XvERD15, an early-responsive gene to stress from *Xerophyta viscosa*

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Declaration

The experimental work described in this report was carried out in the Department of Molecular and Cell Biology, University of Cape Town, under the supervision of Assoc. Prof. SG Mundree, Prof. JA Thomson and Prof. JM Farrant.

The results presented here are the original, unaided work of the author. Where use has been made of the work of others it is duly acknowledged in the text.

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Abbreviations

ABA	abscisic acid
ABRE	ABA-responsive element
bp	base pair(s)
cDNA	copy DNA
DEPC	diethylpyrocarbonate
DRE	dehydration responsive element
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
g	grams
HCl	hydrochloric acid
kb	kilobase(s)
kD	kilodalton(s)
λ	lambda
l	litre(s)
m	molar concentration
µg	microgram(s)
mg	milligram(s)
$MgSO_4$	magnesium sulphate
µl	microlitre(s)
ml	millilitre(s)
mM	millimolar
min	minutes
mRNA	messenger RNA
NaCl	sodium chloride
Ni-NTA	nickel-nitrilotriacetic acid

nm	nanometre(s)
ORF	open reading frame
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RWC	relative water content
SDS	sodium dodecyl sulphate
u	unit(s) of enzymatic activity
UV	ultraviolet
w/v	weight per volume

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Abstract

Genes that are upregulated in the early response to stress are not well understood. *ERD15* (early-responsive to dehydration) in *Arabidopsis* and its homologues in various other plants have been shown to be upregulated within 1 hr post-exposure to dehydration and high salinity stress treatments. There is however limited literature on the functionality of ERD15. A cDNA showing homology to *ERD15* was isolated from a library generated by low temperature stress treatment of *Xerophyta viscosa* and was subsequently named *XvERD15*. Analysis of the *XvERD15* sequence suggested it may be a PABP-CT (poly(A)-binding protein carboxyl terminus) interacting protein. Southern analysis further suggested that there may be two copies of *XvERD15* in the *X. viscosa* genome. This was verified by the isolation of *XvERD15* genomic sequence. The expression patterns of *XvERD15* for abscisic acid, high salinity, low temperature and water-deficit stress indicate that transcripts accumulated in the early phases of stress at the mRNA level. Recombinant XvERD15 proteins were expressed and used as an antigen to generate antibodies for western blotting. Western blot analyses indicate that XvERD15 accumulated under abscisic acid, high salinity and water deficit stress. Two *XvERD15* promoter sequences were isolated and several putative cis-regulatory elements were present. This work contributes to the elucidation of stress tolerance in *X. viscosa*.

Chapter One

Introduction and Literature Review

1.1 Global food 'insecurities'

It is projected that the world population will increase to 9.3 billion by 2050. At present approximately 840 million people are suffering from acute or chronic malnutrition. Thirty-nine countries in the world are facing food security crises. Of these, 25 countries are in Africa [9;24]. Fifteen million people in southern Africa alone are faced with malnutrition and food insecurity [78].

Dramatic environmental changes are one of the leading causes of numerous drought episodes experienced in Africa. Global warming has elevated environmental temperatures and this may induce drought conditions [28;47]. It has been postulated that the erratic climate changes will cause more droughts in Africa and an additional 30 million people will be affected by the year 2050 [9]. The majority of people affected by these periodic events are often the economically disadvantaged in developing nations. The availability of arable land for agriculture is decreasing due to factors such as soil erosion, desertification, deforestation and environmentally damaging agricultural practices [57]. Declining resources such as water would decrease crop yields even further [26]. Factors affecting the growth and development of crops are significant, since they affect the quantity and quality of food produced.

To meet the future increases in world food requirements, plant biotechnologists are pursuing the option of genetically manipulating agronomically important crops, imparting to them traits such as drought tolerance or disease/pest resistance. This strategy aims to increase agricultural output using existing land resources or to exploit land resources currently unavailable for agriculture. The investigation of drought tolerant plants at a genetic level may be an essential tool in developing crops that could withstand harsh environmental conditions.

1.2 Resurrection plants, a possible solution?

A small group of angiosperms, known as resurrection plants, are unique models of desiccation-tolerance. Up to 330 resurrection species have been found to tolerate desiccation [19;55]. Resurrection plants are relatively widely distributed and are found in southern Africa, eastern South America and western Australia (except Antarctica) [19]. These extremophiles have developed mechanisms that allow them to withstand a loss over 90 % of their relative water content and recovers rapidly when water becomes available [2;18;71]. They often grow in shallow soil on rocky outcrops where water is limiting [70]. The growth and reproduction of these plants occurs when water is available, but upon drying they remain quiescent for long periods of time [67].

Desiccation-tolerant species are classified into two categories as either fully-desiccation tolerant or modified-desiccation tolerant plants [51;52]. Fully-desiccation tolerant species such as algae, bryophytes and lichens experience extreme drying rates and may reach air dryness within an hour. They can withstand a total water loss and their internal water content rapidly equilibrates to that of the environment [52]. As for the modified-desiccation tolerant species such angiosperms and ferns, the rate of water loss is critical for its survival as tolerance to desiccation is only achieved over several hours or days. Various essential events and protective measures are established in this period to prepare the plant for desiccation tolerance.

Xerophyta viscosa (Family *Velloziaceae*) is a monocotyledonous plant that belongs to this sub-group of resurrection angiosperm [18;49]. Understanding desiccation tolerance in *X. viscosa* may contribute ultimately to increasing the stress tolerance in crops such as maize and rice. *X. viscosa* can be dehydrated down to 5% relative water content (RWC) (*Figure 1.1*). When watered, the plant 'resurrects' within 80 hours and resumes full metabolic functions [72].

Figure 1.1 Xerophyta viscosa in its fully hydrated (left) and dehydrated state (right)

1.2.1 Surviving water deficit

Any environmental stresses affect plant water status and impair the availability of water in plant as a solvent, transport medium and evaporative coolant [4]. Water is essential for plant survival. The mechanisms by which plants respond to water deficit range from whole plant level to cellular level.

During extreme water deficit, subcellular damage such as denaturation of proteins, concentration of solutes, changes in cell shape and loss of membrane integrity occur [5]. The mechanisms through which these damages occur vary, but often occur via free-radical imposed denaturation [76]. Chloroplasts are susceptible to photo-oxidative damage during water-deficit stress. This results in a decrease photosynthesis and ultimately, a decrease in all metabolism [77].

To counteract the damaging consequences of water-deficit, some resurrection plants, such as *X. viscosa* have developed mechanisms that are known to prevent chlorophyll-light interactions by degrading chlorophyll and dismantling thylakoid membranes. These species are poikilochlorophyllous. During water-deficit, homoiochlorophyllous species utilise mechanisms such as leaf folding and accumulation of anthocyanin to protect chloroplasts, as seen in *Craterostigma* spp [71;72;82;83].

Resurrection plants are unique models to study water-deficit tolerance since they have developed mechanisms to counteract this and other abiotic stresses. These plants house genes, of which many are currently being studied, to enable stress tolerance. It is the

contribution of many mechanisms, both at a whole plant level as well as the integration of cellular responses that allows plants to respond and survive cellular water-deficit [5;73].

1.3 Perception of stress and regulation of gene expression

In response to stress conditions, specific genes are induced at a transcriptional level and encode proteins that play essential roles in counteracting the stress conditions and increasing stress tolerance [73;75;85]. Stress-inducible genes can be classified into two major groups. The first is involved directly in the protection of the plant cell, membranes and proteins. These include genes that code for putative proteinases, chaperones, water channel proteins and late embryogenesis abundant (LEA) proteins. The second group is involved in signalling cascades and regulation of gene expression where gene products include transcriptional factors, protein kinases and enzymes that participate in phosphoinositide metabolism [69;85].

Abiotic stresses such as water-deficit, extreme temperatures (high/low) and high salinity are often interconnected, resulting in water-deficit which cause cellular damage [85] (*Figure 1.2*). A cellular signal transduction pathway is initiated when stress is perceived by plant sensors. A physical stress is converted into a biochemical response. This is known as signal perception [5].

The responses of plants during stress are regulated by multiple pathways that activate transcription [85]. In response to abiotic stresses, genes encoding proteins that are involved in signal transduction cascades are up-regulated [73-75;85]. These are known as stress-inducible transcriptional factors. Most belong to multi-gene families such as the dehydration-responsive binding proteins (DREB), mitogen-activated protein (MAP) kinases, C-repeat binding factors (CBF) family, the basic-domain leucine zippers (bZIP) and the ethylene-responsive binding factors (ERF). Individual transcriptional factors of the same family respond to various stress stimuli differently and could regulate various stress-inducible genes, separately or co-operatively [75;85]. The molecules involved in the activation of signal cascades during abiotic stress have not been well studied.

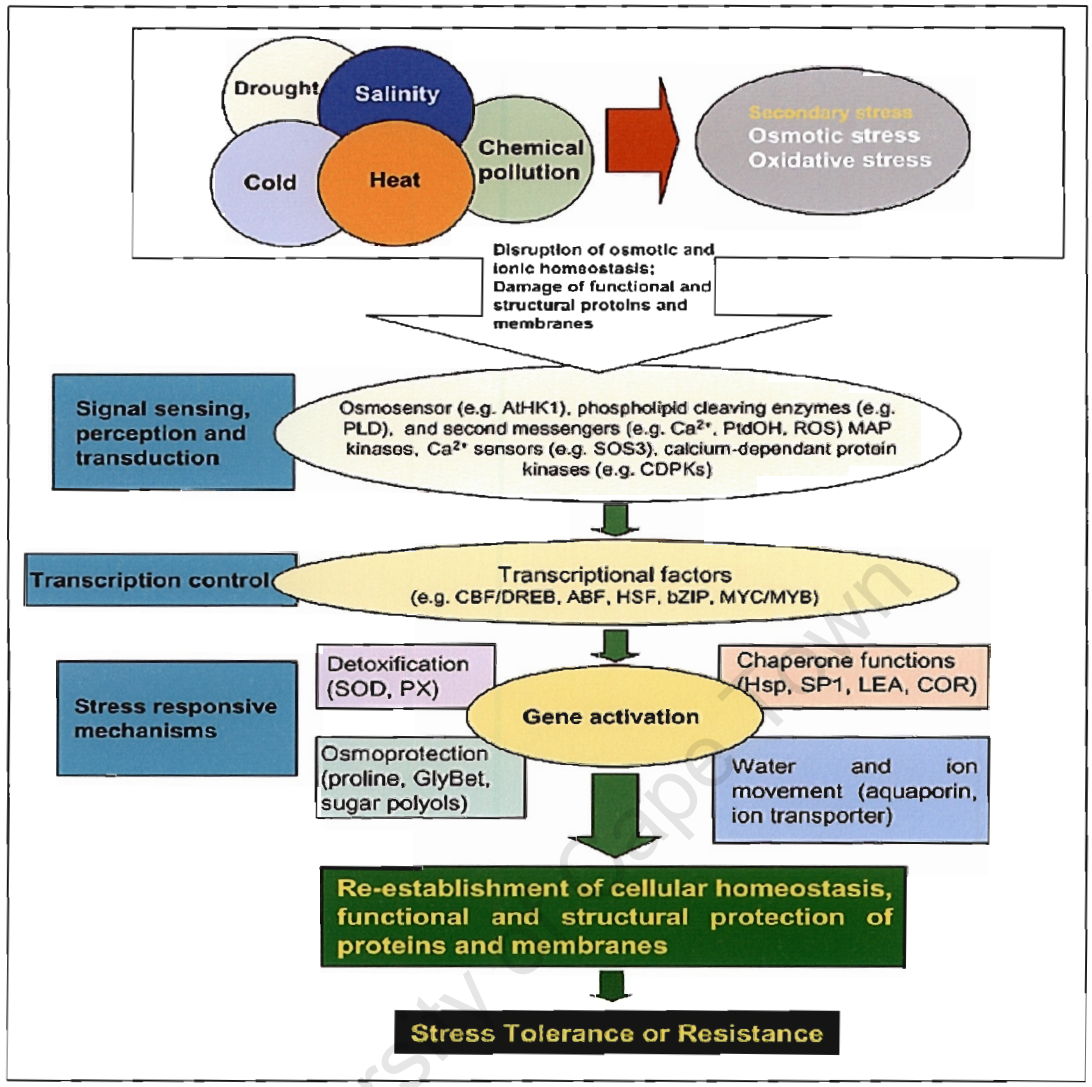

Figure 1.2 The signal transduction pathways of plant response to abiotic stress (adapted from Wang *et al.* [85])

1.3.1 Abscisic acid dependent and independent gene regulation

Abscisic acid (ABA) is a phytohormone and signal transmitter that plays a key role in many plant stress responses. Elevated levels of ABA have been observed mainly under water-deficit and high salinity stress but not under low temperature stress [75;88]. ABA is involved in stomata closure and the induction of many genes [5;75]. Some genes are induced very rapidly while others are induced less rapidly after the onset of ABA accumulation [73;88]. Certain genes induced by dehydration stress were not induced by exogenous ABA treatment, therefore it has been hypothesised that both ABA-dependent

pathway and ABA-independent pathways exist. The complex regulation of gene expression in response to water-deficit and cold stress is highly interconnected in both the ABA-dependent and independent pathways, as schematically represented in *Figure 1.3*.

Two major *cis*-acting elements, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE)/C-repeat (CRT), are usually present in the promoter regions of most stress-inducible genes [75] (*Figure 1.3*). Many ABA-independent genes share the DRE/CRT *cis*-acting element. The dehydration-responsive transcription factors (DREB/DREB2) and C-repeat binding factors (CBF) bind to the DRE/CRT *cis*-acting elements and activate ABA-independent gene expression. Both the DREB1A/CBF3 and DREB2 contain the same motif (GCCGAC) [73]. The expression patterns of DREB genes suggests that DREB1 proteins are cold-responsive while DREB2 is induced by dehydration [21;45]. Transcriptional activators DREB1a and DREB2A bind specifically to DRE/CRT. Transgenic plants overexpressing DREB1/CBF showed improved tolerance when exposed to low temperature, drought and high salinity [64]. Other genes that are regulated by the ABA-independent pathway are the *rd19* and *rd29* encoding cysteine proteases [41]. Transgenic plants overexpressing *ICE1* (Inducer of CBF Expression1) showed an improved tolerance to low temperature stress. The activation of *ICE* proteins requires a cold signal but the mechanism of this process is still not understood [75].

Many of the ABA-inducible genes share an ABRE *cis*-acting element, with the ACGTGGC consensus sequence [85]. The transcription factors ABRE-binding protein (AREB)/ABRE-binding factor (ABF) bind to the ABRE *cis*-acting element and activate the ABA-dependent gene expression [75] (*Figure 1.3*). During drought stress, a gene encoding 9-cis-epoxycarotenenoid dioxygenase (NCED) involved in ABA biosynthesis is highly induced. The regulatory factors concerning the expression of *NCED* during drought stress are not clearly understood [75]. One possibility discussed by Xiong *et al.* [88] is that the *NCED* could be activated through a Ca^{2+}-dependent phospho-relay cascade experienced during drought and salinity stress. A drought inducible gene, *rd22*, is mediated by the presence of ABA [89]. The co-operative binding of MYC (from

Myelocytoma, a virus induced tumor), *rd22BP*, and MYB (from Myeloblastoma), *AtMYB2* transcriptional factors, to the MYBR and MYCR *cis*-elements of the *rd22* promoter region, is required to activate *rd22* [1]. Recent studies have shown that SnRK2 and SnRK3 protein kinases may be involved in the activation of transcription factors for ABA-responsive gene expression [90] (*Figure 1.3*).

The DRE/CRT and ABRE cis-acting elements are thought to work independently. However, it has been observed that DRE/CRT functions cooperatively with ABRE in *RD29A* gene expression as a coupling element in response to drought stress [50]. This suggests there is a degree of cross-talk between different stress signalling pathways [75] (*Figure 1.3*). The complexities of these pathways are further being elucidated.

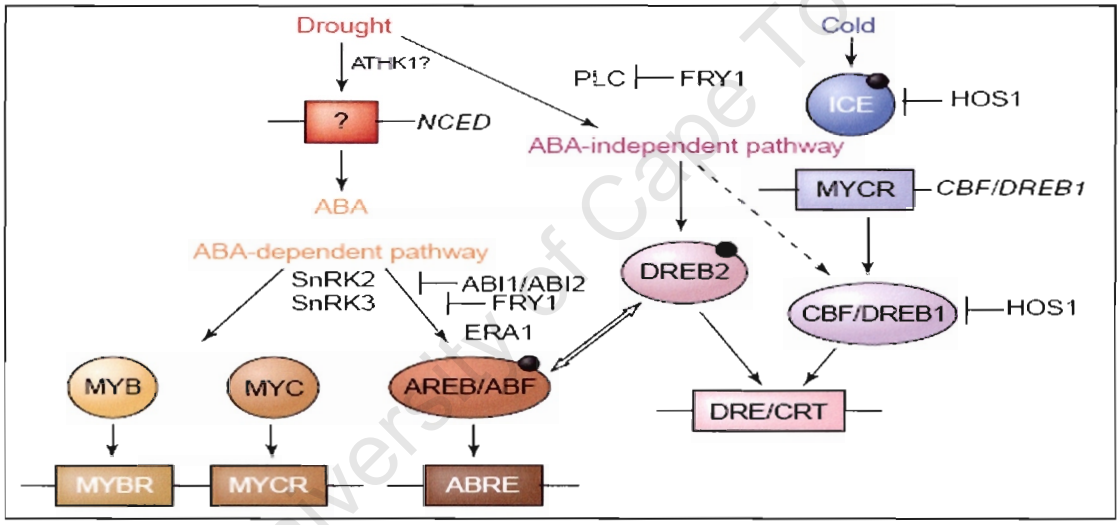

Figure 1.3 Regulatory network of gene expression in response to drought and cold stress (adapted from Shinozaki *et al.* [75])

1.4 Early response to dehydration

The mechanisms by which plants respond to dehydration in the early stages are not well understood at a cellular level. The expression patterns of around 7000 *Arabidopsis* genes were monitored. It was found that transcript of 245, 54, 213 and 299 genes increased by more than 5-folds while a total of 34, 77, 79 and 70 genes were down-regulated after ABA, dehydration- , salt- and cold-stress treatments respectively [75].

Genes up-regulated in the early response to stress were isolated from *Arabidopsis thaliana* L. (Columbia ecotype) dehydrated for 1 h. Twenty-six cDNA clones were isolated by differential screening. The clones were classified into 16 groups based on Southern blot hybridisation and were named ERDs (early-responsive to dehydration) [37]. Sequences of ERD clones were compared to DNA sequences stored in the GenBank and putative functions for the different ERD genes were assigned (*Table 1.1*). Analysis of the sequences revealed that these ERD proteins have different functional roles and must be characterised individually to uncover how each counteract stress conditions.

Table 1.1 Characterisation of ERDs from Arabidopsis and their respective inductions under salinity (green), abscisic acid (yellow), low temperature (blue) and dehydration (red) stress treatments. Up-regulation (✓) and no up-regulation (×)

Gene	Feature	Up-regulation under stress			
ERD1	ClpA/BATP-dependent protease [35]	✓	×	×	✓
ERD2	Heat shock protein [37]		✓		✓
ERD3	No homology [79]				✓
ERD4	Membrane protein [79]				✓
ERD5	Proline dehydrogenase [39]				✓
ERD6	Sugar transporters [34]			✓	✓
ERD7	No homology [79]				✓
ERD8	Heat shock protein [37]		✓		✓
ERD9	Glutathione S-transferase [79]				✓
ERD10	Group II Lea protein [36]		✓	✓	✓
ERD11	Glutathione S-transferase	✓	×		✓
ERD12	No homology [79]				✓
ERD13	Glutathione S-transferase	✓	×		✓
ERD14	Group II LEA protein [36]		✓	✓	✓
ERD15	Hydrophilic, acidic, lacks Cys [38]				✓

The gene of interest in this study is the homologue of *ERD15* found in *X. viscosa* [38]. Due to limited literature on *ERD15*, not much is understood of the functional roles it plays in the early stages of stress. Up-regulation of *ERD15* expression has been observed in various studies and its function is currently being elucidated.

1.4.1 Expression of *ERD15*

ERD15 is a small 163 amino acid protein, acidic (isoelectric point of 4.36), hydrophilic and lacks cysteine residues. Kiyosue *et al.* [38] observed an increase in *ERD15* transcripts in dehydrated *Arabidopsis* plants within 1 h and the levels remained elevated for up to 24 hrs.

Dunaeva and Adamska [17] isolated genes that were upregulated from light stressed leaves of *Arabidopsis*. A cDNA clone identical to *ERD15* was termed *Lsr1* (light stress-regulated). *Lsr1* transcripts increased 8-fold after 2 hrs of light stress and an increase in transcripts by 3.4- and 3.1-fold was observed for low temperature (4 ^{o}C) and water-deficit stress treatments respectively. In addition, the transcript increased 1.9-fold in oxidative (H_2O_2) stressed leaves. However, no increase in *Lsr1* transcripts was observed for wounding, salinity or UV-A stress treatments. The authors concluded that *Lsr1* was not specifically regulated by light stress due to the increase in transcripts caused by other stress conditions. Furthermore, a significantly higher transcript level was observed in light stress as compared to other abiotic stress conditions imposed.

Differential expression of *ERD15* was also noticed when *Arabidopsis* was treated with the plant growth-promoting rhizobacterium, *Paenibacillus polymyxa*. The mRNA levels for *ERD15* using RNA-differential display was 50 times higher in treated plants compared to the untreated [81]. A possible explanation for this could be that the treatment with the rhizobacterium may have led to stunted growth of the roots, which may have affected the availability of intracellular water and led to a dehydration response by inducing *ERD15* gene, since *ERD15* was previously shown to be drought-stress responsive.

The unique early responsive nature of *ERD15* has also been observed in salinity stressed (150 mM NaCl) maize roots [84]. From EST-based microarray analysis conducted by Wang *et al.* [84], it was observed that 916 of 7943 ESTs had changes in transcript levels throughout different time points. A clone encoding a protein similar to *ERD15* increased in transcript levels by 1.5-fold in 1 h and was elevated to 2.3-fold within 3 hrs post-exposure.

Surprisingly, an increase in *ERD15* transcripts was also observed in hypergravity treated *Arabidopsis* hypocotyls when seedlings were grown in centrifuge tubes under basipetal hypergravity at 1 *g* or 300 *g* for 57 h [91]. The authors suggested that the transmission pathway of gravity signals could be connected with environmental signalling such as temperature and dehydration.

1.4.2 Putative functions of genes sharing conserved sequence domains with ERD15

Two proteins, PCI6 (PABP-CT interacting) and PCI243 that interact with the carboxyl-terminus (CT) of poly(A)-binding proteins (PABPs), were identified from a two hybrid cDNA library by Wang and Grumet [86] using C-terminal 254 amino acids of *Cucumis sativus* (cucumber) [87]. A ubiquitously conserved 12 amino acid motif, SxLnpnApxFxP, shared amongst most PABP-CT interacting proteins from humans, yeast, *Drospholia*, *Xenopus* and even viruses, was also found towards the N-terminus of *ERD15*. Furthermore, the authors demonstrated that *Arabidopsis* ERD15 interacts with cucumber PABP [86]. In order to understand the functional roles PABP-CT interacting protein and thus the possible roles of *ERD15,* an overview of PABPs is given below.

1.4.3 What are PABPs?

PABPs are a class of proteins that bind to the poly(A) tail of mRNAs in eukaryotic cells. They are well documented in a broad range of organisms [3]. The inhibition of PABP in yeast either by promoter inactivation or deletion of PABP-coding region leads to a decrease in translation initiation, poor cell growth and the deletion of the *PAB1* gene leads to a lethal phenotype [62;63]. Studies by Kerekatte *el al.* [31] have shown that infection of HeLa cells by coxsackievirus, a virus responsible for the cleavage of PABP, resulted in the inhibition of host translation.

PABPs are surprisingly abundant: 0.6 % of total cellular proteins of eggs and embryos from sea urchins are of PABPs [16] and there are as high as 8 x 10^6 PABPs per *HeLa* cell resulting to approximately six molecules per ribosome [22]. PABPs are phylogenetically conserved, sharing the four RNA recognition motifs (RRMs) or ribonucleoprotein (RNP) domains. The four RRMs are arranged in tandem and are connected to each other by short linker sequences. The proline-rich C-terminal domain

is associated with the four RRMs by a proline- and glutamine-rich linker. X-ray crystallography studies have revealed that the RRMs consists of four anti-parallel beta strands backed by two alpha-helices [43;53] (*Figure 1.4*). Another highly conserved domain of 75 amino acids is present at the C-terminal portion. The RRMs containing two conserved sequence motifs, RNP1 and RNP2, come in direct contact with the RNA while the C-terminus does not contribute to the binding to the poly(A) [6].

The functions of the C-terminus domain are not well understood. However studies by Coller *et al.* [10] suggest that the C-terminal 50 amino acids in yeast PAB1 are required to assist cell viability, indicating that they are essential for cellular functions. NMR and X-ray crystallography has characterised the C-terminal domain to be an independently folded unit consisting of four or five alpha-helices [14] where PABP-CT interacting proteins carrying the 12 amino acid motif associate [86].

Various studies have shown PABPs to have essential roles in eukaryotic cellular functions and participate in the vital steps of pre-mRNA processing for the initiation of translation [63]. PABP is an essential precursor of mRNA translation and plays a vital role in the initiation of translation.

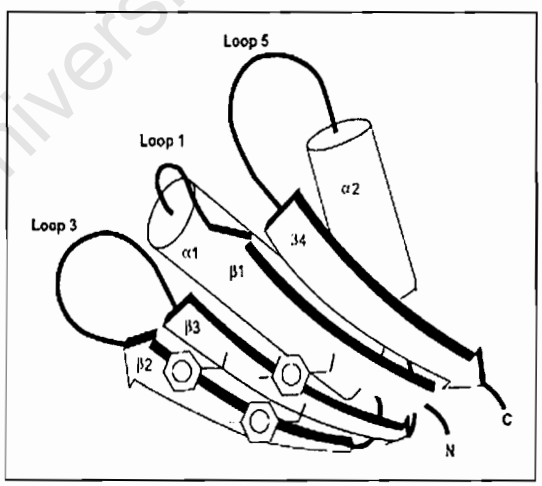

Figure 1.4 The tandem arrangement of RNP or RRM fold of poly(A) binding proteins (adapted from Kühn and Wahle [43])

1.4.4 Overview on initiation of translation

Most mRNA in eukaryotes undergo modification that includes the addition of the cap structure (7-methyl-guanosine, m^7GpppN, where m is a methyl group and N represents any nucleotides) at the 5'-terminus and the poly(A) tail at the 3'-terminus by poly(A) polymerase [56]. The result of this nuclear processing allows the binding of various cap-binding proteins including the conserved eukaryotic initiation factors (eIFs) and poly(A)-binding proteins to the mRNA termini. The recruitment of these elements synergistically promotes the initiation of translation [48].

The steps of translation initiation are illustrated in *Figure 1.5*. The formation of the 43S complex which consists of the Met-$tRNA_i$, GTP and eIF2, whereby it associates with the 40S ribosomal subunit, is one of the first steps in the initiation of translation. Another complex, eIF4F (also known as eIFiso4F in plants), which consists of eIF4E, eIF4G and eIF4A, assists the recruitment of the 43S complex to the 5' cap structure.

The eIF4E, a modulator of cell growth and proliferation, binds directly to the 5' cap structure. The eIF4A, an RNA-dependent RNA helicase, with eIF4B unwinds the 5'-UTR region of the mRNA with energy provided by ATP hydrolysis (*Figure 1.5*). This promotes ribosome binding and allows efficient scanning of the 5' leader [25;48].

The eIF4G serves as a modular scaffolding protein for binding of eIF4E, eIF4A, eIF3 and Mnk, a MAPK-activated protein kinase that is responsible for the phosporylation of eIF4E [58]. The eIF4G also interacts with PABP at the second RRM [32]. Once the eIF4G and the eIF3 recruit the 43S complex to the 5' terminus, the complex scans the 5' leader to locate the initiation codon (*Figure 1.5*).

The 60S ribosomal subunit subsequently joins, forming the 80S ribosome and allowing translation to begin.

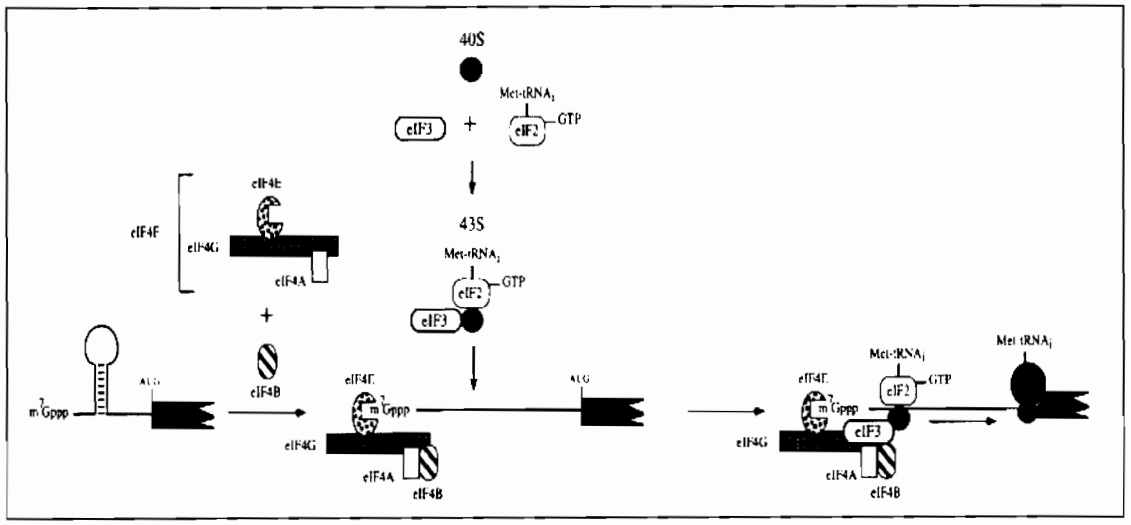

Figure 1.5 The summary of steps during translation initiation (adapted from Gallie [20])

1.4.5 Circularisation of mRNA

It has been well documented that PABPs participate in the circularisation of mRNA, referred to as the closed-loop model *(Figure 1.6a).* The physical interaction between PABP and eIF4G results in mRNA circularisation by bridging the 5'-cap and 3'-tail of mRNA. This protein-protein interaction at the extremities promotes synergistic enhancement of initiation and stimulates translation [30].

Three essential mechanisms have been proposed for the circularisation to increase translation: Firstly, the circularisation promotes the recycling and reinitiation of ribosomes on the same mRNA for enhanced translation. Secondly, PABP enhances the joining of the 60S ribosomal subunit to the translation complex. Lastly, PABP stabilises the circular structure by its interaction with eIF4G in the eIF4F complex and subsequently promotes 43S ribosomal subunit binding [80]. PABP in humans do not interact with eIF4G directly but through the interaction of an PABP-CT interacting-protein Paip1 and eIF4A [11] (*Figure 1.6b*) .

1.4.6 PABP-CT interacting proteins regulate PABP

In mammalian cells, PABP is regulated by two interacting proteins, Paip1 and 2. Paip1 shares homology with the central domain of human eIF4G. An increase in the

intracellular concentration of Paip1 enhanced the translation of a reporter mRNA in cultured-mammalian cells by 2.8-fold [11]. Paip1 is part a multi-protein complex involved in mRNA turnover. This complex binds to the major protein-coding-region determinant of instability (mCRD) of the *c-fos* proto-oncogene mRNA which stabilises the mRNA and impedes deadenylation [23].

Paip2 is a highly acidic protein which preferentially inhibits PABP-dependent translation of poly-adenylated mRNA [33]. The over-expression of Paip2 in *Drosophila melanogaster* repressed host translation, which resulted in a size reduction phenotype in various fly tissues [61]. Two possible mechanisms for this inhibitory activity on translation were proposed. Firstly, Paip2 may displace PABP from the poly(A) tail as observed in an RNA-binding assay and thus disrupts the circularisation of mRNA (*Figure 1.6c*). Secondly, Paip2 may compete with Paip1 for binding to PABP. Khaleghpour *et al.* [33] suggested that the Paip2 binding leads to a conformational change of PABP resulting in a decreased affinity of PABP for the poly(A) tail. Alternatively, Paip2 competes with the RNA for the RNA-binding sequence located on the PABP. Furthermore, since the binding sites of Paip2 and eIF4G on PABP overlap, it suggests that they compete for binding to PABP [30;61].

Glutathione-S transferase pull down experiments revealed that the PABP-interacting motif, PAM1 of Paip1 and 2 interacts with RRMs 1-2 and RRM 3-4 of PABP respectively. PAM2 of Paip1 and 2 interacts with the conserved C-terminal domain (last 138 aa) of PABP [60]. PAM2 is a conserved motif shared by various PABP-CT interacting proteins including the eukaryotic polypeptide chain releasing factor, eRF3 [27]; Ataxin2, a protein implicated in spinocerebellar ataxia [46]; the inducer of Erb*B*-2, Tob [14] and the Drosophila shuttle craft protein [42].

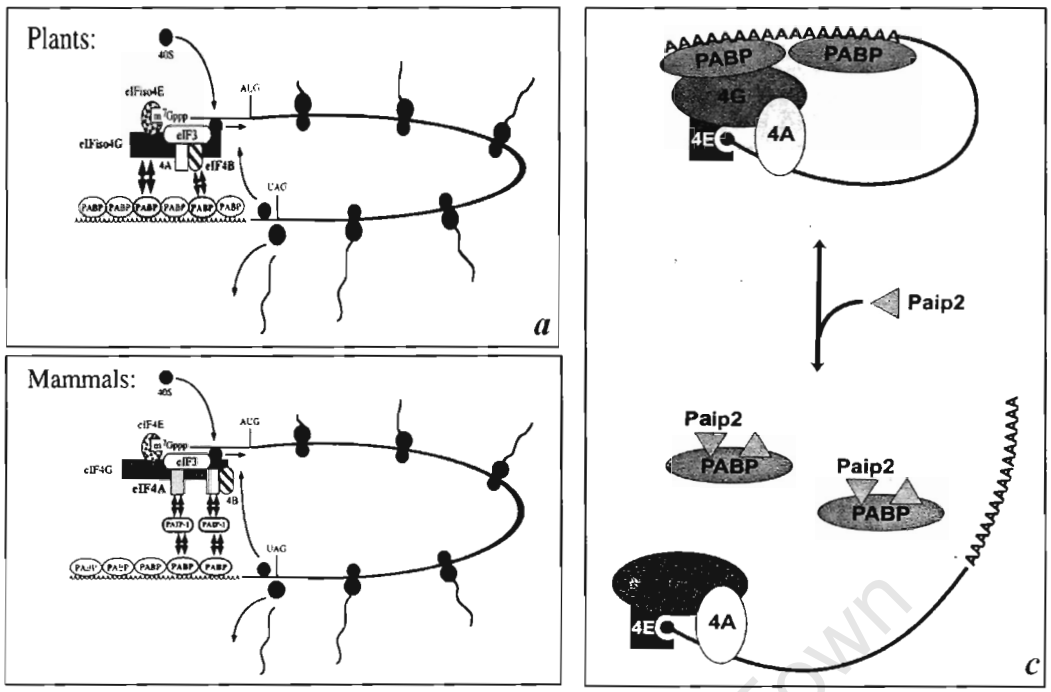

Figure 1.6 (a) The close-loop model of mRNA as result of PABP interaction. (adapted from Gallie *et al.* [20]) *(b)* The interaction of PABP and eIF4G via Paip1. (adapted from Gallie *et al.* [20]) *(c)* Model for the inhibition of translation by Paip2. (adapted from Kahvejian *et al.* [30])

Even though there is limited literature on PABP-CT interacting proteins, studies show that they play a vital role in post-transcriptional modification and modulation of translation. Translation control is an important means of regulating gene expression and plays various roles including response to stress. The earlier a plant responds to stress both at a whole plant level and cellular level, the better it is prepared for downstream mechanisms required to tolerate the stress conditions.

1.5 Aims of this dissertation

This work contributes to the elucidation of how *X. viscosa* tolerates abiotic stress at a cellular level. A cDNA library was generated from low temperature stressed leaves of *X. viscosa* and 60 random cDNA clones were selected for sequencing (Govender pers. comm.). One of the cDNA clones shared high similarity with *ERD15* from *Arabidopsis* and hence it was named *XvERD15*.

The aim of this work was the molecular characterisation of *XvERD15* which entailed:

1. Performing a sequence analysis and prediction a putative function for *XvERD15*
2. Determining gene-copy number of *XvERD15* in *X. viscosa*
3. Investigating the expression patterns for *XvERD15* under various abiotic stress conditions (high salinity, low temperature and dehydration) and exogenous ABA imposed on *X. viscosa*
4. Isolating the 5'-UTR sequences of *XvERD15* and locate potential promoter elements
5. Expression of recombinant XvERD15 and the generation of antibodies
6. Verifing if XvERD15 is present under various abiotic stress treatments by western hybridisation
7. Development of transgenic *Arabidopsis* over-expressing *XvERD15*

Chapter Two

Materials and Methods

2.1 Plant material and stress treatments

2.1.1 Plant collection

Xerophyta viscosa Baker plants were collected from the Cathedral Peak Nature Reserve (Kwazulu Natal Province, South Africa), potted and maintained under glasshouse conditions [71].

2.2 General methods

General methods described below were used in experimental procedures.

2.2.1 Quantitation and visualisation of nucleic acids

The concentration and purity of the samples were determined spectrophotometrically [65] using a UV spectrophotometer (GeneQuant, Amersham Pharmacia Biotech). The nucleic acids were electrophoresed through agarose gels stained with ethidium bromide (EtBr) and viewed at 260 nm on a UV transilluminator (Protea Laboratory Services, South Africa) where the image was captured using Image Store 5000 Gel Documentation System (Ultraviolet Products Ltd, UK).

2.2.2 Restriction endonuclease digestion and ligations

Digestions were carried out using 1 ug of DNA in a 20 ul volume (1X restriction buffer (v/v), 2 U endonuclease (v/v)) at 37 ^{o}C for 3 hours. For ligations, a 4:1 ratio of insert to vector DNA was used in a 20 ul volume (1X ligation buffer (v/v), 2U T4 DNA ligase (v/v)) and incubated at 15 ^{o}C overnight.

2.2.3 Bacterial transformations and plasmid isolation

Ten nanograms of the plasmid vector construct was added to chemically competent cells [12] and transformation heat-shock was carried out using the pGEM-T Easy protocol (Promega). High Pure Plasmid Isolation Kit (Roche) was used to isolate plasmids from 5 ml overnight culture grown in Luria Broth (LB (0.5 % yeast extract (w/v), 1 % tryptone (w/v), 85 mM NaCl)) with the appropriate antibiotics at 37 ^{o}C.

2.2.4 Polymerase Chain Reaction (PCR)

PCR was carried out using 10 ng of DNA template in a 50 ul reaction (1 X PCR reaction buffer (v/v), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U Taq polymerase (v/v), 0.2 uM primers). For colony PCR screening, the template would be substituted by bacterial cells. The initial denaturation temperature was 95 °C for 3 min. This was followed by 30 cycles of denaturation and elongation at 95 °C (30 s) and 72 °C (1 min) respectively with a final elongation (72 °C) for 5min. The various primer annealing specifications were used for different experiments. PCR products were purified directly or from agarose gel slices using High Pure PCR Product Purification Kit (Roche Diagnostics, USA).

2.2.5 PCR primers and sequencing

All oligonucleotides were synthesized on a Beckman 1000M DNA synthesizer using a high purity program (Synthetic DNA Laboratory, University of Cape Town). Sequencing of plasmids and PCR products were carried out on a MegaBACE 500 Automated Capillary DNA Sequencing System (Amersham Biosciences) using the DYEnamic ET Dye Terminator Cycle Sequencing kit [66] at the Specialist Sequencing Services (University of Cape Town). Nucleotide sequences were edited in-silico using DNAMAN (v.4.13, Lynnon Corporation), BioEdit sequence alignment editor (v.7.0.2, Tom Hall) and Chromas (v.2.01, Technelysium) software packages.

2.3 Plant stress treatments and relative water content

X. viscosa plants selected for stress treatments were transferred to a phytotron (27 °C, 16/8 hrs light (500 $\mu mol.m^{-2}.s^{-1}$)/dark cycle, relative humidity of 50 %) and allowed to acclimatise for 30 days prior to imposition of stress treatments. Each stress treatment was carried out in duplicate. All leaf weight measurements for abscisic acid, high salinity, low temperature, water-deficit stress treatments and rehydration were conducted in triplicates. The initial weight (W_i) of the leaves was taken for each sampling point before immersing it in dH_2O for 24 hours. The weight at full tugor (W_t) was taken and subsequently the fully-hydrated leaf samples were wrapped in tinfoil and allowed to dry in an oven at 80 °C. The dry weight (W_d) of the leaf samples was measured after 2 days. The RWC was determined using the formula RWC = ((W_i - W_d) /

W_d)/((W_t - W_d) / W_d))*100 [29]. Excised leaf samples were frozen with liquid nitrogen and stored at -70 °C.

2.3.1 High salinity treatment

A salt solution (150 mM NaCl) was applied to roots of whole plants. Samples were taken at 0, 1, 3, 6, 12, 24, 48 and 72 hrs.

2.3.2 Abscisic acid treatment

Excised leaves were placed in ABA (cis and trans, Sigma) solution (100 µM) and were sampled at 0, 1, 3, 6, 12, 24, 48 and 72 hrs. For the control, leaves were placed in equivalent volumes of dH_2O.

2.3.3 Low temperature shock

Whole plants were exposed to a low temperature shock of -20 °C for 2 hrs. Samples were taken after 0, 15, 30, 60, 90, 120 mins.

2.3.4 Water-deficit and rehydration

Water was withheld from plants for the duration of the treatment and leaves were sampled every two days over a period of 27 days until the plant reached a RWC of 5%. The plant was maintained in a totally dehydrated state for four days and subsequently hydrated to full turgor by soil watering daily. Samples were taken at regular intervals.

2.4 Nucleic acid work

2.4.1 Genomic DNA extraction and Southern blot analysis

DNA preparations were carried out using the procedure described by Dellaporta *et al.* [13]. Fifteen micrograms of DNA were digested with different restriction enzymes (Roche) EcoRI, HindIII or EcoRV. After complete digestion, the samples were electrophoresed through a 0.8 % agarose gel stained with EtBr.

2.4.2 RNA extraction and Northern blot analysis

Total RNA extraction was performed using Trizol ® LS Reagent (Invitrogen) according to the manufacturer's instructions. The samples were electrophoresed through a 1.2 % agarose gel stained with EtBr.

2.4.3 Nucleic acid membrane transfer

The electrophoresed nucleic acids were transferred onto a nylon membrane (Hybond XL, Amersham Pharmacia Biotech) using the downward capillary method as described by Koetsier *et al.* [40]. The transfer buffers used were 0.4 M NaOH/1 M HCl and 10X SSC (1.5 M NaCl, 0.3 M sodium citrate, pH 7.4) for DNA and RNA respectively. The nucleic acids were fused to the membrane using a UV crosslinker (Hoefer UVC 500, Amersham Pharmacia Biotech)

2.4.4 Nucleic acid hybridisation and (α-^{32}P)dCTP-labelled probe detection

All hybridisation and washing steps were conducted in a hybridisation oven (Amersham Biosciences) at 65 °C with agitation. The membrane was incubated in hybridisation buffer (1 % BSA (w/v), 1 mM EDTA, 0.5 M NaH_2PO_4, 7 % SDS (w/v)) for 3 hours. Denatured (α-^{32}P)dCTP-labelled probe (conducted on *XvERD15* cDNA using primer ERD15F 5'-ATGAGTACCATGGCAGTCGCCAC-3' and ERD15R 5'-TCAACGCGG CTGGTGAATGACG-3' with 50 µCi α^{32}P-dCTP at an annealing temperature of 55 °C) was subsequently added and allowed to hybridise overnight. The membrane was washed with a stringent wash buffer (0.5 % SDS (w/v), 2X SSC) until the desired radioactive count was detected with a Geiger counter (Mini-Monitor 900, Amersham AEC). The membrane was then exposed to a phosphor screen (Amersham Biosciences) for 4 hours to overnight. The screen was scanned with an automated documentation system (Molecular Imager, Bio-Rad Laboratories). The image was analysed using quantitation software (Quantity One® version 4.1, Bio-Rad Laboratories) provided.

2.5 5'UTR and genomic sequence isolation

2.5.1 5' UTR sequence isolation of *XvERD15*

The 5'-UTR sequence was obtained using the Splinkerette protocol as described by Devon *et al.*[15] with modifications. Individual aliquots of *X. viscosa* genomic DNA

were digested with restriction endonucleases BamHI, EcoRI, DraI, BglII, EcoRV, AseI, NdeI, HindIII or XbaI. Expand High Fidelity PCR System (Roche) was used for amplification reactions. The annealing and elongation temperatures for the primary PCR using reverse primer Promo 1R 5'- GCTTCATCCAGCTCAGCTTCAAAACTG-3' were 65 ºC for 3 min and 68 ºC for 6 min, respectively. Secondary PCR using internal reverse primer Promo 2R 5'-CTGGAGAATTCAGCCTCAATATCGAGATCTAC-3', was performed using 1 ul of purified primary PCR product with annealing and elongation temperatures of 65 ºC for 1 min and 68 ºC for 3min, respectively. The PCR products were purified and sent for sequencing.

2.5.2 Genomic sequence isolation of XvERD15

The full-length *XvERD15* DNA was isolated using similar PCR conditions as above (2.4.1). *XvERD15* genomic DNA was used as the PCR template. Primer pairs Geno1F 5'-CTGGAGAATTCAGCCTCAATATCGAGATCTAC-3' and Geno1R 5'-GCTGGT GAATGACGCGGCG-3' (R) were used for the primary PCR. Primer pairs Geno2F 5'GATCGCCGGCTTCACAGATATTTTCC-3' and Geno2R 5'-GCTTGCACTTTGG GCTCACGC-3 (R) were used for the secondary PCR. The PCR products were purified and sent for sequencing.

2.6 Protein work

2.6.1 Recombinant protein expression and antibody generation

XvERD15 recombinant proteins were expressed in BL21(DE3)pLysE chemically competent *E. coli* using the pCR® T7 TOPO® TA Expression Kit (Life Technologies, Invitrogen) following the manufacturer's instructions. The recombinant proteins were purified using Ni-NTA agarose under denaturing conditions. The *XvERD15* antibodies were generated using the purified recombinant protein as described by Chart *et al.* [7] and purification of polyclonal antibody from antisera was performed using the PEG (polyethylene glycol) precipitation method [54].

2.6.2 Protein extraction and western blot analysis

Soluble proteins were extracted from 1 g of liquid-nitrogen frozen tissue using protein extraction buffer (0.5 M Tris-HCl (pH 7.5), 10 mM EDTA, 2 % mercaptoethanol (v/v)).

A protease inhibitor cocktail tablet (Complete Mini EDTA-free, Roche) was added for every 10 ml extraction buffer. Protein concentrations were determined using the Bradford Dye Reagent (BioRad) carried out according to manufacturer's instructions. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 5 % stacking gel and a 12.5 % resolving gel. Protein integrity was determined by staining gels with Coomasie blue (0.05 % Coomasie Brilliant Blue R-250 (w/v), 40 % ethanol (v/v), 10 % glacial acetic acid (v/v)). The proteins were transferred onto nylon membranes (Hybond-P; Amersham Pharmacia Biotech) using the Hoefer® Electro-transfer System (Bio-Rad Laboratories, USA) according to manufacturer's instructions. The membranes were stained with ponceau S solution (2 % ponceau S (w/v), 1.1 M sulfosalicyclic acid, 1.8 M tri-chloroacteic acid) to determine transfer and loading efficacy.

2.6.3 Immuno-blotting

The membranes were incubated in blocking solution (3% bovine serum albumin (BSA) in 1X TBS (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) for 2h with agitation. The appropriate dilution (1:2000) of XvERD15 antiserum was added and incubated overnight at 4 ^{o}C. Membranes were subsequently washed twice with a wash buffer (20 mM Tris-Cl, 0.5 M NaCl, 0.05 % (v/v) Tween 20, 0.2 % (v/v) TritonX 100, pH 7.5) for 10 min followed by a wash with TBS. The membranes were incubated with secondary antibody (anti-rabbit IgG, peroxidase linked whole antibody from goat, Sigma) at a dilution of 1:5000 and washes were carried out as before. Detection of antibody cross-reactivity were conducted using ECL detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Films were exposed to Biomax XL Kodak film (Sigma).

2.7 *Agrobacterium*–mediated transformation of *Arabidopsis thaliana*

Competent *Agrobacterium tumefaciens* strain GV3101 were prepared as described by Clough *et al.* [8] and transformed with the binary plasmid vector construct pBINPLUS-*XvERD15*. The culturing of Agrobacterium harbouring pBINPLUS-*XvERD15* vector construct, inoculation of flowering-stage *Arabidopsis* and selection of putative transformants using 50 ug ml^{-1} kanamycin were conducted according to Clough *et al.*

[8]. Total genomic DNA was isolated from putative transformants and further screening was performed by PCR with primers pairs ERD15F and ERD15R.

Chapter Three

Results

3.1 In silico analysis of *XvERD15*

Sequence analysis indicates that *XvERD15* cDNA consists of 882 nucleotides including the 5' and 3' UTR regions (including polyadenylated tail) with an open-reading frame (ORF) of 519 nucleotides. The deduced polypeptide sequence has 172 amino-acid residues having a predicted molecular weight of 19.7 kD (*Figure 3.1.1*). It is an acidic protein with a predicted isoelectric point of 4.51 containing 11 aspartate and 17 glutamate residues which accounts for 16 % of the total amino acids. The hydropathy plot revealed that the majority of amino acids in the polypeptide are hydrophilic with two hydrophobic regions near the N-terminus (*Figure 3.1.2a*). Secondary structure predictions indicated numerous α-helices throughout the polypeptide (*Figure 3.1.2b*). A tyrosine (amino acid postion 104), a threonine (position 140) and four serine (positions 34, 88, 135, 159) are potential phosphorylation sites observed throughout the polypeptide (*Figure 3.1.2c*).

The deduced amino-acid sequence was found to share high similarities with predicted amino acids of ERD15 homologues from *O. sativa* (rice) (XP479417), *L. esculentum* (tomato) (AAF75749), *A. thaliana* (BAA06384) and two PABP-CT interacting proteins from *C. sativus* (cucumber), *CS-PCI243* (AAQ18142) and *CS-PCI6* (AAQ18141) in a BLAST search (*Figure 3.1.3a*). An overall identity of 50 % over a 100 amino-acids region at the N-terminus was observed for the aligned sequences. The conserved 12 amino-acid motif, SxLnpnApxFxP, shared among PABP-CT interacting proteins of human Paip1 and Paip2 [11;33], the eRF3 [27], cucumber PCI6 and PCI243, Ataxin-2 [46], the transducer of *ErbB-2* (Tob) [14] and the *Drospholia* shuttle craft protein is present in the predicted XvERD15 amino acid sequence (positions 12 to 24) and its homologues (*Figure 3.1.3b*).

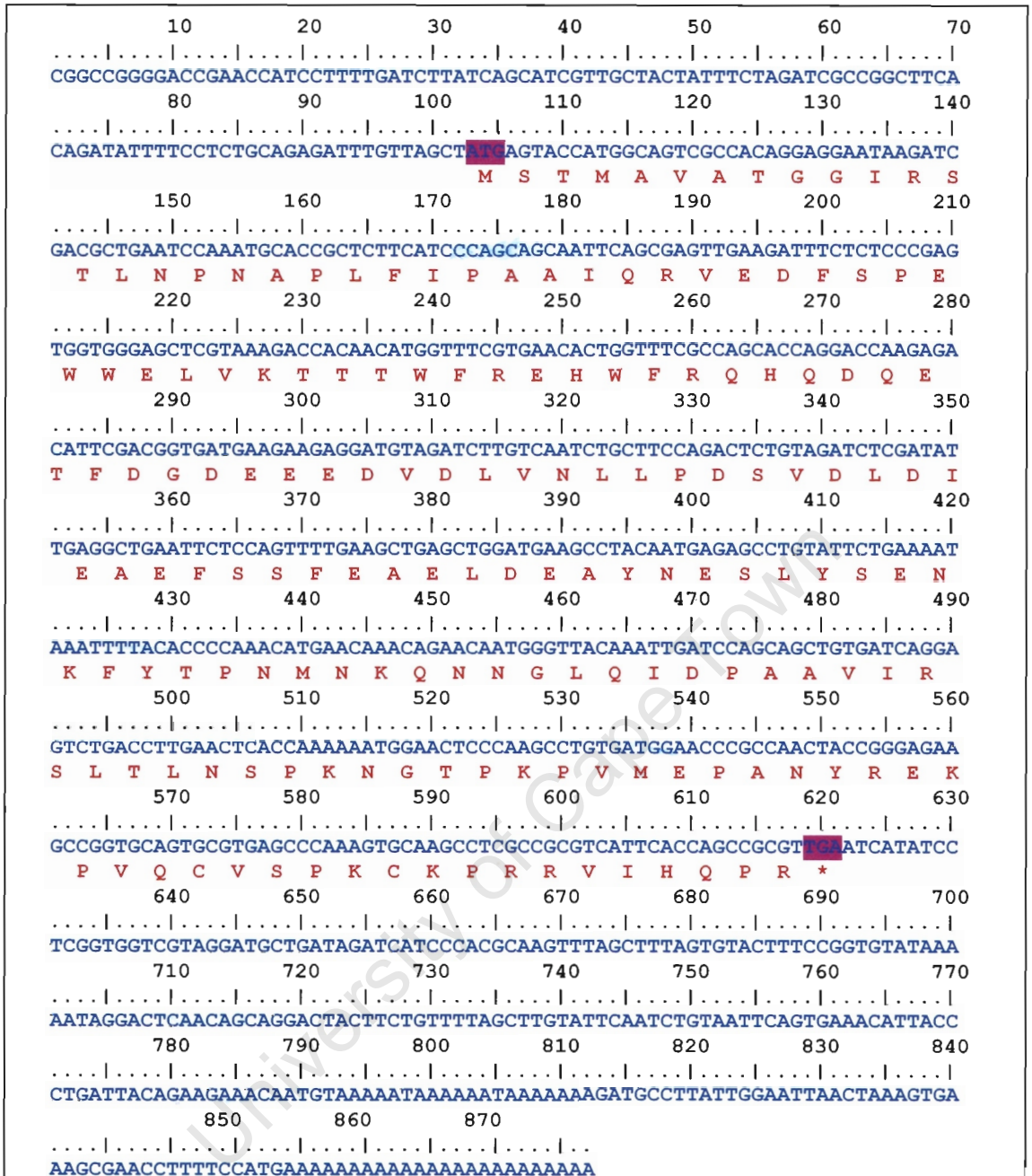

Figure 3.1.1 Nucleotide (blue) and corresponding predicted amino acid sequence (red) of *XvERD15*. The in-silico analysis was conducted using BioEdit software.

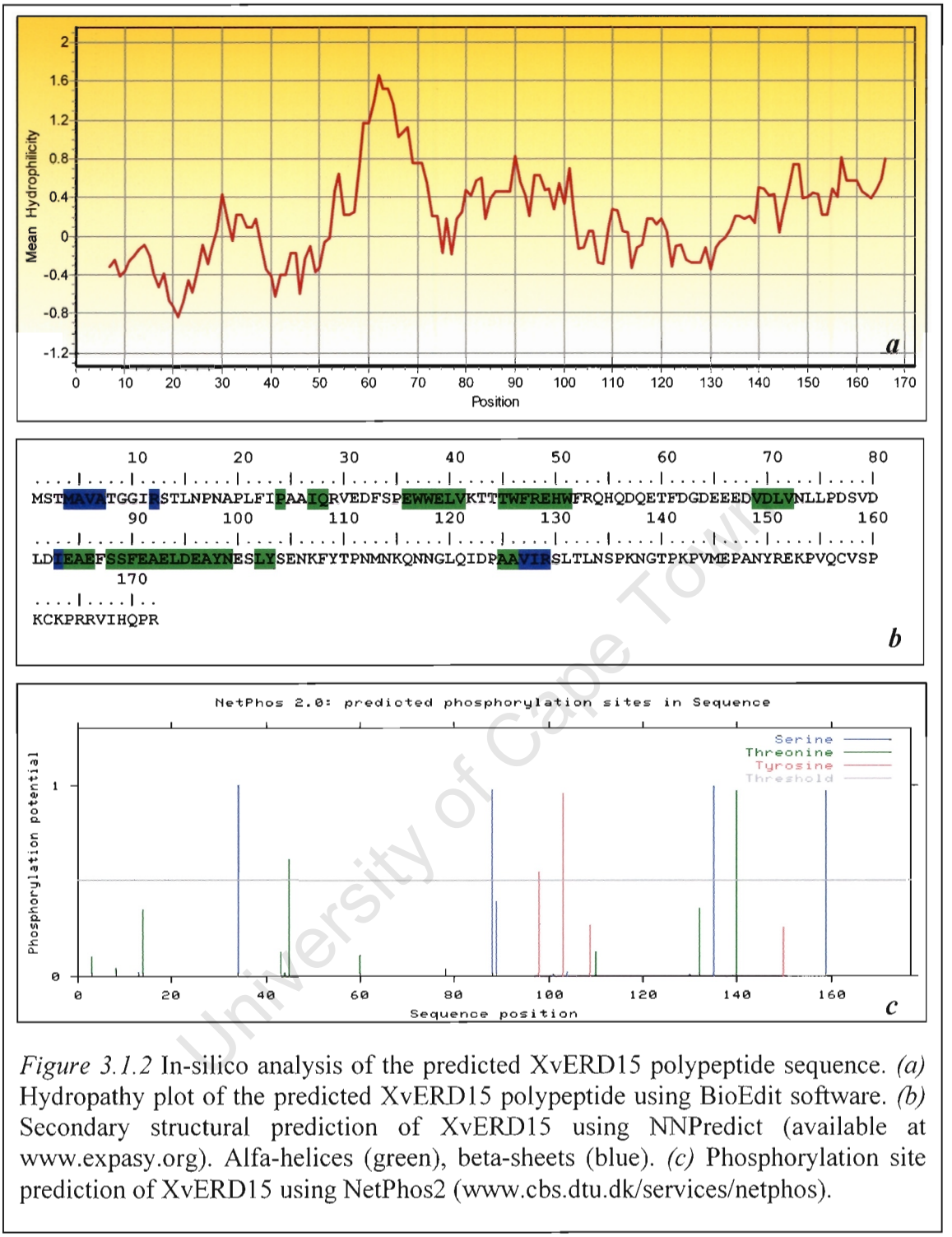

Figure 3.1.2 In-silico analysis of the predicted XvERD15 polypeptide sequence. *(a)* Hydropathy plot of the predicted XvERD15 polypeptide using BioEdit software. *(b)* Secondary structural prediction of XvERD15 using NNPredict (available at www.expasy.org). Alfa-helices (green), beta-sheets (blue). *(c)* Phosphorylation site prediction of XvERD15 using NetPhos2 (www.cbs.dtu.dk/services/netphos).

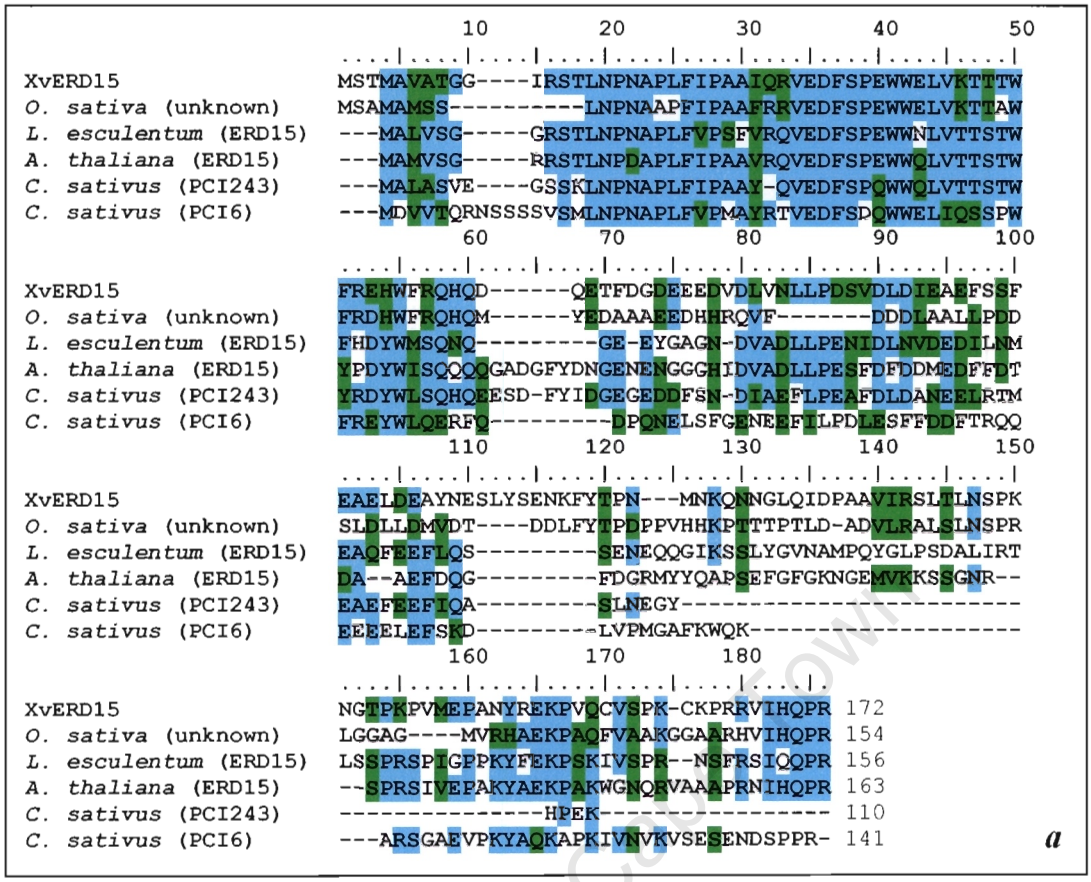

Figure 3.1.3 Amino acid sequence alignment. Sequence identity (blue shading) and similarity (green shading). (*a*) Alignment of *XvERD15* amino acid sequence to similar proteins from rice, tomato, Arabidopsis, and cucumber. (*b*) The 12 amino acid motif [11;33] conserved among PABP-CT interacting proteins from plants, humans and insects.

3.2 Southern blot and *XvERD15* genomic sequence analysis

Genomic DNA isolated from *X. viscosa* was digested with restriction endonucleases chosen from a restriction map of the *XvERD15* cDNA (*Figure 3.2.1a*). Of the restriction enzymes used, only EcoRI had a recognition sequence within the cDNA. HindIII and EcoRV endonucleases did not cut within the *XvERD15* cDNA sequence. Analysis of the blot (*Figure 3.2.1b*) revealed that EcoRI digestion resulted in three hybridising bands while digestion with HindIII resulted in two hybridising bands. The double-digestions with EcoRI/EcoRV and HindIII/EcoRV resulted in five and two hybridising bands respectively. Only one hybridising band was observed for EcoRV digestion. The *XvERD15* genomic sequence (*Figure 3.2.2a*) was isolated with specific primers located at the 5'- and 3'-UTR regions followed by nested primers. Sequence analysis revealed a 231 bp intronic region with two (ScaI and SpeI) restriction sites present (*Figure 3.2.2b*). This suggests the gene is present in two copies in the *X. viscosa* genome.

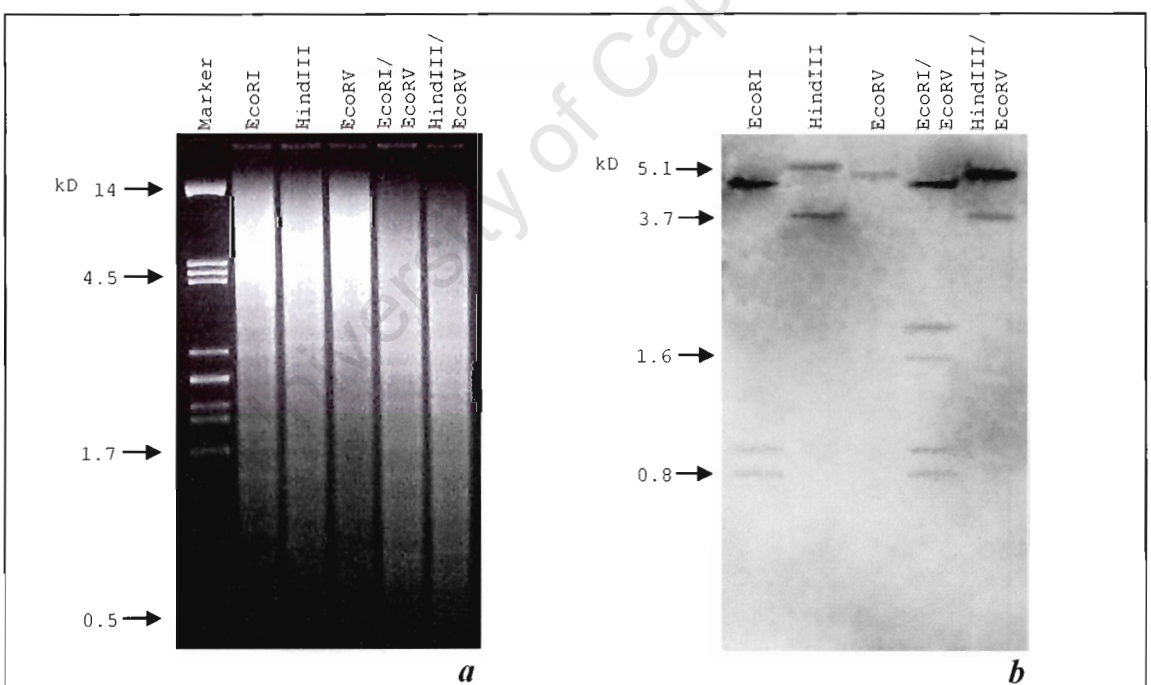

Figure 3.2.1 Complete restriction of *X. viscosa* genomic DNA with selected restriction endonucleases. (*b*) Southern hybridisation with radioactively-labelled *XvERD15*

```
         10        20        30        40        50        60        70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATGAGTACCATGGCAGTCGCCACAGGAGGAATAAGATCGACGCTGAATCCAAATGCACCGCTCTTCATCC
ATGAGTACCATGGCAGTCGCCACAGGAGGAATAAGATCGACGCTGAATCCAAATGCACCGCTCTTCATCC
         80        90       100       110       120       130       140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAGCAGCAATTCAGCGAGTTGAAGATTTCTCTCCCGAGTGGTGGGAGCTCGTAAAGACCACAACATGGTT
CAGCAGCAATTCAGCGAGTTGAAGATTTCTCTCCCGAGTGGTGGGAGCTCGTAAAGACCACAACATGGTT
        150       160       170       180       190       200       210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCGTGAACACTGGTTTCGCCAGCACCAGGACCAAGAGACATTCGACGGTGATGAAGAAGAGGATGTAGAT
TCGTGAACACTGGTTTCGCCAGCACCAGGACCAAGAGACATTCGACGGTGATGAAGAAGAGGATGTAGAT
        220       230       240       250       260       270       280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTGTCAATCTGCTTCCAGACTCTGTAGATCTCGATATTGAGGCTGAATTCTCCAGTTTTGAAGCTGAGC
CTTGTCAATCTGCTTCCAGACTCTGTAGATCTCGATATTGAGGCTGAATTCTCCAGTTTTGAAGCTGAGC
        290       300       310       320       330       340       350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGGATGAAGCCTACAATGAGAGCCTGTATTCTGAAAATAAATTTTACACCCCAAACATGAACAAACAGAA
TGGATGAAGCCTACAATGAGAGCCTGTATTCTGAAAATAAATTTTACACCCCAAACATGAACAAACAGAA
        360       370       380       390       400       410       420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAATGGTTGGTTTACCCTCTAATTAAGAAGATTGTCCTTATTGAGTACTCATGGCTAGTTCAGGTTCTAA
CAATGG------------------------------------------------------------
        430       440       450       460       470       480       490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACGTCCTTTGGTGGTAGATGGGAGTGGCCTAGAACCTTATATAGACTTGAACTAGTCCCCGACTATCCAA
------------------------------------------------------------------
        500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGTCTATTCATTTCAACACTTATTTATACAATTCTACATATAGTTTTGAGAAGTTTTAATTGACAGTTCC
------------------------------------------------------------------
        570       580       590       600       610       620       630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTTGTGCATTGTTGATTGTTTGCAGGGTTACAAATTGATCCAGCAGCTGTGATCAGGAGTCTGACCTTG
--------------------------GTTACAAATTGATCCAGCAGCTGTGATCAGGAGTCTGACCTTG
        640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACTCACCAAAAAATGGAACTCCCAAGCCTGTGATGGAACCCGCCAACTACCGGGAGAAGCCGGTGCAGT
AACTCACCAAAAAATGGAACTCCCAAGCCTGTGATGGAACCCGCCAACTACCGGGAGAAGCCGGTGCAGT
        710       720       730       740       750
....|....|....|....|....|....|....|....|....|....|
GCGTGAGCCCAAAGTGCAAGCCTCGCCGCGTCATTCACCAGCCGCGTTGA
GCGTGAGCCCAAAGTGCAAGCCTCGCCGCGTCATTCACCAGCCGCGTTGA
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a

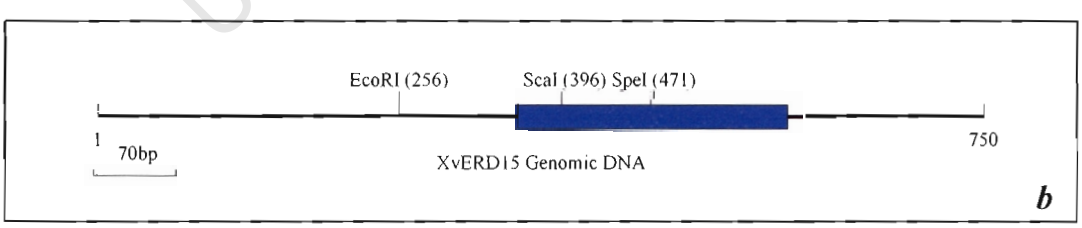

Figure 3.2.2 (*a*) Alignment of the genomic and cDNA sequence of *XvERD15*. XvERD15 intronic region (blue). (*b*) Restriction analysis of *XvERD15* intronic region

3.3 Northern blot analysis

X. viscosa plants were subjected to exogenous ABA (100 µM), salinity (150 mM), low temperature (-20 °C) and water-deficit stress treatments. Northern blot analysis on total RNA extracted from leaves of stressed plants indicated that the transcript was not present in leaves of untreated, hydrated plants but there was an early increase in *XvERD15* transcripts to all stresses imposed.

The transcript levels were at a maximum between 6 and 12 h post-exposure to exogenous ABA and high salinity stress and thereafter levels decreased (*Figure 3.3.1a and b*). *XvERD15* was significantly induced when exposed to low temperature shock for 15 min and levels remained similar thereafter (*Figure 3.3.1c*). In response to water-deficit, transcript levels increased significantly at RWC of 74 % and decreased thereafter until no transcripts were detected at RWC of 7 % (*Figure 3.3.1d*). Furthermore, no transcripts were observed during the rehydration process and wounding (data not shown). *Figure 3.3.2* represents the RWC for the respective stress treatments conducted. This shows that, with the exception of the dehydration stress, there was no significant change in water content during the stress.

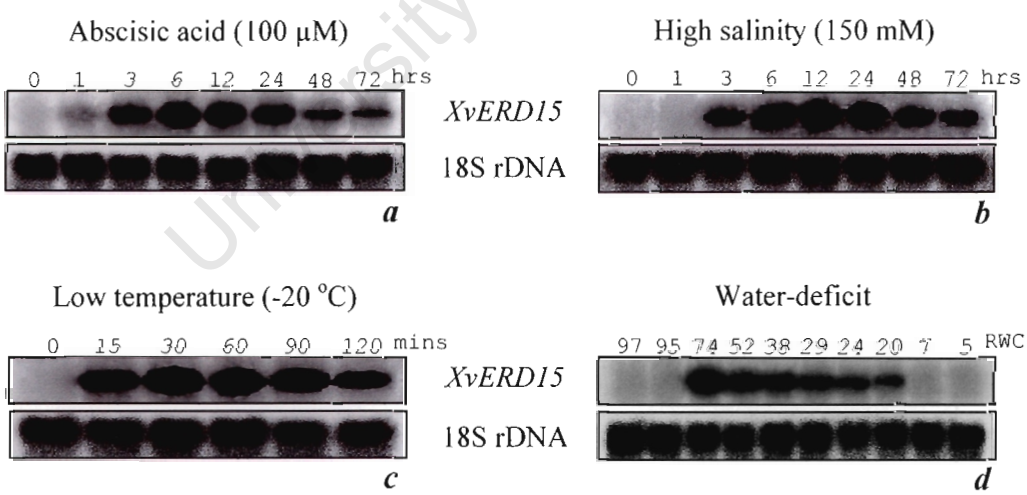

Figure 3.3.1 Northern hybridisation with radioactively-labelled *XvERD15* on total *X. viscosa* RNA (*a*) exogenous abscisic acid (100 µM) treatment (*b*) high salinity (150 mM), (*c*) low temperature (-20 °C) and (*d*) water-deficit stress treatments

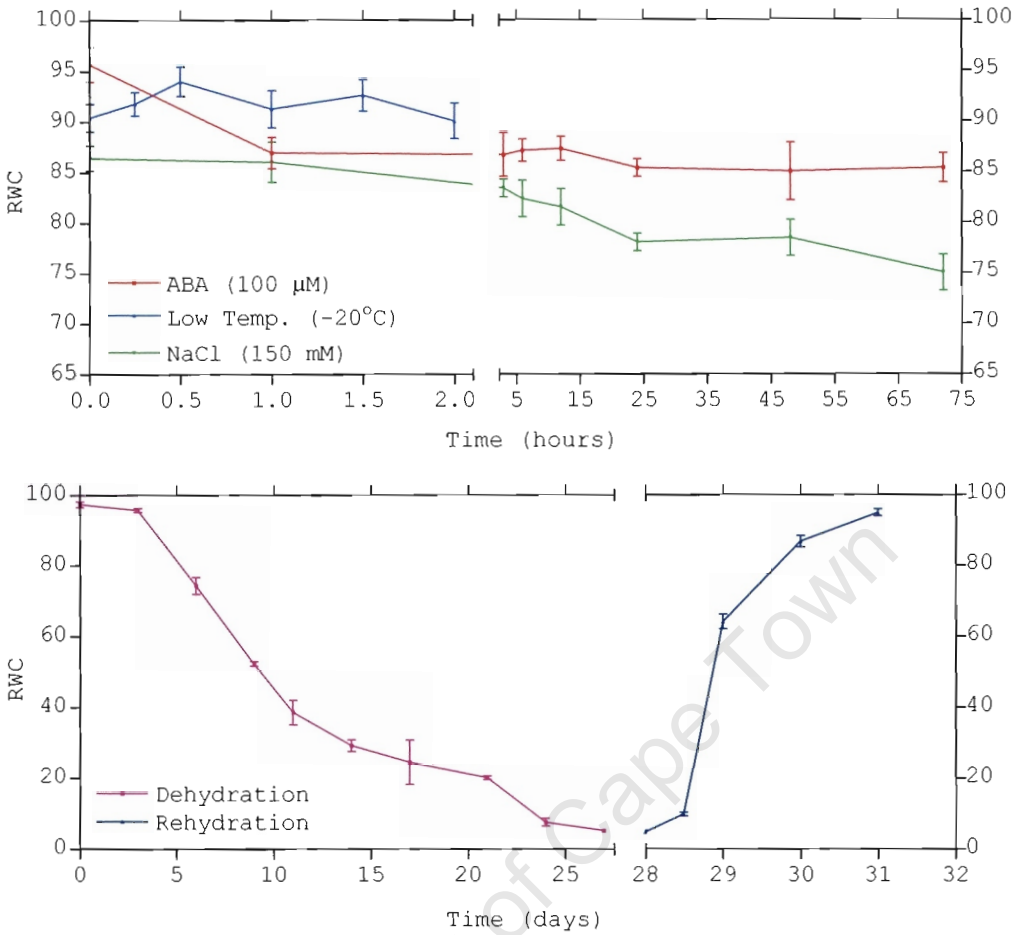

Figure 3.3.2 Relative water contents (RWC) of the respective stress treatments

3.4 Overexpression and purification of recombinant protein HisXvERD15

The recombinant protein was overexpressed in a prokaryotic expression system. With the overexpression parameters optimised, the highest yield was observed after four hours post-induction (*Figure 3.4a*). A higher molecular mass of 35.5 kD was observed for the HisXvERD15 compared to the predicted size of 23.9 kD (including His-tag). A large-scale induction was conducted and the fusion protein was purified using the affinity of Ni-NTA resin under denaturing conditions (*Figure 3.4b*). Anti-Histidine monoclonal antibodies were used to confirm that the purified protein contained the poly-histidine tag (data not shown). In order to purify large quantities of HisXvERD15, cells were lysed under denaturing conditions even though the majority of amino acid in the predicated XvERD15 polypeptide was hydrophilic (*Figure 3.1.2 a*). However, the two hydrophobic groups near the N-terminus could affect the solubility to an extent.

When recombinant proteins are being overexpressed in high quantities in a prokaryotic system, the protein levels become too high for the bacterium to tolerate even becoming toxic. To counteract the increasing levels, the proteins may be compartmentalised into inclusion bodies rendering them insoluble. With the combination of sonication and lysing of the cell under denaturing conditions, large amounts of HisXvERD15 were extracted. The histidine tag in HisXvERD15 allowed its purification by affinity to Ni^{2+}-NTA agarose beads. Under physiological conditions (pH 7.0), the histidine tag is protonated. When the histidine tag is deprotonated at pH 8.0, binding of the protein occurs. Thus to release the maximum yield of the fusion protein, an alkaline solution (pH 6.3), which protonates the histidine tag, containing imidazole (a competitor for Ni^{2+} sites), is used to dissociate the histidine tagged proteins from the agarose. The proteins were further concentrated using filter columns which also served to desalt the solution. The purified recombinant protein was used as an antigen for HisXvERD15 antiserum production in rabbits. Polyclonal antibodies were purified from the antiserum and high specificity of HisXvERD15 antibodies was observed on immuno-blots when tested against HisXvERD15 using a chromogenic detection method (*Figure 3.4c*).

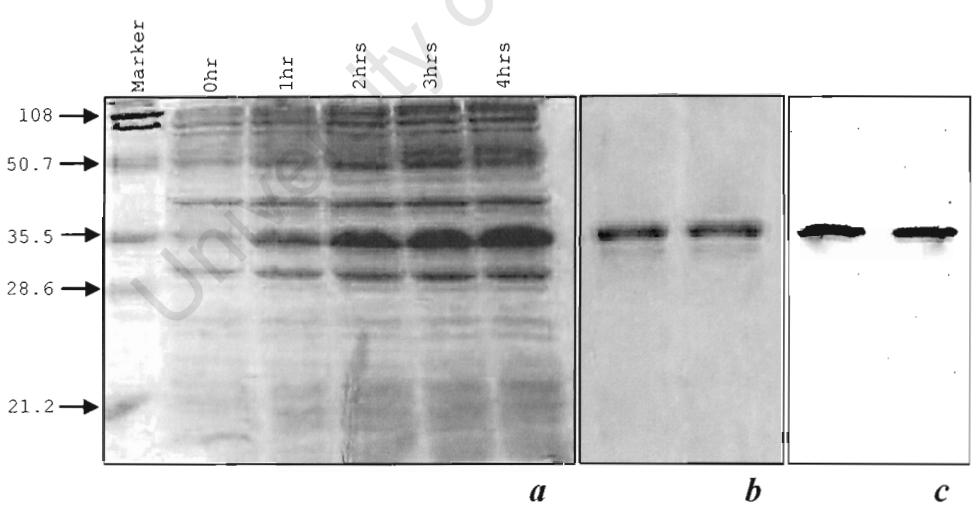

Figure 3.4 (*a*) SDS-PAGE gel showing the induction of recombinant *XvERD15* protein over time (*b*) SDS-PAGE gel of the purified recombinant *XvERD15* protein (*c*) Immuno-blots of purified recombinant *XvERD15* proteins using XvERD15 polyclonal antibodies

3.5 Western blot analysis

Total proteins were isolated from exogenous ABA (100 µM), high salinity (150 mM), and water-deficit stress treated *X. viscosa* leaves. Western blots revealed an increase in XvERD15 protein 3 h post-exposure to ABA and high salinity stress treatments. Protein levels remain fairly constant thereafter. Western blots for water-deficit stress indicated that XvERD15 protein was present at RWC of 74 % and levels decreased slightly towards RWC of 29 %. Protein isolation for samples below the RWC of 29 % proved to be difficult as protein levels were very low.

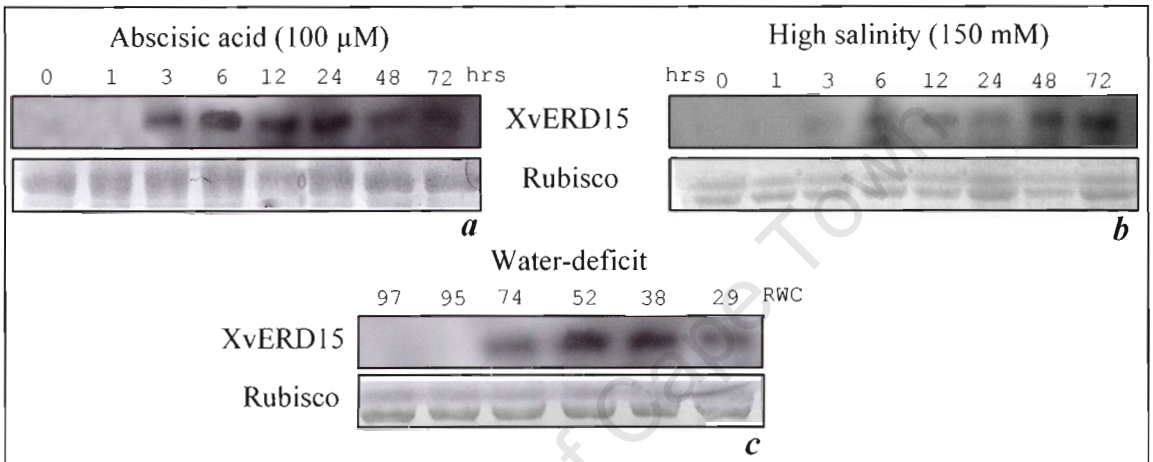

Figure 3.5 Immuno-blots of total protein from excised leaves of *X. viscosa* stress treatments (*a*) exogenous abscisic acid (100 µM) treatment (*b*) high salinity (150 mM) and (*c*) water-deficit stress treatments. Rubisco bands were represented by Ponceau staining.

3.6 Analysis of *XvERD15* 5'-UTR

Two upstream 5'-UTR *XvERD15* sequences, 1.03 kb (*Figure 3.6.1*) and 0.53 kb (*Figure 3.6.2*), were isolated using the Splinkerette [15] method from AseI and DraI digestions respectively (*Figure* 3.6). The respective bands were sequenced and analysis indicated that the 1.03 kb band represented the 5'-UTR sequence of *XvERD15*. It is possible that the 1.03 kb sequence is representative for the 5'-UTR of *XvERD15* studied here because the 0.1 kb upstream sequence from the start codon of *XvERD15* cDNA was identical to that of the isolated sequence. The 1.03 kb sequence was compared to the 5'-UTR of *AtERD15* of equivalent size and only one ABRE element was found in *XvERD15* while four ABRE elements were present in the *AtERD15* sequence. This sequence was compared to the 5'-UTR of *AtERD15* (*Figure 3.6.3*) of equal size and sequence alignment revealed shared identity of 34.53 %. The three 5'-UTR sequences were scanned for cis-acting regulatory elements using PlantCARE [59]. Putative regulatory elements were selected based on their significance to abiotic stress (*Table 3.6*). Comparison of the 1.03 kb 5'-UTR sequence of *XvERD15* to *AtERD15* revealed that both had differences in the number of selected regulatory elements though putative gibberellin- and salicylic acid- responsive elements were present in *XvERD15*.

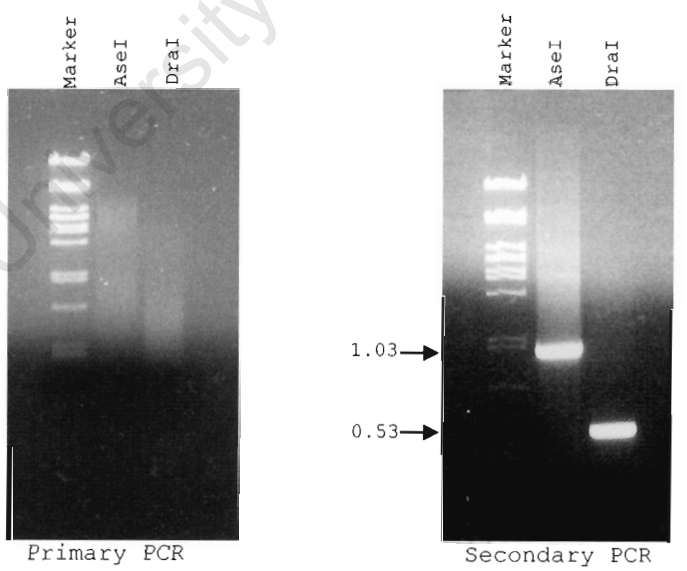

Figure 3.6 5'-UTR isolation of XvERD15 using the Splinkerette [15] method.

Table 3.6 Cis-acting regulatory elements of the two 5'-UTR sequence of *XvERD15* and the *AtERD15*. The upstream sequences were scanned using PlantCARE [59]

Promoter elements	Functions	Number of elements		
		XvERD15 1.03 kb	*XvERD15* 0.53 kb	*AtERD15* 1.03 kb
ABRE	cis-acting element involved in the abscisic acid responsiveness	1	2	4
CAAT-box	common cis-acting element in promoter and enhancer regions	35	18	29
EIRE	elicitor-responsive element	4	1	1
ERE	ethylene-responsive element	3	0	1
GC-motif	enhancer-like element involved in anoxic specific inducibility	2	1	3
GCN4-motif	cis-regulatory element involved in endosperm production	1	2	1
HSE	cis-acting element involved in heat-stress responsive	2	1	5
MRE	MYB binding site involved in light responsiveness	5	0	3
P-box	gibberellin-responsive element	2	1	0
SARE	cis-acting element involved in salicylic acid responsiveness	1	0	0
TATA-box	core promoter element around -30 of transcription start	25	2	30

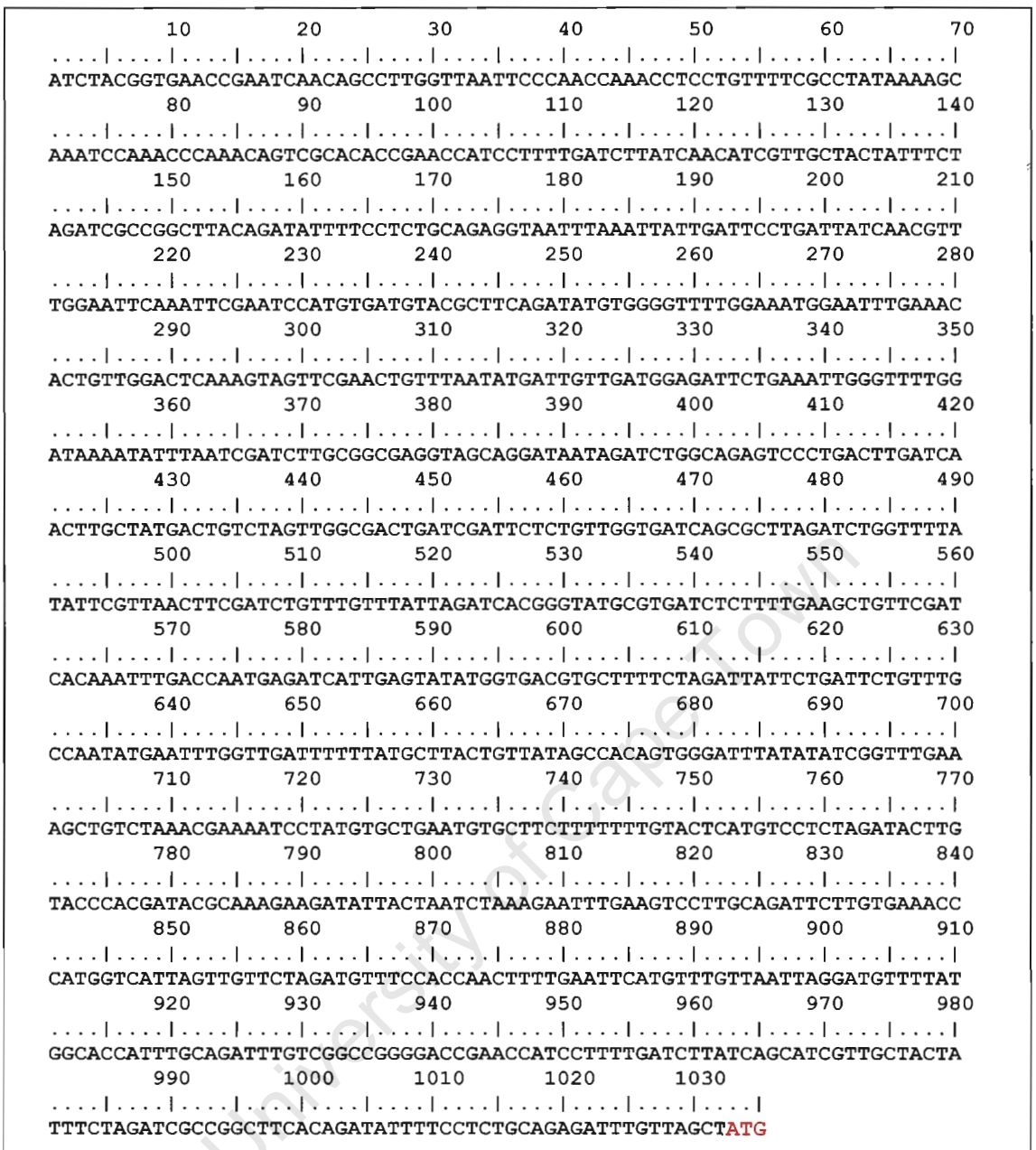

Figure 3.6.1 5'-UTR 1.03 kb sequence of *XvERD15* in *X. viscosa*

```
         10        20        30        40        50        60        70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCCTCTCCAACGAGCCAGATAATTATCGGTTTGAAGCTGTCCATCTGATCCTATATGCTGAGTATGATC
         80        90       100       110       120       130       140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTCAATTGTTGTGTTCCTTTTGTGCTTGTGTCCATCTGAAACCTGTAGCCACCACGTGCAAAGAAGCTAT
        150       160       170       180       190       200       210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TACTGATCTAGAGAGTTTGAAGTCCYTGCAGATTAGTGAAATCTATGTTCAGTAGTTGGGTCAAAATTTG
        220       230       240       250       260       270       280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCCACCAAATCACGAATTCATGTTGGTTAATTATCATGTTTTCTGGTGGCATCTGCAGATTTGTGAGCTA
        290       300       310       320       330       340       350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGAGTACCATGGCAGTCGCCACAGGAGGAATAAGATCGACGCTGAATCCAAATGCACCGCTCTTCATCCC
        360       370       380       390       400       410       420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGCAGCAATTCAGCRAGTTGAAGATTTCTCTCCCGAGTGGTGGGAGCTCGTAAAGACCACAACATGGTTT
        430       440       450       460       470       480       490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTGAACACTGGTTTCGCCAGCACCAGGACCAAGAGACATTCGACGGTGATGAAGAAGAGGATGTAGATC
        500       510       520       530
....|....|....|....|....|....|....|....|....|
TTGTCAATCTGCTTCCAGACTCTGTAGATCTCGATATTGAGGATG
```

Figure 3.6.2 5'-UTR 0.53 kb sequence of *XvERD15* in *X. viscosa*

```
         10         20         30         40         50         60         70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATCCTAGTTGTTCGACACCTTAATGATCTTGGTGTCCTTGTATAGATTACCTTAGAATTTGGTTCTTCA
         80         90        100        110        120        130        140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTCATTGGTTAGCTTTTCAAACTTGATGTCCTATAAAAATGCACATCTCAGACATTACGTAAATCCTA
        150        160        170        180        190        200        210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAATCTACTTTTGGTACTTACACGATACAATACCGAAAACGTAAGTACCAAAAGTAGATTCTAGGATTAG
        220        230        240        250        260        270        280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCCCAAAATGATGATGTAAAACAAAATCATGAGTGGGTTAGGCCCATACGGTCATAAATGGGCTTATATT
        290        300        310        320        330        340        350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGATTAGTTTCCTCCTACCTTATCCTCTTTCCGACCAAATTTAAGATATTTTATAGAAAGAATAATAATT
        360        370        380        390        400        410        420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAACTTTTTAGTGTGAGTAGCCAACAGGATTGGAACGAAAAAGAAAGTCAATAAAAGCCAACAGTAACGC
        430        440        450        460        470        480        490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTCTACACGTGGACGGTTGATTACTCAGAACCATGTTAGCTATGGGTCCAGACCTACCCACTTGTGTCTC
        500        510        520        530        540        550        560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACCTTTAATCCTGCCTCGACGTTACGAACCCCAGCTTCGTCACCGAACCTTACTTTATATATTAGTCCTT
        570        580        590        600        610        620        630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTCTTCTCATCTTCTCGCAACGTAGGTTCTGGTGAATGATCCTAATCCGTTTAGCTTTTAAAAAAATAA
        640        650        660        670        680        690        700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAAGACATATTTATCAACTTGATCAACTTGAGCAAGTTGCAGCAGATATTTTCCTCGGCCAAGGTAAAGA
        710        720        730        740        750        760        770
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCTGATGTTGTCTTCTTCTCTACTCGATCAATTTCTATATTTGAAGTTCGTGGATTTTGAAGGATTTGAT
        780        790        800        810        820        830        840
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGGTTTCCATGGTATTAGATCTCTGTGATGGTTGTTGATCTATCTTAAAACTTGATTTATCTTCAGTTCT
        850        860        870        880        890        900        910
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTCGATTTCAAACTTGTACTTGTCCTACTTCAAGTGATCTAATCGGAAACTTATTGTTGCTATGTTTACT
        920        930        940        950        960        970        980
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GATCGTACAGATCTATTGATCTAAGGATTAAGCTATGTTGTGATTGAGTTGTTAGATTTGAATTTGAATT
        990       1000       1010       1020       1030
....|....|....|....|....|....|....|....|....|....|
TGAACTGAGCTTGTTTGTTTGTGTGTGCAGATCTAGAGTTTTGAGAATGGCGATG
```

Figure 3.6.3 5'-UTR sequence of *AtERD15* in *Arabidopsis*

3.7 Transgenic plants overexpressing *XvERD15*

Transgenic *Arabidopsis thaliana* were generated using *Agrobacterium* [8] carrying pBINPLUS-*XvERD15*. The seeds were harvested from the transformed plants and putative transformants were selected on kanamycin (*Figure 3.7a*). Subsequently, the seeds were harvested from the first generation transgenic (T1) plants and genomic DNA was isolated from 18 plants for PCR screening with *XvERD15* specific primers. Of the 18 plants that were screened, 15 were carrying *XvERD15* (*Figure 3.7b*).

Figure 3.7 (*a*) Transgenic plants overexpressing *XvERD15* on kanamycin selection (*b*) PCR-screening for putative transformants overpressing *XvERD15*

Chapter Four

Discussion

The Southern blot conducted on *X. viscosa* genomic DNA suggests that *XvERD15* is likely to be a double copy gene in the *X. viscosa* genome. However, since the restriction endonucleases were chosen based on the cDNA sequence of *XvERD15*, it is difficult to determine the gene copy number accurately as the restriction endonucleases sites that may be present in the intronic region could not be accounted for. To counteract the problem, the genomic sequence was isolated using primers situated on the 5'- and 3'-UTR regions and nested primers. Analysis of the intronic region revealed that none of the restriction endonucleases chosen was present (*Figure 3.2.1c*). Therefore, this suggests that there may be two *XvERD15* gene copies in *X. viscosa*. Furthermore, two 5'-UTR sequences of *XvERD15* were isolated.

The unique feature of ERD expression is that transcripts only increase at the onset of stress. *XvERD15* adheres to this expression pattern. When leaves of *X. viscosa* plants were subjected to exogenous ABA (100 μM) and high salinity stress (150 mM NaCl), an early increase in *XvERD15* transcripts was observed at 1 h and 3 h post-exposure respectively (*Figure 3.3.1a and b*). Thereafter, transcript levels decreased. *XvERD15* transcripts increased dramatically in response to low temperature shock after 15 min exposure at -20 ^{o}C (*Figure 3.3.1c*). Transcripts levels remained relatively the same thereafter. However, it has been observed cellular metabolism ceases within 1 hr of such intense stress. By the time it reaches 1 h, the plant cells freeze and any *XvERD15* transcripts should be considered artifacts of transcript upregulated at 15 min. Nevertheless, *XvERD15* transcript increased at low temperature shock. The early response of *XvERD15* was also observed in water-deficit stress as indicated by increase in transcripts at RWC of 74 % and thereafter, transcript levels decreased (*Figure 3.3.1 d*). *XvERD15* transcripts were previously found to be up-regulated in ABA and high salinity treated leaves of *X. viscosa* plantlets [44]. The early up regulation of *XvERD15* is consistent with published ERD15 from *Arabidopsis* [17;38] and maize [84] when exposed to water-deficit and high salinity respectively. A higher molecular mass of 35.5

kD was observed for the HisXvERD15. Similar finding have been observed in acidic proteins which tends to run slower than the predicted molecular size [86]. Immuno-blots of total proteins isolated from stress treatments indicated that XvERD15 protein levels increased when exposed to exogenous ABA and stress treatments, NaCl and water-deficit. Protein levels increased significantly after 3 h post-exposure to ABA and NaCl stress treatments (*Figure 3.5 a and b*). Protein levels increased in leaves with RWC of 74 % (*Figure 3.5 c*) and decreases at RWC of 29 %. This verifies the presence of XvERD15 proteins during stress. These results correlate with the northern blot analyses of *XvERD15* transcripts.

Several putative cis-regulatory elements are present in the *X. viscosa* (*table 3.6*) including at least one ABRE element. This could account for response to ABA treatment. ABRE elements found in *AtERD15* suggest it may be up-regulated when exposed to exogenous ABA treatment as previously described for *XvERD15*. In addition, putative elements found in *XvERD15* suggest it could be responsive to gibberellin and salicylic acid (*Table 3.6*).

It is important to note that to date, there is little literature available on the homologues of *ERD15* and their functional roles have not been elucidated. However, its presence as an early responsive gene to stress has been observed in various plant species.

XvERD15 is a small, acidic protein with a predicted molecular mass of 19.7 kD and has amino acid sequence similarity to ERD15 homologues in rice (46 %), tomato (41 %) and *Arabidopsis* (38 %). However the functions of these proteins are unknown. A 50 % shared identity over a 100 bp region from the N-terminus was observed between XvERD15 and PABP-CT interacting proteins PCI243 and PCI6 from cucumber [86] (*Figure 3.1.3 a*). Within this region, the conserved 12 amino acid motif found in PABP-CT interacting proteins is present in XvERD15 (*Figure 3.1.3b*) suggesting a possible function for ERD15 homologues. This conserved motif, PAM2 motif, found in human Paip1 and Paip2 [33;60] is shared among PABP-CT interacting proteins in a wide range of species. Many studies have shown that the PAM motif is necessary for the interaction with the C-terminal of PABP [60;86]. Mutations introduced in the motif decreased the

affinity of mutant PCI6 to PABP-CT and migration of the mutant in SDS-PAGE was faster compared to the wild-type suggesting a conformational change in the protein [60;86]. Furthermore, the deletion of the PAM motif from PCI6 abolished interaction with PABP. *Arabidopsis* ERD15 was also found to interact with the C-terminus of PABP [86]. The acidic nature and size of *XvERD15* is similar to that of PCI243 and PCI6. PABP-CT interacting proteins can be grouped into two categories. The first group includes *XvERD15*, Paip2, PCI243 and PCI6 characterised by their small size and high acidity. This could suggest similar function. The other group includes proteins of larger sizes, more neutral pI's and the presence of one or more RNA recognition motifs that can interact with both RNA and PABP [86].

The hydropathy plot indicates the PAM motif (amino acids position 13 to 24) in *XvERD15* is hydrophobic (*Figure 3.1.2a*). Interestingly, the pocket formed between helices 2 and 3 at the C-terminal of PABP in which the PABP-CT interacting proteins interact is hydrophobic. It was proposed that the interacting proteins orient themselves vertically in such a way that the phenylalanine in the 12 amino acid motif slots into the hydrophobic core [42]. A mutation at the phenylalanine residue in the conserved motif of PCI6 highly decreased its binding affinity to PABP-CT suggesting that the residue is critical for binding [86].

With the finding in the in-silico analysis, XvERD15 may play two functional roles. As a PABP-CT interacting protein, it could regulate PABP either by enhancing translation or repressing it. The characteristics of XvERD15 as a small and highly acidic protein are similar to that of Paip2 and PCI6, with PCI6 highly similar to AtERD15 at the N-terminal [86]. Both Paip2 and PCI6 repress translation and in vitro competition experiments have shown that Paip2 competes with Paip1 for binding to PABP [33]. Similarly, PCI6 was shown to inhibit translation in vitro in mouse ascites Krebs-2 cell-free and wheat germ translation system [86].

Paip1 and Paip2 are phosphoproteins and whether phosphorylation modulates their function is currently unknown [30]. Putative phosphorylation sites prediction revealed

that XvERD15 has six potential sites (*Figure 3.1.2c*). This suggests that functions of XvERD15 may be modulated by phosphorylation at a post-transcriptional level.

A possible explanation why XvERD15 and its homologues are upregulated early in response to stress is to inhibit expression of certain genes that are not required for stress tolerance. Housing-keeping and other constitutively-expressed genes are highly energy consuming and the stressed plant needs to channel the energy for other important processes. Since *X. viscosa* disassembles thylakoid membranes within chloroplasts, and chlorophyll, to protect itself from oxidative damage during water-deficit [72], proteins that repress translation may play a functional role in shutting down genes responsible for photosynthesis. Many photosynthesis-related genes, such as ribulose 1.5-bisphosphate carboxylase and chlorophyll a/b-binding proteins, were found to be downregulated in water-deficit stressed *Arabidopsis* using microarray analysis [68].

The T2 transgenic *Arabidopsis* plantlets overexpressing *XvERD15* did not survive well on kanamycin selection and all plantlets died shortly (data not shown). This was not expected. It is only towards the later stages of this research that literature suggesting that ERD15 homologues may regulate PABPs was avaliable. Therefore, it is hypothesized that *XvERD15* and *ERD15* homologues may regulate PABPs by repressing translation.

4.7 Concluding remarks

From this research, it is observed that *XvERD15* is early responsive to stress in *X. viscosa*. The research presented here serves as an initial phase into elucidation of a potential functional role of *XvERD15*. Experiments should however be conducted to determine if *XvERD15* associates with the C-terminus of PABPs and whether it represses translation. Furthermore, Since there is little literature available on the functional roles of ERD15 homologues in stress tolerance, the role of *XvERD15* remains speculative.

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