of various prokaryotic and eukaryotic organisms. The genome of Arabidopsis has already been completely sequenced and contains an estimated 26 000 genes (Arabidopsis Genome Initiative, 2000). The human and yeast genomes were also sequenced through the efforts of several groups and the sequencing of other

organisms including rice is nearing completion. The ultimate goal of these various initiatives is to identify and ascertain the biological function of every gene in the genome of these organisms (Chory et al., 2000). Techniques such as sequence homology have been used for this purpose but with limited success. This is because there are many genes without homology to functionally characterized genes and the precise function of any gene cannot necessarily be determined from sequence homology alone. In some cases, the function of some genes has been investigated through specific assays but the use of reverse genetics has yielded by far the most information. Reverse genetics is used to determine the function of a gene by examining the phenotypes of individuals with alterations in the gene of interest (Sessions et al., 2002). Loss-of-function mutants resulting from transposable elements or insertional mutagenesis have assisted greatly in the functional characterization of many genes. Large insertion mutant collections have been established for the study of gene function in Arabidopsis (Aspiroz-Leehan and Feldmann, 1997; Parinov and Sundaresan, 1999; Sussman et al., 2000) and yeast (Ross-Macdonald et al. 1999). The identification of mutations in the genes of interest is a laborious and time-consuming procedure which involves PCR-based screening of pooled mutant populations (Sussman et al., 2000). It has been estimated that about 35 000 independent transformants would have to be generated in order to have a 90% chance of finding one specific 1-kb gene in Arabidopsis using T-DNA insertional mutagenesis (Krysan et al., 1999). Alternative, less laborious methods such as sequencing of regions flanking insertion sites in individual plants have been described (Tissier at al., 1999; Sessions, 2002).

Post-transcriptional gene silencing (PTGS) offers another alternative tool to knock out expression of specific genes in a variety of organisms. Initially regarded as a bizarre phenomenon limited to

petunias and other plant species, PTGS is now considered an invaluable tool in functional genomics. PTGS came to light when Jorgensen et al., (1996) attempted to deepen the purple colour of petunias by increasing the activity of chalcone synthase, an enzyme involved in the production of anthocyanin pigments. Instead of exhibiting a deeper purple colour, the resulting flowers were variegated or even white in colour. The introduction of the gene coding for chalcone synthase under the control of a powerful promoter into petunias actually caused the silencing of both the introduced gene and the endogenous gene, a phenomenon that Jorgensen et al. termed co-suppression. They observed that the white colour obtained could be passed on to the next generation suggesting that the co-suppression was mediated by a trans-acting molecule that could passed on from generation to generation.

Researchers working on nematodes obtained similar curious results. Whilst attempting to silence the *par-1* gene which controls symmetry in the nematode *Caenorhabditis elagans* using antisense RNA, Guo and Kemphues (1995) found that not only did injecting antisense RNA into the worms result in silencing, but injecting sense RNA into the control worms also had the same effect. These results were a puzzle until Fire et al. (1998) showed that injection of double-stranded RNA (dsRNA) formed from the annealing of sense and antisense strands present in *in vitro* RNA preparations, completely silenced the target endogenous genes in *C. elegans* in a phenomenon they termed RNA interference (RNAi). From this and other subsequent work it is now known that the trans-acting factor responsible for PTGS or RNA interference is dsRNA. RNAi was subsequently described as a process whereby dsRNA silences endogenous gene expression by the highly specific cleavage of both the foreign RNA and the target RNA. RNAi has since been shown to work in a

variety of organisms including animals, plants, fungi and protists (Fire, 1999; Sharp, 2001; Waterhouse et al., 1998).

The most interesting aspects of RNAi are that dsRNA, rather than single-stranded antisense RNA, is the interfering agent and that the interfering activity can be detected in cells and tissues far removed from the site of introduction. Furthermore, RNAi is highly specific and potent since only a few dsRNA molecules per cell are required for effective interference. A complete understanding of how dsRNA results in the loss-of-function of the targeted homologous gene has remained elusive.

Studies by a few groups have provided clues on the mechanism of RNAi. Hamilton and Baulcombe (1999) identified RNAs of ~25 nucleotides complementary to both the sense and antisense strands of the gene being silenced, which were present only in silenced plants. Zamore et al., (2000) discovered that dsRNA added to *Drosophila* embryo lysates was cleaved at 21-23 nucleotide intervals corresponding to the homologous endogenous mRNA which was also simultaneously cleaved. These key discoveries assisted in developing the current RNAi model. It is thought that in an initiation step, dsRNA is digested into 21-23 nucleotide small interfering RNAs (siRNAs) or "guide RNAs". Indications are that an enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, specifically degrades dsRNA in an ATP-dependent manner. Successive cleavage events degrade the RNA to 19-21 duplexes each with 2-nucleotide overhangs.

In what is referred to as the effector step, the siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or RISC. An ATP-dependent unwinding of the siRNA duplex is required for activation of RISC. The active RISC then targets the homologous transcript by pairing interactions and cleaves the mRNA ~12 nucleotides from the 3'terminus of the siRNA. The RISC contains a single siRNA and an RNase that appears to be distinct from Dicer (Hammond et al., 2001; Hutvagner and Zamore, 2002; Sharp, 2001). Figure 5.1 shows a simplified version of how RNAi is thought to work.

Figure 5.1 A simplified diagram of how RNAi interference is thought to work (Gura, 2000).

It has become increasingly clear that PTGS probably evolved as a defense mechanism against RNA viruses and transposons in both plants and animals (Hammond, 2001). However, it is the use of RNAi as a tool in functional genomics that has generated excitement in research. The function of many genes in *Drosophila, C. elagans* and several plant species has already been ascertained using RNAi (Fraser et al., 2000; Wang and Waterhouse, 2000). In most cases injection or transfection of dsRNA into the cells and organisms has been the main method of inducing gene silencing. The silencing effect is temporary and does diminish with time. A number of expression vectors that facilitate direct silencing of specific genes in a stable manner have been developed. These include transgenes designed to express dsRNA or single-stranded self-complementary hairpin RNAs (hpRNA) which get processed *in vivo* into siRNAs-like molecules that are also capable of carrying out gene-specific silencing in plants and animals (Paddison et al., 2002; Wang and Waterhouse, 2000; Waterhouse et al., 1998).

In an attempt to ascertain the function of the *XVSAP1* protein, the genes coding for the Arabidopsis orthologues termed ATCAPs were silenced using RNA interference. This chapter describes the creation and characterization of Arabidopsis plants expressing a gene construct designed to silence at least four orthologues in the plants.

5.2 MATERIALS AND METHODS

5.2.1 Plant Material and Growth Conditions

A. thaliana ecotype Columbia seeds were stratified in 0.1% agar at 4°C prior to sowing to promote even germination either in soil or on plates. For the salt and mannitol stress treatments, Arabidopsis seeds were germinated on plant nutrient agar (PNA, Haughn and Somerville, 1986) and then transferred to media containing either 75 mM NaCl, 100 mM mannitol or no supplementation. For plate assays, Arabidopsis seeds were surface sterilized as described in section 4.2.2. All plants were grown in a growth room (16-h day, 8-h night, 22°C, and 65% humidity). T2 seeds were used for all assays.

5.2.2 cDNA synthesis

Arabidopsis plants grown in soil were rinsed to remove soil particles and dehydrated on filter paper in a petri dish overnight. RNA was prepared from the wilted plants using the Trizol reagent as described in section 2.2.7. DNA contamination was removed by treating the RNA samples with DNAseI. cDNA was synthesized from the RNA preparations using the Omniscript Reverse transcription kit (Qiagen GmbH, Germany) according to the manufacturer's indications. The RNase inhibitor was obtained from Roche Diagnostics GmbH, Germany.

5.2.3 Transgene Constructs

As there is at least 75% identity between the genes coding for the first three ATCAPs (ATCAP1-3) (Fig. 2.4), an oligonucleotide primer pair designed to amplify a ~500-bp region from the three genes coding for these proteins was synthesized. The primer pair used was as follows: CTACTAGTGGCGCGCCGCGTTACTCATTCCGATAT (forward primer, *Spe*I site underlined

and *Asc*I site bold) and CGGGATCC**ATTTAAAT**CGATGCTCGAATATGTCTTGA (reverse primer, *Bam*HI site underlined and *Swa*I site bold). Another primer pair designed to amplify a 520-bp gene sequence from *ATCAP4* was also prepared with the following sequences: GGACTAGT**GGCGCGCC**TCTGAATGAGATCCAAGCCG (forward primer, *Spe*I site underlined and *Asc*I site bold) and CGGGATCC**ATTTAAAT**AGTAGAAGAGGGCAGCAAATGA (reverse primer, *Bam*HI site underlined and *Swa*I site bold). The enzyme restriction sites included were for later cloning into a binary vector designed for double stranded RNA production. The primer sets were used in a 50-µl PCR reaction with 5 µl of cDNA. Reactions were conducted using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, CA, USA) thermocycler under the following conditions: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 63°C for 40 s and 72°C for 45 s and a final extension step for 7 min.

The PCR products were gel-purified and cloned into pGEM-T Easy (Promega, USA) to create pGEM-*ATCAP1-3* and pGEM-*ATCAP4*, according to the manufacturer's protocol. The ligation mix was used to transform *E. coli* DH5α as described in section 3.2.4. Transformants were selected on LA supplemented with 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. Colony PCR was used to screen the white colonies with M13 forward and reverse primers. Reactions were conducted using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, CA, USA) thermocycler under the following conditions: 94°C for 2 min followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s and a final extension step for 7 min. Recombinant plasmids containing the targeted *ATCAP* inserts were purified using the High Pure plasmid mini-preparation kit (Roche, Germany) according to the manufacturer's recommendations. Colony PCR and restriction digests

with appropriate enzymes were done to ensure that the pGEM-T vector contained the *ATCAP* inserts. pGEM-*ATCAP* constructs were sequenced using the M13 forward and reverse primers to confirm that the correct gene fragments had been cloned.

The *ATCAP* inserts were excised from the pGEMT-*ATCAP* constructs using *Asc*I and *Swa*I and then separately cloned into the binary pFGC1008 vector (http://ag.arizona.edu/chromatin/fgc1008.html) (Fig. 5.2) between the *Swa*I and *Asc*I sites in an antisense orientation. The constructs were then used as templates for the another round of PCR using the original primer pairs. The resulting PCR products were then cloned into the *Bam*HI – *Xba*I site in the sense direction to create inverted repeats of the cDNAs, separated by the 335-bp GUS gene.

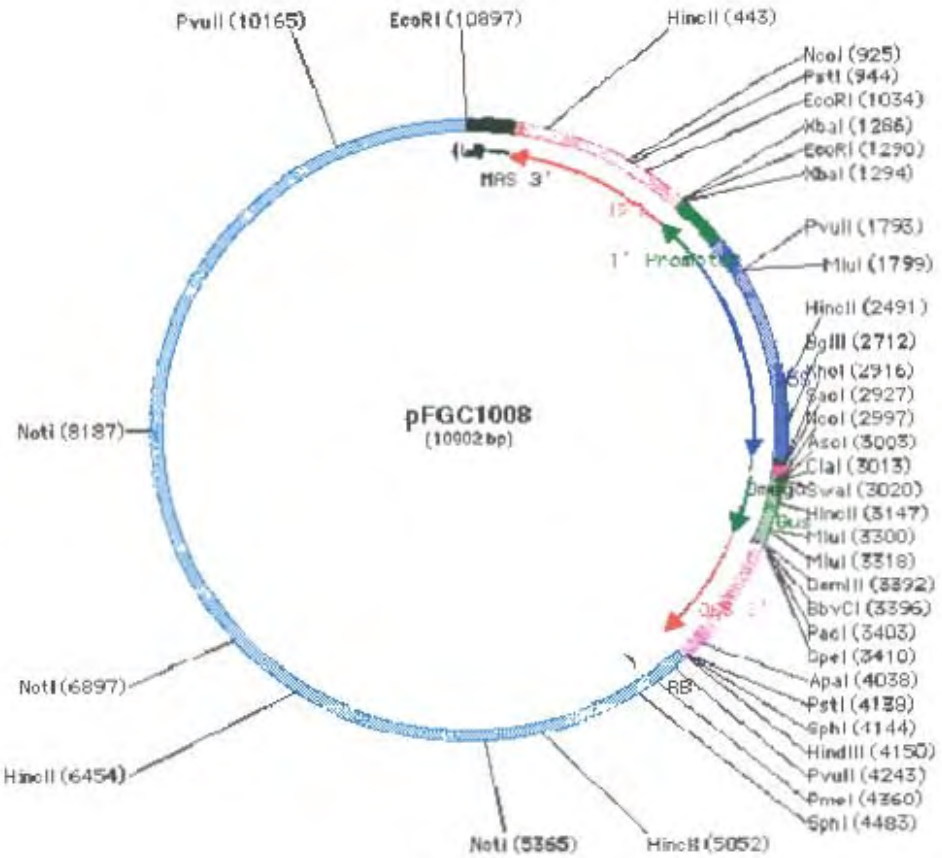

Figure 5.2 Map of the binary pFGC1008 vector (http://ag.arizona.edu/chromatin/fgc1008.html)

5.2.4 Plant Transformation

To create RNAi transgenic lines, the pFGC1008-*ATCAP* (pFGC-*ATCAP*) constructs and the pFGC1008 vector were introduced into *A. tumefaciens* (GV3103) by electroporation (Bio-Rad Gene Pulser, Hercules, California). Arabidopsis wild type plants were transformed using the floral dip method (Clough and Bent, 1998) and seed was collected. Selection of transgenic Arabidopsis was carried out on plates containing hygromycin (20 mg/L) and cefotaxime (200 mg/L). Transgenic Arabidopsis plants were initially screened by PCR for the presence of the specific gene constructs or for the vector hygromycin gene sequence. The following primer pairs were used: (1) forward primer, 5' – CGTCTGCTGCTCCATACAAGC –3'; reverse primer, 5'-TGTCACGTTGCAAGACCTGC-3') specific for the *HPT* (hygromycin) gene in pFGC1008; (2) forward primer, 5' – TTCCAACCACGTCTTCAAAGC –3'; reverse primer, 5'-AGTCTGAGAACAACCGCAACG-3' specific for the pFGC-*ATCAP1-3* construct; and (3) forward primer, 5' – TTTCATTTGGAGAGGACACGC–3'; reverse primer, 5'-GAAGGCACACATCACCTCCC-3' specific for the pFGC-*ATCAP4* construct.

5.2.5 Southern Blot and RT-PCR Analysis of Transgenic Plants

To confirm integration and also to determine copy numbers of the pFGC –*ATCAP* constructs, Southern blot analysis was carried out. Genomic DNA was isolated from Arabidopsis plants using the Nucleon Phytopure DNA extraction and purification kit (Amersham, Buckinghamshire, England) according to the manufacturer's indications. Approximately 500 mg of leaf tissue was used for DNA extraction. Five micrograms of genomic DNA from transgenic plants were restricted

with *Hind*III, *Eco*RI and *Pst*I, separated on a 1% agarose gel and blotted onto a nylon membrane (Amersham, 045 μM). Probes specific for either the pFGC-*ATCAP1-3* construct, the pFGC-*ATCAP4* construct or the pFG1008 vector were prepared using the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The primers described in section 5.2.3 were used for the PCR reactions. Hybridization detection was as described in section 2.2.5.

To determine the levels of silencing of the endogenous *ATCAP* genes, RT-PCR analysis was carried out. RNA from the transgenic plants was isolated using the Trizol reagent. In each case, 1 μg RNA was reverse-transcribed as described in section 2.2.7. A 1:10 dilution of the cDNA from the RT step was used in 50-μl PCR reactions with the primers described in section 5.2.3.

5.2.6 Plate Assays

To assess the effect of osmotic and salt stress on Arabidopsis plants expressing pFGC-*ATCAP*, plate assays as described in section 4.3.5 were carried out. Transgenic and control seeds were placed in single rows on each plate. The seeds were allowed to germinate with the plates placed vertically to permit easier observation of growth patterns of the roots. Osmotic stress was imposed by growing seedlings on PNA containing 0, 50, 100 and 200 mM mannitol. Salt treatment was carried out on PNA containing 0, 50, 75 and 100 mM NaCl. Observations were made over a period of eight days.

5.3 RESULTS

5.3.1 Creation of RNAi Gene Constructs

Sequence alignments showed that the first three orthologues of *XVSAP1* in Arabidopsis corresponding to *ATCAP1-3* (Fig. 2.3) were at least 75% homologous whilst the identity between the four orthologues was 50%. It was decided to create two constructs, one targeted at *ATCAP1-3* and another at *ATCAP4* with restriction sites for cloning into the pFGC1008 vector. As *XVSAP1* is induced by dehydration, it was reasonable to assume that *ATCAP1-4* would also be induced by dehydration. This indeed proved to be the case as was shown by RT-PCR. The *ATCAP1-3* primers produced a product slightly smaller than that produced by the ATCAP4 primers (Fig 5.3A and B). The PCR products were designated *ATCAP1-3* and *ATCAP4*, respectively. *ATCAP1-3* was ~500 bp whilst *ATCAP4* was ~590 bp.

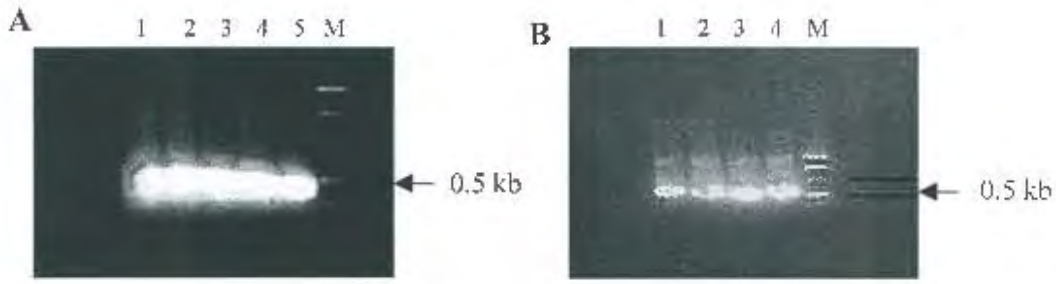

Figure 5.3. Isolation of *XVSAP1* orthologues in Arabidopsis by RT-PCR. RNA isolated from dehydrated Arabidopsis leaves was reverse-transcribed and PCR performed with primers specific for four orthologues of *XVSAP1* in Arabidopsis. (A) RT-PCR using primers specific for the three Arabidopsis orthologues (*ATCAP1-3*) with closest identity to *XVSAP1*. Lanes 1-5 are PCR products from different RT-PCR reactions. (B) RT-PCR using primers specific for *ATCAP4*. Lanes 1-4 are PCR products from different RT-PCR reactions. M refers to marker VIII (Roche, Germany).

Both sets of PCR products were slightly larger than the size calculated based on the gene sequences due to the extra nucleotides introduced by the enzyme restriction sites. The products from the RT-PCR step were cloned into the pGEM-T Easy vector to create pGEM-*ATCAP1-3* and pGEM-*ATCAP4*. To confirm successful cloning of the *ATCAP* gene fragments, the pGEM-*ATCAP* plasmids were restricted with *Eco*RI (Fig. 5.4) to release the inserts. Although in some cases, the plasmid preparations had been shown to harbour an insert by PCR, restriction with *EcoR*I failed to release an insert (Fig 5.4, lane 4). This could be the result of rearrangement of the *Eco*RI sites in the pGEM constructs. Such preparations were discarded. Sequencing of the pGEM constructs also confirmed that the sequences of the inserts matched the gene sequences of the *ATCAP* genes.

Figure 5.4 Restriction analysis of pGEM-*ATCAP4* constructs. pGEM-*ATCAP4* plasmid preparations were restricted with *Eco*RI to release the inserts and the products run on a 1% agarose gel. The gel was stained with ethidium bromide. Lanes 1-4 are different pGEM-*ATCAP4* plasmid preparations. M1 is the λ *Pst* I DNA marker and M2 is marker VIII (Roche, Germany).

5.3.2 Plant Transformation

Plasmid DNA was extracted from the *Agrobacterium* cultures before plant transformation and restricted with *Pst*I to confirm stability of the inverted repeat constructs. The pFGC1008 binary vector has two *Pst*I restriction sites flanking the *Asc*I, *Swa*I and *Bam*HI, *Spe*I restriction sites (Fig

5.2). Digestion of the vector with *Pst*I resulted in two fragments, one 7.7 kb and another 3.3 kb long. Figure 5.5 shows the results of a digestion of four preparations of pFGC-*ATCAP1-3*. Sub-cloning of two copies of *ATCAP1-3* resulted in the addition of ~1 kb to the smaller vector fragment. Restriction fragments in agreement with the calculated sizes were obtained. Similar results were obtained with the pFGC-*ATCAP4* construct.

To assess the *in vivo* role of *XVSAP1*, dominant negative ATCAP-mutant lines were generated in Arabidopsis by RNAi. Primary transformants were selected on hygromycin in culture. Analyses were carried out using T_2 generation plants. Although a large number of transgenic plants were obtained, a maximum of four independent lines from each of the transformation events was selected for further analysis. Transgenic plants were initially screened by PCR to confirm the presence of the *ATCAP* transgenes as well as the vector sequence. Figure 5.6 shows one of the results obtained in these screens using selected plants. The pFGC1008 vector and wild type Arabidopsis genomic DNA were used as controls in the reaction.

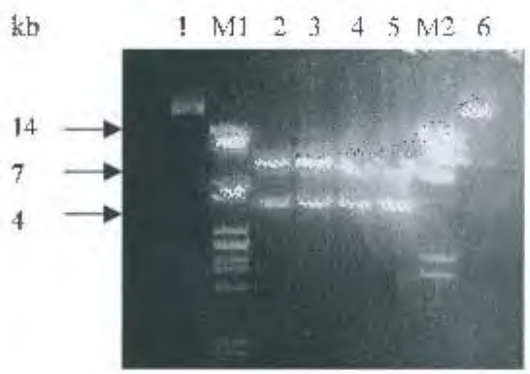

Figure 5.5 Restriction analysis of pFGC-*ATCAP1-3* constructs. pFGC-*ATCAP1-3* plasmid preparations were restricted with *Pst*I and the products run on a 1% agarose gel. The gel was stained with ethidium bromide. Lane 1- undigested pFGC-*ATCAP1-3* plasmid DNA. Lanes 2-5 are four different pFGC-*ATCAP1-3* plasmid preparations. Lane 6- pFGC1008 vector. M1 is the λ *Pst* I DNA marker and M2 is marker II (Roche, Germany).

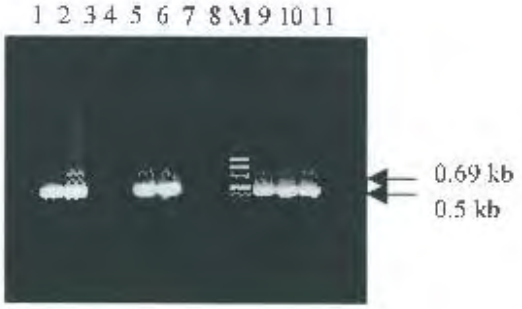

Figure 5.6 PCR screening of Arabidopsis plants transformed with the pFGC-*ATCAP* constructs. Genomic DNA was isolated from selected transgenic Arabidopsis plants and PCR used to confirm the presence of the pFGC-*ATCAP* constructs. Primers spanning part of the pFGC1008 *hpt* gene and part of the *ATCAP* genes were used for the PCR. Lanes 1and 2 are PCR products from plants transformed with pFGC-*ATCAP1-3*. Lanes 3 and 7 - pFGC1008 control. Lanes 4 and 8 - water control. Lane 5 and 6 are PCR products from plants transformed with pFGC-*ATCAP4*. Lane 9-11 are PCR products from plants transformed with (9) pFGC1008, (10) pFGC-*ATCAP1-3* (11) pFGC-*ATCAP4*. M is Marker VIII (Roche, Germany) using primers specific for the pFGC1008 vector.

5.3.3 Southern Blot and RT-PCR Analysis of Transgenic Plants

The integration and gene copy number of the *ATCAP* inverted repeats were determined by Southern blot analysis of Arabidopsis genomic DNA isolated from PCR-positive transgenic lines. The blots were hybridized with an HPT probe derived from part of the hygromycin gene sequence. Of the restriction enzymes chosen, *Hind*III had only one restriction site within the target genes. *Eco*RI and *Pst*I were used to perform a double digestion to ensure complete restriction of the genomic DNA. Two of the three pFGC-*ATCAP*1-3 lines (ATC-10, ATC-21) as well as the vector control line (VC-5) chosen had at least three copies of the transgene (Fig. 5.7). One of the lines (ATC-9) had a single copy of the *ATCAP* gene construct. Arabidopsis lines transformed with the *ATCAP*4 (ATC4-10, ATC4-16) also had three copies of the transgene whilst the vector control

(VC-3) appeared to have only one copy (Fig. 5.8). These results confirmed that the *ATCAP* constructs had been successfully integrated into the genome of the Arabidopsis plants.

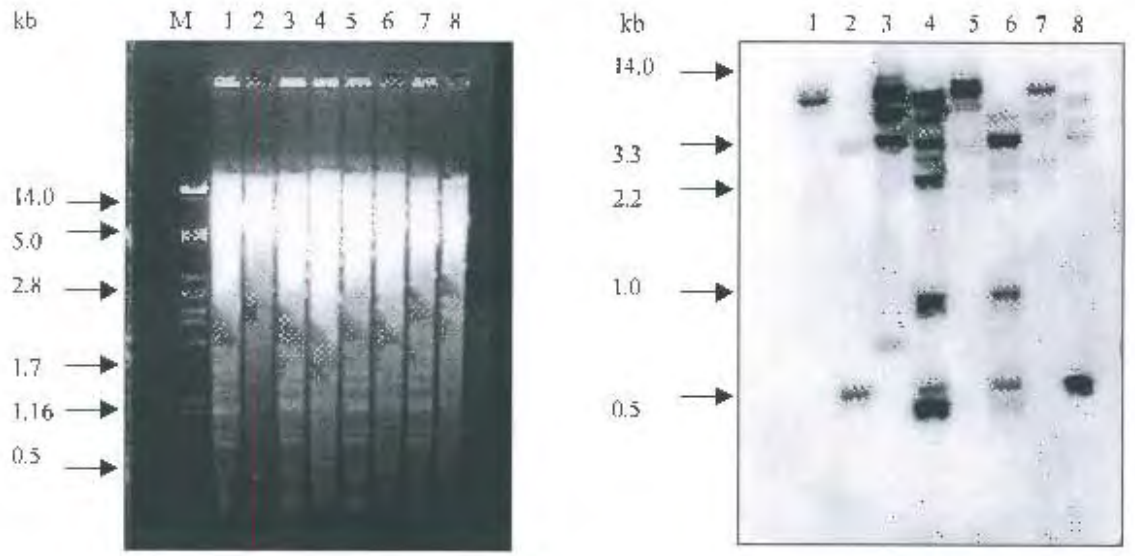

Figure 5.7 Southern blot analysis of genomic DNA from transgenic Arabidopsis transformed with pFGC-*ATCAP1-3* gene constructs. Five micrograms of genomic DNA were digested with *Hind*III and *Eco*RI and *Pst*I, separated on a 1 % agarose gel and blotted onto a nylon membrane. The blot was probed with a DIG-labelled probe derived from ~500 bp of the hygromycin gene of the vector. (A) Gel picture of the Arabidopsis DNA after staining with ethidium bromide. (B) ATC-9 (Lanes 1 and 2), ATC-10 (lanes 3 and 4), ATC-21 (lanes 5 and 6) are transgenic lines, and VC-5 (lanes 7 and 8) refers to the vector control. Odd number lanes – *Hind*III digestions; even number lanes – *Eco*RI/*Pst*I digestions. M refers to the λ *Pst* I DNA marker. The DNA size markers are shown in kb on the left.

In order to assay target gene reduction, RT-PCR on RNA isolated from PCR-positive plants was carried out. Of the four plants assayed for *ATCAP1-3* gene silencing, ATC-21 showed a dramatic reduction in mRNA levels (Fig 5.9A). Two other lines (ATC-9 and ATC-25) also had reduced

expression levels of the target gene although on a smaller scale, whilst line ATC-10 appeared to have the same expression levels as the controls. Similar results were obtained with the *ATCAP4* lines with ATC4-10 showing significant silencing although not to the same extent as the ATC-21 line (Fig. 5.9B). Lines ATC4-3 and ATC4-5 exhibited some silencing of the target genes whilst line ATC4-2 appeared to have the same mRNA levels as that of the control plants.

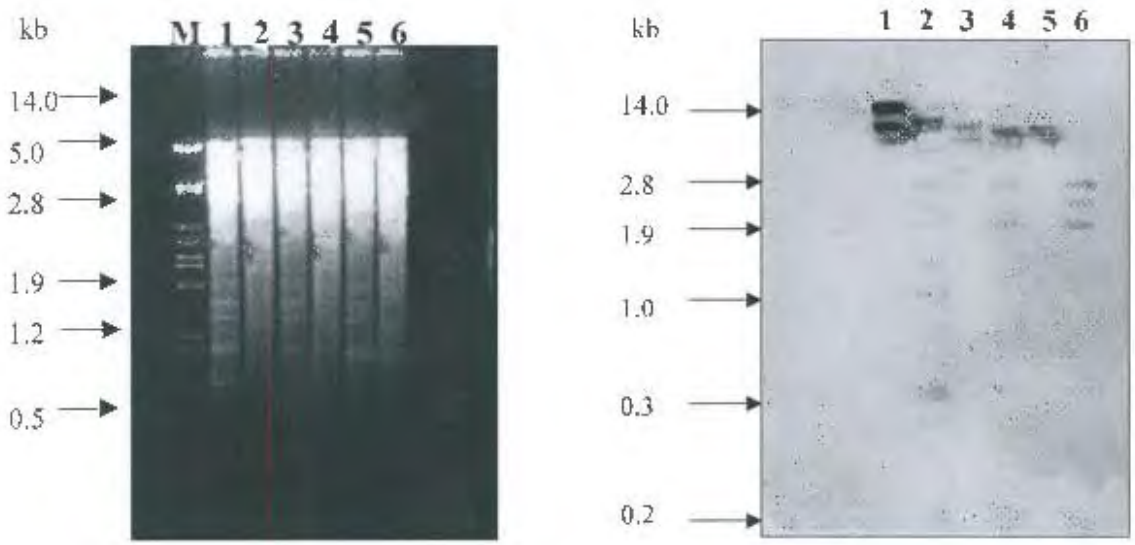

Figure 5.8 Southern blot analysis of genomic DNA from transgenic Arabidopsis transformed with pFGC-*ATCAP4* gene constructs. Five micrograms of genomic DNA were digested with *Hind*III and *EcoR*I and *Pst*I, separated on a 1 % agarose gel and blotted onto a nylon membrane. The blot was probed with a DIG-labelled probe derived from ~500 bp of the hygromycin gene of the vector. (A) Gel picture of the Arabidopsis DNA after staining with ethidium bromide. (B) ATC4-9 (Lanes 1 and 2), ATC4-16 (lanes 3 and 4), are transgenic lines ,VC-3 (lanes 5 and 6) refers to the vector control. Odd number lanes – *Hind*III digestions; even number lanes – *EcoRI*/*Pst*I digestions. M refers to the λ *Pst* I DNA marker. The DNA size markers are shown in kb on the left.

Figure 5.9 RT-PCR to determine the levels of silencing of the *ATCAP* genes in transgenic Arabidopsis. RNA was isolated from transgenic Arabidopsis plants that were expressing the pFGC-*ATCAP* constructs. (A) PCR was carried out using primers specific for the *ATCAP1-3* genes. Lane 1 is DNA marker VIII (Roche, Germany). Lanes 2-7 are ATC-9, ATC-10, ATC-21, ATC25, respectively. Lane 6- wild type control, Lane 7- vector control, Lane 8- water control, Lane 9- plasmid positive control. (B) PCR was carried out using primers specific for the *ATCAP4* gene. M is DNA marker VIII (Roche, Germany). Lanes 2-7 are ATC4-2, ATC4-3, ATC4-5, ATC4-10 respectively. Lane 6- wild type control, Lane 7- vector control, Lane 8- water control, Lane 9- plasmid positive control.

5.3.4 Phenotype of the RNAi Transgenic Lines

The effects of osmotic and salt stress were assessed in the RNAi transgenic lines by growing the transformed Arabidopsis on plates containing various concentrations of mannitol and NaCl respectively. After eight days of growth there were no differences seen in the root growth of the lines growing on unsupplemented PNA or PNA containing 50 mM NaCl. However, there were some very clear differences in the root elongation of the silenced lines and the controls on 75 mM and 100mM NaCl. Figure 5.10 shows the phenotype of the Arabidopsis plants growing on 75 mM NaCl. All plants exhibited the chlorosis and the reduction in leaf expansion typically seen as a result of salt stress. The root growth of lines ATC-21, ATC-9 and ATC4-10 was retarded whilst

that of the wild type and vector control plants was inhibited to a lesser extent. Similar results were observed on 100 mM NaCl except that overall growth for all the lines was significantly reduced.

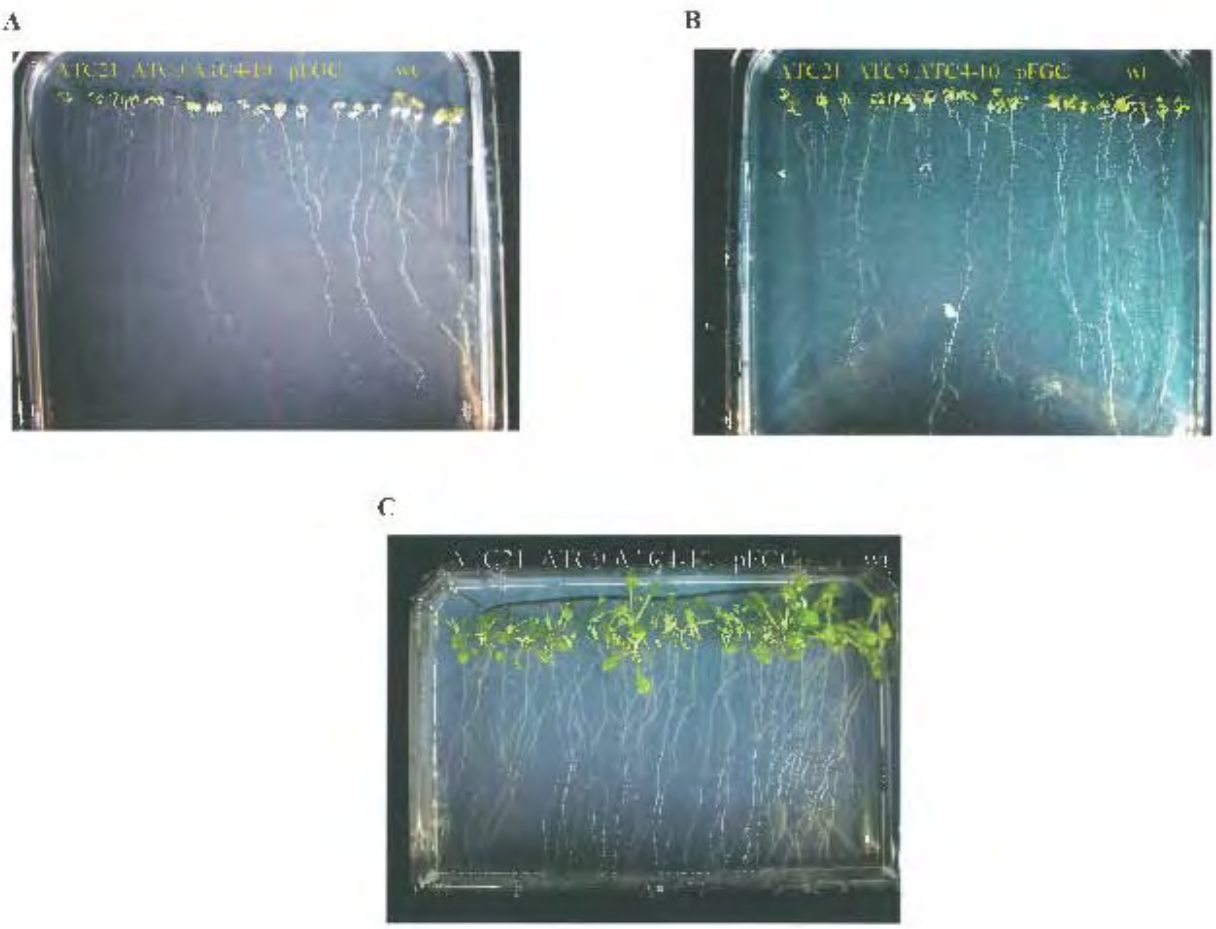

Figure 5.10 Phenotype of Arabidopsis RNAi lines growing on salt stress, osmotic stress and control media. Four-day-old seedlings were transferred to PNA supplemented with (A) 75 mM NaCl, (B) 100 mM mannitol or (C) unsupplemented PNA. ATC21 and ATC9 are transgenic lines transformed with the pFGC-*ATC1-3* construct and ATC4-10 is a transgenic line transformed with the pFGC-*ATC4* construct. pFGC is the vector control and wt refers to the wild type control. The pictures were taken 8 d after the commencement of the treatments.

Plants growing on mannitol media had a similar growth pattern to that of plants growing on salt stress media. Root growth was inhibited in the ATC-21, ATC-9 and ATC4-10 RNAi lines on 100 mM (Fig. 5.10B) and 200 mM mannitol but not on 50 mM mannitol. All roots exhibited significant branching as a result of mannitol stress. It was noted that the transgenic Arabidopsis lines growing on control PNA media appeared a little smaller than the wild type Arabidopsis (Fig. 5.10C). However this trend was not observed in plants grown in soil

It was noted that the lines harbouring the *ATCAP* inverted repeats exhibited an early flowering phenotype when grown in soil. In almost all cases these lines flowered 2-4 days before the untransformed controls (Fig 5.11).

Wt ATC-9

Figure 5.11 Early flowering phenotype exhibited by the Arabidopsis RNAi lines. Arabidopsis lines transformed with either the pFGC-*ATCAP1-3* or pFGC-*ATCAP4* constructs flowered up to 2-4 d earlier than the wild type controls. A pFGC-*ATCAP1-3* line, ATC-9 and the wild type (Wt) Arabidopsis are shown.

5.4 DISCUSSION

XVSAP1 was shown to be a stress-responsive gene in *X. viscosa* (Garwe et al., 2003). The gene was also shown to belong to a small family of genes denoted as cold-responsive genes. To further elucidate the function of *XVSAP1*, it was decided to create knock-outs of the gene in the model plant Arabidopsis. The reasoning behind this was that: firstly there is 56% identity between XVSAP1 and one of the ATCAP proteins in Arabidopsis making it possible that the proteins from the two plant systems have the same or similar functions. Secondly, although the identity of the wheat and rice orthologues with the XVSAP protein is about 50%, transformation of these monocotyledonous plant systems is relatively difficult. On the other hand, the transformation procedures for Arabidopsis are well established making it simpler to create loss-of-function mutants. To date there is no transformation procedure that has been described for *X. viscosa.* It was therefore not possible to silence *XVSAP1* in the original plant system.

Mutants of specific individual genes have been created by chemical or insertional mutagenesis or by the use of transposons. The identification of the subsequent mutants and their corresponding genes is both a slow and laborious. Post-transcriptional silencing of plant genes has also been achieved using anti-sense or co-suppression constructs but the numbers of individuals with the target genes silenced are usually relatively low. It has been shown that transgenes designed to express double-stranded or single-stranded complementary hpRNA efficiently silenced genes in plants (Wang and Waterhouse, 2000). Almost 100% silencing of the target gene was achieved using an intron-containing hpRNA construct (Smith et al., 2000). Using this information, Vasher Wesley et al. (2001) designed vectors that allowed the creation of intron-containing constructs by

PCR that achieved between 90% and 100% silencing of the target genes. Other vectors designed on the same principle, including pFGC1008 which was used for the work described in this chapter, have been described (http://ag.arizona.edu/chromatin/procedure/html). To silence the *ATCAP* orthologues of *XVSAP1* in Arabidopsis, intron-containing hpRNA constructs were created using the pFGC1008 vector. This necessitated the amplification of the target genes from a cDNA population derived from dehydrated Arabidopsis plants.

Successful cloning of the target genes was confirmed by sequencing of the insert sequences in pGEM-T. Transgenic Arabidopsis plants were created by Ti-mediated transformation. These plants were screened using ATCAP primers designed to span part of the 35S promoter region of the pFGC1008 vector and part of the *ATCAP* gene sequence to ensure that the endogenous genes would not be amplified. These same primers however could not be used for Southern blot analysis because they always resulted in multiple hybridization bands probably because the probes were at least 500 bp long. This would result in hybridization with the complete *ATCAP* genes. It is therefore quite conceivable that the probes were binding to the endogenous genes as well as the transgenes. Primers specific for the HPT gene were therefore designed. Multiple hybridization bands were also obtained with this probe although some of the screened lines did contain only one copy of the gene. As the GV3101 *Agrobacterium* strain had been used for the transformation this result was not unexpected. GV3101 gives a higher frequency of multi-copy transgenes than other strains such as LBA4404. It is usually desirable to have only one copy of a transgene in order to reduce the possibility that the silenced lines will become unstable in later generations. For the purposes of this study, plants from the T2 generation were used and the number of copies of the *ATCAP* genes did not seem to affect the level of silencing.

RT-PCR was used to assess the mRNA levels of the genes corresponding to the four *ATCAPs* with the closest identity to *XVSAP1*. Although no internal control was used in this set of experiments, it is still quite clear that the constructs proved capable of almost totally silencing the target genes. The lines showing the highest levels of silencing had at least three copies of the transgenes. It is difficult to conclude that silencing is more efficient when there are more copies of the transgene since the sample size used in these experiments was small. Except for line ATC21, total silencing of the endogenous *ATCAP* genes was not observed. This result was not unexpected since complete silencing is not frequently observed with the vector system used (ChromDB website). Instead, a several-fold decrease in the target RNA is usually expected. A reduction in expression, rather than the complete silencing of a gene can be a useful feature. For example, the complete silencing of genes involved in basic cell development or growth could prove lethal at the embryo stage thus making it difficult to use traditional DNA-tagging approaches to determine gene function. Partial silencing of a gene could give a gradation of phenotypes making it still possible to discover the role of a particular gene (Varsha Wesley et al., 2001).

Partial silencing of the *ATCAP* genes in Arabidopsis resulted in hypersensitivity to osmotic and ionic stress induced by mannitol and NaCl, respectively. This result suggests that these genes are involved in the tolerance of Arabidopsis to these stresses. There is, however, a potential conflict of results here. When *XVSAP1* was expressed in Arabidopsis (see Chapter 4), as expected, transgenic plants were more tolerant to osmotic and salt stress than the wild-type plants. These results suggested that the *ATCAP* genes in Arabidopsis do not function under normal circumstances in increasing the tolerance of the wild type plants to abiotic stress. So the question that arises is why

does silencing of the same apparently non-functional *ATCAP* genes result in plants with increased sensitivity to salt and osmotic stress? The explanation probably lies in the regulation of the genes. Expression of both *XVSAP1* and the inverted repeat *ATCAP* genes was achieved using the constitutive and powerful 35S CAMV promoter. The endogenous promoter for the *ATCAP* genes may be inducible only under certain conditions or may not be induced at all. The signalling pathway leading to the activation of these genes could have, through evolution, become blocked or inactive. This is quite plausible since there are at least six copies of the *ATCAP* genes in Arabidopsis. Such a high level of redundancy may make it possible that that the genes are not required in the tolerance of Arabidopsis to abiotic stresses. This argument on the regulation of the *ATCAP* genes is further reinforced by the fact that the ATCAP protein levels increase dramatically on introduction of the *XVSAP1* transgene (see Fig 4.5). However, no such increase is seen in the levels of the ATCAP1 protein the wild type Arabidopsis. The introduction of *XVSAP1*, *ugonder* the control of the 35S promoter, may release the *ATCAP* genes from their internal control and allow their expression. Promoter studies of both the *X. viscosa XVSAP1* gene and the Arabidopsis *ATCAP* genes should assist in elucidating the differences in the response of these genes to abiotic stresses.

Another possibility is that the *ATCAP* mRNA is only required at low levels even during stress conditions. The levels of the transcript product are probably not enough to confer high tolerance to stress but loss of the mRNA results in increased sensitivity. As the mRNA levels of the genes in transgenic and wild type Arabidopsis before and during stress were not studied, the actual expression patterns are unknown. Further studies are required to unravel the differences in the expression of *XVSAP1* and *ATCAP* genes in Arabidopsis.

It is also possible that the *ATCAP* genes have a totally different function to that of *XVSAP1* and, therefore, are involved in a different regulation pathway.

The silencing of the *ATCAP* genes resulted in an early flowering phenotype. The reason for this is unknown. However, a universally observed phenomenon is the early induction of the reproductive stage by various stresses in both plants and animals.

The results presented in this chapter are preliminary. Studies are required to assess the effect of silencing *ATCAP* genes in Arabidopsis in more detail. An internal control needs to be constructed to more accurately determine the levels of gene silencing. However, it is clear from the results obtained that silencing the *ATCAP* genes results in Arabidopsis plants that are more sensitive to osmotic and salt stress than the wild type. The early flowering phenotype also needs further investigation. This study has nonetheless demonstrated the potential of RNAi as a tool to silence specific genes. Most published work has focused on RNAi in animal systems. This work displays how the same technique can be adapted successfully in plant systems.

CHAPTER SIX

GENERAL CONCLUSIONS

In order to provide and maintain adequate food stocks for a growing world population, new means of improving crop productivity are required. The productivity and distribution of plants, particularly those of agronomic and horticultural importance, is greatly influenced by environmental stresses such as drought, salinity and extremes of temperature (Boyer, 1982). Genetic engineering has been used to improve the tolerance of plants to environmental stresses, with major advances having been made in the last decade. Methods for stable genetic transformation as well as for the regulation of introduced transgenes have been established improved significantly. Most strategies rely on the transfer of one or several genes that encode biochemical pathways or endpoints of signalling pathways that are controlled by a constitutively active promoter. These gene products protect the plant directly or indirectly against environmental stresses.

Angiosperms known as the resurrection plants offer a potential rich source of genes that could used to confer tolerance to abiotic stresses. These plants have developed mechanisms that allow them to withstand severe water deficit and are unique in their ability to tolerate drying of their vegetative tissues. Genes, which could be used to improve the tolerance of crop plants to abiotic stresses and consequently improve their yield, have been isolated from species such as *Craterostigma plantagenium* (Bartels and Salamini, 2001), *Tortula ruralis* (Oliver et al., 1998) and *Xerophyta viscosa* (Mowla et al., 2002; Mundree et al., 2000; Ndima et al., 2001). This study has shown that

the expression of *XVSAP1*, a gene isolated from *X. viscosa* in Arabidopsis and tobacco, improves the tolerance of these plant systems to salinity, heat, osmotic stress and drought.

6.1 XVSAP1 is a hydrophobic protein

XVSAP1 was isolated using the strategy of "complementation by functional sufficiency" from a cDNA library constructed from dehydrated *Xerophyta viscosa* Baker leaves. Analysis of the cDNA sequence indicated a highly hydrophobic protein with six transmembrane regions suggesting that XVSAP1 is probably an integral membrane protein.

6.2 XVSAP1 is similar to other stress-associated proteins

The deduced amino acid sequence showed 49% identity to WCOR413, a low temperature-regulated protein from wheat and 53% identity with another cold associated protein in rice. The protein also showed between 25% to 56% identity to WCOR413-like proteins from *Arabidopsis thaliana*. Southern blot analysis revealed that there are at least two copies of *XVSAP1* in *X.viscosa*. Taken together with the similarity to the stress-associated proteins in other plant systems, this data indicates that XVSAP1 belongs to a small gene family.

6.3 *XVSAP1* expression in *X. viscosa*

Analysis of gene expression using semi-quantitative RT-PCR revealed that *XVSAP1* is induced by dehydration, salt stress (100 mM), both low (4°C) and high temperature (42°C) and high light treatment (1500 μmol m^{-2} s^{-1}). Western blot analysis showed that the gene expression correlated with the accumulation of XVSAP1 protein in the stressed plants suggesting that *XVSAP1* had a significant role to play in the response of *X. viscosa* to abiotic stresses.

6.4 Expression of *XVSAP1* in other biological systems

The effect of expressing XVSAP1 protein in *E.coli*, *S. cerevisiae*, Arabidopsis and tobacco was determined. Expression of *XVSAP1* in *Escherichia coli (srl*::Tn10) conferred osmotic stress tolerance when the cells were grown in 1 M sorbitol. However expression of the cDNA in yeast did not complement the mutations in the strains used. Expression of *XVSAP1* in both Arabidopsis and tobacco plants led to constitutive accumulation of the corresponding protein in the leaves. Transgenic Arabidopsis grown in tissue culture and tobacco grown hydroponically were more tolerant to salt and osmotic stress. In addition to showing a greater tolerance to drought stress when grown in soil, transgenic tobacco plants achieved a higher fresh and dry weight than the untransformed controls when grown hydroponically.

6.5 XVSAP1 and the recovery from stress damage

An interesting feature of the response of Arabidopsis plants to heat stress was the fact that both transgenic and wild type plants showed initial damage by heat. However the transgenic plants quickly recovered from the damage, unlike the wild type plants. This suggests that XVSAP1 is actually involved in the recovery of plants from stress damage rather than in preventing the injury.

6.6 Further evidence of the role of XVSAP1 in abiotic stress

In order to further elucidate the role of *XVSAP1* in the tolerance to abiotic stresses, RNAi was used to silence the *XVSAP1* orthologues in Arabidopsis. The results obtained showed that silencing the *ATCAP* genes resulted in Arabidopsis plants that were more sensitive to osmotic and salt stress than the wild type. It was also noted that the transgenic plants also showed an early flowering phenotype. Further studies are required to understand the genetic and molecular basis for this

phenomenon. Taken together, the results obtained using transgenic plants, clearly demonstrate that *XVSAP1* confers tolerance to environmental stresses such as high salinity, heat, water deficit and osmotic stress.

6.7 XVSAP1 and membrane integrity

The work presented herein shows that *XVSAP1* is involved in the response of *X. viscosa* to environmental stresses. Characterization of the gene product has shown that XVSAP1 is likely to be an integral membrane protein. It is proposed that the protein could be involved in the protection of membranes possibly by maintaining structural integrity of plant cells or XVSAP1 could be one of the components involved in the repair of the membrane damage that results from severe water deficit.

6.8 Is XVSAP1 an ion transporter?

Although the current speculation is that the gene product works in the stabilization of membranes during dehydrative stresses, perhaps the protein could also be an ion transporter since *XVSAP1* shows some homology to K^+ transporters. The regulation of intracellular ion concentrations is a fundamental property of living cells. Potassium, a major cellular cation is actively retained so that it is present in very high concentrations internally. The cation is the principal determinant of physiological parameters such as cell volume and turgor. Various proteins appear to mediate the transport of potassium across cell membranes and a number of K^+ channels including the Na^+/K^+-ATPase have been characterised in eukaryotes. It is possible that XVSAP1 is one these ion transporters. Attempts to establish whether XVSAP1 could complement mutant yeast cells lacking the TRK1-TRK2 potassium transporter were unsuccessful. This could have been due to non-

expression of XVSAP1 in yeast or simply that XVSAP1 is not involved in the TRK1-TRK2 transport system. Future work could focus on investigating whether XVSAP1 is involved in the transport of ions across the plasma membrane in *X. viscosa*

6.9 Improvement of stress tolerance in monocotyledons using *XVSAP1*

The evidence presented has demonstrated that *XVSAP1* distinctly improves the tolerance to water deficit, high salinity and high temperature of the model dicotyledonous systems used in this study. It would be interesting to see if the gene confers greater tolerance in monocotyledonous plants since it was isolated from a monocotyledon. This is likely although there is the possibility of gene silencing in wheat, for example, due to the homology of XVSAP1 to WCOR413. However the identity between the two proteins is only around 50% and this might not be close enough to induce post-transcriptional gene silencing.

6.10 Increasing the level of expression of *XVSAP1*

The results presented in this work demonstrate the effect of expressing a single gene isolated from a monocotyledonous resurrection plant in dicotyledonous model plants. Although the improvement in stress tolerance in tobacco and Arabidopsis was striking, perhaps a higher level of expression of *XVSAP1* could have been obtained by using a stress-inducible promoter or better still, the *XVSAP1* promoter from *X. viscosa*. Future work could focus on isolating the *XVSAP1* promoter from *X. viscosa* and using it to obtain better expression of the gene in crops such as maize which form the staple diet of most southern African people. It is established that tolerance to abiotic stresses is a multi-genetic trait as is evidenced by the large number of transcripts that are altered and the concomitant changes in protein profiles in response to stress (Meyer et al., 1990; Nguyen et al.,

1997). It is therefore possible that stress tolerance can be improved by pyramiding different stress-responsive genes instead of using just one gene. Expression of *XVSAP1* in tandem with other genes isolated from *X. viscosa* such as *XVT8* (Ndima et al., 2001), *ALDRXV4* (Mundree et al., 2000) and *XvPer1* (Mowla et al., 2002) could result in a very significant increases in tolerance by crops to abiotic stresses.

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