Characterization of *XVEF* and *XvCaM*, two calcium-binding proteins isolated from the resurrection plant *Xerophyta viscosa*

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Abbreviations

°C	degrees Celsius
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
ABA	abscisic acid
ATP	adenosine triphosphate
BLAST	basic logarithm alignment search tool
bp	base pair(s)
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
cyclic GMP	guanidine monophosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
H	hours
H^+	proton
HCl	hydrogen chloride
His	histidine
IP_3	inositol triphosphate
IPTG	isopropyl-β-D-thiogalactoside
Kb	kilobase pair
KCl	potassium chloride
kDa	kiloDalton
LB	Luria-Bertani
M	molar
mg	milligram
$MgCl_2$	magnesium chloride

min(s)	minute(s)
ml	millilitre
mM	millimolar
Na^+	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometre
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric focusing point
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RWC	relative water content
SDS	sodium dodecyl sulphate
SSC	sodium citrate
V-ATPase	vacuolar ATPase
w/v	weight per volume

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Abstract

Xerophyta viscosa (Baker) is a resurrection plant with the ability to survive desiccation and rehydrate upon watering with minimal tissue damage. *XVEF* was isolated by differential screening of a *X. viscosa* dehydration stress cDNA library. Reconstruction of the full length *XVEF* cDNA was conducted utilizing overlap extension PCR. *XVEF* codes for a putative calcium-binding protein and sequence analysis indicated that it has a 708 bp ORF corresponding to a protein with a molecular mass of 26.95 kDa, has a conserved calcium-binding EF-hand motif, potential phosphorylation sites, a pI of 6.49 and a putative conserved transmembrane domain spanning residues 90-107. Northern blot analyses of total RNA indicated that *XVEF* transcript increased 48 hours after 100μM ABA application and was present between 12 and 48 hours in response to a low temperature stress (4°C). A second gene, *XvCaM,* was isolated from a low temperature (4°C) cDNA stress library. Sequence analysis indicates that it has a 450 bp ORF corresponding to a 16.39 kDa protein, a pI of 3.90 and potential phosphorylation sites. It apparently encodes a calcium-binding protein with putatively three EF-hands which showed the highest similarity to plant calmodulins. XVCAM was heterologously expressed in *E. coli* as a fusion protein with a 6X His-tag and it was shown to be a functional protein that binds Ca^{2+} by utilizing a $^{45}CaCl_2$ overlay assay. Northern blot analyses of *XvCaM* using total RNA under low (4°C) and high temperature treatment (42°C) showed constitutive expression levels of the transcript. Under the dehydration/rehydration treatment transcript levels decreased between 40% RWC dehydration and 26% RWC rehydration. The northern blot conducted with polysomal

RNA isolated from 150 mM NaCl treatment also showed constitutive expression. Western blot analyses using anti-XVCAM polyclonal antibodies showed that the protein accumulated at 24 hours during the NaCl stress and at 15% RWC (dry) to 40% RWC (rehydration) during the dehydration/rehydration stress. The northern and western analyses results suggest that XVCAM undergoes post-translational modifications and *XvCaM* mRNA is possibly stored for rapid recovery processes upon rehydration. These results indicate that *XVEF* and *XvCaM* are possibly calcium-binding proteins most likely involved in modulating stress-responsive calcium-signaling cascades.

Chapter 1

General introduction

By the year 2025, the world's population will have grown from six to eight billion (Dyson, 1999). A third will live in areas that endure extreme water shortages (Salamini Bartels &, 2001). This factor coupled with a decline in available farming land and an increase in adverse environmental conditions has placed the agricultural industry under pressure to maintain high crop yields not only to remain economically viable, but also to provide sufficient food for an ever-growing worldwide population. The study of stress tolerance mechanisms in plants has become the focus of a growing network of researchers who endeavour to utilize the knowledge gained about these mechanisms to genetically engineer crops to withstand unfavorable environmental conditions.

Plants utilize many mechanisms to address their changing environments, including responses to factors such as fluctuations in water availability (Scott, 2000). Water is needed in all biological systems and acts as a solvent, a medium of transport and as an electron donor (Bohnert et al., 1995). It is crucial to plants and vegetative growth can only occur within the limits of their water status. Turgor pressure is maintained by plant cells by regulating the osmotic potential to adapt to changes in the environment, affecting the availability of water to those cells (Xiong & Zhu, 2002).

Water-deficit stress is generally experienced as a mild form of water loss and minor cellular and organellar changes occur in response to it. On the other hand, desiccation describes the condition where the 'bulk/free' water of the protoplasm is lost and only water associated with the cell matrix remains (Ramanjulu & Bartels, 2002). Plants experience osmotic stress when exposed to water deficit, low temperatures and an increase in salinity. Seeds represent the desiccation-tolerant phase in the life cycle of most plants, but very few plants have or retain these mechanisms during vegetative growth phases. Plants with the ability to survive desiccation are able to habituate extreme environments. Such desiccation-tolerant plants restrict cellular damage to a repairable level, having the ability to sustain physiologically in the dry state, yet being able to repair damage on rehydration themselves. Plants that employ such physiological and biochemical adaptations are termed resurrection plants (Scott, 2000; Oliver et al. 1998).

1.1 Resurrection plants

Resurrection plants are able to withstand a loss of up to 95% of their water content in the vegetative state (Gaff, 1971) and enter a stage of anhydrobiosis where metabolic activity is low and intracellular water is minimal (Hoekstra et al., 2001), yet there is negligible tissue damage during the dehydration and rehydration cycle (Scott, 2000). *Xerophyta viscosa* is an example of a resurrection plant and has been used to isolate and characterize genes upregulated during dehydration (Mundree & Farrant, 2000). It is a monocotyledonous angiosperm and is a representative of the group called poikilochlorophyllous plants, which dismantle their photosynthetic machinery upon drying. *Craterostigma wilmsii* exemplifies the homoiochlorophyllous group of

angiosperms that retain chlorophyll and thylakoids during dehydration (Scott, 2000; Sherwin & Farrant, 1996).

1.2 Plant responses to stress

Stress tolerance is invoked by the expression of genes whose products allow adjustment of the metabolism of the plants under severe stress conditions (Bohnert et al., 1995; Bray, 1993). The duration and severity of the response is a function of the plant species, the stage of its growth and the type of stress. Subtle physiological changes enable the first phase of defence against a stress before any metabolic adjustments are made. Morphological adaptations would include changing leaf orientation to reduce transpiration, reducing leaf surface area, having sunken stomata and a modified root system to increase water uptake (Ramanjulu & Bartels, 2002). Upon drying, *X. viscosa* loses its chlorophyll and produces pigments called anthocyanins to reduce damage caused by UV and oxygen free radicals that accumulate during desiccation (Sherwin & Farrant, 1998).

Osmotic potential can be maintained or increased by the synthesis and accumulation of compatible solutes or osmolytes such as sucrose, glycine betaine, polyols and aldose (Bray, 1993; Zhang et al., 1999; Bohnert et al., 1995; Xiong & Zhu, 2002). Osmolytes have been found to maintain protein-protein complexes, stabilise membranes by replacing water molecules and to maintain an osmotic balance. For example, sugars can substitute water at hydrogen bonding sites to preserve native protein confirmation (Hoekstra et al., 2001) and prevent inactivation of enzymes by ions, which accumulate

when water is lost from the protoplasm (Hartung et al., 1998; Zhang et al., 1999). Sugars also facilitate the formation of the 'glassy state' of water in cells, which mimics the mechanical properties of solids (Leopold et al., 1994; Crowe et al., 1998). Through the composition of sugars and compatible solutes, vacuoles and intracellular spaces, which arise through water loss, are filled during desiccation to maintain the shape of the cell, to prevent mechanical damage and suspend certain metabolic events (Ingrams & Bartels, 1998; Farrant, 2000). Compartmentation of the vacuoles and subsequent filling with compatible solutes has been shown to occur in *X. viscosa* under dehydration stress (Mundree & Farrant, 2000).

Plants are aerobic organisms that utilize an array of photosensitizing pigments to drive the synthesis of ATP and NADPH by splitting water to produce oxygen. Reactive oxygen species (ROS) are produced as a result of photosynthetic metabolism (Foyer et al., 1994). These metabolites are potentially harmful if they are allowed to accumulate within chloroplasts and other cellular compartments, as some ROS such as the hydroxyl radical can damage cellular components such as proteins, nucleic acids or unsaturated lipids, resulting in organic peroxides. The latter reaction results in the production of organic peroxides that can initiate or exacerbate lipid peroxidation cascades. However, ROS are kept at a very low level within plant cells because of the presence of highly efficient antioxidant systems that prevent the build up of ROS to levels where damage is caused. Superoxide dismutases (SOD), catalases, glutathione reductase and ascorbate peroxidase are examples of enzymes used to sequester ROS (Bohnert et al., 1995; Sherwin & Farrant, 1998). A repair mechanism used by plants involves the use of enzymes to

degrade unnecessary or damaged proteins to free up resultant amino acids for synthesis of new proteins. Ubiquitin and thiol proteases have been identified as candidates for such roles (Ingram & Bartels, 1998; Oliver et al., 1998). Osmotic stress can also result in ion imbalances in the cell, especially when caused by an increase in salinity. Sequestering, export and transport of ions are important processes needed in ion homeostasis (Xiong & Zhu, 2002).

The stress mechanisms discussed here have been intensively reviewed and will not further be elaborated upon, as they are not the current area of interest. This work will focus on plant signal transduction mechanisms that form part of the stress response activation. Calcium and calcium-binding proteins are well-characterized signaling components and will form the main focus of the rest of this introduction.

1.3 Perception and regulation of stress signals

Perception of the stress is vital to initiating the correct response. Therefore, the stress stimulus has to be conveyed from receptors exposed to the environment to the cells where the relevant biochemical and molecular pathways are initiated to express genes whose products will bring about the required response (Shinozaki & Yamaguchi-Shinozaki, 1996). The cell could sense changes due to water loss through a reduction in water potential or the activity of cellular water activity. A decrease in turgor pressure may occur because of change in osmotic potential across the plasma membrane. Alterations in membrane fluidity caused by a decrease in temperature may also occur as well as changes in concentration of small molecules, cell volume fluctuations and even conformational

changes of cellular macromolecules (Shinozaki & Yamaguchi-Shinozaki, 1996; Bray, 2002). After the stress is sensed, signal transduction cascades relay the stress signal to the nucleus via phosphorylation pathways, sensor proteins and a multitude of secondary messenger molecules link together various combinations of pathway components to produce a defined message that results in the correct physiological response (Figure 1.1).

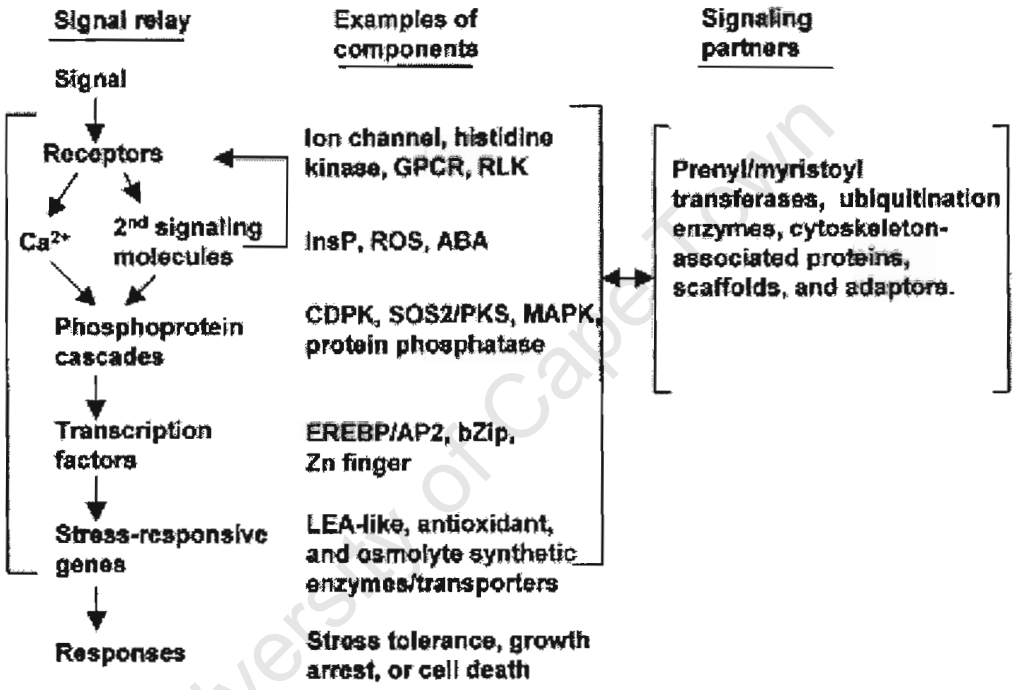


Figure 1.1 Schematic diagram representing components of signal transduction cascades used in abiotic stress signaling (Xiong et al., 2002)

Signals transduced from the cell membrane to the nucleus are mainly carried out via MAP (mitogen-activated protein) kinase pathways, resulting in the expression of genes used in downstream processes (Chinnusamy et al., 2004). These pathways form a primary link in the network of receptors and sensors involved in signal transduction cascades. Cross-talk can roughly be defined as when one intermediate or element is present in different signaling events and MAPKs are prime candidates for such signaling

components. Plant MAPK signaling cascades are not only used in developmental signaling, but increasing evidence has shown that they are part of stress-responsive signaling as well (Shinozaki & Yamaguchi-Shinozaki 1997; Tena et al., 2001).

Responses pertaining to water loss brought about by abiotic stress such drought, low temperature and salinity involve the phytohormone abscisic acid (ABA) and an accumulation of ABA would initiate the transcription of ABA-requiring genes (Bray, 1993; 2002). There is ABA-dependent and ABA-independent signaling pathways and not all stress-induced genes respond to the accumulation of ABA (Shinozaki and Yamaguchi-Shinozaki, 1997).

1.4 Calcium: A universal secondary messenger in signaling

Plants utilize a number of secondary messengers to allow the cells to process the multitude of stimuli they experience. Signaling pathways use intermediates such as Ca^{2+}, pH, lipids, inositol triphosphates (IP_3), cyclic guanidine monophosphate (GMP) and ROS (Sanders et al., 1999; Bowler & Fluhr, 2000), yet Ca^{2+} responds to more stimuli than any other messenger does. Besides calcium's vital role as a messenger element, it is also required as a structural element in cell walls and membranes. Calcium concentrations in apoplastic regions (Hirschi, 2004), such the cell wall, and in intracellular stores, such as the vacuoles, are kept at quite high levels. The cytosolic concentration of free calcium is maintained at low levels, since elevated concentrations are cytotoxic and can even initiate cell death. Due to this disparity in calcium concentration between cytosolic and

organellar calcium stores, a steep electrochemical gradient is set up, promoting rapid responses to signaling by increasing cytosolic Ca^{2+} through activation of membrane channels (Stryer, 1996).

Transient increases in cytosolic Ca^{2+} occur in response to signals from development, growth, cold induced stress, drought, high light, phytohormones (e.g. ABA) and even mechanical stimuli such as gravity and touch. Calcium also has the capacity to bind proteins and ligands with high affinity because of its ability to coordinate uncharged oxygen atoms. Remote regions of proteins bind Ca^{2+} and by binding to different oxygen atoms on proteins, conformational changes occur. The utilization of such changes can result in downstream events in signaling pathways (Sanders et al., 1999). The question always arises about how such a ubiquitous and abundant element can be utilized to elicit the plethora of cellular functions it is apparently involved in. The changes in cytosolic calcium is mediated by tight networks of sensory proteins, membrane pumps and ion channels, which allow the spatial and temporal management of the resultant calcium signal (Hirschi, 2004; Pittman & Hirschi, 2003; White & Broadley, 2003; McAinsh & Hetherington, 1998).

1.4.1 Calcium transport in the cell

Plants use various mechanisms to regulate calcium fluxes. Depending on the type of signal and its cellular localisation, internal or external stores of Ca^{2+} may be used to elevate cytosolic Ca^{2+}. For example, intracellular Ca^{2+} can be supplied by the ER, vacuoles, mitochondria, chloroplast, and nucleus (Reddy, 2001). Membrane proteins of

the plasma, vacuolar and endoplasmic reticulum membranes are integral in controlling Ca^{2+} movement across membranes, but are not responsible for processing the Ca^{2+} signal (Carafoli, 2002).

Calcium does not behave as an easily transportable ion (Trewavas, 1999) and active transport is required to remove Ca^{2+} from the cytosol to reinstate the 'resting' Ca^{2+} concentration. This is needed to prevent chaotropic effects that could be caused by sustained elevated cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and to restore the electrochemical gradient that exists across all membranes. The maintenance and restoration of calcium levels is mediated by a system of energy-requiring pumps or proton pumps and passive entry ion channels that include Ca^{2+}-ATPases and Ca^{2+}/H^{+}-antiporters. These also allow the exchange of other ions out of the cytosol to maintain correct ion homeostasis (Ward et al., 1995; Sanders et al., 2002).

1.4.1.1 Active transport of calcium

Two families of Ca^{2+}-ATPases exist. The first lacks an N-terminal autoregulatory domain and members of the second possess an N-terminal domain and comprised a Ca^{2+}-CaM binding site and a serine-residue phosphorylation site. An important and well characterized example of Ca^{2+}/ H^{+}-antiporters is the *CAX* gene family as identified in Arabidopsis. CAX1 was demonstrated to function in response to the plant hormone auxin and was shown to be a vacuolar Ca^{2+}/H^{+}-antiporter (Cheng et al., 2003). A V-ATPase mutant Arabidopsis line, *Det3,* displayed impaired stomatal closure when exposed to oxidative stress and exogenous calcium, suggesting that the antiporter is necessary for

correct calcium signaling in guard cells (Allen et al., 2000). ATPase and antiporter isoforms span the tonoplast, vacuole and plasma membrane and differentially combine with sensor protein isoforms to produce a range of distinct calcium signals (White and Broadley, 2003).

1.4.1.2 Calcium waves

Calcium signatures are unique calcium responses to a particular stimulus and result in the correct physiological effect. Thus, calcium's spatial orientation, oscillation profile, magnitude, duration and relevant effectors all contribute to produce the desired 'signature' effect (McAinsh & Hetherington, 1998, Hirschi 2004 and Trewavas 1999).

White and Broadley (2003) summarized the variation in $[Ca^{2+}]_{cyt}$ signatures in response to environmental stresses as well as developmental stages and studies. In addition, studies in Arabidopsis have illustrated these examples of cellular calcium flux (Nordin Hendriksson and Trewavas, 2003; Allen et al., 2000; Knight et al., 1996). By changing the magnitude of the stimulus, the calcium profiling was adapted and this in turn affected downstream gene expression. The expression kinetics of the low temperature induced gene, *LTI78*, were monitored as cooling rates were varied. This demonstrated a direct relationship between the cooling rates used, the changes in $[Ca^{2+}]_{cyt}$ and the changes in transcript levels of *LTI78* (Nordin Hendriksson and Trewavas, 2003). In wild-type Arabidopsis, the variation in the magnitude and frequency of the cytosolic calcium oscillations caused by exposure to ABA, cold and oxidative stress leads to stomatal closure of guard cells. The V-ATPase mutant, *Det3*, produced calcium waves lacking in

oscillatory behaviour when exposed to external calcium and oxidative stress (Allen et al., 2000). Using another cold-induced gene, *kin1*, evidence was provided that a low temperature exposure elicits $[Ca^{2+}]_{cyt}$ changes as well as *kin1* expression. Calcium tracking techniques using the protein aequorin revealed that the calcium stores were derived from the vacuole. Cold acclimation also resulted in a change in the calcium signature (Knight et al., 1996). These data support the theory that induced calcium oscillations needs to be distinctive to a particular stimulus to produce the desired physiological response.

1.4.1.3 Ca^{2+} channels/Passive transport

The opening of calcium permeable channels is dependant on the type of cell, the type of organelle and the nature of the signal. There are many varieties of membrane channels situated on calcium storage vesicles using either voltage or ligand manipulation. An effective example of ligand-gated channels is those that use IP_3 to control the flux of calcium in the cell (Trewavas, 1999 and White & Broadley, 2003). Phospholipase C occurs at plasma membranes and when a Ca^{2+} signal is received, the channels are activated, converting its substrate, PIP_2, into diglyceride and IP_3, where IP_3 is a mobile ion. Channels are opened by binding of IP_3 and this activates a positive feedback system to maintain spatially localized Ca^{2+} signals. If not for this system, IP_3 would open all IP_3-dependant channels because of its high diffusion rate. The feedback loop exploits calcium-binding by exposing a calcium-binding site when IP_3 binds the channel. Thus, IP_3 only transiently opens the Ca^{2+} channel and calcium is further responsible for its own transport.

1.4.2 Calcium sensors

Calcium-binding proteins are a primary example of calcium sensors. They respond to transitory cytoplasmic increases in Ca^{2+} concentration to decipher the initial Ca^{2+} signal (Reddy, 2001) and act as adapters of the Ca^{2+} signal (Sanders et al., 1999). In plants, there exist four principal groups by which most calcium-binding proteins may be classified: 1) calmodulin; 2) EF-hand containing Ca^{2+} binding proteins; 3) Ca^{2+} -regulated protein kinases; 4) Ca^{2+} -binding proteins without EF-hand motifs. This review focuses on calmodulin and other EF-hand-containing proteins.

1.4.2.1 Calmodulin

The EF-hand binds Ca^{2+} with high affinity via its helix-turn-helix motif (Figure 1.2) (Reddy, 2001).

Figure 1.2. Model of EF-hand motif showing helices in red and the binding pocket in blue (http://www-structure.llnl.gov/Calmod/ef.gif)

Helix E and F form a calcium-binding site, which are orientated similar to the forefinger and thumb of the right hand (Stryer, 1996). Calmodulin (CaM) is highly conserved and ubiquitous as a Ca^{2+} receptor in plants. CaM typically has four EF-hand motifs and is composed of 148 amino acid residues (Figure 1.3).

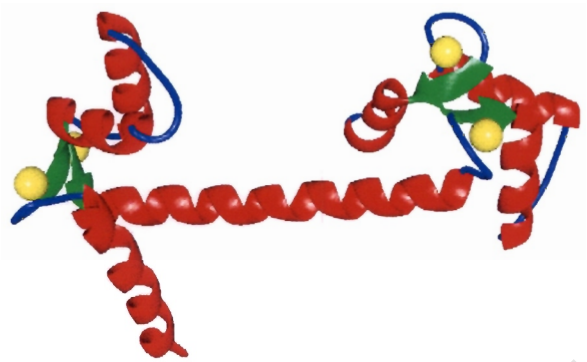


Figure 1.3. Schematic diagram of calmodulin. Calcium ions (yellow) are bound by EF-hands (red, green and blue) (www.cs.stedwards.edu/.../ Calmodulin/calm_lg.gif)

Four Ca^{2+} ions can bind CaM and the conformational change exposes hydrophobic regions that interact with target proteins. To display localization of calmodulin in respect to the stress stimulus, aequorin was used in tobacco to show that cold and wind stimuli induced spatially different calcium signatures (Van der Luit et al., 1999). Cold responses were seen in the cytoplasm and wind-responsive calcium signaling was localized to the nucleus. The calmodulin gene, *NpCaM-1* was similarly differentially expressed in either the cytoplasm or the nucleus in response to respective stimuli, suggesting that *NpCaM-1* is regulated by different calcium signaling pathways. Besides differential expression, calmodulin has a number of isoforms. Townley and Knight (2002) observed associations between the CaM3 isoform and gene expression of the *COR* (*c*old *o*n *r*egulated) group of

genes in Arabidopsis. The *COR* genes studied was the cold-regulated *KIN1-2* and *LTI78* (Knight et al., 1996; Nordin Hendriksson & Trewavas, 2003). The calmodulin genes, *CaM1 & 2* from wheat is upregulated in response to heat shock (37°C), including *hsp26* and *hsp70. Hsp* (heat shock proteins) genes were positively regulated by $CaCl_2$ and negatively regulated by calcium chelators (Liu et al., 2003).

Lee et al. (2000) targeted calmodulins by testing the activation kinetics of the two CaM isoforms from soybean, SCaM-1 and SCaM-4. The activation kinetics of the permutations between the two CaMs and five target enzymes were investigated. It was discovered that neither isoform activated the enzymes to the same extent and that certain isoforms could even inhibit enzymes. These CaMs can act in a Ca^{2+}-dependant and Ca^{2+}-independent manner suggesting that calmodulin is a multifunctional protein with vital roles to play in modulating activity of target proteins (Reddy, 2001).

Other examples of calcium-binding proteins are calcineurin B-like (*CBL*) proteins and caleosin (Hernandez-Pinzon et al., 2001) which contain EF-hands but have little homology to calmodulin. They are larger than calmodulin and contain between one and six EF-hand motifs (Snedden & Fromm, 1999). A *CBL*-like protein involved in abiotic signaling is the SOS3 (salt overly sensitive) protein, which is part of the SOS pathway in *Arabidopsis*. Salt stress causes an increase in Na^+ and affects ion homeostasis. In response to this change, the subsequent calcium spike activates SOS3. The activated sensor protein then complexes with the SOS2 protein, a protein kinase. This activated complex mediates SOS1, the Na^+/H^+ antiporter, thereby initiating transport of Na^+ out of the cell or into vacuole to reinstate the correct balance of ions in the cell (Zhu, 2001;

Xiong & Zhu, 2002). *SCaBP5* and *PKS3* are SOS3-like and SOS2-like homologues from Arabidopsis, respectively. They behave similarly to that displayed in the SOS pathway, but were found to be negative regulators of the ABA signaling pathway (Guo et al., 2002).

The EF-hand-containing proteins may possess other Ca^{2+}-binding motifs such C2 domains, which was shown in the protease calpain, having seven Ca^{2+} -binding sites in its large subunit. Four of these are EF-hands, one is a C2 domain and the remainder is novel domains (Carafoli, 2002).

1.4.2.2 Caleosin

Oleosins were initially characterised as proteins that are distinctly associated with oil body surfaces and when a calcium-binding protein was discovered, it was therefore termed caleosin (Figure 1.4) (Chen et al., 1999).

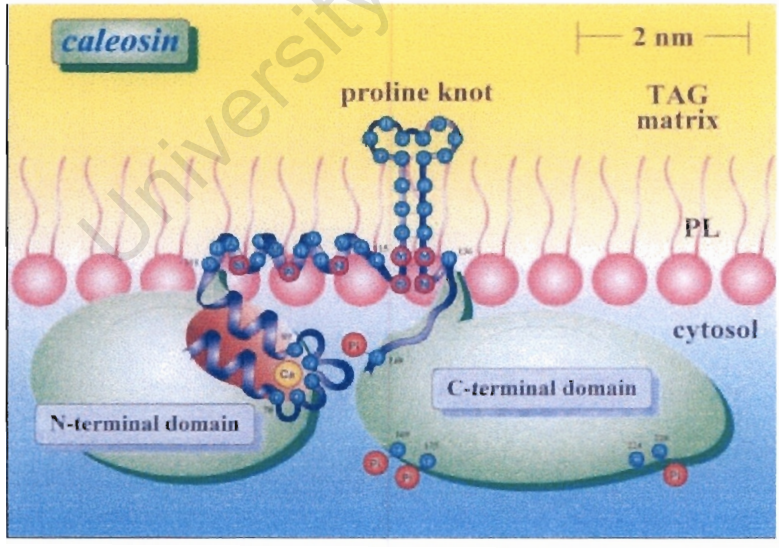


Figure 1.4. Schematic representation of caleosin targeted to an oil body showing the various domains (Frandsen et al., 2001).

Recently it was found that approximately 5% of oleosins were associated with the endoplasmic reticulum (ER) near oil bodies. Oleosins have a central hydrophobic domain characterized by a 'proline knot' motif. They play a role in stabilizing oil bodies and aid in its degradation to release stored triacylglycerols (TAGs) (Frandsen, 2001; Abell, 2004; Chen et al., 1999). Structurally, caleosins can also be divided into three regions where the N-terminal is hydrophilic and contains a single EF-hand motif (Figure 1.4). The central region is hydrophobic and contains a putative transmembrane domain with conserved proline residues forming a targeting signal (Chen et al., 2001). The C-terminal is hydrophilic with an array of phosphorylation sites (Murphy et al., 2000; Frandsen et al., 2001). Localization studies have shown that it has lipid body and ER isoforms (Murphy et al., 2000; Chen et al., 2001; Hernandez-Pinzon et al., 2001).

Frandsen et al. (1996) isolated a CaM-like protein, which was induced by ABA, salt stress and water deficit in rice seedlings. It has one EF-hand and was called EFA27. Later the protein was given a new designation, OsClo, as a rice caleosin and was found in late embryogenesis stages (Frandsen et al., 2001). HvCaBP1, a calcium binding protein from barley, displayed similarity to EFA27/OsClo having a conserved EF-hand and a transmembrane domain (Jang et al., 2003). Similarly, another orthologue was discovered in sesame called SiCLO1, which is also seed-specific. The mRNA was most abundant 2 weeks after flowering and disappeared from mature seeds. A small gene family was reported in Arabidopsis with orthologues to caleosin-like genes (Næsted et al., 2000). *AtClo1-5* was tested for expression using various tissue samples, but *AtClo1* was the only gene to behave in a similar manner to the oleosins. At*Clo2-5* was expressed in all tissues at low levels, but At*Clo1* was expressed greatly in embryos and root tip cells. Sequence

showed similarity to the OsEFA27 and the protein also contains the EF-hand followed by the hydrophobic membrane anchor.

Localization studies using *Brassica napus,* demonstrated caleosin to the lipid bodies of mature dry seeds and germinating rapeseed. Immunolocalization using antibodies to organellar marker proteins suggested that caleosin co-localized with oleosin, BiP, which is an ER marker and α-TIP, a storage vacuole marker protein (Næsted et al., 2000). Two caleosin isoforms were reported in *Brassica napus*, where the 25-kDa protein localized to lipid bodies and the 27-kDa protein to the ER (Hernandez-Pinzon et al., 2001).

1.5 Aims of dissertation

This dissertation aims to describe the functional role of two putative genes isolated from the resurrection plant *X. viscosa* Baker using a range of techniques. These genes were identified as *XvCaM* and *XVEF*. The techniques used toward the objectives include a bioinformatics analysis, Southern blot analysis, northern and western blot analysis. Heterologous expression of the recombinant proteins was attempted and the functional role of the recombinant proteins was tested by conducting a radioactive calcium-binding assay.

Chapter 2

Sequence analysis of *XvCaM* and *XVEF*:

Bioinformatics and Southern blot analysis

2.1 Introduction

The full-length cDNA of *XvCaM* was isolated from a cDNA library made from *X. viscosa* leaf material exposed to a 4°C low temperature stress (Iyer, pers. comm.). *XVEF* was initially identified from a differential screen using dehydrated and rehydrated *X. viscosa* leaf material. The original designation of the 5' truncated *XVEF* was *CBPXV* and its isolation was detailed by Mundree & Farrant (2000). The 5' fragment of *XVEF* was isolated using SMART RACE (SMART RACE cDNA Amplification kit, Clonetech, BD Biosciences, USA) (Schonrock, Honours Thesis). The full-length *XVEF* needed to be produced as one DNA fragment and this procedure is described in this chapter. Sequence analysis of each gene was done in silico to predict the probable functional properties of the gene and the corresponding protein using computer software and internet databases. A Southern blot was conducted to verify whether the cDNAs in question originated from the *X. viscosa* genome and to estimate the copy number of each gene.

2.2 Materials and methods

2.2.1 Full-length construction of *XVEF*

The cDNA templates used were the 5' truncated fragment in the vector pGEM® -T EASY (Promega, USA) and the 3' truncated fragment in the vector pProEXa (Invitrogen, Life Technologies, USA) (Schonrock, Honours Thesis). DNA templates were prepared by growing *E. coli* (DH5α) transformed with the appropriate vectors, harvesting cells by centrifugation and extracting the plasmid DNA using the High Pure Plasmid Purification Kit (Roche, Germany). Resultant plasmid DNA was subjected to restriction digests to release relevant fragments from plasmids. The pGEM® -T EASY construct was cut with the restriction endonuclease EcoRI and the pProEXa construct with EcoRI and XhoI. The released fragments were gel purified using the High Pure PCR Purification Kit (Roche, Germany).

Primers EF1203F (5' GAT GGA CAC GAA AGA GGG 3') and MIDREV (5' CCT TTG TGA ATG TTG CAG ATA TAG A 3') were used with the 5' truncated fragment as the template (Figure 2.2.1). This produced the fragment called MID 1. Primers MIDFOR (5' ATC TAT ATC TGC AAC ATT CAC AAA GG 3') and EFREV (5' GCT AGA TCA GCT AAG AAG CTA GTT TCC 3') were used with the 3' truncated fragment as the template to produce MID2. Duplicate reactions of each primer set were set up. Negative controls consisted of PCR reactions incorporating all PCR reagents, but lacking template. The PCR cycling conditions used were an initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 53.1°C for 45 seconds, elongation at

72°C for 1 minute and a final elongation at 72°C for 7 minutes. The PCR cycle was repeated 25 times.

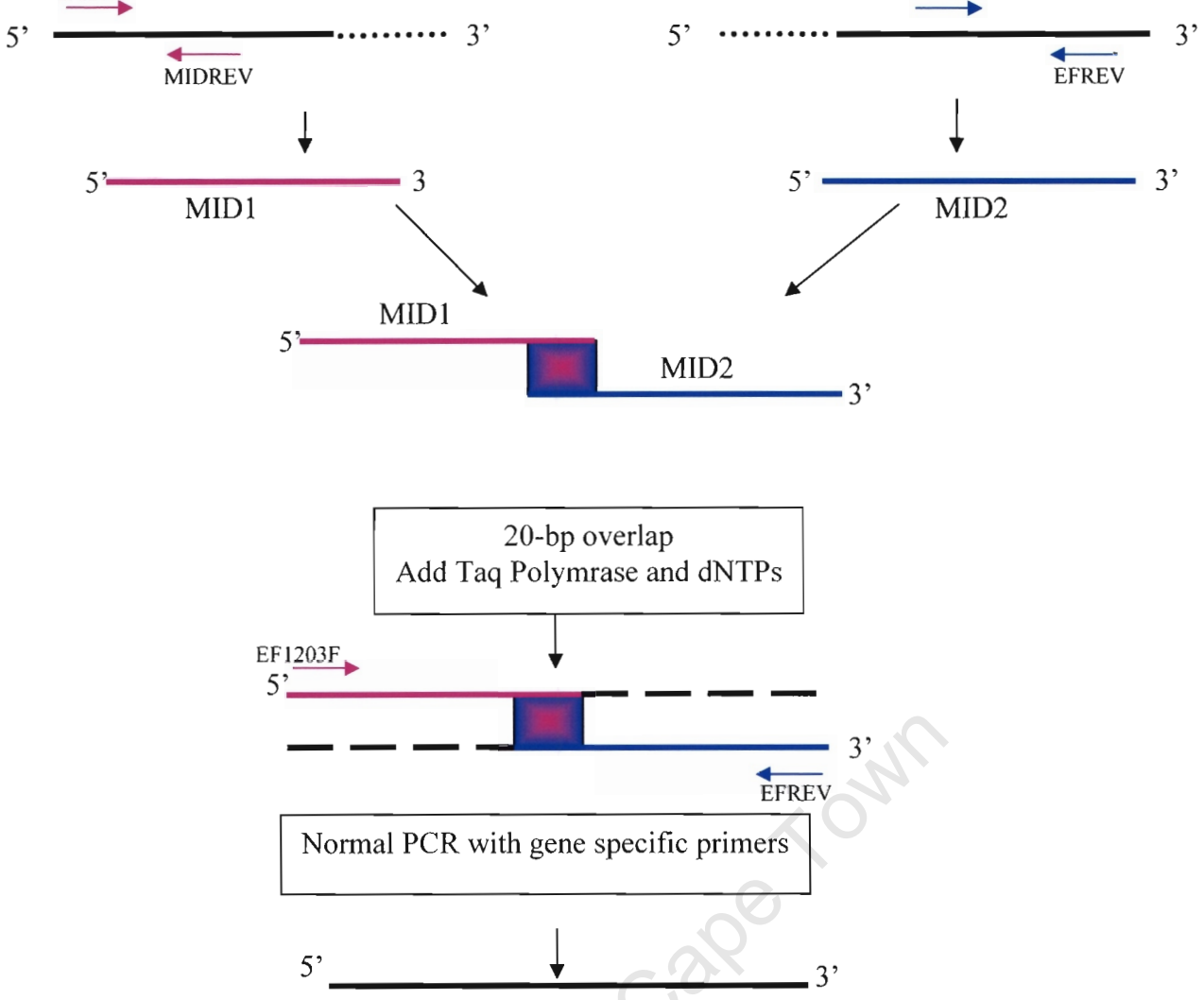


Figure 2.2.1. Schematic representation of PCR method to produce full-length *XVEF*

PCR products were visualized by electrophoresis using a 1.2 % agarose (w/v) TBE (tris/borate/EDTA) gel stained with ethidium bromide. Gels were viewed using a UV transilluminator (UVP, USA). These fragments were gel purified using a High Pure PCR Purification Kit (Roche, Germany) and cloned into the pGEM® -T EASY vector (Promega, USA) to simplify sequencing. The fragments were sequenced on a MegaBACE 500 (Molecular Dynamics, Amersham Biosciences, UK) which uses an automated capillary DNA sequencing system. The sequencing kit used was the DYEnamic ET Dye terminator Cycle sequencing Kit. All sequencing reactions were performed according to the manufacturer's instructions and cycle sequenced on a

GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems, UK). The primers used to amplify inserts from the pGEM® -T EASY vector in the PCR reaction were standard M13 forward and reverse primers. The gel matrix used was the LPA long-read.

Equimolar amounts of each purified fragment, namely MID1 and MID2, were added to dNTPs, PCR buffer and $MgCl_2$. This was boiled for 10 minutes and placed on ice for 2 minutes. Supertherm (SR products, UK) or Expand Long Range (Roche, Germany) Taq polymerase was added and incubated for 10 minute at either 68°C or 72°C respectively. 1µl of this reaction was used as the template for the PCR reaction using the gene specific EF1203F and EFREV under the same conditions as stated previously. Negative controls consisted of PCR reactions incorporating all PCR reagents, but lacking template. The resultant PCR product was visualized, gel purified and subcloned into the vector pGEM® -T EASY. The resultant clones were screened via colony PCR and one positive colony was verified by sequencing.

The success of this method was the inclusion of the 20 bp overlap between MID1 and MID2. This overlap was designed to act as internal primers so that when the two templates are added together with dNTPs and polymerase, the overlap will prime the 'filling in' of the sequence in the 5' and 3' direction. A small population of full-length DNA fragments is produced and the subsequent rounds of PCR using 5' and 3' gene specific primers are used to amplify the fragment.

2.2.2 Sequence analyses

What was determined by preliminary analysis to be the forward sequence of *XvCaM* (Iyer, pers. comm.) and *XVEF* was translated in three possible reading frames using DNAMAN software (v 4.13 Lynnon Biosoft © 1994- 1999). The sequence of XVCAM and XVEF were compared to related proteins by conducting a BLAST search (http://www.ncbi.nih.nlm.gov) and a multiple sequence alignment was conducted with putative relatives.

The XVCAM and XVEF sequences were subject to a hydrophobicity plot forecast using DNAMAN. The pI and total protein charge were also predicted. Amino acid sequences were submitted to the PredictiProtein website (http://cubic.bioc.colombia.edu) (Rost & Lui, 2003) and potential phosphorylation sites, transmembrane domains and functional motifs predicted.

2.2.3 Southern blot analysis

Genomic DNA extractions

X. viscosa plants were collected from the Cathedral Peak Nature Reserve in Kwazulu-Natal, South Africa. The *X. viscosa* plants were potted in a similar volume of soil, whose composition had a sandy soil:vermiculite:loam soil ratio of 1:1:1, respectively. Plants were maintained under glasshouse conditions as described by Sherwin and Farrant (1996).

Genomic DNA was isolated according to the method described by Dellaporta et al., (1983) with the following modifications. Approximately five to six leaves were cut from a fully hydrated *X. viscosa* plant, necrosed tips removed, and experimental material washed briefly in sterile distilled water. The leaves were then cut into smaller pieces and ground under liquid nitrogen. Quantitation of the genomic DNA was done spectrophotometrically (Sambrook et al., 1989).

The restriction endonucleases EcoRI, EcoRV and HindIII (Roche, Germany) were selected to cut aliquots of approximately 10 µg of *X. viscosa* genomic DNA. These were electrophoresed and blotted (see below) and probed with radiolabelled *XVEF* (5' truncated) cDNA. For probing with radiolabelled *XvCaM* cDNA, EcoRI, HindIII and BglII were used and a double digest was done with BglII and EcoRI. Digestions were conducted in a 50 µl reaction volume [enzyme (1 U/µl), buffer (1x), spermidine (250 µM), BSA (1 µg/µl)]. Reaction tubes were incubated at 37°C overnight. The DNA was electrophoresed on a 0.8% (w/v) agarose gel (containing ethidium bromide) at 20 V for 18 hours. Prior to transfer, gel images were captured using a UV transilluminator (UVP, USA). DNA was transferred onto Hybond XL (Amersham Biosciences, UK) nylon membranes as described by Sambrook et al., (1989). DNA was crosslinked to the membrane using an UV crosslinker (Amersham Biosciences, UK).

DNA probes were labeled with 50 µCi α-^{32}P-dCTP with a specific activity of 3000 Ci/mmol, using a Megaprime DNA labeling kit (Amersham Biosciences, UK), according to the manufacturer's protocol. Between 25-100 ng of purified *XVEF* (3' truncated) or

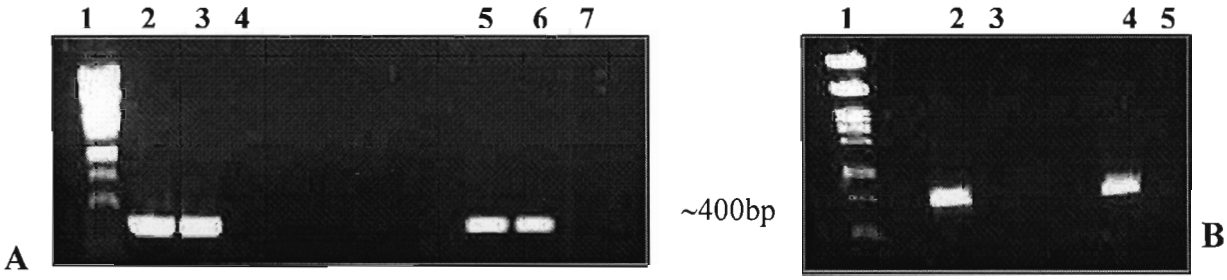
XvCaM insert was used in the labeling reaction. Unincorporated α-^{32}P-dCTP was removed using the SigmaSpin™ Post-Reaction Purification Columns (Sigma, UK) according the manufacturer's instructions.

The resultant membrane was incubated with the radiolabelled probe under conditions described by Sambrook et al., (2002). For the *XVEF* Southern blot, excess probe was removed from the membranes with high stringency washes at 65°C using ~0.1x SSC (wash buffer C) for 60 minutes. Buffer was changed after every 10 minutes for 30 minutes followed by a 30 minute wash. For the *XvCaM* Southern blot, lower stringency was used by washing with 0.5xSSC, 0.1% SDS (wash buffer B) for 10 minutes at 65°C. Membranes were sealed in plastic bags and exposed to a storage phosphor screen (Kodak, USA) and scanned using the BioRad Personal Molecular Imager Fx (BioRad, USA) or exposed to X-ray film (Hyperfilm, Amersham Biosciences, UK) in an autoradiograph cassette at -70°C for 1-10 days. Multiple films were used and developed manually after varying exposure times.

2.3 Results and discussion

Full-length production and sequence analysis of *XVEF*

Two fragments of the putative *XVEF* were available with an approximate 523-bp overlap. These fragments together produced what appeared to be the full-length cDNA of *XVEF*. To produce a single DNA fragment incorporating the entire open reading frame (ORF), truncated fragments of specific lengths with an optimal overlap of 20-bp were produced (D. Chopera, pers. comm., adapted from SMART RACE, BD Biosciences, USA).



A B

Figure 2.3.1. **A** Results of PCR amplification to produce two truncated fragments. Lanes 1, DNA marker (λ *Pst*I); 2 and 3, MID1; 4 and 5, MID2; 4 and 7, negative controls. **B** Full-length PCR products. Lanes 1, DNA (λ *Pst*I); 2, full length PCR product using Expand Taq polymerase; 4, full-length product using Supertherm Taq polymerase; 3 and 5, negative controls.

A PCR product of ~400bp resulted from the first rounds of PCR to produce the fragments MID1 and MID2 (Figure 2.3.1 A) with lanes 4 and 7 representing the negative controls. After the extension of MID1 and MID2, the PCR reaction using gene specific primers targeted to the 5' and 3' ends resulted in a PCR product of ~800bp (Figure 2.3.1 B) where lane 2 and 4 represents the full-length PCR product using Expand Taq polymerase and Supertherm Taq polymerase, respectively. Cloning and sequencing of the PCR product confirmed the presence of the full-length open-reading frame.

Sequence analysis of *XVEF*

The sequence analysis showed that *XVEF* consists of a 708 bp ORF (Figure 2.3.2) encoding a putative polypeptide of 26.9 kDa. This has a calculated pI of 6.49 and contains a putative transmembrane domain spanning from residues 90 and 107 (Rost et al., 1996; Rost 1996). According to the PHD (Profile network prediction HeiDelberg) prediction, the reliability of this prediction is 5, where 9 is high and 0 is low. Several

possible phosphorylation sites (PROSITE, Bairoch et al. 1997) are also indicated. The most distinguishing features uncovered by the sequence analysis were the conserved EF-hand domain and the putative transmembrane domain. The hydrophobicity plot generated by DNAMAN (Figure 2.3.3) substantiates this prediction as it shows a possible hydrophobic region between residues 90-107.

```
1          ATGGACACGAAAGAGGGGCTTCAGACGGTGGCGGCCAAGGCGCCTGTCACGGCGGAGCGT
1           M  D  T  K  E  G  L  Q  T  V  A  A  K  A  P  V  T  A  E  R

61         CCGGTGCCCACCGACCTCGATGATAGCATTTCCAAACCTTATATGGCGAGAGCTCTAGCT
21          P  V  P  T  D  L  D  D  S  I  S  K  P  Y  M  A  R  A  L  A

121        GCAGTTGACAGGTACCACCCTGAGGGAACAAAGGACCACAATCCAGATAACATGAGTGTG
41          A  V  D  R  Y  H  P  E  G  T  K  D  H  N  P  D  N  M  S  V

181        TTGCAACAACATGTTGCTTTTTTTGATCGTAATGGTGATGGCATTGTTTATCCATGGGAA
61          L  Q  Q  H  V  A  F  F  D  R  N  G  D  G  I  V  Y  P  W  E

241        ACTTATGCAGGTTCAAGAGCACTGGGGTACAACCCCTTCATATCATTTCTCTTGGCTATT
81          T  Y  A  G  S  R  A  L  G  Y  N  P  F  I  S  F  L  L  A  I

301        TCTGTGAATGTATTTTTGAGCTATGCAACTCTGCCTCACTGGCTACCATCTCCTTTATTT
101         S  V  N  V  F  L  S  Y  A  T  L  P  H  W  L  P  S  P  L  F

361        CCCATCTATATCTGCAACATTCACAAAGGCAAGCACGGCAGTGACTCCCGCACCTATGAT
121         P  I  Y  I  C  N  I  H  K  G  K  H  G  S  D  S  R  T  Y  D

421        ACCGAGGGCAGATTCGAACCTACGAGCTTCGAGAACATTTTTAGCAAATATGCACACACA
141         T  E  G  R  F  E  P  T  S  F  E  N  I  F  S  K  Y  A  H  T

481        GTCCCAGACAAGCTCAGTTTGAGAGACATTTGGAGGATGACTGAAGGCAACCGGAAGGCA
161         V  P  D  K  L  S  L  R  D  I  W  R  M  T  E  G  N  R  K  A

541        TGGGACTTCGTCGGCTGGATAGGAGCAAAAGGGGAGTGGTTCCTCCTCTATATTTTGGCC
181         W  D  F  V  G  W  I  G  A  K  G  E  W  F  L  L  Y  I  L  A

601        AAGGATGACGATGGTTACTTATCGAGGGAAGCAATCAGGCGTTGCTTCGACGGAAGCTTG
201         K  D  D  D  G  Y  L  S  R  E  A  I  R  R  C  F  D  G  S  L

661        TTCGAGTACATCGAAAAACAGAGGATCACTGCACACATGAAGAAATGTTAG
221         F  E  Y  I  E  K  Q  R  I  T  A  H  M  K  K  C  *
```

Figure 2.3.2. ORF of *XVEF*. Stop and start codons are highlighted in pink, the EF-hand domain in blue. The putative transmembrane domain is underlined in green, the putative casein kinase II phosphorylation sites in black.

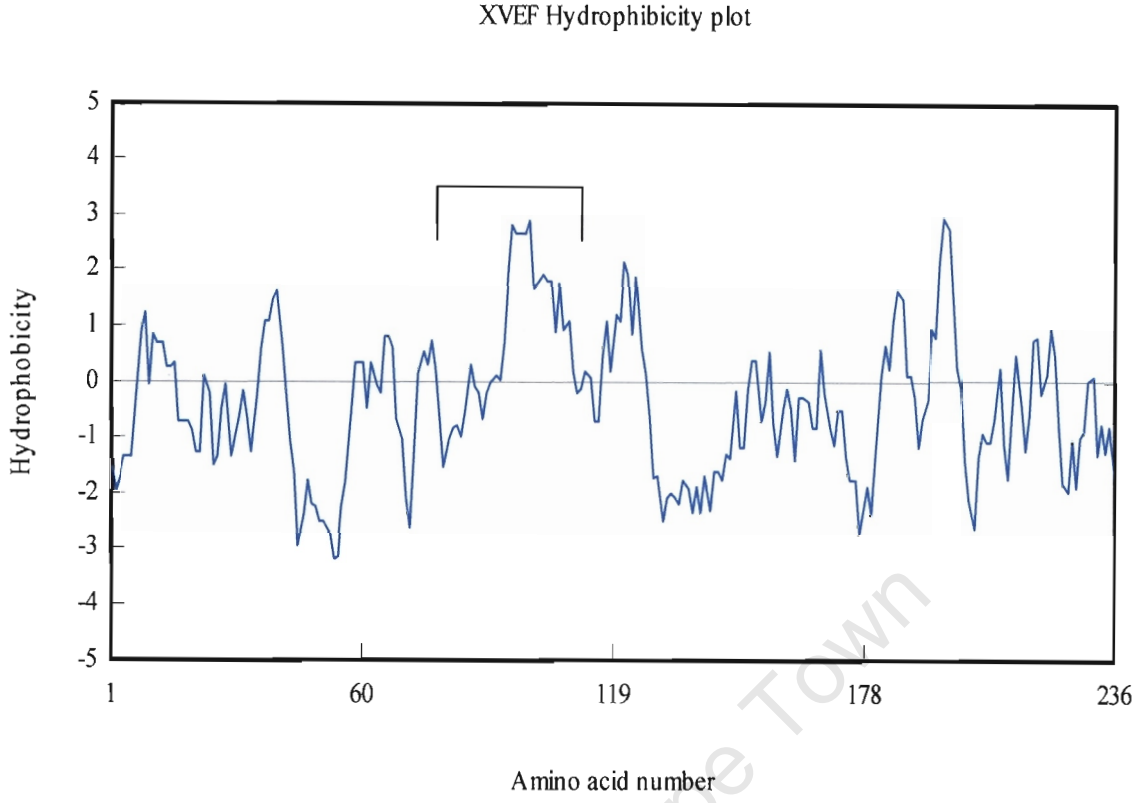


Figure 2.3.3. Hydrophibicity plot of XVEF (DNAMAN). Possible hydrophobic regions appear above the zero line and indicated by black bar.

The multiple alignment of XVEF with the orthologues showing highest similarity is shown in Figure 2.3.4. The rice orthologue (Frandsen et al., 1996; Frandsen et al., 2001) has the highest similarity to XVEF at 64%. Arabidopsis, sesame and barley also contain the conserved EF-hand and the putative transmembrane domain. The rice orthologue received the new designation OsClo as it was found that it was similar to a group of proteins called caleosins. Caleosins are calcium-binding proteins similar to the lipid body proteins oleosins (Murphy et al., 2000; Næsted et al., 2000). Structurally they have three regions and the N-terminal is hydrophilic and contains a single EF-hand motif. The

central region is hydrophobic and contains a putative transmembrane domain. The C-terminal is hydrophilic with an array of phosphorylation sites (Frandsen et al., 2001).

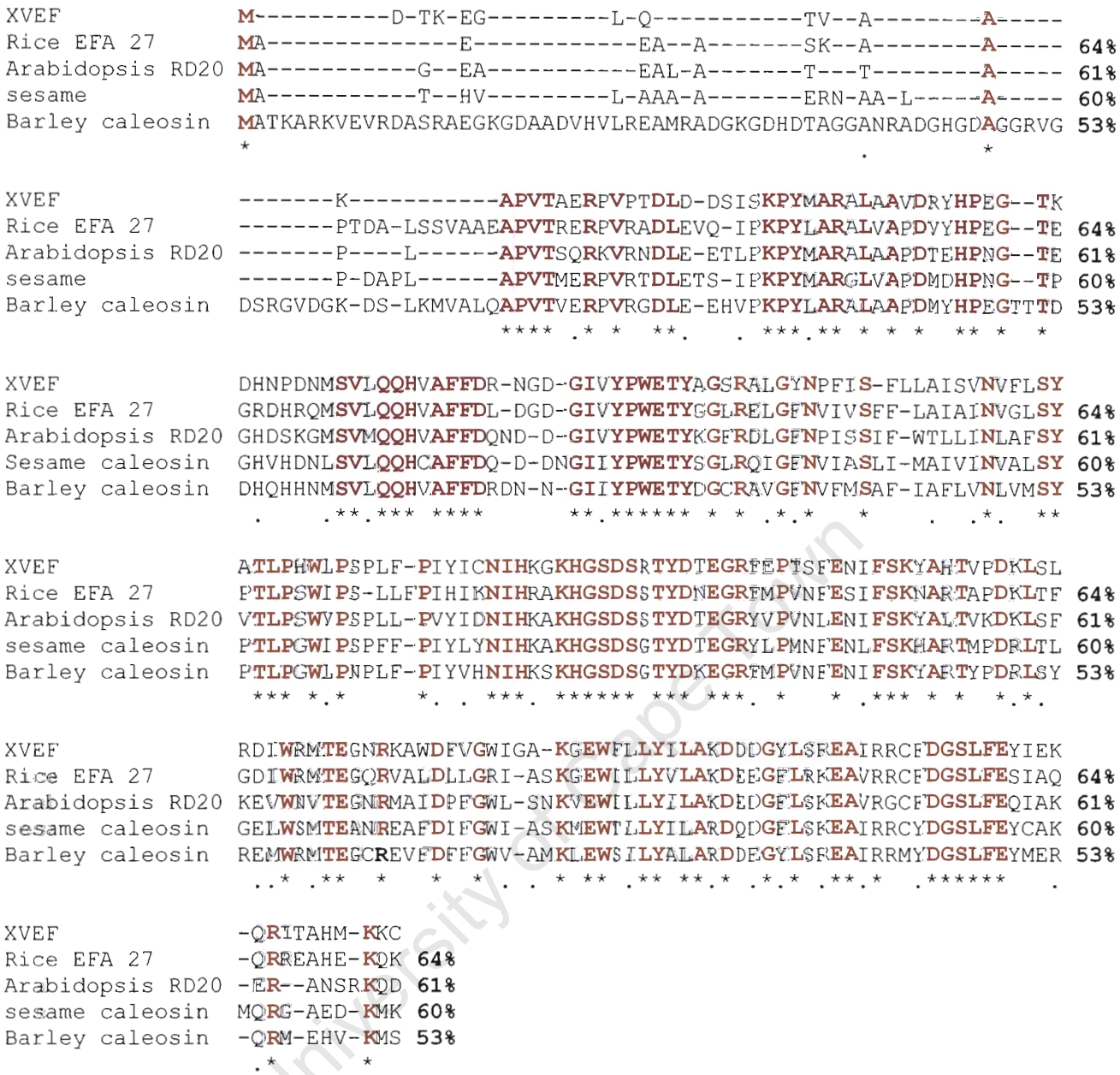


Figure 2.3.4 Amino acid sequence alignment of *X. viscosa* XVEF with orthologues from *Oryza sativa* (rice EFA 27), *Arabidopsis thaliana* (RD20), *Hordeum vulgare* L. (barley caleosin) and *Sesamum indicum* (sesame caleosin). Percentages indicate identities of orthologues to XVEF.

Sequence analysis of *XvCaM*

Bioinformatic analysis of the *XvCaM* sequence revealed that it has a single ORF of 450 bp (Figure 2.3.5) corresponding to a putative polypeptide product of 149 amino acids with a molecular weight of 16.39 kDa. It has a predicted pI of 3.90 and three EF-hands No putative transmembrane domains were found. A range of potential phosphorylation sites were found using the software program DNAMAN, with the most abundant being those for the casein kinases II (CKII). Multiple alignments with similar sequences indicated by the BLAST search demonstrated that XVCAM represents one of a highly conserved family of calmodulins amongst a wide range of plant species, having percentage identities of between 95 and 99% (Figure 2.3.6). The highest sequence conservation was found with *Capsicum annuum*, having 99.33% similarity. Orthologues were also identified from wheat (Lui et al., 2003) and soybean (Lee et al., 2000).

```
1          TAGTCAGTCGACTAATGGCAGACCAGCTCACCGACGATCAGATCTCCGAGTTCAAGGAGG
1             V  S  R  L  M  A  D  Q  L  T  D  D  Q  I  S  E  F  K  E

61         CCTTCAGCCTCTTTGACAAGGACGGCGATGGTTGCATCACCACCAAAGAATTGGGAACTG
20         A  F  S  L  F  D  K  D  G  D  G  C  I  T  T  K  E  L  G  T

121        TGATGCGCTCTCTAGGCCAGAACCCCACAGAGGCAGAGCTGCAGGACATGATAAATGAAG
40         V  M  R  S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E

181        TGGATGCTGATGGTAACGGAACAATCGACTTTCCTGAGTTCCTCAACCTGATGGCCCGCA
60         V  D  A  D  G  N  G  T  I  D  F  P  E  F  L  N  L  M  A  R

241        AGATGAAGGACACCGACTCAGAGGAAGAGCTGAAGGAGGCTTTCCGTGTGTTCGACAAGG
80         K  M  K  D  T  D  S  E  E  E  L  K  E  A  F  R  V  F  D  K

301        ATCAGAACGGGTTCATTTCTGCTGCTGAACTTCGCCACGTCATGACCAATCTTGGTGAGA
100        D  Q  N  G  F  I  S  A  A  E  L  R  H  V  M  T  N  L  G  E

361        AACTCACGGATGAAGAGGTTGATGAGATGATTCGTGAGGCTGACGTTGATGGCGATGGCC
120        K  L  T  D  E  E  V  D  E  M  I  R  E  A  D  V  D  G  D  G

421        AGATCAACTACGATGAGTTTGTCAAAATCATGATGGCCAAATAA
140        Q  I  N  Y  D  E  F  V  K  I  M  M  A  K  *
```

Figure 2.3.5 ORF of *XvCaM*. Start and stop sites are highlighted in pink, EF-hands in blue. The casein kinase II phosphorylation sites are underlined in black.

```
X. viscosa     MADQLTDDQISEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG
C. annuum      MADQLTDDQISEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 99.33%
O. sativa      MADQLTDDQIAEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 98.66%
T. aestivum    MADQLTDDQIAEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 97.66%
G. max         MADQLTDDQIAEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 97.99%
Z. mays        MADQLTDEQIAEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 97.32%
S. oleracea    -AZZLTDEQIAEFKEAFSLFDKDGDGCITTKELGTVMRSLGQNPTEAELQDMINEVDADG 95.30%
                *  ***.**.**************************************************

X. viscosa     NGTIDFPEFLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE
C. annuum      NGTIDFPEFLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 99.33%
O. sativa      NGTIDFPEFLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 98.66%
T. aestivum    NGTIDFPEFLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 97.66%
G. max         NGTIDFPEFLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 97.99%
Z. mays        NGTIDFPEFLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 97.32%
S. oleracea    NGTIDFPEFLNLMARKMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTNLGEKLTDE 95.30%
               ************************************************************

X. viscosa     EVDEMIREADVDGDGQINYDEFVKIMMAK
C. annuum      EVDEMIREADVDGDGQINYDEFVKVMMAK 99.33%
O. sativa      EVDEMIREADVDGDGQINYDEFVKVMMAK 98.66%
T. aestivum    EVDEMVREADVDGDGQINYDEFVKVMMAK 97.66%
G. max         EVDEMIREADVDGDGQINYEEFVKVMMAK 97.99%
Z. mays        EVDEMIREADVDGDGQINYDEFVKVMMAK 97.32%
S. oleracea    EVDEMIREADVDGDGQINYEEFVKVMMAK 95.30%
               *****.*************.*****.****
```


Figure 2.3.6 Amino acid sequence alignment of *X. viscosa* XVCAM with orthologues from *Capsicum annuum* (pepper), *Oryza sativa* (rice), *Triticum aestivum* (wheat),*Glycine max* (soybean), *Zea mays* (maize) and *Spinacia oleracea* (spinach).

Southern blot analysis of *XVEF* and *XvCaM*

Probing *X. viscosa* genomic DNA membranes with radiolabelled *XVEF* cDNA revealed that EcoRI digestion produced a single prominent band corresponding to a DNA fragment of 3.9 kb (Figure 2.3.7 B).

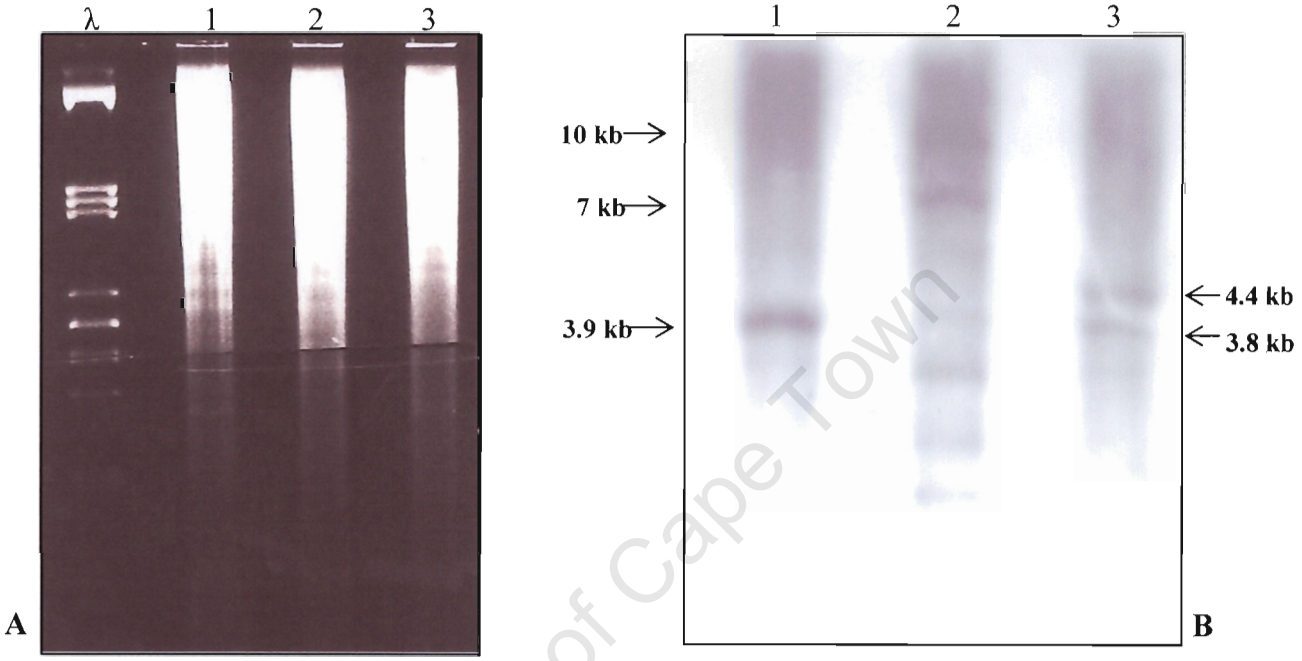


Figure 2.3.7. **A** Gel photograph of digested genomic DNA of *X. viscosa*. Lanes 1, EcoRI; 2, EcoRV; 3, HindIII. **B** Autoradiograph of Southern blot of *X. viscosa* genomic DNA probed with radiolabelled *XVEF*. Lanes 1, EcoRI; 2, EcoRV; 3, HindIII. Relative sizes are indicated by arrows on either side of autoradiograph.

The EcoRV digestion resulted in two prominent bands at 10 kb and 7 kb along with fainter bands at 4.75, 3.2, 2.5 and 2.1 kb. HindIII digestion produced two bands at 4.4 kb and 3.8 kb. These bands are again the most prominent, but fainter bands can be seen on the autoradiograph. According to sequence analysis, EcoRI has no restriction sites within the *XVEF* cDNA and both EcoRV and HindIII cut the cDNA once. A single copy gene would produce one band for the EcoRI digestion and two bands for the EcoRV and

HindIII digestions. Therefore, *XVEF* can be confirmed to be present in the *X. viscosa* genome. Since the genomic DNA sequence of *XVEF* is not known, the appearance of additional bands on the autoradiograph could also be attributed to introns within the genomic sequence containing restriction endonuclease sites. The concentration of large molecular weight DNA fragments seen in Figure 2.3.7 A, suggests that undigested DNA was still present on the gel. Therefore partial digestion of the genomic DNA could have also resulted in non-specific bands being hybridized by the radiolabelled probe. The less prominent bands on the autoradiograph may be attributed to the presence of homologous sequences to *XVEF* in the genome.

Homologues have been shown to exist in Arabidopsis where a small caleosin-like gene family was discovered in Arabidopsis. At*Clo1-5* was investigated where At*Clo2-5* was expressed in all tissues at low levels, but At*Clo1* was expressed in embryos and root tip cells. The sequence was similar to OsClo and contained the EF-hand followed by the hydrophobic membrane anchor (Næsted et al., 2000).

XvCaM sequence analysis predicted that there were no EcoRI and HindIII recognition sites within the cDNA, with one interval BglII recognition sequence. The *XvCaM* Southern blot analysis suggests that it is a single copy gene in the *X. viscosa* genome as EcoRI digestions of genomic DNA produced one band at approximately 1.6 kb (Figure 2.3.8 B, lane 1). Digestion of genomic DNA with BglII produced two bands as expected at approximately 4 and 3.5 kb (Figure 2.3.8 B, lane 3).

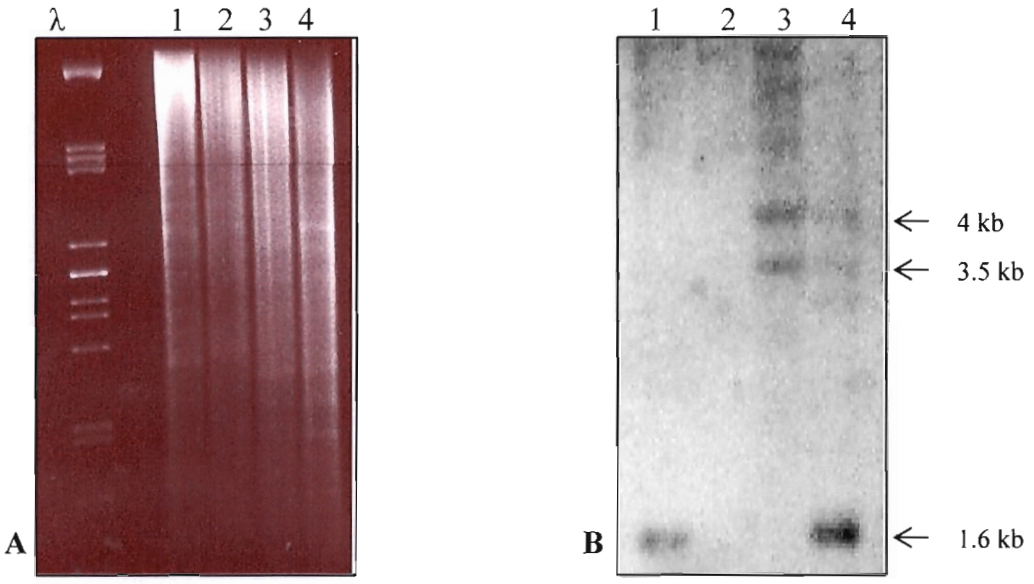


Figure 2.3.8: **A** Gel photograph of digested genomic DNA of *X. viscosa*. Lanes 1, EcoRI; 2, HindIII; 3, BglII; 4, BglII/EcoRI double digest. Lanes 1, EcoRI; 2, HindIII; 3, BglII; 4, BglII/EcoRI double digest. **B** Autoradiograph of Southern blot of *X. viscosa* genomic DNA probed with radiolabelled *XvCaM*. Relative sizes are indicated by arrows on either side of autoradiograph.

Non-specific bands are visible at higher molecular weights, but this could be due to undigested DNA. The double digests with EcoRI and BglII (Figure 2.3.8 B, lane 4) gave the same sized products as resulted from the individual digests with EcoRI and BglII. This could be due to partial digestions as the resultant banding pattern of the double digest should be different from that of the individual enzymes. EcoRI should cleave within the fragments that resulted from the BglII digest. Distinct bands were not seen in lane 2 of Figure 2.3.8 B, which represents the HindIII digestion. From the corresponding agarose gel (Figure 2.3.8, A) it is clear that DNA was present in lane 2. It could be possible that the DNA did not transfer efficiently to the membrane and therefore not enough DNA was present for the *XvCaM* probe to bind. The other bands present on the

autoradiograph could be the result of homologues within in the genome. These results are putative and should be verified with repeat experiments to confirm the analysis presented here. It has been previously shown that calmodulin homologues exist, as in wheat, *CaM1* and *CaM2* was found to be involved in heat shock signal transduction cascades (Liu et al., 2003). This supports the theory that homologues could occur within the genome.

Chapter 3

Heterologous expression of *XvCaM* in *E. coli* and antibody synthesis

3.1 Introduction

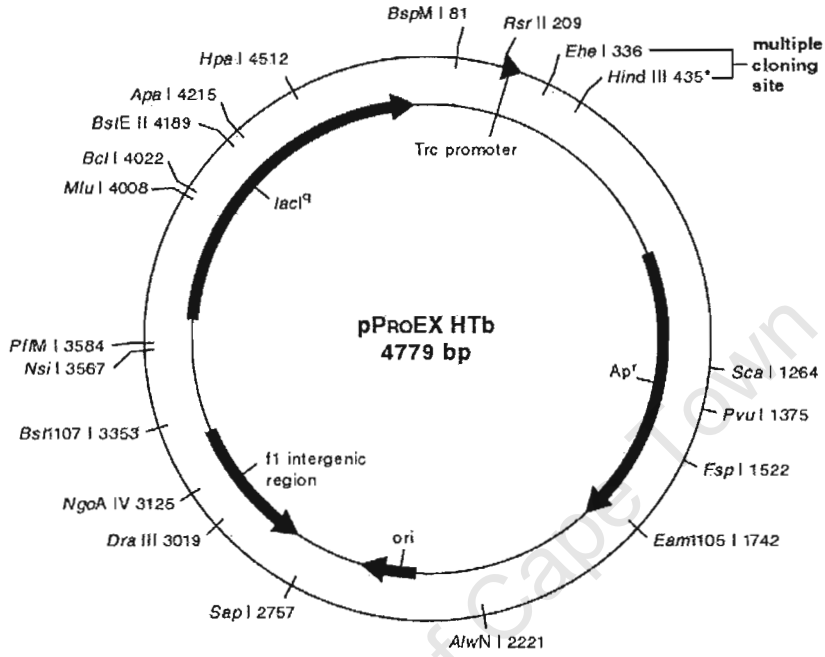
The vector pProEX HT (Life Technologies, Invitrogen, USA) was used for expression of *XvCaM*. This system has three variations where each provides a different expression frame and six histidines upstream of the multiple cloning site allowing for nickel-based purification from bacterial extracts. There is a wide range of expression systems available and the pProEX HT system was selected because of its established protocols and methods. Recombinant production of a desired protein allows for large enough quantities of a pure protein product to be made. With this protein antibodies can be raised which can be used for western blot analysis. The protein can also be used to conduct in vitro functional assays, so as to verify its function.

3.2 Methods and materials

3.2.1 Cloning of XvCaM into the expression vector

XvCaM was cloned into pProEXb HT (Invitrogen, Life Technologies, USA) (Figure 3.2.1) between the EcoRI and XhoI restriction endonuclease sites. The resultant constructs were sequenced to check whether the cDNA was in frame with the His-Tag

coding region. Constructs were then transformed into *E. coli* DH5α cells and cultures were incubated at 37°C with shaking.



pPROEX HTb multiple cloning site and primer binding region: 235-463

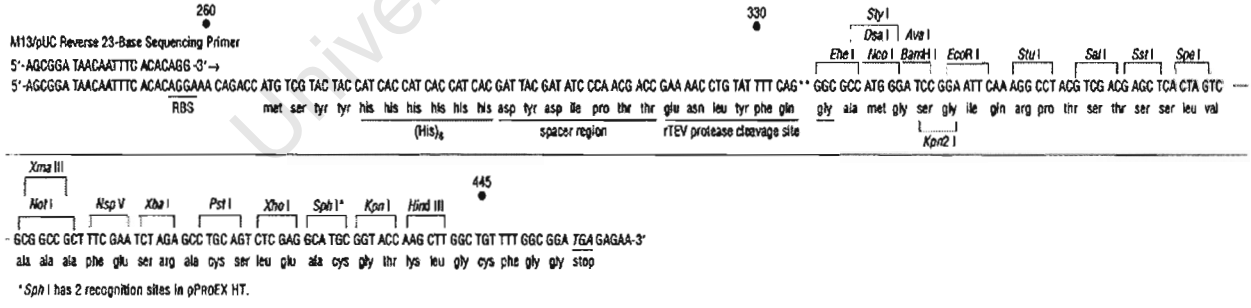


Figure 3.2.1 Vector map and polylinker region of the pProEXb vector (Invitrogen, Life Technologies)

3.2.2 Induction of protein expression

The expression of *XVCAM* was induced by the addition of IPTG (0.6-1 mM) to 100 ml of bacterial culture at an absorbance of 0.5-1 OD units measured at a wavelength of 590 nm. Samples were taken prior to induction as a control and every hour for three hours. Harvested bacterial cells were stored at -20°C. The induced XVCAM protein extracts were separated by 15% polyacrylamide gel electrophoresis using the Hoefer® miniVE electrophoresis apparatus (Amersham, Biosciences, UK). Protein samples were prepared by adding 5X SDS gel loading buffer, samples were boiled for 10 minutes and cooled on ice. Pre-stained SDS-PAGE Standards Low Range (BioRad, USA) was used as molecular weight markers. Where applicable the proteins were stained in gels with Coomassie blue or transferred onto nitrocellulose membrane (Osmonics, UK) using the Hoefer® Electrotransfer System (Amersham Biosciences, UK) following the manufacturer's instructions. The transfer was conducted at 300 mA for 1-2 hours or 30 V overnight at 4°C and membranes were stained with Ponceau S (Sambrook et al., 1989) for 10 minutes to confirm transfer of the proteins. To check the presence of the His-tagged protein, western blot analysis was conducted using the 6X His monoclonal antibody according to the manufacturer's instructions (BD Biosciences, Belgium). Reactions were visualized by application of the chemiluminescence agent, NBT/BCIP (Roche, Germany).

The 6X His-tagged proteins were purified manually under native conditions using the MagneHis™ Protein Purification System (Promega, USA). Small scale purifications were done using the microfuge tube magnetic separation stand with multiple bacterial culture samples. Primary elution was conducted with 50 µl elution buffer per 1ml of

culture and a secondary elution was performed with 20 µl buffer. Purified protein was concentrated and desalted using a centrifugal filter, Amicon Ultra-15 (Millipore, USA).

3.2.3 Antibody and purification synthesis

The purified recombinant protein was used to raise polyclonal antibodies in rabbits. Standard protocols were used according to Rybicki (1979). An enzyme linked immuno-sorbant assay (ELISA) was conducted using anti-serum from rabbits.

Recombinant XVCAM protein was diluted in a range from 1:10 to 1:1000 in phosphate-buffered saline [PBS (1.8 mM KH_2PO_4, 2.7 mM KCl, 10 mM $Na_2HPO_4.7H_2O$, 137 mM NaCl, pH 7.4]. XVCAM antiserum was diluted in a range from 1:10 to 1:2000 in blocking buffer [1X PBS; 3% (w/v) skim milk powder]. The various recombinant protein dilutions were innoculated into the wells of a Nunc-Immuno™ 96 MicroWell™ Plate, Maxisorp™ surface (Nunc, Denmark) and incubated at 4°C O/N. The bound protein was washed three times with 1X PBS and incubated with blocking buffer for 2 hours at room temperature. These samples were washed again thrice with 1X PBS and incubated O/N at 4°C with the various XVCAM antiserum dilutions. The recombinant protein-antiserum complex was washed thrice with 1X PBS and incubated with anti-rabbit IgG, alkaline phosphatase conjugate (1:10 000 dilution) (Sigma, Germany) for 2.5 hours at 37°C. This was washed three times with 1X PBS. The detection was done utilizing ρ-nitrophenyl phosphate tablets (Sigma Fast™, Germany), according to the manufacturers recommendations. The absorbance at 405 nm was determined using a Titertek Multiskan® Plus MK II (Flow laboratories, UK).

To purify the antibody, two volumes of 0.037 M borate-buffered saline pH 8.6 were added to one volume of anti-serum. 14% (w/v) of crushed PEG 6000 was added to the diluted serum and gently mixed by inversion. This was centrifuged at 12 000g for 10 minutes at 4°C. The pellet was dissolved in the original serum volume using phosphate buffer pH 7.4 (PBS). PEG 6000 was added again at a 14% (w/v) concentration and dissolved. It was centrifuged as before and the pellet was dissolved in half the original serum volume using PBS containing 60% (v/v) glycerol. Aliquots were stored at -20°C (Polson et al., 1964).

3.3 Results and discussion

Production of XVCAM recombinant protein

A prominent band was evident 1 hour after induction with IPTG and increased in intensity after 2 and 3 hours (Figure 3.3.1, lanes 7-9), indicating that *E. coli* DH5α transformed with pProEXb::*XvCaM* overexpressed XVCAM. The simultaneous time course induction of the pProEXb vector control confirmed that this was a protein unique to the XVCAM-containing *E.coli*. Taking into account additional residues from the 6X His-tag, the expected size of recombinant XVCAM is ~21kDa. This roughly corresponds to the position of molecular weight marker.

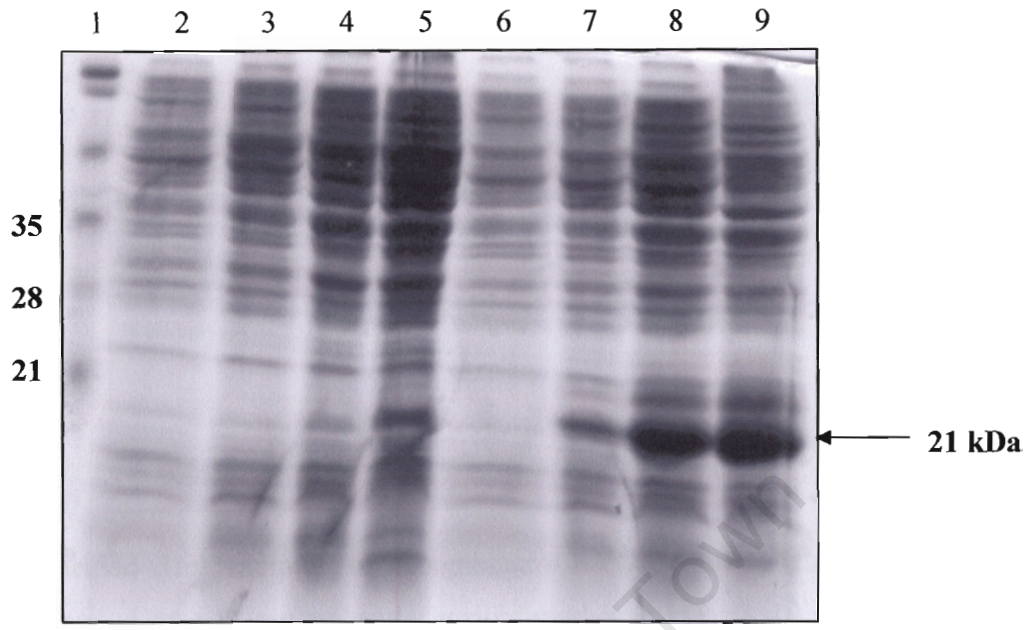


Figure 3.3.1 Protein extracts of *E.coli* DH5α transformed with either pProEXb or pProEXb::*XvCaM*. Lanes 1, low molecular weight marker in kDa (size shown); 2-5, non-recombinant vector-transformed culture 0, 1, 3 & 24 after induction with IPTG, respectively; 6-7, recombinant vector-transformed culture 0, 1, 3 & 24 hours after induction with IPTG. The arrow denotes the recombinant protein accumulation at the predicted size.

The recombinant protein was purified using the MagneHis™ Protein Purification System and a resulting band of ~28 kDa was seen (Figure 3.3.2). The molecular weight marker ladder could possibly not have separated sufficiently during electrophoresis thus the size relative to the marker was not correct. Some of the paramagnetic nickel particles remained after purification, therefore non-specific bacterial proteins at higher molecular masses were present. However, XVCAM was discernible in excess quantity. In retrospect, it would have been advisable to have increased the number of wash steps from three to five in order to maximise the removal of contaminants, but this could have risked

a decline in the amount of recombinant protein and possibly negatively affect its visualization.

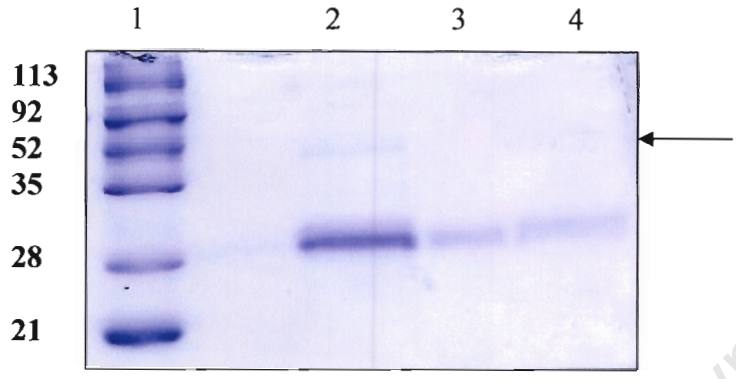


Figure 3.3.2 Purified 1ml samples of recombinant XVCAM using the MagneHis Purification system. Lanes 1, low molecular weight marker in kDa; 2, 10 µl of purified protein after first elution; 3 & 4, 10 µl of purified protein after second elution. The top arrow indicates non-specific protein.

Polyclonal antibodies were raised by injecting XVCAM into rabbits. The reactivity of the antiserum with XVCAM is represented in Figure 3.3.3. Various dilutions of protein and antiserum were used, with the graph illustrating that the antiserum positively cross-reacted with the protein, even at an antiserum dilution of 1:1000 and a protein dilution of 1:500. At lower protein dilutions the reactivity dropped slightly. These results were useful in selecting a range of dilutions to be used in future experiments such as western blot analysis, as described in Chapter 5. Following antibody purification, empirical tests were conducted (data not shown) to establish the ideal dilution factor that would yield the best results.

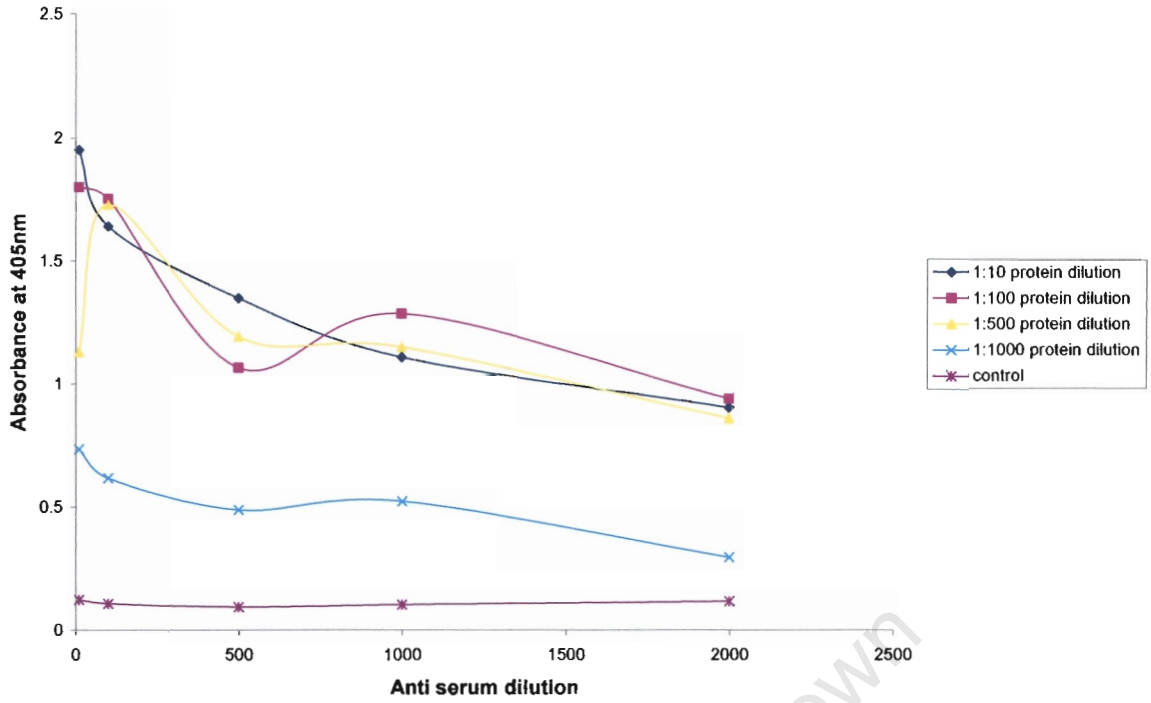


Figure 3.3.3 ELISA results after a 30 minute development of substrate.

Recombinant protein production of XVEF

An attempt was made to express XVEF recombinantly, but was unsuccessful. Therefore no antibodies were raised, western blot analysis could not be conducted and a calcium-binding assay could not be conducted.

Chapter 4

Testing the functional role of XVCAM using a calcium-binding assay

4.1 Introduction

The designation of a novel cDNA from a library with an annotated gene name is usually based mostly on sequence data and in silico functional research. Calmodulin is said to be a universal calcium sensor which binds calcium with high affinity due the EF-hand motifs. Even if XVCAM shows high similarity to genes in the database, it is still necessary to verify whether the calcium-binding protein actually binds calcium. The possibility of mutations and non-functional domains occurring in the protein sequence is a possibility. In this chapter, a simple radioactive overlay assay was used to ascertain whether the recombinantly produced XVCAM binds calcium.

4.2 Methods and materials

Approximately 0.5 µg of purified recombinant XVCAM, BSA (negative control) and the human calmodulin CAM*I* (positive control) (Santa Cruz, USA) were electrophoresed on a 15% SDS polyacrylamide gel in duplicate using a Hoefer® Mighty Small Electrophoresis apparatus (Amersham Biosciences, UK). One gel was stained with Coomassie blue and the other was transferred to a nitrocellulose membrane. Protein samples were prepared by adding 5 X SDS gel loading buffer to protein samples, boiling for 10 minutes and placing on ice immediately. Pre-

stained SDS-PAGE Standards Low Range (BioRad, USA) were loaded alongside the protein samples. The proteins were transferred onto nitrocellulose membranes (Osmonics, UK) using the Hoefer® Electrotransfer System (Amersham Biosciences, UK) following the manufacturer's instructions. The transfer was conducted at 300 mA for 2 hours. The membranes were stained with Ponceau S (Sambrook et al., 1989) for 10 minutes to visualize the proteins.

After the western transfer onto the membranes, they were washed thrice for 20 minutes in buffer (60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole-HCl (pH 6.8)) to remove the excess electrode buffer. The isotope $^{45}CaCl_2$ (PerkinElmer, USA) was diluted to 1 mCi/litre in hybridisation buffer and used to incubate the membrane for 10 minutes. It was then washed with 50% ethanol for 5 minutes. Membranes were sealed in plastic and exposed to autoradiograph film (Pierce, USA) for 1-5 days at -70°C (Maruyama et al., 1984).

4.3 Results and discussion

XVCAM binds calcium with high affinity (Figure 4.3.1 B) as the autoradiograph showed a good signal after only 2 days exposure to radioactive calcium. When comparing the autoradiograph to the duplicate gel (Figure 4.3.1 A) it is evident that XVCAM binds calcium specifically as there are non-specific bands present in the sample due to possible bacterial contamination from the purification process. Calcium was only bound to the major band corresponding to XVCAM.

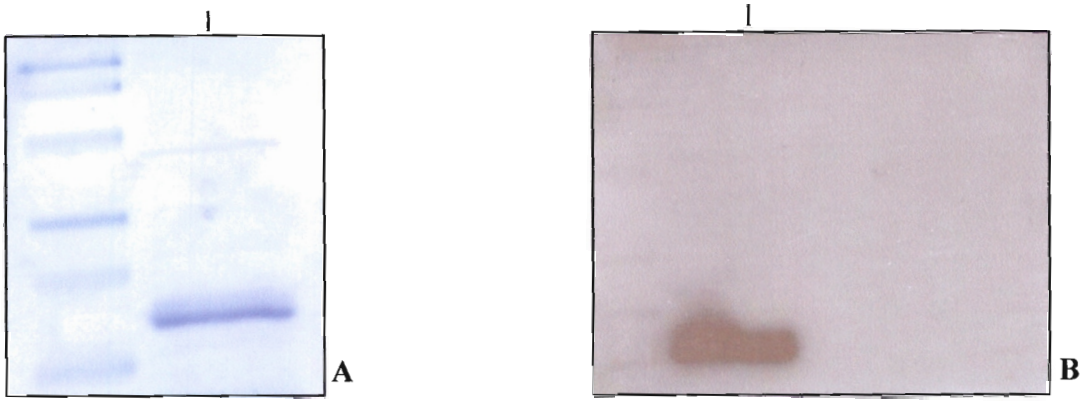


Figure 4.3.1: **A**- 15% polyacrylamide gel with 0.5 µg of purified recombinant XVCAM. Prestained marker is loaded alongside protein. **B** Autoradiograph of XVCAM incubated with ^{45}Ca and exposed for 2 days

In the initial experiment, where the controls were incubated with XVCAM, the protein sequestered all the $^{45}CaCl_2$, and no band was visible for CAM I. The positive (CAM I) and negative (BSA) controls were probed separate from the experimental proteins to circumvent this problem. The resultant image however was not of a suitable quality due to unavoidably high background signal (results not shown). These control experiments would have to be repeated to support the results presented here.

It was decided to use this protocol because of its ease of use. This method was used by Fransden et al. (1996), Takahashi et al. (2000) and Reddy et al. (2004) and it was developed to specifically test the EF-hand domain in the protein those authors were studying. KIC is a protein isolated from Arabidopsis and has one EF-hand. It was shown that the entire protein and a truncated KIC protein isolating the EF-hand, both specifically bound calcium (Reddy et al., 2004). In the present

study range of 2, 4 and 6 µg of protein was loaded, but 0.5 µg of XVCAM was needed to produce the desired result. All the above indicate that XVCAM has a very high affinity for calcium.

Takahashi et al., (2000) also used an electrophoretic mobility shift assay to indicate calcium-binding activity. Calmodulin usually has four EF-hands that cooperatively bind calcium in pairs, so it is always imperative to check the binding capabilities of a protein that does not have pairs of EF-hands viz. XVCAM has four EF-hands (Chapter 2.3).

Chapter 5

Expression profiles of *XvCaM* and *XVEF*

5.1 Introduction

It is necessary to investigate the response of a stress-response related gene to abiotic stress conditions. This can be done by using northern and western blot analysis to determine when the RNA and protein levels, respectively, amass in response to the given stress. Total RNA extracts from leaf tissue were used for most northern blots, and to test the potential of post-transcriptional regulation in response to salt stress, polysomal RNA was used. Western analysis was conducted for XVCAM as only anti-XVCAM antibodies was available. In this chapter, the northern blot analyses of *XvCaM* and *XVEF* will be illustrated under various stress conditions. Western blot analysis for XVCAM was also conducted dehydrated/rehydrated conditions, NaCl and ABA stressed conditions.

5.2 Methods and materials

5.2.1 Plant stress treatments

Maintenance of plants was previously described (chapter 2.2.3). All stress treatments were conducted in a phytotron at a constant temperature of 25°C, 50% relative humidity, a photon flux density (PFD) of 300 µmol $m^{-2}s^{-1}$ and a day/night cycle 16/8h, unless otherwise stated. Plants were moved from glasshouse conditions to the phytotron, where they were equilibrated for a month prior to use for experimental purposes. Whole plants

were subjected to dehydration stress, 150 mM NaCl solution application, 100 µM abscisic (ABA) solution and heat shock at 42°C. A low temperature stress was conducted by placing plants in a chamber set at 4°C with adequate lighting. At regular intervals after initiation of the stress, harvested plant tissue was placed into foil pockets, flash frozen in liquid nitrogen and stored at -70°C until RNA extraction. Unless otherwise indicated, leaves were randomly sampled.

The dehydration stress was induced by withholding water from whole plants for over a 30 day period. Leaves were sampled at the same time during the day and at various RWC throughout the period of drying. RWC was determined by the using the equation $[(W_i-W_d)/W_d]/ [(W_t-W_d)/W_d]$ X 100 (Jin et al., 2000) where W_i=initial weight, W_t=full turgor (leaf material immersed in water for 24 hours) and W_d=dry weight (leaf material dried for 24 hours at 95°C) with three replicates from a given sample. The control sample was represented by hydrated leaves taken before the dehydration stress commenced. Salinity stress was imposed by watering the soil with approximately 1 litre of 150 mM NaCl solution at the same time every day for 4 days. The ABA stress was conducted by watering the soil and leaves with a 100 µM ABA solution. A spray bottle was utilized to effectively cover the leaves. The low temperature stress was conducted by placing plants in a chamber set at 4°C. The heat shock stress was imposed by increasing the ambient temperature in the phytotron to 42°C. Leaf samples were collected every 24 hours for 4 days and stored at -70°C.

5.2.2 RNA extraction and membrane transfer

Total RNA was extracted from *X. viscosa* leaf tissue using TRIzol LS Reagent (Life Technologies, GibcoBRL) according to the manufacturer's instructions. Plant tissue was flash frozen using liquid N_2 and ground in a sterile mortar and pestle. Polysomal RNA extraction was conducted according to Wood and Oliver (1999) with the following modifications. Approximately half a leaf was used at every sampling point and ground in a sterile mortar and pestle using liquid nitrogen. A Ti50 rotor was used for the ultracentrifugation step, the speed was set at 40 000rpm and centrifuged for 2 hours. The resultant pellet underwent a phenol/chloroform clean-up according to the protocol in Sambrook et. al. (1989). Extracted total and polysomal RNA was quantified spectrophotometrically using the relationship A_{260} = 1 OD unit for 40 µg/ml of single stranded RNA.

Approximately 10 µg of RNA was loaded equally in 1.2% (w/v) agarose gels using either TBE or formaldehyde as the buffer. RNA sample loading buffer (10xMOPS, 3.1 µl formaldehyde, 10 µl formamide and 2 µl sample buffer) was added to each RNA sample then the mixture was boiled for 5 minutes. All reagents were prepared using RNAse-sterile techniques, which included the use of 0.01% diethyl pyrocarbonate (DEPC) and ethanol rinses of equipment where possible. Gel images were captured using an UV illuminator (UVP, USA). Electrophoresed RNA was transferred onto nylon membranes (Hybond XL, Amersham Biosciences, UK) by capillary transfer. The membrane was air-dried for 30 minutes and then subsequently UV cross linked (Amersham Biosciences, UK).

The RNA-bound membranes were probed with [α-^{32}P]dCTP labelled *XvCaM* cDNA or *XVEF* (full-length) cDNA using the Megaprime DNA Labelling System (Amersham Biosciences, UK) according to the manufacturer's specifications, except that 100 ng of probe was labelled in the initial step. Thereafter, membranes were re-probed with the *X. viscosa* 18S gene, which was radiolabelled using the Megaprime DNA Labelling System (Amersham Biosciences, UK) according to the manufacturer's instructions. In both cases, hybridization was conducted at 65°C in hybridization buffer. Excess un-bound probe was washed off using wash buffer A (2 x SSC, 0.5% SDS) and wash buffer B (0.5 x SSC, 0.1% SDS). Membranes were sealed in plastic bags and were either exposed to storage phosphor screen (Kodak, USA) and scanned using the BioRad Personal Molecular Imager Fx (BioRad, USA), Packard Instant Imager (Packard, USA) or exposed to x-ray film (Hyperfilm, Amersham Biosciences, UK) in an autoradiograph cassette at -70°C for 1-10 days. Multiple films were used and developed manually after varying exposure times.

5.2.3 Protein isolation

Total protein was isolated using the TRIZOL LS reagent (Life Technologies, USA) according to the manufacturer's instructions with the following modifications. Instead of guanidine hydrochloride in 95% ethanol, 0.1 M ammonium acetate was used. The protein pellet was washed with 2 ml of cold acetone and thereafter air-dried. Proteins were resuspended in 50 µl of Laemmli buffer (625 mM Tris-HCl pH 8.0, 2% SDS, 10% glycerol, 0.02 v/v β-mercaptoethanol) and incubated at 65°C overnight to facilitate

resuspension. Proteins were stored at -20°C until needed. Proteins were quantitated using the Bradford reagent (BioRad, USA).

5.2.4 Protein electrophoresis and transfer

A 15% polyacrylamide separating gel and a 5% stacking gel was used for separation of total protein. Protein samples were prepared by adding 5x SDS gel loading buffer to protein sample, boiled for 10 minutes and cooled on ice. Equal quantities of protein were loaded along with 6µl of pre-stained SDS-PAGE Standards Low Range (BioRad, USA) and electrophoresed at a constant current of 40 mA for 2 hours using the Hoefer® Mighty Small™II SE 250 electrophoresis apparatus. The proteins were transferred onto a nitrocellulose membrane (Osmonics, UK) using the Hoefer® Electrotransfer System (Amersham Biosciences, UK) following manufacturer's instructions. Transfer was conducted at 300 mA for 1-2 hours or 30V overnight at 4°C. The membrane was stained with Ponceau S (Sambrook et al., 1989) for 10 minutes to visualize proteins.

Immunoassay was performed according to Sambrook et al., (1989). Blocking solution consisted of 5% non-fat dried milk in 1X TBS pH 7.5. Primary antibody (anti-XVCAM) was added at a dilution of 1:1000 and incubated overnight at 4°C with agitation. This was followed by 3 X 10 minute washes using blocking solution. Secondary antibody (anti-rabbit IgG peroxidase-linked whole antibody from goat; Sigma, UK) was added at a dilution of 1:5000 and incubated at room temperature for ±2 hours with agitation. Final washes were performed with 1 X TBS (pH 7.5). Antibody reactivity was detected using the SuperSignal® West Pico/Femto Chemiluminescent Substrate (Pierce, USA) and by

exposure to X-ray film (Pierce, USA). Multiple films were used and developed manually after varying exposure times.

5.3 Results and discussion

A range of techniques was used to investigate the expression of *XvCaM* in response to the range of stress treatments. Initially, northern blot analyses were conducted to ascertain when and how mRNA transcripts accumulated. Western blot analyses were performed to verify the presence of protein during the stress treatments.

Stress treatments

Figure 5.3.1 represents the RWC of leaves from plants subjected to the various stress treatments. In NaCl, ABA and temperature (high and low) treated plants remained at relatively constant water contents throughout the treatment. Figure E and F represents the dehydration and rehydration curves respectively and represents a typical drying profile as previously reported for this species (Mowla et. al., 2002; Garwe, et al., 2003).

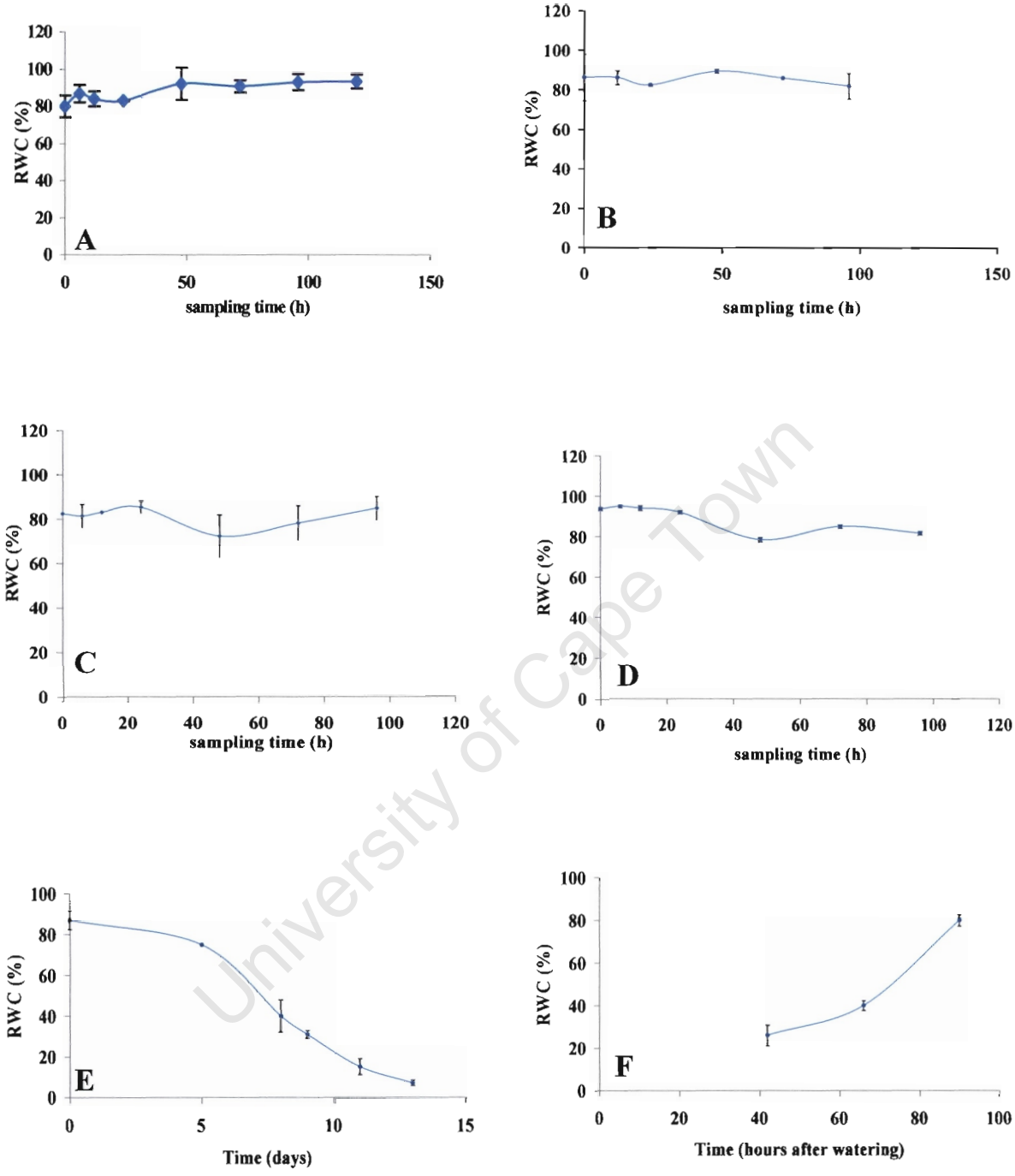


Figure 5.3.1: Relative water contents (RWC %) of leaf tissues at each sampling point. **(A)** low temperature treatment (4°C), **(B)** ABA (100 µM) treatment, **(C)** NaCl (150 mM) treatment, **(D)** heat shock (42°C) treatment, **(E)** dehydration treatment and **(F)** rehydration treatment.

***XVEF* Northern analysis**

The *XVEF* transcript was only seen after 48 hours of the ABA treatment but RNA apparently did not persist throughout treatment and after 72 hours there was no further indication of its presence (Figure 5.3.2 A). During the cold treatment, *XVEF* transcript accumulated between 12 and 24 hours exposure to cold, but once again did not persist thereafter. From the loading control image (Figure 5.3.2 B, lower panel), it is evident that at t=24 hours the 18S band has electrophoresed differently to the rest of the samples. This is possibly due to high amounts of polysaccharides that remained with the RNA after isolation and this in return negatively affected the migration of the RNA sample. There was no *XVEF* transcript present at t=0 hours in either the ABA or cold treated plants. This suggests that *XVEF* is a stress-inducible gene with transcription occurring only after specific duration into the stress treatment. The loading controls also indicate that unequal quantities of RNA were loaded and this could affect the transcription profile and should be repeated to confirm results.

It is clear that transcription only occurs at very distinct times, alluding to the possibility that this calcium-binding protein is temporally regulated. Frandsen et al., 1996 demonstrated that the *EFA27* (new designation, *OsClo*) orthologue from rice was responsive to ABA (50 µM) and NaCl (200 mM) treatment at the RNA level in various tissue specimens such as roots and shoots. However, a higher level of transcript was found in the shoots. Transcripts of another orthologue, *HvCaBP1* from barley, also accumulated in response to ABA and other hormone treatments such as giberrillic acid (GA), methyl jasmonate (MeJA) and 6-benzylaminopurine (6-BAP) (Jang, et al. 2003). The effect of changes in $[Ca^{2+}]_{cyt}$ in response to cold has been well documented with

respect to the calcium sensor calmodulin (Knight et al., 1996; Allen et al., 2000), so it is likely that *XVEF* could be involved in modulating calcium signals brought on by stress adaptations in response to the low temperature stress.

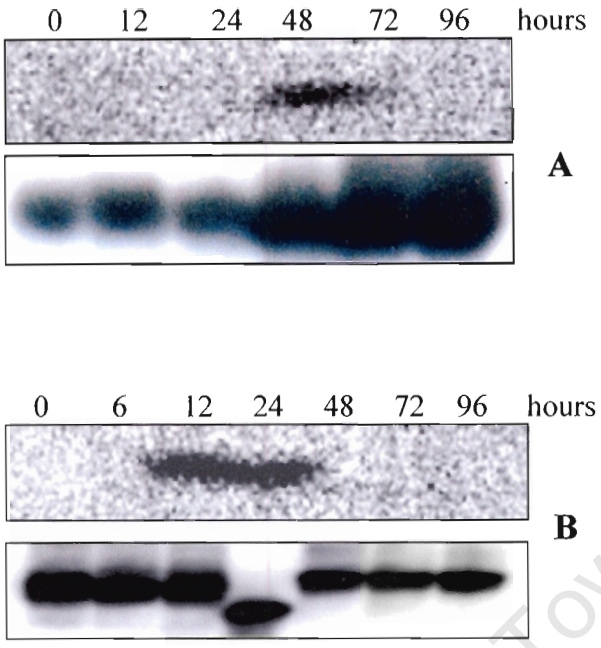


Figure 5.3.2: Northern blots of *XVEF* expression after leaves were subjected to 100 μM ABA (A) or a low temperature stress at 4°C (B). Upper panel represents blots probed with radiolabelled *XVEF* DNA and lower panel represents blots probed with radiolabelled 18srDNA.

The study of the expression of other orthologues was mainly dedicated to localizing the protein during specific developmental stages and by cellular position to ascertain where the protein is being expressed (Næsted et al., 2000). Western blot analysis using antibodies raised against the Arabidopsis orthologue was used to localize caleosin isoforms to the ER and lipid bodies in *Brassica napus* (Hernandez-Pinzon et al., 2001). These studies would imply that the *XVEF* orthologues are responsive to a range of stress treatments, but the location of that protein is important to infer its role in stress responsive signaling. Perhaps studies such as immunolocalization involving electron microscopy (EM) could be used to ascertain where *XVEF* was situated in the cell.

XvCaM Northern and Western analysis

The expression of *XvCaM* (total RNA) was investigated in response to heat shock (42°C), low temperature stress (4°C) and a dehydration/rehydration treatment as shown in Figure 5.3.3 A, B and C. Transcripts were present at t=0 hours and no quantitative variation was seen in response to the heat shock (A). A slight increase in transcript was seen at and after 24 hours, with a decrease after 96 hours in response to low temperature (B). This was not seen as highly distinctive when compared to the loading control, which indicated that there was a significantly lower amount of RNA present at 96 and 120 hours. *XvCaM* transcript seemed constitutively expressed as no significant variation in total RNA was seen. However, transcript quantities varied in response to the dehydration stress (Figure 5.3.3 D). Transcripts were first seen at 75% RWCs and remained until 31%, where a disappearance of transcript was maintained into the dry state. Upon rehydration, transcripts were readily evident, even as early as 26% RWC. At 85% RWC, no transcript is seen on the autoradiograph. It is likely that not enough RNA was loaded on the gel to produce detectable levels of *XvCaM.* The corresponding blot probed with 18S supports this idea as there is much lower levels of 18S transcript present at 85% RWC than at any other sampling point. It is also possible that because autoradiograph film was used to expose the northern blot instead of a storage phosphor screen, the lowered sensitivity of the film could have resulted that no transcript was visible on the final image. Thus, it appears that *XvCaM* transcript is present under non-stress conditions.

It was then decided to investigate whether there is variation in the polysomal RNA in response to salt stress. Total RNA is also referred to as 'steady state' RNA and it

represents all RNA that is being generated and degraded. Polysomal RNA represents those transcripts that are actively being translated and are attached to ribosomes. Figure 5.3.3 C shows a relatively constitutively expressed profile of RNA was apparent except for a potential decline in transcript at t=24 hours. This could be attributed more to a degree of RNA degradation than to the possibility of down-regulation.

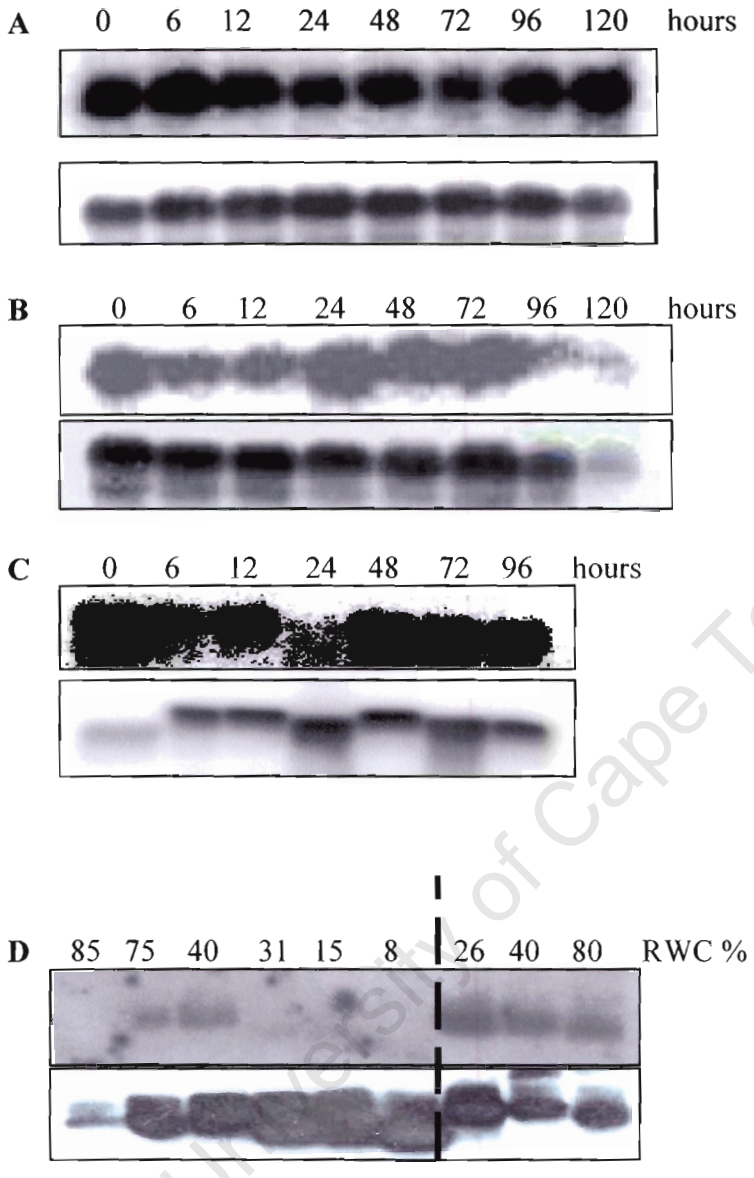


Figure 5.3.3: Northern blots probed with *XvCaM*. Upper panel represents blots probed with radiolabelled *XvCaM* and lower panel represents blots probed with radiolabelled 18srDNA. **(A)** heat shock (42°C) treatment, **(B)** low temperature treatment (4°C), **(C)** NaCl (150mM) treatment, polysomal RNA was used **(D)** dehydration/rehydration treatment. ▬ ▬ ▬ denotes where the rehydration samples were loaded on the gel.

XVCAM protein expression was investigated only under dehydration/rehydration stress, NaCl and exogenous ABA (Figure 5.3.4). No protein was detected under ABA stress (results not shown) and under the NaCl stress a clear accumulation of protein was present only after t=24 hours (Figure 5.3.4 B). XVCAM accumulated under dehydration when the plant reached 15% RWC, and the protein was retained until rehydration to a RWC returned to 40%. At 80% RWC after watering protein was considerably reduced (Figure 5.3.4 A). These results suggest that XVCAM is highly stress-responsive at a protein level. Interestingly, the corresponding northern blots for the NaCl and dehydration stress do not follow the conventional processes of an increase in transcript corresponding to an increase in protein. It seems that the RNA is present at 75 to 40 % RWC and the protein is then made. The protein appears at low RWC (15 and 8% RWC) and remains until rehydration to 40% RWC. From the loading controls (Figure 5.3.4, lower panel) it is evident that varying amounts of protein was present on the western blots. This could hinder interpretation of results and should be repeated to confirm results.

It is possible that during drying, the transcript is sequestered by storage proteins called mRNPs (messenger ribonucleoprotein particles). Studies conducted in the moss *Tortula ruralis,* demonstrated that during slow desiccation, mRNA is stored to speed the recovery process upon rehydration (Oliver et al. 1998; Wood & Oliver, 1999). This could represent a critical stage where the transcript had already been made and stored, but the protein is not yet required. Perhaps the mRNAs are stored during drying so that rapid recovery upon rehydration can occur as all elements for recovery are available (Cooper & Farrant, 2002).

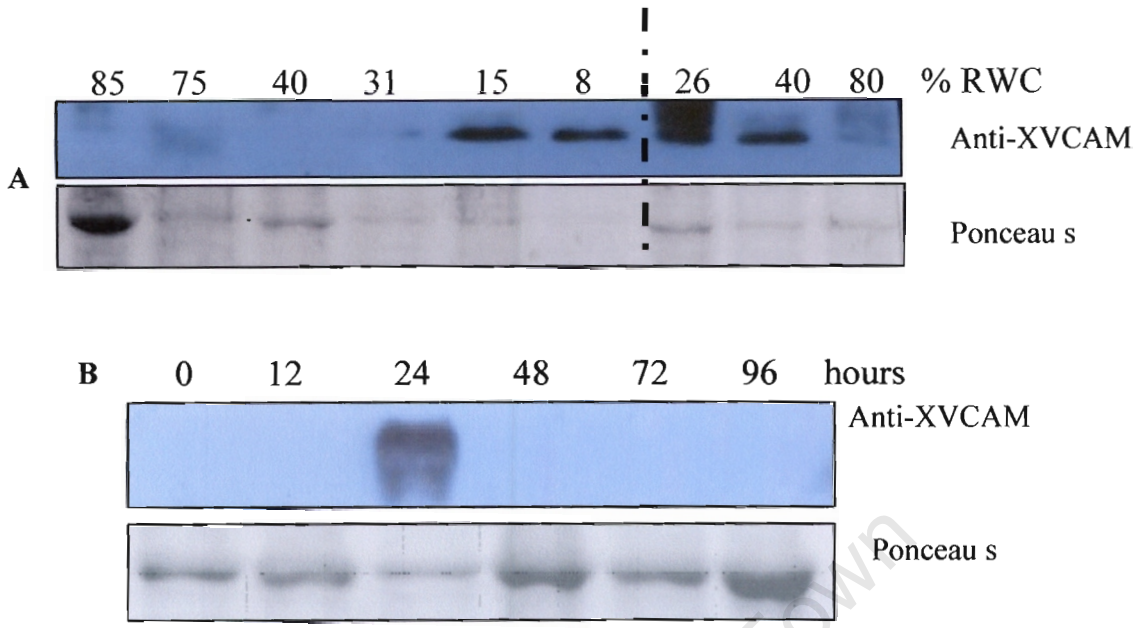


Figure 5.3.4: Western blot of leaves at various water contents (A) and NaCl treated (B) probed with anti-XVCAM antibodies in upper panel. Lower panel represents membrane stained with Ponceau S and acts as loading control. Each set of panels is labelled with a box indicating the stress treatment used. - - - denotes where rehydration samples were loaded on the gel

The XVCAM expression pattern for the NaCl treatment also poses a few questions. The northern blot for this treatment was performed using polysomal RNA. Transcripts were present at all sampling points, but when compared to the western blot, the protein is only present at 24 hours. This could be attributed to post-translational control via turnover regulation. Protein degradation has been cited as a key factor to manage proteins in the cell, for example if proteins are damaged during drying, proteases could degrade the protein to make available amino acids for new protein synthesis (Ingram and Bartels, 1996). Similarly, ubiquitination also plays a role in desiccation tolerance (O'Mahony and Oliver, 1999). These processes could be degrading XVCAM shortly after translation and therefore RNA transcript is present in the polysomal fraction but not the protein. Having

a calcium sensor such as calmodulin readily availability seems logical as transient increases in cytosolic calcium occurs at rapid rates (Trewavas, 1999). These calcium signals need to be modulated by sensor proteins that are activated by calcium and mitigate downstream cellular processes such as gene expression (Pittman & Hirschi, 2003; Zielinski, 1998). Perhaps under specific stress conditions XVCAM is modified to reduce its turnover rate. If for example, XVCAM undergoes a cycle of translation and ubiquitination, then conceivably XVCAM could experience modification such as phosphorylation, which could block ubiquitination sites and render XVCAM available for calcium signaling pathways (Roberts et al., 1992; Parag et al., 1993).

These discrepancies between the appearance of RNA transcript and protein during the dehydration and NaCl stress treatments, suggest that the type and possibly extent of stress dictates the pattern of the protein expression. These results are an indication of the various levels of regulation that occur in the cell. Calcium-binding proteins sense changes in cytosolic calcium, which are transient reactions in the cell. It is possible that post-transcriptional and post-translational modifications are necessary to effectively manage the function of this protein and its role in stress responsive calcium signaling.

Chapter 6

General Discussion

Resurrection plants are known to use stress responsive protection mechanisms that give them the capacity to withstand harsh environments. These mechanisms are controlled as a result of signaling cascades initiated by the stress perception. The role of calcium-binding proteins as modulators of transient increases in cytosolic calcium in calcium signaling (Snedden & Fromm, 1998; Zielinski, 1998; Reddy, 2001) and their role in the stress responses in *X. viscosa* is the primary focus of this body of work.

The *XVEF* cDNA was isolated from a differential screen made from dehydrated *X. viscosa* leaf material. The full-length gene was generated using PCR techniques adapted from the SMART RACE kit (D. Chopera, pers.comm.) (Figure 2.3.1). Southern blot analysis confirmed the presence of the gene in the *X. viscosa* genome and it also suggested that *XVEF* could possibly be present as a single copy gene (Figure 2.3.7). Sequence analysis revealed that XVEF has one EF-hand, a putative transmembrane domain and possible phosphorylation sites (Figure 2.3.2). These features are highly conserved in genes termed caleosins (Chen et al., 1999) (Figure 2.3.4). Caleosins are calcium-binding proteins similar to oleosins, which were found to be specific to lipid bodies and most are seed-specific (Chen et al., 1999; Jang et al., 2003). Expression of the

rice orthologue *OsClo* was found to be upregulated in response to ABA and osmotic stress in vegetative organs, seedlings as well in late embryogenesis (Frandsen et al., 1996). Sesame caleosin (SiCLO1) is seed-specific as the mRNA is most abundant in embryonic tissue two weeks after flowering and disappears from mature seeds. Arabidopsis also possess orthologues to caleosin-like genes and a small gene family was reported (Næsted et al., 2000).

It was shown that the *XVEF* transcript was stress inducible in the leaves of *X. viscosa* and was present transiently after exposure to the imposed stress of low temperature (4°C) and ABA (100µM) (Figure 5.3.2). The calcium-binding capacity and possible phosphorylation sites of XVEF suggest that the protein can modulate its own function. Caleosins have been localized to lipid bodies and the ER and therefore possibly mediate calcium signals originating from ER calcium stores (Næsted et al., 2000; Hernendez-Pinzon et al., 2001). It is known that caleosins also aggregate to lipid bodies during seed desiccation (Chen et al., 1999). Since *XVEF* is present in vegetative tissues in response to stress, this adds to the growing evidence that *X. viscosa* utilizes seed-specific genes to confer stress tolerance in vegetative tissues (Mowla et al., 2002). A further study that may be undertaken to better understand the role of XVEF in vegetative tissues of *X. viscosa* is to repeat the northern analysis to confirm the upregulation in response to dehydration as this gene was isolated from a dehydration cDNA library. If antibodies are available, western blot analysis and immunolocalization should also be done to confirm the protein's presence and location. Membrane protein fractions could be probed in western analysis as the putative transmembrane domain may possibly target XVEF to

membranes. Studies should be attempted to localize the protein within the cell, which would help to predict its function.

XvCaM was isolated from a low temperature cDNA library. It codes for a calmodulin, atypically has three EF-hands (Figure 2.3.5) along with a range of phosphorylation sites, with casein kinase II (CKII) having four sites. CKII is a serine/threonine protein kinase and is repeatedly present across many species (Zielinski, 1998; Benaim & Villalobo, 2002). Sequence analysis indicated that it has high identity to calmodulins from a range of plant species (Figure 2.3.6). XVCAM was successfully expressed in a bacterial system. The resultant 6X His-tagged fusion protein was used for a $^{45}CaCl_2$ overlay assay to confirm that the XVCAM positively binds calcium (Figure 4.3.1). The protein was also used to raise polyclonal antibodies, which were subsequently used in the western analysis.

Northern blot analysis was conducted for *XvCaM* mRNA transcript expression under high (42°C) and low (4°C) temperature, 150 mM NaCl and dehydration/rehydration stress conditions. Total RNA from leaf material was used for all treatments except NaCl, for which polysomal RNA was used. *XvCaM* is constitutively expressed under most conditions, except dehydration. The constitutive expression of *XvCaM* could be attributed to the fact that it was not isolated from a differential screen (as was the case with *XVEF*) where gene expression under unstressed conditions is accounted for. This could explain the presence of constitutively expressed *XvCaM* throughout the stress treatments. The differences in transcript and protein expression profiles are very interesting and a number

of factors could contribute to it. It is probable that the DNA probe for *XvCaM* could detect other homologues in the genome (see Chapter 2) and could conceal possible changes in the transcript pattern of *XvCaM.*

The apparent decrease in transcript under dehydration could be attributed to sequestration into storage machinery such as mRNPs, which would promote the recovery process upon rehydration (Oliver et al., 1998; Wood & Oliver et al., 1999). Interestingly, transcript was present in northern analysis, even when polysomal RNA was isolated after the NaCl treatment although westerns blot analysis showed that XVCAM was present only at 24 hours post-exposure to NaCl treatment (Figure 5.4.4). It is possible that XVCAM could be degraded, for example via ubiquitination, shortly after translation if it is not needed immediately. A stress signal could possibly cause modification of XVCAM to block those ubiquitination sites and decrease the turnover rate (Roberts et al., 1992; Parag et al., 1993; O'Mahoney & Oliver, 1999). Studies in Arabidopsis to elucidate cold stress signaling pathways has revealed that HOS1, a negative regulator of gene expression in response to low temperature, was found to be a RING finger protein with ubiquitin E3 ligase activity. Thus providing further evidence that protein degradation plays a role in signaling pathways (Xiong et al., 2002). Further studies that should be performed are to confirm expression studies. Expression *XvCaM* of under dehydration/rehydration conditions using polysomal RNA should be investigated. The results could indicate whether the mRNA is stored between 31 and 8% RWC when total RNA decreases (Figure 5.3.3 C), and whether any active translation takes place in *X. viscosa* as the plant prepares for the dry state.

To postulate the role of XVEF and XVCAM in stress-responsive signaling in *X. viscosa,* a comparison can be drawn to one of the best characterized stress-responsive calcium-dependant signaling pathways, which is the Salt Overly Sensitive (SOS) pathway found in Arabidopsis (Zhu, 2002). The increase in cytosolic calcium that results after an increase in Na^+ is perceived is mediated by the calcium-binding protein SOS3. The calcium-modulated protein activates its target, SOS2, which is a serine/threonine protein kinase. This complex activates the vacuolar Na^+/H^+ antiporter to drive the removal of excess Na^+.

There are also SOS3 and SOS2 homologues in Arabidopsis called SCaBP (SOS3-like Ca^{2+}-binding protein) and PKS3 (protein kinase), respectively (Guo et al., 2003). It was illustrated that they are negative regulators of the ABA-signaling pathway.

It is therefore plausible that XVEF and XVCAM could behave in a similar manner to SOS3. They possess calcium-binding domains and numerous phosphorylation sites and thus would be able to interact with a protein kinase. An important question that remains relates to what the targets of both proteins are. Many proteins have been identified as targets of calcium-binding proteins and an example of a method to isolate calmodulin-binding proteins is to probe cDNA expression libraries with radiolabelled calmodulin. This method was used to isolate *AtCaMBP25* from Arabidopsis (Perruc et al., 2004) and *MCamb1* and *MCamb2* from *Physcomitrella patens* (Takezwa & Minami, 2004). *AtCaMBP25* was shown to be involved in stress signaling pathways by observing the

stress tolerance of antisense and overexpressing mutant lines. However, the antisense lines showed higher tolerance to stress and this indicated that *AtCaMBP25* negatively regulates stress tolerance. *MCamb1* and *MCamb2* were reported to be novel transporter-like proteins and highly stress responsive to low temperature, osmotic stress and ABA.

The fact that two stress responsive calcium-binding proteins were isolated from *X. viscosa* indicates that calcium sensors play an important role in stress tolerance signaling. These findings are important to our understanding of how resurrection plants function to bring about stress tolerance, but overexpressing such proteins in a crop might not have the desired result of stress tolerance (Sneddon & Fromm, 1998). It was shown that a higher level of stress tolerance was achieved when all three components of the SOS pathway were coexpressed in yeast than in single gene transformants (Xiong et al., 2002). The elucidation of downstream effector proteins of XVEF and XVCAM is imperative if these genes are to be used in transgenic studies. Therefore the study of calcium signaling components such as *XVEF* and *XvCaM* would be useful in understanding the stress tolerance responses required to generate stress tolerant crops.

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