Molecular characterisation of *XvRG6 and XvRF17*, genes isolated from the resurrection plant *Xerophyta viscosa*

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Declaration

I declare that 'Molecular characterisation of *XvRG6* and *XvRF17*, genes isolated from the resurrection plant *Xerophyta viscosa*' is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Mba Medie Felix

19th February 2007

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List of abbreviations

ABA	abscisic acid
ABF	ABA-responsive element binding factors
ABRE	ABA-responsive element
AGP	arabinogalactan protein
bp	base pair(s)
cDNA	copy DNA
Cys	cysteine
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DRE	drought responsive element
EDTA	ethylenediaminetetra-acetic acid
ETR	ethylene receptors
g	grams
h	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
JA	jasmonic acid
JIP	jasmonate-induced proteins
kb	kilobase(s)
kDa	kilodalton(s)
LEA	late embryogenis abundant
MTs	metallothionein
λ	lambda
l	litre(s)
LB	Luria-Bertani
M	molar concentration
μg	microgram(s)

mg	milligram(s)
µl	microlitre(s)
ml	millilitre(s)
µm	micrometer(s)
mM	millimolar
min	minutes
mRNA	messenger RNA
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometre(s)
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsufonyl fluoride
PRPs	proline-rich protein
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
RWC	relative water content
SA	salicylic acid
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TBE	tris borate EDTA
TE	tris-EDTA
TEMED	N,N,N',N' - tetramethylethylene diamine
Tris	tris(hydroxymethyl)aminomethane
U	unit(s) of enzymatic activity
UTR	untranslated region
w/v	weight per volume

Abstract

A number of genes up-regulated in response to abiotic stress have been isolated from *X. viscosa* by various methods, one of which includes differential screening of cDNA libraries. Of these genes, a large number are undescribed, with no identity to known plant genes. Two such genes, *XvRG6* and *XvRF17*, isolated from a *X. viscosa* cDNA library have been partially characterised. Southern blot analysis confirmed the presence of both *XvRG6* and *XvRF17* genes in the *X. viscosa* genome. *In silico* analyses predicted XvRG6 to be a proline-rich protein, since it possesses many of the features common to PRP such as a signal peptide, proline repeats, a cysteine residue and possible phosphorylation sites. It also possesses a tyrosine residue present in some of the repeats, and this residue is believed to play a role in protecting the plant against environmental stresses. The XvRF17 protein was predicted by *in silico* analyses to belong to the family of metallothioneins, a family of metal-binding proteins. Analysis of the *XvRG6* mRNA transcript showed that the gene was not dehydration stress inducible, but was induced by ethylene, endogenous ABA, SA and JA application. In contrast, the *XvRF17* mRNA transcript was shown by RT-PCR to be induced by dehydration stress, endogenous ABA, SA and JA application but not by ethylene treatment. Interestingly, western blot analyses revealed that XvRG6 protein levels increased only during dehydration and not during any other imposed stress. This study has shown that both *XvRG6* and *XvRF17* are responsive to stress. Since there are no known orthologues of these genes, their respective roles in *X. viscosa* remains speculative.

Chapter 1

Literature review

1.1 BACKGROUND

Over 35% of the world's land surface is considered to be arid or semi-arid, experiencing precipitation that is inadequate for most horticultural uses. Water deficit has a major impact on the area of land available for cultivation and is one of the most commonly experienced environmental stress in southern Africa. In America, soil water deficits are estimated to depress agricultural crop yields by about 70% compared with maximum achievable yields and similar problems are encountered worldwide (Neumann, 1995).

1.1.1 Water-deficit stress

Plants have several strategies to deal with water deficit, desiccation tolerance is but one of these. Desiccation tolerance is the ability of an organism to survive the loss of most (>95%) of its cellular water (Illing et al., 2005). Desiccation tolerance is a rare phenomenon in the vegetative tissues of angiosperm, but is common, although not ubiquitous to reproductive structures (pollen and seeds). Desiccation tolerance is found among mosses and pteridophytes, occurs in certain animals such as nematodes and the encysted embryos of brine shrimps, as well as in lichens terrestrial micro-algae and micro-organisms.

Land plants suffer from dehydration or water stress not only under drought and high salt-concentration conditions but also under low temperature conditions (Shinozaki and Yamaguchi-Shinozaki, 1997). They respond to water stress by using various biochemical and physiological adaptations. Physiological adaptations take many forms ranging from partial senescence of tissues, to structural adaptations such as water storage organs and restrictions in the surface area of aerial tissues as seen in *Cactaceae* and *Euphorbiaceae* (Scott, 2000). All of these forms are effective and allow plants to inhabit a wide range of arid environments, but when subjected to prolonged lack of water these plants will dehydrate and die (Scott, 2000).

Virtually all plant species, at some point in their life cycle are partially tolerant to desiccation, for example, seeds and pollen frequently lose large quantities of water during their

maturation process (Bewley, 1979; Scott, 2000). But the ability of mature tissue, such as roots and leaves to survive virtually complete desiccation is very rare.

1.1.2 Desiccation-tolerant plants

Plants survive under various kinds of environmental stresses in nature. Consequently, they have developed numerous sophisticated adaptations, some of which are unique in the biological world (Boyer, 1982). Most flowering plants cannot survive exposure to a water deficit equivalent to less than 85 to 98% (v/v) to relative humidity during their vegetative growth period. Only a few plants possess desiccation tolerant vegetative tissues, these include a small group of angiosperms termed resurrection plants (Gaff, 1971). Resurrection plants are unique among angiosperms in their ability to survive protoplasmic desiccation to less than 2% relative water content (RWC) (Bartels et al., 1990). Resurrection plants survive the loss of their tissue water content until a quiescent stage is achieved, but upon watering the plants rapidly revive and are restored to their former state (Scott, 2000). This resurrection capacity is not restricted to the main meristems of these plants. Fully mature leaves can lose up to 95% of their water content and then, upon watering, the leaves are rehydrated and are fully photosynthetically active within 24 h (Bernacchia et al., 1996). Desiccation in resurrection plants prevents growth and reproduction over the dehydrated period (Scott, 2000). Resurrection plants are found in ecological niches with limited seasonal water availability, usually on rocky outcrops at low to moderate elevation in tropical and subtropical zones (Porembski and Barthlott, 2000). Tissue damage appears to be minimal to non-existent and the plant is preserved until water becomes available.

Resurrection plant species are represented in most taxonomic groups, ranging from pteridophytes to dicotyledonous (Hartung et al., 1998). These include terrestrial species such as the monocotyledonous *X. viscosa*, the dicotyledonous shrub *Myrothamnus fabellifolia*, and the aquatic species *Chamaegigas intrepidus*. The terrestrial species are often found on rocky outcrops in shallow sandy soil (Sherwin and Farrant, 1995). It has been estimated that around 300 species of resurrection plants may exist, mainly in southern Africa, Australia, India, and South America (Bartels and Salamini, 2001). The physiological basis of desiccation tolerance in resurrection plants is complex in that some mechanisms may vary between different species (Farrant, 2000). Resurrection plants may be subdivided into species whose dry leaves contain chlorophyll (homoiochlorophylous) such as *C. plantagineum* and those which do not (poikilochlorophyllous)

such as *X. viscosa* (Hallam and Gaff, 1978; Alamillo and Bartels, 2001; Phillips et al., 2002). In *X. viscosa*, the loss of chlorophyll in the leaves is accompanied by the accumulation of anthocyanin (Sherwin and Farrant, 1998; Fig. 1.1).

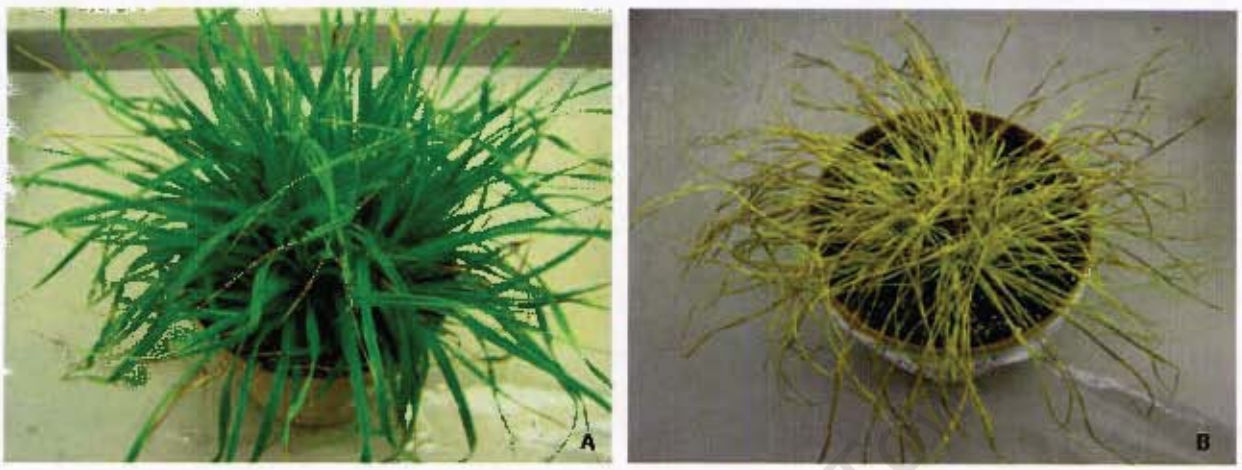

Figure 1.1 The resurrection plant *X. viscosa* in its fully hydrated (A) and dehydrated state (B).

Desiccation tolerance depends on the ability of the cells to maintain integrity of the cell membranes and prevent denaturation of proteins (Phillips et al., 2002). In resurrection plants desiccation tolerance is both molecularly and physiologically complex. This is due to the multiple stresses that are imposed on plant tissues during severe dehydration. The establishment of tolerance must overcome problems such as minimisation of mechanical damage associated with turgor loss, maintenance of the functional integrity of macromolecules and organelles, minimisation of toxin accumulation and free-radical damage, and initiation of repair mechanisms upon rehydration (Phillips et al., 2002).

Mundree and Farrant (2000) have shown that in response to dehydration, instead of losing water and shrinking, the central vacuole of *X. viscosa* subdivides into a number of smaller vacuolar structures. It has been hypothesised that these structures become filled with compatible solutes such as ions, small proteins and sugars. Because of their unique ability to withstand desiccation, resurrection plants are valuable in our study on the mechanisms involved in achieving desiccation tolerance in plants.

Desiccation tolerance has been investigated using three main approaches in plants: (i) examining tolerant systems, such as seeds and resurrection plants; (ii) analyzing plant mutants

from genetic model species; and (iii), analyzing the effects of stress on agriculturally relevant plants (Ingram and Bartels, 1996). Resurrection plants provide excellent models to study water deficit tolerance at the physiological, genetic and biochemical levels. There are therefore being analysed to further our understanding of plant responses to water deficit. In addition,these plants are a potential source of genes to enhance stress tolerance. The underlying mechanisms together with the genes involved in desiccation tolerance can be exploited to improve the drought tolerance of drought sensitive crops.

Another critical approach in researching dehydration tolerance has been to analyze the plant response after drought stress of important agricultural species. This type of study is useful because through intensive breeding or *in vitro* selection, lines are available with differing degrees of tolerance and thus correlated evidence can be sought for genes putatively involved in the drought response (Ingram and Bartels, 1996). A particular advantage of these plants in studies at the molecular level is that desiccation tolerance can be investigated in both whole plants and undifferentiated calli (Ingram and Bartels, 1996).

However with respect to *Xerophyta* species, very few genes have been characterised and the genomes of the respective species have not been sequenced. Furthermore plants such as *Arabidopsis* and *Oryza*, which have been sequenced and whose genes have been extensively studied are not vegetatively desiccation tolerance.

1.1.3 Molecular studies in resurrection plants.

Tightly regulated programs of gene expression, both at the spatial and temporal levels occur in vegetative tissues during drying (Phillips et al., 2002). The basic pattern of changes in gene expression that occur in response to dehydration can be summarised for *Craterostigma plantagineum* as follows: (a) transcripts accumulate to high levels during dehydration and disappear early during rehydration; (b) transcripts accumulate transiently during the initial dehydration phase; (c) transcripts decline during dehydration; and (d) transcripts remain unchanged in response to dehydration (Bartels et al., 1990). This means that in *C. plantagineum* the majority of changes in gene expression occur during dehydration, and not during the rehydration phase of the resurrection process, which in turn leads to a very efficient protection system against desiccation (Phillips et al., 2002). This is in contrast to what is observed for

Tortula. ruralis and indicates that differing mechanisms of desiccation tolerance probably exist in vascular higher plants versus bryophytes (Phillips et al., 2002).

Collet et al. (2004) using small-scale microarray and reverse northern blot experiments on 424 cDNA from *X. humilis* identified 55 genes that were up-regulated and 79 genes that were down-regulated during dehydration. Among the 79 genes that were down-regulated 25% were involved in photosynthesis and metabolism. Ingle et al. (2007) used 2D SDS-PAGE to investigate the protein expression patterns of *X. viscosa* in response to dehydration. The proteomic analyses revealed that a RNA-binding protein, chloroplast FtsH protease, glycolytic enzymes and antioxidant proteins were increased significantly during drying while photosynthetic proteins decreased at RWC below 65%.

A number of interesting genes isolated from *X. viscosa* have also been identified as up-regulated in response to various stresses. A few of these include *XvPer1* which is a stress-inducible antioxidant enzyme (Mowla et al., 2002); the *XvCaM* gene, which was isolated from dehydration stressed *X. viscosa* leaves and which codes for a classic calmodulin with 4 EF-hands (Conrad, 2005); the XvSAP1 protein, which is highly hydrophobic and is postulated to play a signalling role during abiotic stress (Garwe et al., 2003); the *XvIno1* gene, which encodes a myo-inositol-1-phosphate synthase, which catalyses the conversion of glucose-6-phosphate to myo-inositol-1-phosphate (Chopera, 2006). These genes could potentially improve the drought tolerance of agriculturally important plants such as maize and wheat. Several other stress-inducible genes from *X. viscosa* have been characterised and their ability to improve abiotic stress tolerance in model plants is under investigation (Mundree et al, 2006).

1.1.4 Plant stress response.

The plant stress response is not well understood, as complex regulatory and signalling cascades dictate the expression of stress response genes (Bray, 1993). Not enough is known about the means by which plants detect drought or the functioning and identity of the various response mechanisms that are activated (Scott, 2000). Activation of these pathways results in the expression of genes believed to function in stress tolerance and those that regulate the signal transduction pathways involved in the stress response (Shinozaki and Yamaguchi-Shinozaki, 1997; Fig. 1.2).

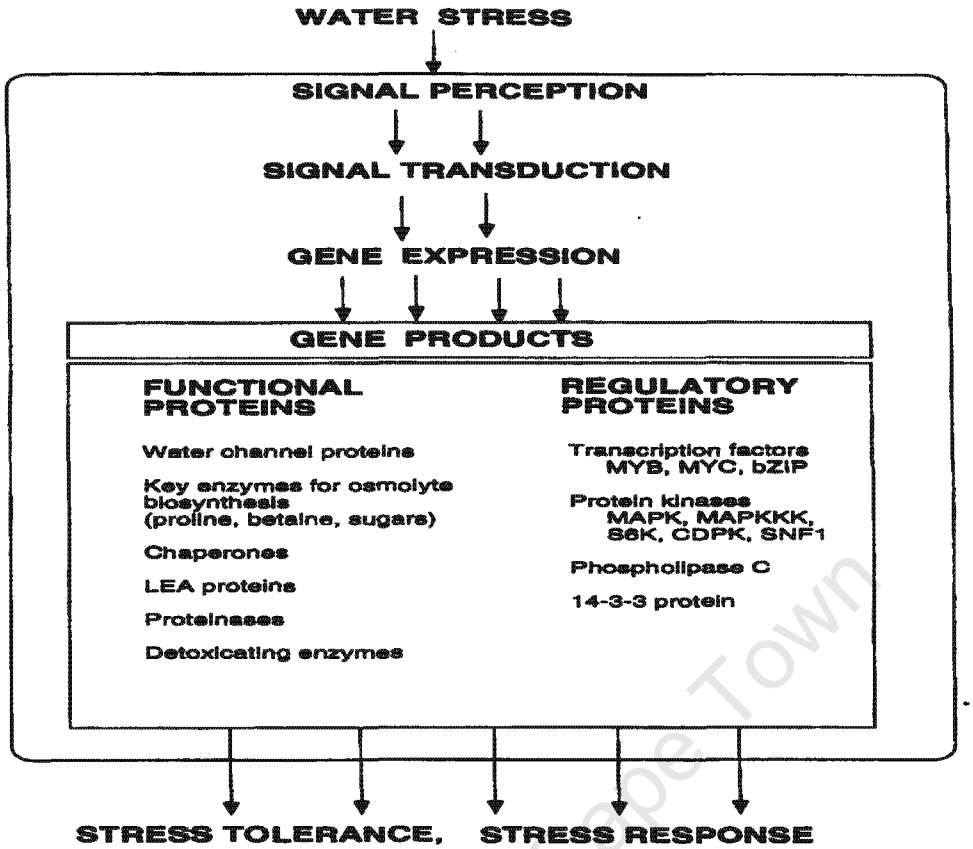

Figure 1.2 Function of water stress-inducible gene products in stress tolerance and stress response. The gene products are classified into two groups: (i) functional proteins that are involved in water-stress tolerance and cellular adaptation; and (ii) regulatory proteins that may function in gene expression and signal transduction in stress response (adapted from Shinozaki and Yamaguchi-Shinozaki 1997).

The above scheme applies to desiccation sensitive plants in response to water-deficit, while a similar general response will happen to desiccation tolerance plants, the nature of the genes and protection responses will differ. One specific mechanism does not confer tolerance on its own, but the interplay of several mechanisms simultaneously is required. This requires an integrated response at the whole plant level as well as a cellular response. To understand the mechanisms by which a plant may survive desiccation, it is necessary to know how plant cells sense the loss of water and how the gene products function in stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 1996).

1.2 OBJECTIVES OF THIS STUDY

Differential screening of cDNA libraries, screening of expression libraries and complementation by functional sufficiency has been used to isolate a number of *X. viscosa* genes up-regulated in response to various stresses such as cold and dehydration. A large number of *X. viscosa* genes detected in response to various stresses are undescribed with no homology to known plant genes. These include *XvRG6* and *XvRF17* isolated from a *X. viscosa* cDNA library.

The aim of this research project is the molecular characterisation of *XvRG6* and *XvRF17*. This involves: (a) sequence analysis and bioinformatic studies to predict the probable functional properties of the genes and their corresponding proteins using computer software and internet databases; (b) Southern blot analysis to estimate the copy number of the genes in the *X. viscosa* genome; (c) real time PCR to determine the transcript level of the genes in response to abiotic stresses such as dehydration, ABA, SA, JA and ethylene; and (d) western blot and two dimensional gel electrophoresis analyses to determine the protein expression patterns of the genes.

Chapter 2

Molecular characterisation of *XvRG6* and *XvF17* from *Xerophyta viscosa*

2.1 INTRODUCTION

The *XvRG6* and *XvRF17* genes were isolated from a cDNA library synthesised from *X. viscosa* leaf material exposed to a 4°C low temperature stress (Mundree et al., 2006). This chapter describes the characterisation of *XvRG6* and *XvRF17* based on the gene sequence, including the determination of the *XvRG6* and *XvRF17* gene copy number in *X. viscosa* genome.

2.2 MATERIALS AND METHODS

2.2.1 Plant material and growth conditions

The *X. viscosa* plants were collected from Cathedral Peak Nature Reserve in the Drakensberg Mountains (Kwazulu-Natal, South Africa). The plants were grown under green-house conditions as described by Sherwin and Farrant (1996). Prior to imposing stress treatments, plants were allowed to acclimate in a controlled environment chamber [16 h light (150 $\mu mol.m^{-2}.s^{-1}$); 25°C; 8 h dark] for 1 month. Stress treatments as described in the subsequent chapters were applied under these environmental conditions.

2.2.2 Sequence analyses of *XvRG6* and *XvRF17*

The pDNR-LIB-XvRG6 and pDNR-LIB-XvRF17 plasmids were isolated from *E. coli* DH5α cells using the High Pure Plasmid Isolation Kit (Roche, Germany) according to the manufacturer's instruction. Each plasmid construct was sequenced using the M13 forward and reverse primers (Appendix B). The nucleotide sequence of each of the genes was determined on both forward and reverse strands. Multiple sequence alignment was performed using DNAMAN software (version 5.2.10). The BLAST program of the National Centre for Biotechnology Information (Altschul et al., 1990) was used to search the GenBank database for sequence similarities. Software analyses of both *XvRG6* and *XvRF17* included hydrophobicity prediction (using Expasy: www.expasy.org), pI prediction, total protein charge prediction and the potential phosphorylation site prediction.

2.2.3 Southern blot analyses of *XvRG6* and *XvRF17*

The Southern protocol was adapted from Sambrook et al. (1989). Genomic DNA was extracted from leaves of fully hydrated *X. viscosa* plants according to the procedure described by Dellaporta et al. (1983). Approximately 1 g of leaf tissue was ground to a fine powder in liquid nitrogen. The DNA was precipitated using isopropanol, resuspended in TE buffer (10 mM Tris, pH 7.6; 1 mM EDTA) and quantitated spectrophotometrically. Aliquots of genomic DNA (10 µg) were digested in separate tubes using the following restriction endonucleases: *Eco*RI, *Hin*dIII, *Xba*I, *Sac*I, *Hin*dIII + *Sac*I for *XvRG6*, and *Hin*dIII, *Xba*I, *Bcl*I, *Hin*dIII + *Cla*I for *XvRF17*. Digested DNA was electrophoresed overnight at 20 V on a 1% w/v agarose gel. On completion

of electrophoresis the DNA was transferred by capillary transfer (Sambrook et al., 1989) onto a nylon membrane (Hybond-XL; Amersham Biosciences, USA) and UV cross-linked (UV Crosslinker; Amersham Biosciences, USA) onto the membrane. Approximately 10 ng of purified *XvRG6* or *XvRF17* plasmid insert was used in the labeling reaction. Primers used in the amplification procedure were XvRG6F1 + XvRG6R1 and XvRF17F1 + XvRF17R1 (Appendix B), respectively. Both reactions contained [α-^{32}P]-dCTP at a concentration of 50 uCi. The PCR reactions were conducted using a Hybaid PCR Sprint thermal cycler (Thermo Hybaid, UK) with the following parameters: 95°C for 5 min; 15 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 10 min; and a final extension step at 72°C for 10 min. A longer extension time of 10 min was used to ensure that the 'heavier' radio-labeled dCTP would incorporate during amplification. Unincorporated [α-^{32}P]-dCTP was removed using the SigmaSpin™ Post-Reaction Purification Columns (Sigma, UK) according to the manufacturer's instructions.

The specific activities of the labelled probe were determined in a scintillation counter by counting 1 µL of probe in 2 mL of scintillation fluid. The membranes were pre-hybridised in pre-hybridisation buffer (0.5 M NaH_2PO_4; 1 mM EDTA; 7% w/v SDS; 1% w/v BSA) for 3 h at 65°C. Following pre-hybridisation, the radio-labeled probe was denatured by incubation for 10 min at 55°C in a heating block and immediately thereafter placed on ice. The denatured probes were added to the pre-hybridisation buffer and hybridisations were carried out for 18 h at 65°C with gentle shaking. The membranes were washed once for 12 min at 65°C in Wash Buffer A (0.5% w/v SDS; 2X SSC), followed by a second wash for 10 min at 65°C in Wash Buffer B (0.1% w/v SDS; 0.5X SSC). The membranes were autoradiographed at -70°C onto Hyperfilm MP (Amersham Biosciences, USA). Following 30 days exposure, the films were developed manually using developer and fixer reagents (Amersham Biosciences, UK) according to the manufacturer's instructions.

2.3 RESULTS

2.3.1 Software analysis of *XvRG6*

Sequence analysis using DNAMAN revealed that the *XvRG6* cDNA sequence is 629 bp long. This sequence contains an ORF of 369 bp. The deduced amino acid sequence was observed to encode a protein of 122 amino acids with a molecular mass of 13.4 kDa and a predicted high isoelectric point (pI) of 9.91 at pH 7. The upstream (5'-untranslated region) and downstream (3'-untranslated region) region of the ORF consisted of 24 and 242 bp, respectively. Neural networks and hidden Markov models predicted that XvRG6 has a signal peptide with a cleavage site between amino acid position 20 and 21. This suggests that XvRG6 could be a secreted protein.

```
1          GGGGATCAAGAGCAAATATAATCAATGGCGCCTTCGAAAACCTTCTTCATGGTTGCGATG
1                                   M  A  P  S  K  T  F  F  M  V  A  M

61         CTCTTAGTATCATCTTCTTTCTTCGCATGCTTCGCGAGGGAGCTCCATGAAACTAAAGCA
12          L  L  V  S  S  S  F  F  A  C  F  A  R  E  L  H  E  T  K  A

121        ACAGAAGAAGCAGGAGCCGAAAAAATGGACGCAGCACAGAGGTCTATTCGTCACGGTTCT
32          T  E  E  A  G  A  E  K  M  D  A  A  Q  R  S  I  R  H  G  S

181        GTGAACCCCAAGCGACCTGTTGTTAACCATCCTCTGAACCCCAACCGACCTTATTGTCCT
52          V  N  P  K  R  P  V  V  N  H  P  L  N  P  N  R  P  Y  C  P

241        CCAGGTCGTGGTTGCGGTGTGCACCCGCCTCCCTACCCGCAATACCCACCTTCTTACGGT
72          P  G  R  G  C  G  V  H  P  P  P  Y  P  Q  Y  P  P  S  Y  G

301        CCTTATACTCCACCTGGTGCTACTCCGTCCAGGCCTCCAAAGCGTCCATGCCGACCCCCA
92          P  Y  T  P  P  G  A  T  P  S  R  P  P  K  R  P  C  R  P  P

361        TTTTGCCGTCGAATTCCTCCAGCAACACCTTAGAAAGATGACTGATGCCACAACCCAGCC
112         F  C  R  R  I  P  P  A  T  P  *

421        AGACGGATTAATTAGTTTGCTCTATATATATTAAAGCTGTAGTATTAATCTTACGTGGGA
481        ATAAAGTTGTAATTGCTCCATGTAGAGCTGCATATATAGTCGATGTATACTTGGTAGTCA
541        TGCTGCATAAGGAACATGTATGTTATTTTGTAATGCTACCTATTTTAATGATTTCTAGTT
601        TCTACATTTTAGGAGTCGTACTACTACTC
```

Figure 2.1 Nucleotide and deduced amino acid sequence of XvRG6. The putative start and stop codons as well as the proline residues are represented in red. The cleavage site position of the signal peptide and the glycosylation site are indicated by a green and red line respectively. The stop codon is indicated by an asterisk (*), the primer locations are indicated by a black line. The suspected phosphorylation sites are represented in blue.

The hydropathy plot (Kyte and Doolittle, 1982) predicted that the protein is predominantly hydrophilic (Fig 2.2). The high hydrophilic score of the XvRG6 protein, as indicated by the negative score values makes it unlikely that it spans a membrane.

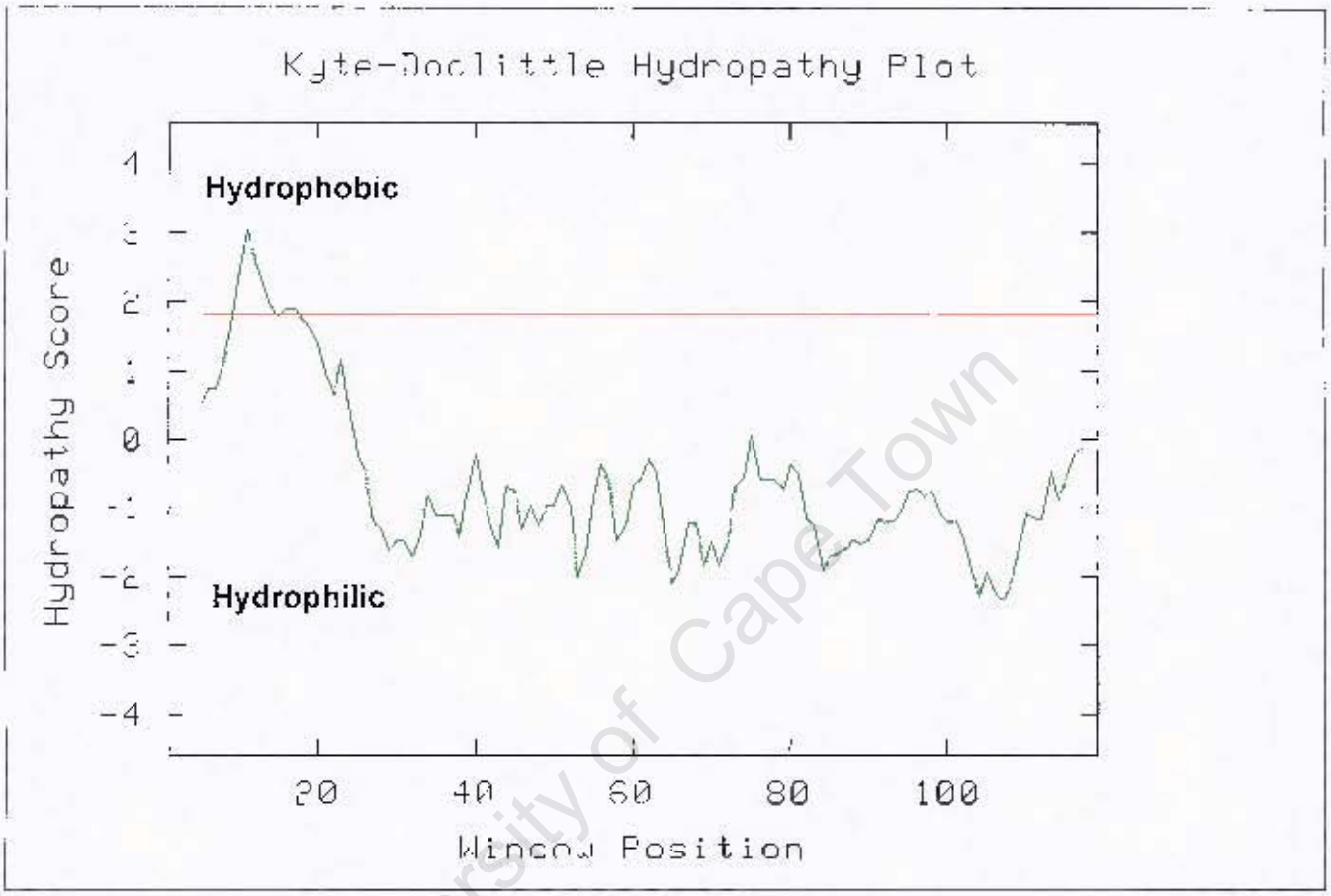

Figure 2.2 A hydropathy plot of XvRG6 as determined by the method of Kyte and Doolittle (1982). The red line represents the cut-off for the hydrophobic and hydrophilic residues.

The XvRG6 protein has 7 potential phosphorylation sites with phosphorylation potentials above the threshold value of 0.5 (Fig. 2.3A). These are two serine residues (amino acid positions 47 and 52), three threonine residues (amino acid positions 96, 100 and 121) and two tyrosine residues (amino acid positions 82 and 94). Parallel software analysis was carried out to predict glycosylation sites (Fig. 2.3B). The XvRG6 protein was found to have five predicted *O*-glycosylation potential sites (amino acid positions 90, 95, 100, 102 and 121). The XvRG6 protein has 26 proline residues and 5 cysteine residues. It is interesting to note that the first domain (the hydrophobic domain) of XvRG6 protein has one proline residue and one cysteine residue. The second domain (the

hydrophilic domain) is rich in proline repeats. The presence of two distinct domains as described above and the fact that XvRG6 amino acid sequence contains both proline and cysteine repeats suggests that it could be a hybrid proline rich protein (HyPRP) (Fig. 2.1). However, using BLAST searches XvRG6 was predicted to be proline-rich protein with an E-value of 2.06e-03.

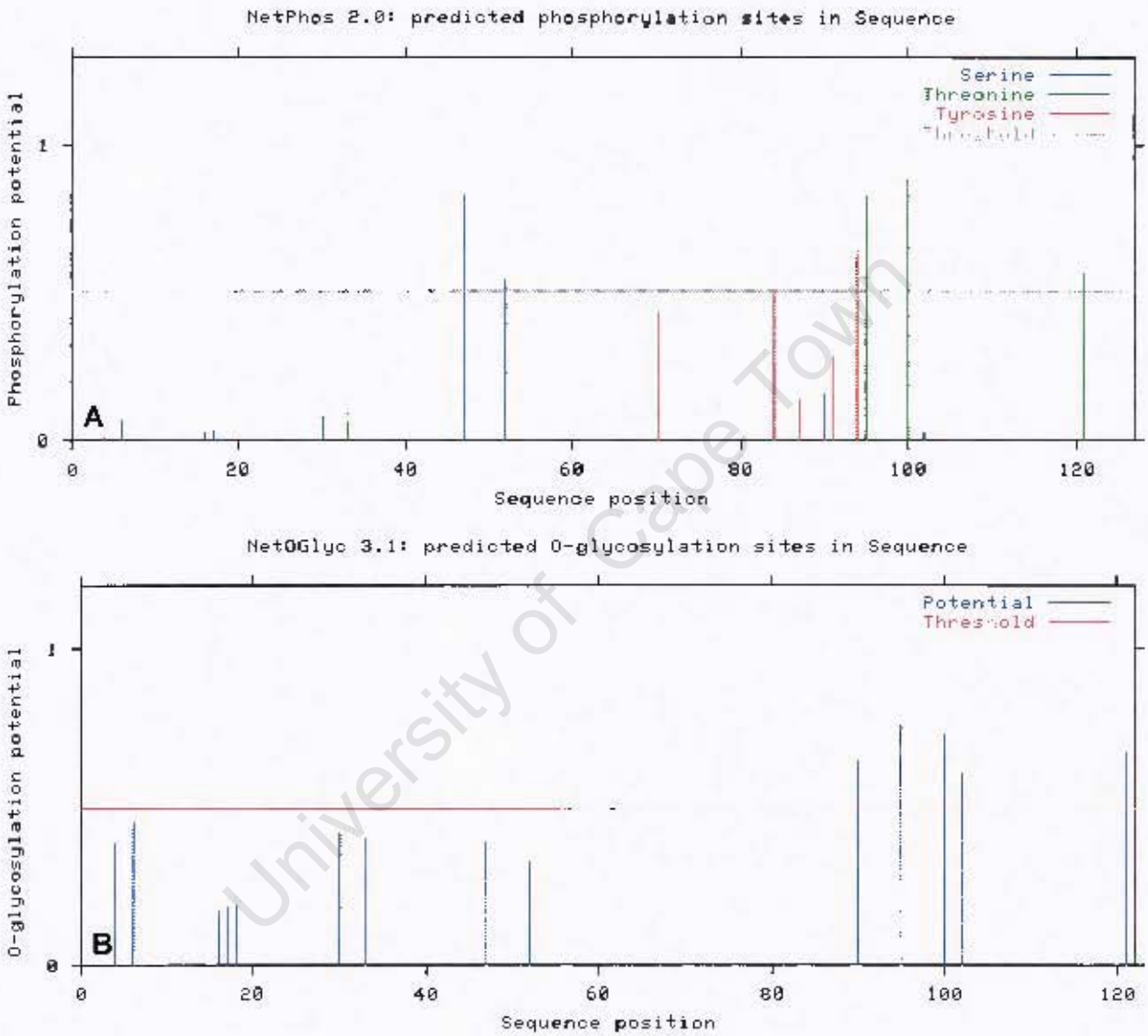

Figure 2.3 Software based prediction of phosphorylation potential (A) and O-glycosylation potential (B) of XvRG6.

2.3.2 Software analysis of *XvRF17*

The full-length *XvRF17* cDNA sequence (647 bp) contains an ORF of 240 bp. The deduced amino acid sequence was observed to encode a protein of 80 amino acids with a molecular mass of 10.78 kDa and a predicted isoelectric point (pI) of 7.95 at pH 7. The upstream and downstream regions of the ORF consisted of 76 and 277 bp, respectively.

```
1       GGGGATCGAAGGAGATAAACAGAGCCGAGTCTTGAGAAATTTCGTGAGATTGTATTTGAA

61      TATCAGGAAATCGATCATGGCGTCTTGCGGAGGAAACTGCGGCTGCGGACCGAACTGCAA
1                        M  A  S  C  G  G  N  C  G  C  G  P  N  C  K

121     GTGCGGTAGCAACTGCAACTGCGGAGGGAACAAGATGTACCCTGGGTTGGCCGAGGAGAG
15        C  G  S  N  C  N  C  G  G  N  K  M  Y  P  G  L  A  E  E  R

181     GAGCACAACCACGCAGACCAACGTGCTCGGCGTGGCACCTCAGCAGGAGCGCCGTGAAGG
35        S  T  T  T  Q  T  N  V  L  G  V  A  P  Q  Q  E  R  R  E  G

241     GTTTGAGGCCGGGCAACGATCAGAGAGCGGTGGCTGCAAGTGCAGTCCCTGCAACTGCAA
55        F  E  A  G  Q  R  S  E  S  G  G  C  K  C  S  P  C  N  C  N

301     CCCCTGCAACTGCAAATGAGCGTCATCTATCTCTCGCTTCGCTTTATGATGCAGAATCAA
75        P  C  N  C  K  *
361     TAGTAAATCAATAGTAATAACTAATAGCCTAGGGGGAAATACATATAAGCTCCGTCTTTC
421     TAAATCATGTGTAAATTAATCCCTGATCAGGGACTTATTTGTATGTTATGGACTGATATG
481     TAAGTTGTCCAATAAACTAGGTTTGTATGTTGGAGTTTTTGTGAGTGCTGCTACTACTAG
541     AAAATAATGGCTTTCTTGTTACTGCTAGGCAAGAGTTGTTTAGAGAGTGATAATATGTAA
601     TAATTGTGTCAATATGTCAATATGTAATAAACTGGTTTTCGGCTTTA
```

Figure 2.4 Nucleotide and deduced amino acid sequence of XvRF17. The putative start and stop codons as well as the cysteine residues are represented in red. The stop codon is indicated by an asterisk (*), the primer locations are indicated by a black line. The suspected phosphorylation sites are represented in blue.

The hydropathy plot (Kyte and Doolittle, 1982) predicted that the XvRF17 protein is completely hydrophilic (Fig 2.5).

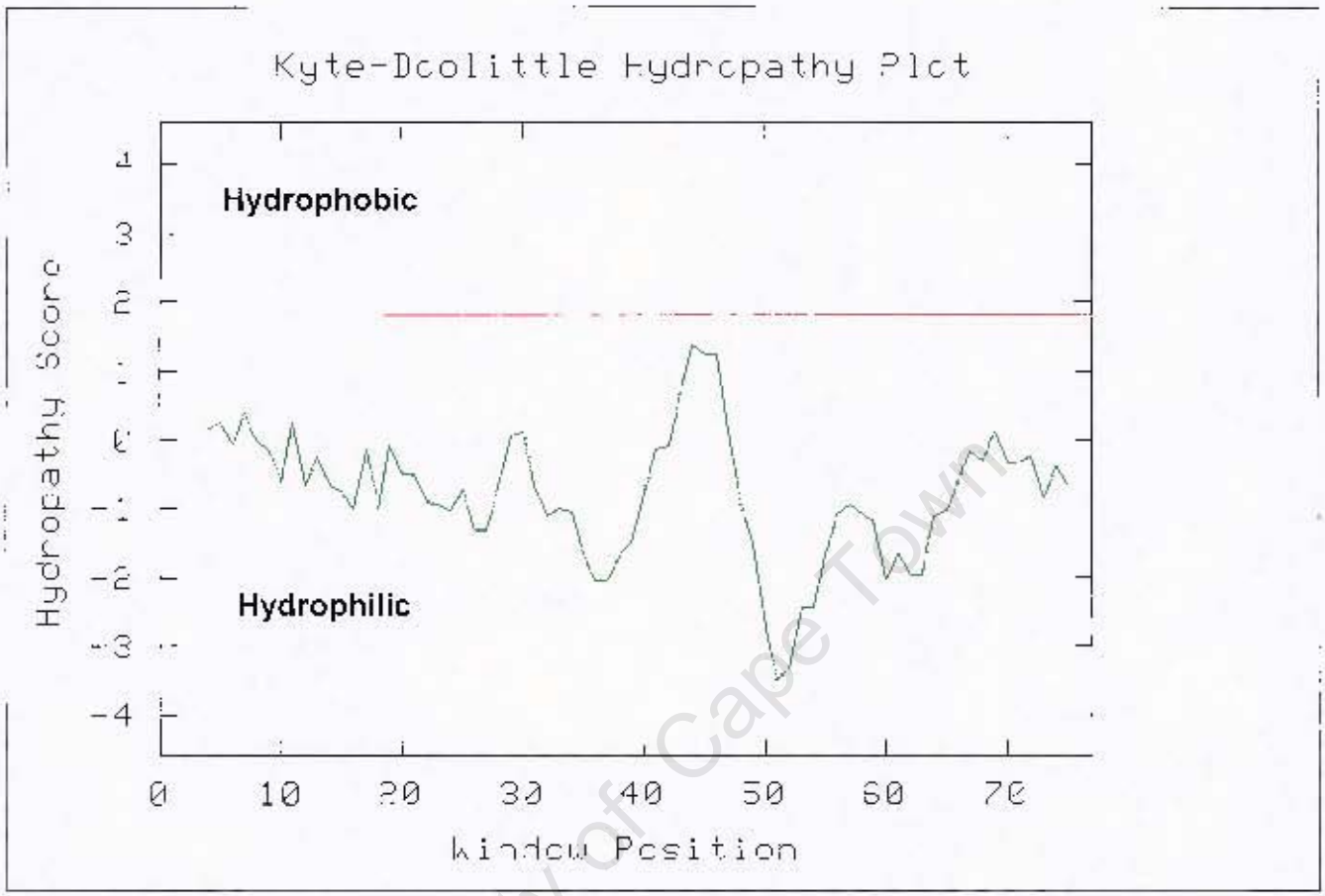

Figure 2.5 A hydropathy plot of XvRF17 as determined by the method of Kyte and Doolittle (1982) The red line represents the cut-off for the hydrophobic and hydrophilic residues.

The XvRF17 protein has 4 potential phosphorylation sites with phosphorylation potentials above the threshold value of 0.5 (Fig. 2.6A). These are two serine residues (amino acid positions 62 and 64), two threonine residues (amino acid positions 37 and 39). No tyrosine residue was detected. Parallel software analysis was carried out to predict glycosylation sites (Fig. 2.6B). No glycosylation site was found. The amino acid sequence of XvRF17 has 13 cysteine residues arranged from the N-towards the C-terminal region with a cysteine-free central spacer. These cysteine residues are arranged as Cys-Xaa-Cys where Xaa represents another amino acid. This residue is common to the type 1 metallothionein protein (Robinson et al., 1993) and suggested that XvRF17 protein could possibly be a type 1 MT (Fig. 2.4). However, using BLAST searches XvRF17 was predicted to be metallothionein with an E-value of 1.7e-31.

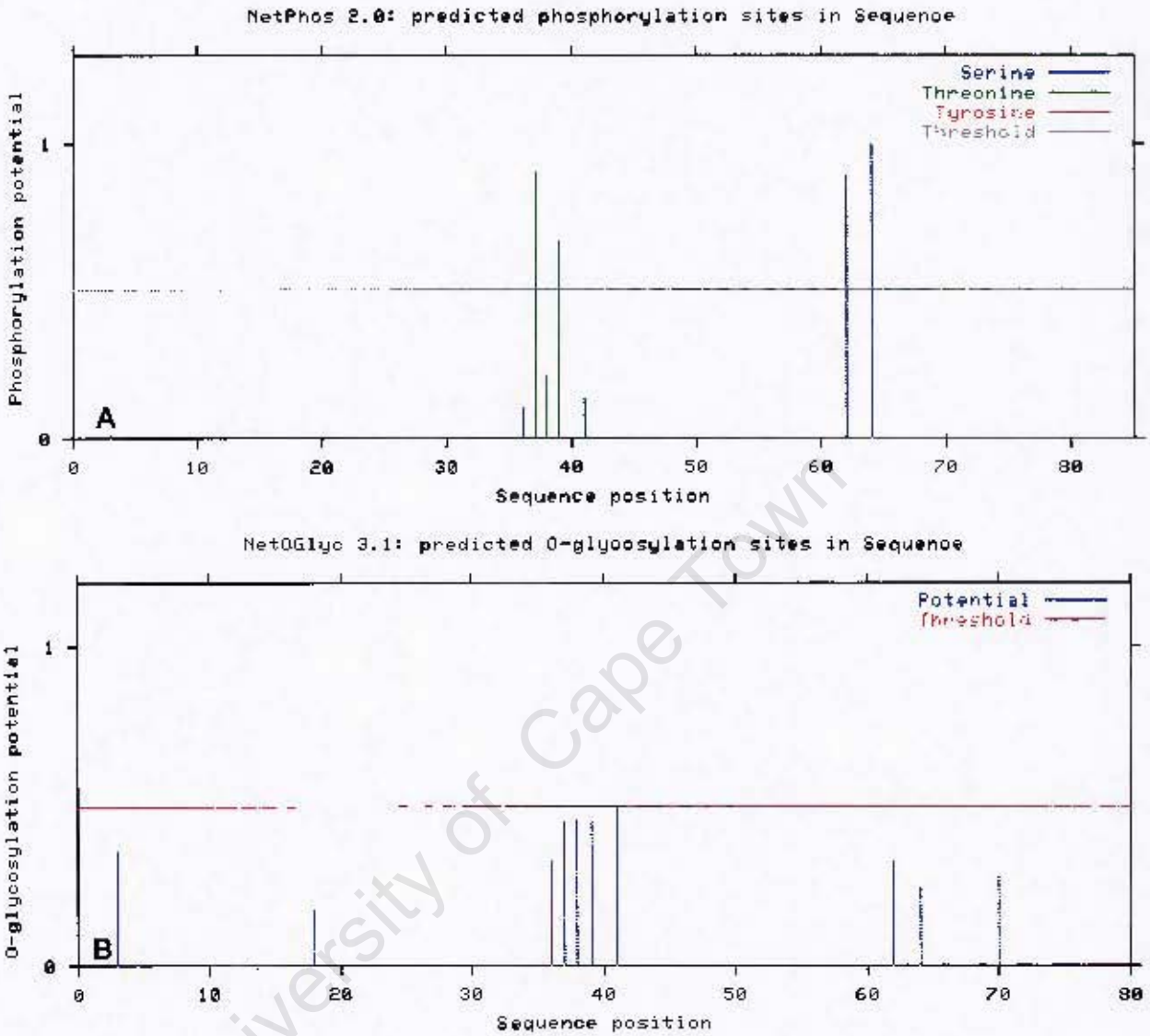

Figure 2.6 Software based prediction of phosphorylation potential (A) and *O*-glycosylation potential (B) of XvRF17.

2.3.3 Southern blot analysis

Southern blot analysis of *X. viscosa* genomic DNA was performed to firstly confirm the presence of the *XvRG6* and *XvRF17* in the *X. viscosa* genome and secondly to estimate the copy number of the gene in the *X. viscosa* genome.

2.3.3.1 Southern blot analysis of *XvRG6*

Analysis of the blot revealed a maximum of 6 bands hybridising to the probe. The DNA digested with *Eco*RI, *Hin*dIII, *Sac*I and the double digestion with (*Hin*dIII + *Sac*I) produced a single band (at 8, 7, 5 and 0.6 kb respectively) hybridising to the probe while digestions with *Xba*I produced 2 bands (at 5 and 5.2 kb) hybridising to the probe (Fig. 2.7B). The enzyme *Eco*RI, *Sac*I have both a single restriction site within *XvRG6* cDNA sequence at position 371 and 104 respectively. These results suggest the presence of *XvRG6* in the *X. viscosa* genome as a single copy.

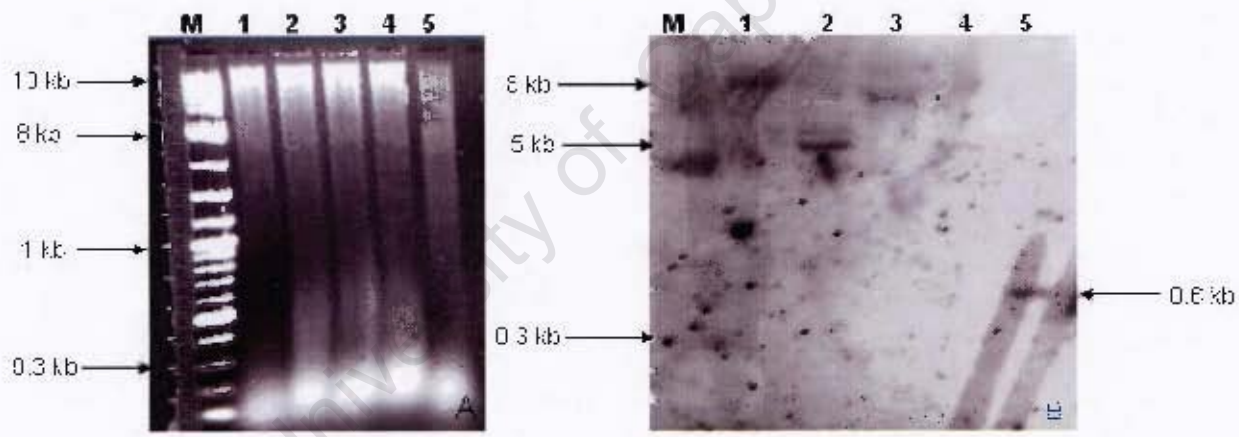

Figure 2.7 Ten micrograms of *X. viscosa* genomic DNA was digested with a number of restriction endonucleases either as single or double digests (A). Autoradiograph following 30 day exposure to membrane probed with radiolabelled *XvRG6* (B). Lanes: M, 2-log DNA molecular weight marker; 1, *EcoR*1; 2, *Xba*1; 3, *Hin*dIII; 4, *Sac*1; 5, *Hin*dIII/*Sac*1.

2.3.3.2 Southern blot analysis of *XvRF17*

DNA digested with either *Hin*dIII, *Bcl*I or *Xba*I produced two bands (at 8 and 0.8 kb; 8 and 7 kb; 8 and 4 kb respectively). The double digested DNA (*Hin*dIII + *Cla*I) produced 2 bands (4.5 and 0.6 kb) (Fig. 2.8B). Among these enzymes only *Bcl*I and *Cla*I have both a single restriction site within *XvRF17* cDNA sequence at position 444 and 71 respectively. These results suggest that there could be at least two copies of *XvRF17* in the *X. viscosa* genome.

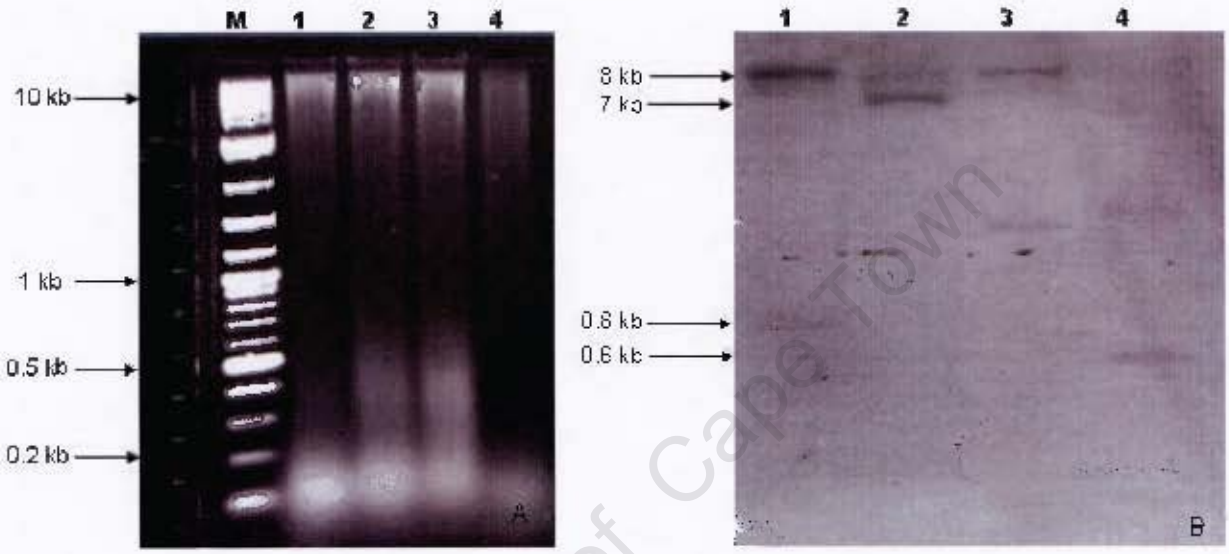

Figure 2.8: Ten micrograms of *X. viscosa* genomic DNA was digested with a number of restriction endonucleases either as single or double digests (A). Autoradiograph following 30 day exposure to membrane probed with radiolabelled *XvRF17* (B). Lanes: M, 2-log DNA molecular weight marker; 1, *Hin*dIII; 2, *Xba*1; 3, *Bcl*1; 4, *Hin*dIII/*Cla*1.

2.4 DISCUSSION

The major amino acid constituent in plant cell wall protein hydrolysates is hydroxyproline (Lamport and Northcote, 1960). In plants there are three classes of glycoproteins containing hydroxyproline. These are lectins, arabinogalactan protein (AGPs) and extensins. The AGPs and lectins are soluble while extensins are insoluble. Extensins which belong to the group of (hydroxyproline-rich glycoproteins HRGPs) can be further classified into a number of different sub-groups among which proline rich protein (PRPs). The PRPs are suggested to be involved in the modification and structure of the cell wall (Showalter and Rumeau, 1990; Darley et al., 2001). This group of proteins constitutes one of the major sub-groups of (HRGPs) (Showalter and Rumeau, 1990). The HRGPs are characterised as extracellular, structural components of the plant cell wall, while PRPs are specifically considered to be involved in cell wall rigidification associated with the cessation of cell expansion (Brownleader et al., 1999; Darley et al, 2001). The PRPs, which accumulate later in cell development, are apparently inserted to lock the extensins, thereby forming a heteropeptide framework (Ye and Varner, 1991). Based on the predicted isoelectric points of PRPs (pI 8.8-10), the PRPs may interact with the acidic pectin network within the cell wall (Satoh and Fujii, 1988).

Despite the proposed role of PRPs in cell wall modification and structure, and the fact that cell wall metabolism is regarded as the main factor in fruit softening and texture, surprisingly little is known about PRPs from ripening fruit (Burger et al., 2004). The PRPs are characterized by unique amino acid compositions and distinct patterns of regulation and localisation, implicating particular roles in the development, structure and function of cell walls of particular cells (Fowler et al., 1999; Davies and Robinson, 2000; Milioni et al., 2001).

The results presented show that XvRG6 is a proline-rich protein. The full-length *XvRG6* cDNA sequence of 629 bp contains an ORF of 329 bp with a 5′ and 3′ UTR of 24 and 242 bp, respectively. The *XvRG6* gene encodes a single polypeptide of 122 amino acids with a molecular mass of 13.4 kDa and an isoelectric point of 9.96. The XvRG6 protein has 26 proline residues and 5 cysteine residues. The hydrophobic domain of XvRG6 protein has one proline residue and one cysteine residue while the hydrophilic domain is rich in proline repeats. The PRPs are arranged in three groups (table 2.1). These are: (i) PRPs with proline repeats along the entire protein sequence and without a cysteine residue; (ii) hybrid PRPs (HyPRP), which contain two domains, the first domain is rich in proline repeats and a second hydrophobic domain which has no proline repeats but is rich

in cysteine. In addition there is a central spacer with no proline repeats between the two domains; (iii) other PRPs, which are similar to the first group (PRPs) in that proline occurs along the entire protein sequence with no cysteine residue; however the proline repeat is different.

Table 2.1 Classification and feature of PRPs, HyPRPs, others PRPs and XvRG6

	PRPs	HyPRPs	Other PRPs	XvRG6
Signal peptide	Present	Present	Present	Present
Proline repeats	Occur along entire sequence	Occur not along entire sequence	Occur along entire sequence	Occur not along entire sequence
Phosphorylation sites	Present	Present	Present	Present
Tyrosine residue	Present	Present	Present	Present
Cysteine residue	Absent	Present	Absent	Present

Despite having features common to proline-rich proteins, such as repetitive sequences containing a high proportion of proline and a signal peptide that might be used for the transport of the protein, XvRG6 appears different from the other reported plant PRPs in that it has a proline residue in the hydrophobic domain. It is possible that XvRG6 is still a HyPRP even though the hydrophobic domain has one proline repeat and there is only one cysteine residue.

Analysis of the XvRG6 amino acid sequence using neural networks and hidden Markov models predict a signal peptide, indicating that XvRG6 could be transported out of the cytoplasm. A hydropathy plot of the XvRG6 amino acid sequence shows that XvRG6 is predominantly hydrophilic. This is unexpected as PRPs are generally insoluble. According to José and Puigdomènech (1993) the insolubilisation of PRPs involves H_2O_2-mediated oxidative cross-linking, probably through tyrosine. This oxidative cross-linking provides a mechanism for rapid hardening of the wall as a protection against environmental stresses (Bradley et al., 1992). The XvRG6 protein also has a tyrosine residue, which might be involved in oxidative cross-linking to protect *X. viscosa* against environmental stresses.

The analysis of XvRG6 protein revealed seven phosphorylation sites. These comprise two putative serine phosphorylation residues. Protein kinase C has been reported in plants and is known to phosphorylate serine residues (Chandok and Sopory, 1998). It is hypothesised that

protein kinase C could recognise the serine phosphorylation sites of XvRG6 and potentially phosphorylate XvRG6 to regulate it's function.

Metallothioneins (MTs) are low molecular weight, cysteine-rich proteins with the ability to co-ordinate metal atoms (Mir et al., 2004). In plants, a correlation has been observed between MT RNA levels and tolerance to heavy metals in different *Arabidopsis* ecotypes (Murphy and Taiz, 1995a), suggesting a role in metal homeostasis in plants. A variety of other stimuli, including ABA, heat shock, cold shock, wounding, viral infection, senescence, salt stress, and suc starvation, have also been shown to influence expression of plant MTs (Buchanan-Wollaston, 1994; Foley and Singh, 1994; Hsieh et al., 1995; Murphy and Taiz, 1995a; Snowden et al., 1995; Choi et al., 1996; Foley et al., 1997). The data available regarding the expression of MT genes from a variety of plant species indicate that each MT gene type exhibits characteristic temporal and tissue-specific expression patterns (Garcia-Hernandez et al., 1998).

In plants, two categories of MT-like proteins are proposed on the basis of the predicted locations of Cys residues and are designated type 1 and 2 (Robinson et al., 1993). In type 1, there are exclusively Cys-Xaa-Cys motifs whereas in type 2 there is a Cys-Cys and a Cys-Xaa-Xaa-Cys pair within the N-terminal domain (Robinson et al., 1993). Based on taxonomic relationships, the current classification consists of 15 MT families, with plant MTs being placed into family 15 (Kojima et al., 1999; Binz and Kagi, 1999). Plants MTs are divided into three major classes (class I, II and III), which are distinguishable on the basis of the distribution of the cysteine free spacer (Robinson et al., 1993). Class I and II polypeptides are direct gene products, whereas class III MTs are non-translational cysteine-rich molecules named phytochelatins (Ma et al., 2002). Metal ions are sequestered by MTs through complexation with the sulphuric group of the Cys rich motifs (Ma et al., 2002). Since plant MTs efficiently bind metals (Kille et al., 1991) and some MT genes are positively regulated by metals, MTs are thought to be involved in cellular metal homeostasis and tolerance mechanisms (Cobbett and Goldsbrough, 2002). Moreover, an increasing number of observations suggest a role for plant MTs in senescing tissues, since expression of MT genes has been shown to be induced in senescing leaves and stems (Buchanan-Wollaston, 1994; Chen et al., 2003), in ripening fruits (Davies and Robinson, 2000), and in tapetum cells (Choi et al., 1996; Butt et al., 1998).

The full-length *XvRF17* cDNA sequence (647 bp) contains an ORF of 240 bp, which encodes a protein of 80 amino acids with a molecular mass of 10.78 KDa and a predicted

isoelectric point (pI) of 7.95 at pH 7. The XvRF17 protein has 13 cysteins residues arranged from the N-terminal toward the C-terminal separated by a cysteine-free central spacer. Furthermore it is interesting to note that these cysteine residues are exclusively Cys-Xaa-Cys where Xaa represents any other amino acid. Class 1 MT proteins are characterized by having two cystein-rich domains which are separated by a cysteine-free central spacer (Giordani et al., 2000). This class 1 MT-like protein is further divided into type 1 and type 2, based on the cysteine spacing within the amino acid sequence (Robinson et al., 1993). In type 1, there are exclusively Cys-Xaa-Cys motifs whereas in type 2 there is a Cys-Cys and a Cys-Xaa-Cys pair within the sequence (Robinson et al., 1993). Consequently, the XvRF17 protein is probably a type 1 metallothionein.

The XvRF17 protein is hydrophilic with six potential phosphorylation sites, which include serine, threonine and tyrosine. Protein phosphorylation is the most important regulatory event and many enzymes are switched on or off by phosphorylation and dephosphorylation (Ishitani et al., 2000). The presence of these putative phosphorylation sites may indicate possible means of regulation of the XvRF17 protein. The XvRF17 protein sequence has 7 and 6 cysteine residues in the N- and C-terminals, respectively. These putative residues might be implicated in the sequestration of metal, suggesting a role for XvRF17 in metal ion homeostasis and development.

Southern blot hybridisation of restriction endonuclease-digested *X. viscosa* genomic DNA confirmed the presence of both *XvRG6* and *XvRF17* in the *X. viscosa* genome. The results suggest the presence of a single copy of *XvRG6* and at least two copies of *XvRF17* in the *X. viscosa* genome. Incidentally, the presence of two copies of *XvRF17* is consistent with the small multigene family nature of MTs genes reported in *Arabidopsis* (Zhou and Goldsbrough, 1995).

2.5 CONCLUSION

Sequence analysis confirmed XvRG6 to be a proline-rich protein, since it possesses many of the features common to PRP such as a signal peptide, proline repeats, a cysteine residue and possible phosphorylation sites. It was also interesting to note the presence of a tyrosine residue present in some repeats of XvRG6. This residue is believed to play a role in protecting the plant against environmental stresses (Bradley et al., 1992). The XvRF17 protein was predicted by software analysis to belong to the family of metallothioneins. In both cases Southern blot analysis confirmed the presence of both *XvRG6* and *XvRF17* genes in the *X. viscosa* genome.

Chapter 3

Real-time PCR analyses of *XvRG6* and *XvF17*

3.1 INTRODUCTION

Understanding responses of plants to their environment in terms of adaptability and performance is of paramount importance. Apart from interest in factors important for ecological and evolutionary distribution, much present day interest is fuelled by practical needs (Swamy and Smith, 1999). Modern agriculture is affected by environmental factors such as water, drought, temperature, light and salt stress. Additional factors are nutrient supply and pathogen attack. Adaptation requires reprogramming of gene expression. Thus, an environmental factor has to be perceived and transduced into an intracellular signal, which upon signal transduction leads locally or systemically to gene expression.

During biotic or abiotic stress, plants produce increased amounts of hormones such as ABA and ethylene (Xiong et al., 2002). In addition, salicylic acid (SA) and perhaps jasmonic acid (JA) may be involved in some aspects of the stress response. These hormones may interact with one another in regulating stress signalling and plant stress tolerance. For example, ethylene has been shown to enhance ABA action in seeds (Gazzarrini and McCourt, 2001), while JA is a plant growth regulator, which functions as a signal of developmentally or environmentally regulated expression of various genes thereby contributing to the defence status of plants (Hamberg and Gardner, 1992; Creelman and Mullet, 1997).

Real-time quantitative PCR (qRT-PCR) is the most sensitive technique for mRNA detection and quantitation currently available (Heid et al., 1996; Livak and Schmittgen, 2001). Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step (Wong and Medrano, 2005).

In this chapter, qRT-PCR was used to analyse the relative changes in gene expression of *XvRG6* and *XvRF17* in plants exposed to dehydration and various hormone treatments.

3.2 MATERIALS AND METHODS

3.2.1 Abiotic treatment of plants

Whole plants were subjected to various abiotic stress treatments. These stresses involved dehydration, ABA, JA, SA and ethylene treatment. These were done in separate experiments and no plant was subjected to dual stress. The experiments were conducted on 3 plants for each treatment and repeated once. Leaf samples were collected and the relative water content (RWC) determined using the formula: RWC = [(FW-DW)/ (WW-DW)] x 100%] where FW is the fresh weight WW is the wet weight and DW is the dry weight. Leaf samples were prepared by cutting off 3 leaves from the treated plant. The excised leaves were dissected into smaller pieces, wrapped in aluminium foil, quickly frozen in liquid nitrogen then stored at -80°C until RNA extractions were performed. For each treatment, samples were collected in triplicate. For ABA, JA, SA and ethylene treatments the RWC was only determined at time 0 and 72 h.

3.2.1.1 Dehydration

Dehydration of *X. viscosa* was conducted by withholding water from the soil for a period of 10 days. Leaf samples were taken at full turgor and thereafter 2, 4, 6, 8, and 10 days without water.

3.2.1.2 Abscisic acid, jasmonic acid and salicylic acid treatments

The ABA was prepared as 10 mM stock in 100% ethanol and diluted to 100 μM with water. The JA and SA were prepared as 10 mM stock in 10% ethanol and diluted with water to the appropriate concentration (100 μM). As the hormones are light sensitive, the solutions were stored in bottles covered with aluminium foil to minimise light inactivation. Plants used in hormone treatments were separated into 4 groups of 3 plants each. Each group was treated by spraying the leaves with ABA, JA, SA or distilled water (control). After spraying, plants were covered for 10 min with black plastic. Plants belonging to the different experimental groups were kept separate to prevent cross-contamination. Samples were harvested at 0, 3, 6, 12, 24, 30, 36, 48, 54, 60 and 72 h after spraying. These plants were kept hydrated throughout the experiment.

3.2.1.3 Ethylene treatment

A whole plant was placed into a plastic bag (Sigma, UK) designed for gas treatment. Ethylene was applied at a concentration of 1 ppm. The plants were removed from the bag 10 min after application and equilibrated under green-house conditions as described by Sherwin and Farrant (1996). Samples were harvested at 0, 3, 6, 12, 24, 30, 36, 48, 54, 60 and 72 h after treatment.

3.3 RNA ISOLATION AND PURIFICATION

3.3.1 RNA isolation

Total RNA was extracted from *X. viscosa* leaves using the EZ RNA Reagent (Bio Basic Inc, Canada). All solutions used were treated with 0.01% v/v diethylpyruvocarbonate (DEPC; Sigma, UK) and all equipment used for isolation were autoclaved twice. Approximately 0.1 g of plant material was ground in liquid nitrogen using a mortar and pestle, keeping the tissue frozen at all times. The ground tissue was transferred to a 2 mL Eppendorf tube containing 1 mL of EZ RNA reagent. The mixture was inverted 6 times, vortexed for 10 min and thereafter incubated for 5 min at room temperature (RT) to allow complete dissociation of nucleoprotein complexes. Thereafter, 0.2 mL of chloroform was added, the mixture was inverted 30 times, then incubated for 3 min at RT, followed by centrifugation for 15 min at 12000 x g at 4°C. The top aqueous layer was carefully transferred to a sterile tube containing 1 mL isopropanol. The mixture was inverted 10 times in isopropanol, incubated for 10 min at RT, followed by centrifugation for 15 min at 12000 x g at 4°C. The supernatant was discarded and the pellet washed with 75% v/v ethanol. Thereafter the pellet was dried for 3-5 min at RT. The dried pellet was resuspended in 50 µL DEPC water by incubating for 3-5 min at 55°C. The RNA was stored at -80°C.

3.3.2 DNase I treatment and RNA purification

To remove contaminating DNA, the RNA was treated with DNase I (New England BioLabs, USA) according to the manufacturer's instructions. After 10 min incubation at 37°C, the sample volume was adjusted to 100 µL with RNase-free water prior to purification. The RNA was purified using the RNA Miniprep Super Kit (Bio Basic Inc, Canada). To the 100 µL sample, 350 µL RLT solution was added and the mixture inverted 30 times, followed by the addition of 250 µL 100% ethanol. After inverting 10 times, the sample was applied to an EZ spin column in

a 2 mL collection tube, centrifuged for 1 min at 4000 x g and washed with 500 µL RW solutions. The flow-through was discarded and 500 µL RPE solution was added to the EZ spin column and centrifuged for 1 min at 8000 x g. The flow-through was discarded. To elute the RNA, the spin column was transferred to a new 1.5 mL collection tube, 50 µL DEPC water was added directly onto the centre of the membrane followed by 2 min incubation at 50°C and centrifugation for 1 min at 10000 x g. A 4 µL aliquot was quantitated using a ND-1000 spectrophotometer (NanoDrop Technologies, USA), separated on a 1.2% w/v agarose gel and stained for 30 min with 0.005% v/v ethidium bromide to verify the concentration of the RNA samples. The remaining RNA sample was stored at -80°C.

3.3.3 Synthesis of cDNA

The RNA concentration was was quantitated using a ND-1000 spectrophotometer. The following components were mixed in a sterile 0.5 mL tube: 1-15 µL RNA (ca 2.5 µg), 5 µL CDS III primer (10 µM; Clontech, UK) and made up to 25 µL with dH_2O. The mixture was centrifuged briefly and thereafter incubated for 2 min at 72°C then cooled for 2 min on ice. Thereafter, 1 µL M-MuLV Reverse transcriptase (Finnzymes, Finland; RNase H,$^-$ 200 U/µL) 5 µL M-MuLV RT buffer (10X) 5 µL dNTP (10 mM) and 14 µL dH_2O was added to the tube prior to incubation for 1 h at 42°C. Thereafter 1 µL RNaseH [Finnzymes, Finland; 5 U/µL] was added followed by 15 min incubation at 37°C. The cDNA was stored at -20°C.

3.3.4 Specificity of real-time primers

The following components were mixed in a sterile 0.5 mL tube: 1 µL cDNA mix, 0.5 µL dNTP (10 mM), 2.5 µL expand buffer (10X), 1.5 µL $MgCl_2$ (25 mM) and 0.2 µL of High Fidelity Taq DNA polymerase, 1 µL (10 µM) each of forward and reverse gene specific primer were used for each PCR reaction. Reactions were conducted using a Gene Amp 9700 thermo cycler under the following conditions: 94°C for 30 s; 94°C for 20 s; 58°C for 30 s; 72°C for 30 s; (30 cycles); 72°C for 5 min; 4 °C for 2 min; 20°C for 2 min. Following PCR, the samples were resolved by electrophoresis on a 2% w/v agarose gel and stained with ethidium bromide. The *XvSAP* gene (Garwe et al., 2003) was also amplified as a control for melting curve comparison.

3.3.5 Real-time PCR for gene expression quantification

The relative expression of *XvRG6* and *XvRF17* in *X. viscosa* treated leaves was determined with the RG-3000A machine (Corbett Research, Australia) using the SensiMix (2X) (dT) DNA Kit (Quantace, Australia). The PCR was performed in a total volume of 25 µL containing 0.5 µL SYBR green solution (50X), 12.5 µL SensiMix (2X) and 9 µL dH_2O. The mixture was spun briefly and 1 µL of both reverse and forward primer (10 µM) was added to each tube followed by addition of 2 µL cDNA. The gene specific primers sets [XvRG6F2 + XvRG6R2 for *XvRG6*; XvRF17F2 + XvRF17R2 for *XvRF17* (Appendix B)] used for qRT-PCR were designed using DNAMAN software. The sequence of the *X. humilis* 18S rRNA was obtained from GenBank (accession number EF418586). All oligonucleotides were synthesised on a 1000M DNA synthesiser (Beckman, USA) using a high purity program (Synthetic DNA Laboratory, University of Cape Town). Real-time PCR was conducted on a RG-3000A machine (Corbett Research, Australia). PCR cycles were as follows: initial enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 5 s, 58°C for 8 s and 72°C for 12 s.

3.3.6 Statistical analysis

PCR efficiencies of target genes were determined by generating standard curves. The PCR efficiencies were achieved based on serial dilutions prepared from plasmid containing a target gene. The C_T value is defined as the cycle number at which the ΔR_n [$\Delta Rn = (R^+_n) - (R^-n)$, where R^+_n is the fluorescence signal of the product at any given time and R^-n is the fluorescence signal of the baseline emission] crosses this threshold. The calculated concentration of the target gene was divided with the calculated concentration of the 18S rRNA. Thereafter the values of the three biological replicates were averaged and used for quantification of transcripts. The quantification of the relative transcript levels was obtained after dividing the sample quantity by calibrator quantity. In this study the calibrator quantity was the average of the three biological reference samples at time zero. The calibrator was designated as 1-fold, with all experimentally derived quantities reported as an n-fold difference relative to the calibrator (Wong and Medrano, 2005).

3.4 RESULTS

3.4.1 RNA extraction

An example of good quality RNA (Fig. 3.1) was isolated from *X. viscosa* plants subjected to various abiotic stresses. After DNAse I treatment, sample 1 and 2 see line 1 and 2 (Fig. 3.1B) respectively were excluded.

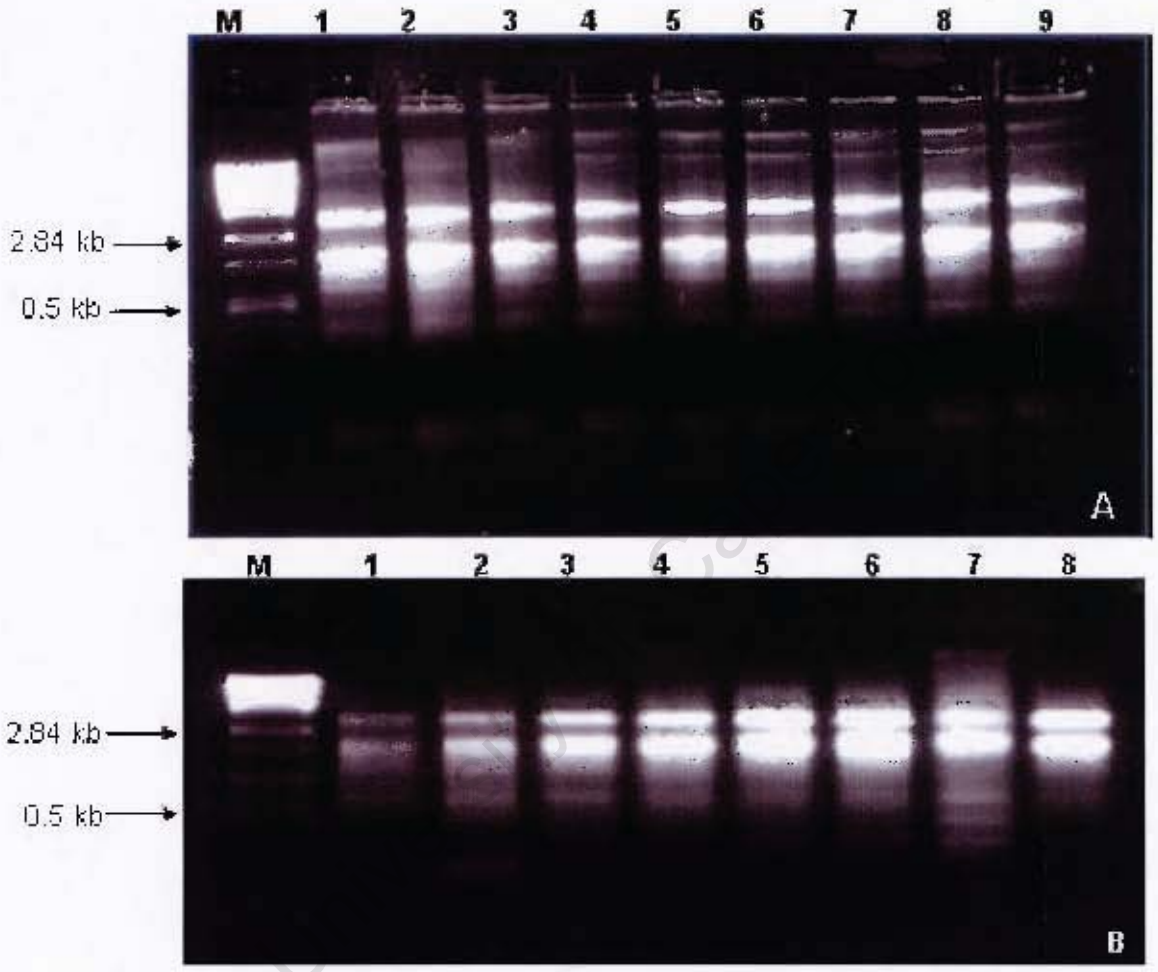

Figure 3.1 Gel electrophoresis of total RNA extracted from *X. viscosa* leaves. A, total RNA; and B, total RNA treated with DNase I. M, λ DNA digested with *Pst*I. Arrows on the left indicate molecular sizes determined from λ *Pst*I markers.

3.4.2 Primer amplification and optimisation of the primer sets

Specificity of each primer set was examined by qRT-PCR using the cDNA mix prepared from RNA extracted from *X. viscosa* leaf samples. Amplification of a specific transcript for all PCR reactions was confirmed by the appearance of a single peak after melting curve analysis following completion of the amplification reaction as indicated by the example below (Fig. 3.2).

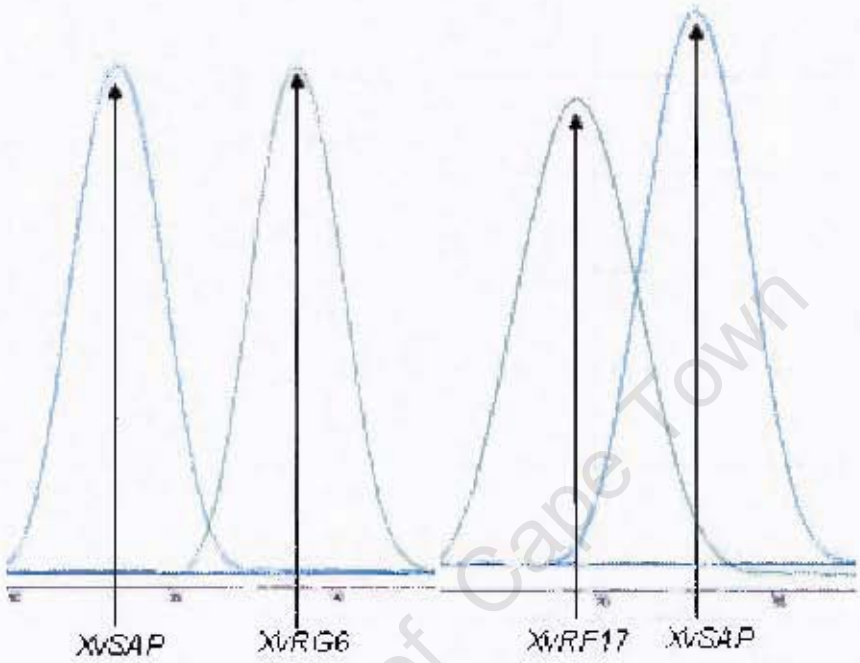

Figure 3.2 Melting curve analyses of *XvRG6* and *XvRF17*. The *XvSap* gene (Garwe et al., 2003) was used as a control.

Amplification products were also analysed by agarose gel electrophoresis and ethidium bromide staining. A single band within the expected size (200 bp for *XvRG6* and 150 bp for *XvRF17*) was detected in each experiment (Fig. 3.3).

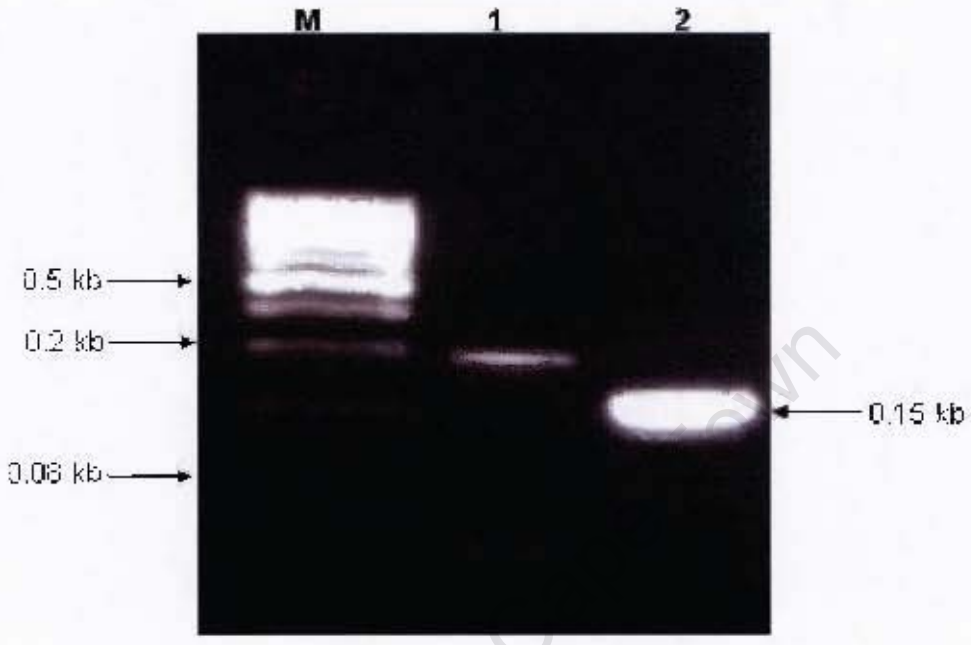

Figure 3.3 Gel electrophoresis of *XvRG6* and *XvRF17* following PCR amplification to determine whether non-specific amplification occurred. Lane: 1, *XvRG6*; 2, *XvRF17*; M, 200 bp DNA ladder (Fermentas, Germany). Arrows on the left indicate molecular sizes.

3.4.3 Real-time PCR for gene expression quantification

To characterise the response of *XvRG6* and *XvRF17* to abiotic treatments, the mRNA level of the two genes was evaluated by qRT-PCR. A PCR efficiency 0.97 and R^2 value of 0.99 for the target genes were obtained by generating standard curves based on a dilution series of plasmid DNA (Fig.3.4).

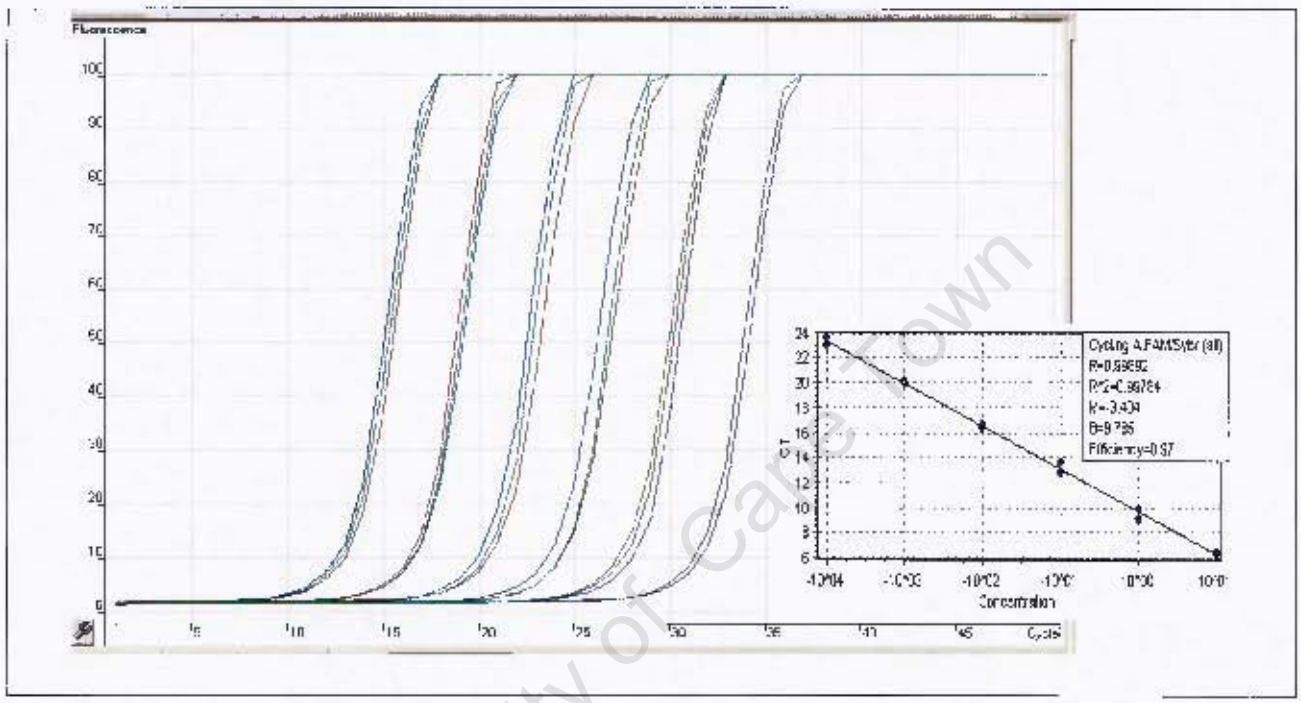

Figure 3.4 Standard curve showing amplification of plasmid DNA with PCR efficiency.

3.4.4 Gene expression during dehydration treatment

No difference in *XvRG6* expression was detected between the control (91% RWC) and dehydrated *X. viscosa* leaves (Fig. 3.5A). The transcript of *XvRF17* was detected 98-fold higher at 38% RWC in comparison with the control (Fig. 3.5B). This suggests that the *XvRF17* gene is induced by desiccation stress specifically whereas *XvRG6* is not.

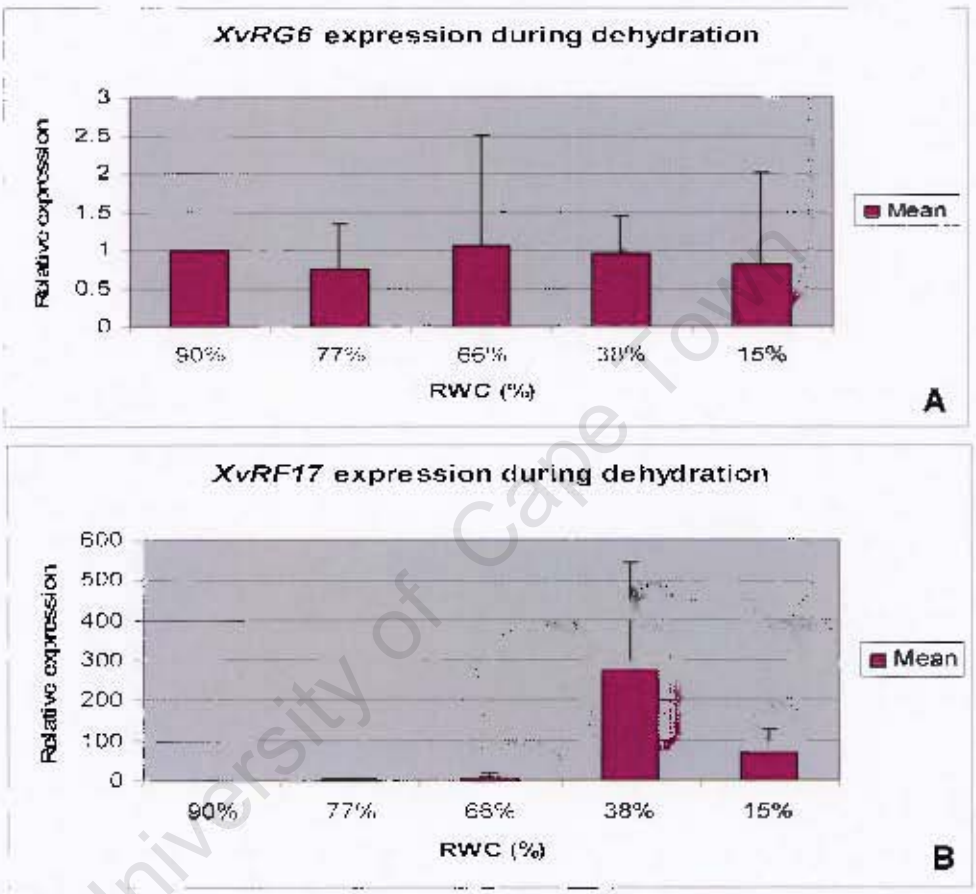

Figure 3.5 Relative expression levels of *XvRG6* and *XvRF17* measured in leaves during dehydration treatment. The cDNA expression levels of *XvRG6* and *XvRF17* were normalised against 18S rRNA (reference gene). After normalisation, the expression levels were measured relative to the calibrator sample (the average of the three biological replicates at 91% RWC). Mean: average of three biological replicates. The error bar is define as Mean ± SD

3.4.5 Gene expression during ABA treatment

Gene expression of *XvRG6* increased 2.5 fold 3 h post-treatment with ABA, then decreased at 6 h post-treatment and appeared to stabilise at 24 h for the remainder of the treatment (Fig. 3.6A). This suggests that the *XvRG6* gene is ABA inducible. The *XvRF17* expression in *X. viscosa* leaves during ABA treatment is shown in Fig. 3.6B. Initially there was a sharp increase 6 h after treatment, followed by a decrease 6 h later (Time: 12 h) and then another slight increase in expression up to 30 h. The *XvRF17* expression reached its peak at 54 h (7-fold higher) in comparison with time zero. This was followed by a sharp reduction in expression to the basal level. This pattern of ABA-induced RNA transcription may indicate a cyclical response with a memory of ABA from the initial application.

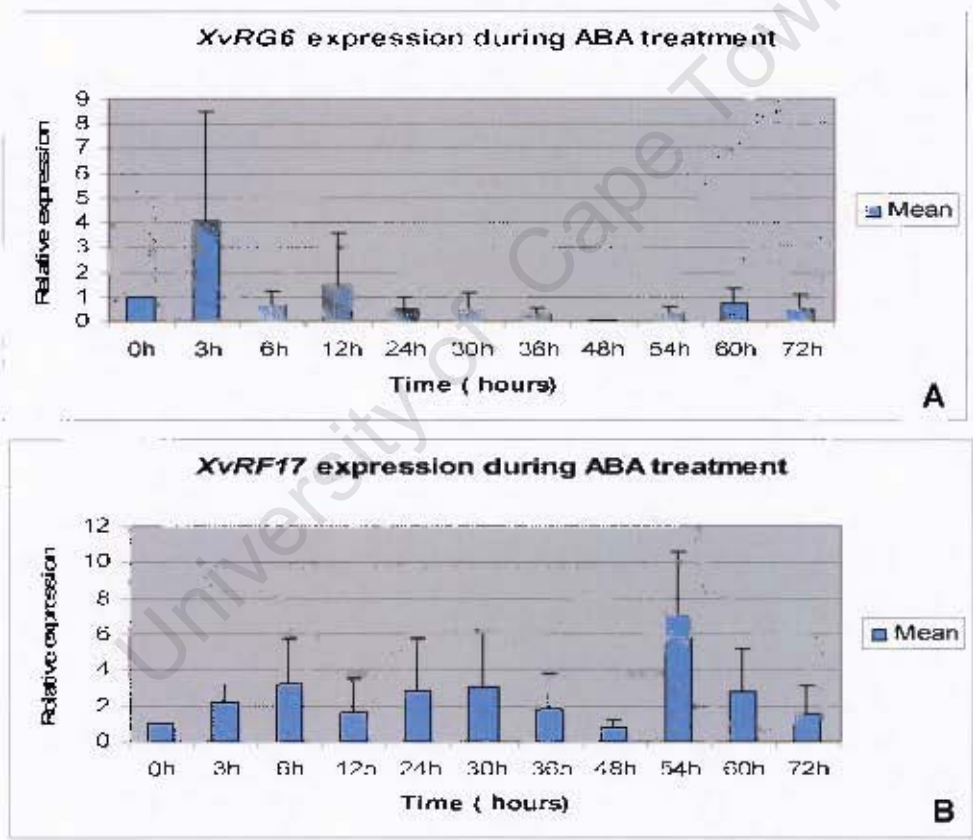

Figure 3.6 Relative expression levels of *XvRG6* and *XvRF17* measured in leaves treated with 100 µM ABA. The cDNA expression levels of *XvRG6* and *XvRF17* were normalised against 18S rRNA (reference gene). After normalisation, the expression levels were measured relative to the calibrator sample (the average of the three biological replicates at time 0). Mean: average of three biological replicates. The error bar is define as Mean ± SD

3.4.6 Gene expression during SA treatment

Expression of both *XvRG6* and *XvRF17* were considerably induced by SA treatment (Fig. 3.7). The *XvRG6* transcript was detected at 6, 36, 48, and 72 h post-treatment with SA. For *XvRG6*, the higher peak (100-fold) was obtained at 6, 48 and 72 h (Fig. 3.7A). For *XvRF17*, the transcript was detected 3, 48, and 60 h post-treatment with SA. The *XvRF17* gene reached its peak at 60 h (24-fold) followed by a slight decrease at 72 h (10-fold; Fig. 3.7B). As observed for ABA, this pattern of SA-induced RNA transcription suggests a cyclical response with a memory of SA from the initial application.

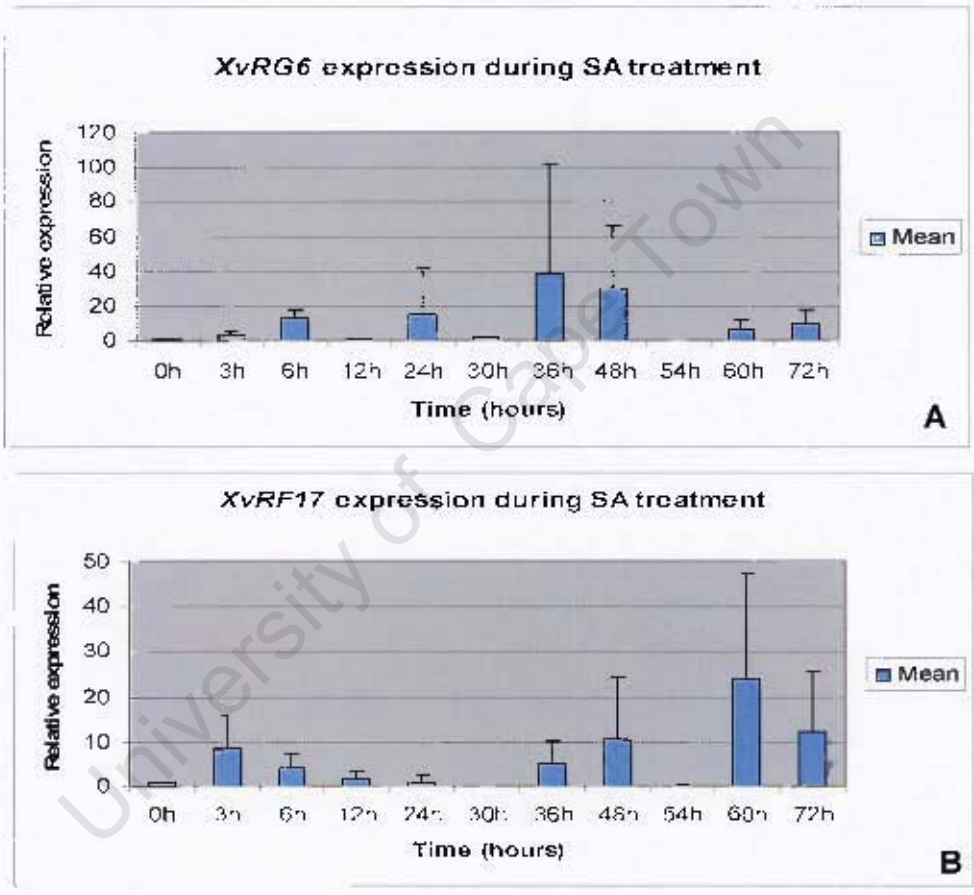

Figure 3.7 Relative expression levels of *XvRG6* and *XvRF17* measured in leaves treated with 100 µM SA. The cDNA expression levels of *XvRG6* and *XvRF17* were normalised against 18S rRNA (reference gene). After normalisation, the expression levels were measured relative to the calibrator sample (the average of the three biological replicates at time 0). Mean: average of three biological replicates. The error bar is define as Mean ± SD

3.4.7 Gene expression during JA treatment

The relative expression of *XvRG6* post-treatment with JA was detected at 24 and 72 h (Fig. 3.8A). The higher peak (7500-fold) was detected at 24 h followed by a sharp decrease to the basal level. The expression of *XvRF17* was detected at 12 and 30 h post-treatment with JA (9-fold and 18-fold higher than control, respectively) (Fig. 3.8B). This suggests that both *XvRG6* and *XvRF17* gene are JA inducible.

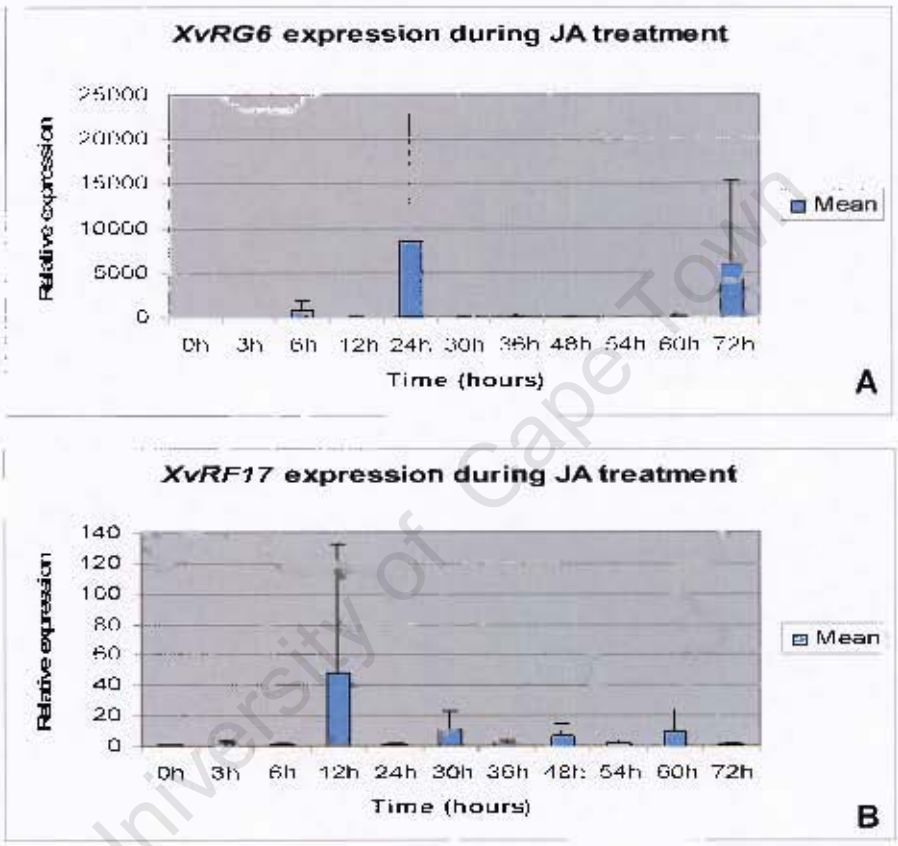

Figure 3.8 Relative expression levels of *XvRG6* and *XvRF17* measured in leaves treated with 100 µM JA. The cDNA expression levels of *XvRG6* and *XvRF17* were normalised against 18S rRNA (reference gene). After normalisation, the expression levels were measured relative to the calibrator sample (the average of the three biological replicates at time 0). Mean: average of three biological replicates. The error bar is define as Mean ± SD

3.4.8 Gene expression during ethylene treatment

The expression of *XvRG6* was found to be induced 3 and 48 h (47.5 fold) after treatment with ethylene (Fig. 3.9A). The expression of *XvRF17* however was down-regulated during ethylene treatment (Fig 3.9B). This suggests that *XvRG6* is possibly a defence induced gene and that *XvRF17* is not.

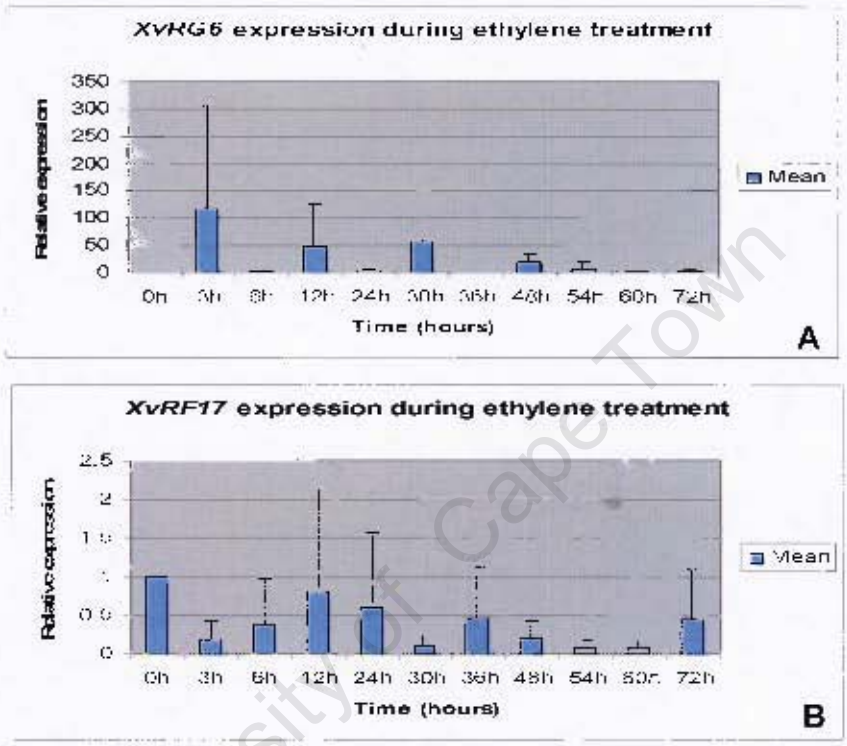

Figure 3.9 Relative expression levels of *XvRG6* and *XvRF17* measured in leaves treated with ethylene. The cDNA expression levels of *XvRG6* and *XvRF17* were normalised against 18S rRNA (reference gene). After normalisation, the expression levels were measured relative to the calibrator sample (the average of the three biological replicates at time 0). Mean: average of three biological replicates. The error bar is define as Mean ± SD

3.5 DISCUSSION

In this study real time quantitative PCR was used to detect the expression levels of two genes, namely *XvRG6* and *XvRF17* with the aim of elucidating how these genes respond to abiotic stress. The data obtained from the biological repeats displayed great differences between individual plants under the same stress conditions due to biological variation. Although plants were grown and treated similarly, biological variation such as plant age, size, soil nutrients and water uptake would have impacted the plants response to stress. Attempts were made to minimise this variation by choosing plants of similar size, which display a similar phenotype. The real-time data was therefore interpreted according to the trend obtained from the means of the three biological replicates.

Several defence response genes are induced by periods of water-deficit (Ramanjulu and Bartels, 2002). The results presented here show that *XvRF17* was up-regulated during dehydration treatment while *XvRG6* was not. Under conditions of water deprivation a 98-fold increase in *XvRF17* transcripts at 38% RWC was observed and this level remained constant throughout the time course. No regulation in *XvRG6* expression was observed (Fig. 3.5A). These results suggest that *XvRF17* might be a desiccation responsive gene. The *XvRF17* gene appears to be a type 1 MT (Chapter 2). Previous studies conducted by Oztur et al. (2002) show that genes belonging to this group are up-regulated in response to drought and salt stress. The function of MTs in plants is not yet clear; however, Moyle et al. (2004) using northern analysis of a small transcriptome from pineapple fruit demonstrated that the up-regulation of MTs during various stress responses could be explained by their involvement in response to oxidative stress. In this regard it is possible that *XvRF17* could also be involved in oxidative stress.

The expression patterns of *XvRG6* and *XvRF17* were also studied in response to signalling molecules, specifically ABA, JA, ethylene and SA molecules, which are known to modulate the activation of defence genes in plants. Depending on the type of stress encountered, host plants switch on the appropriate defence pathways or a combination of pathways (Pieterse and Van Loon, 1999). Complex signal transduction pathways mediated by SA, JA and ethylene are induced by biotic and abiotic stresses in plants (Reymond and Farmer, 1998; McConn et al., 1997; Dempsey et al., 1999) and evidence has also established the involvement of ABA as an important stress regulator (Grill and Himmelbach, 1998). The hormones, ABA, SA and JA all induced the accumulation of *XvRG6* and *XvRF17* mRNA. The phytohormone, ABA plays a regulatory role in many physiological processes in plants (Swamy and Smith, 1999). Both genetic

and biochemical studies have established that ABA is crucial in the response of plants to desiccation, salt and cold (Bray, 1997). Many water stress inducible genes are up-regulated by exogenous ABA treatment and the levels of endogenous ABA increase significantly in many plants under stress conditions (Bray, 1997; Busk and Pages, 1998). Leaf ABA content in wild plants increases with water stress. Upon rehydration, the ABA level returns to pre-stress levels. Substantial evidence suggests that increased ABA levels limit water loss by reducing stomata apertum (Swamy and Smith, 1999). Furthermore, ABA is necessary for the regulation of several events during late seed development (McCarty, 1995). It is believed that the hormone is synthesised in mature leaves and transported in the phloem through the shoot system (Swamy and Smith, 1999).

The functional consequence of JA-induced up-regulation of genes is related to phytoalexin synthesis, N-partitioning, biotic stress response or to developmental events, like seed germination (Wasternack et al., 1998). Exogenous application of JA leads to a decrease in the expression of nuclear- and chloroplast-encoded genes involved in photosynthesis (Weidhase et al., 1987; Reinbothe et al., 1997). Furthermore many stress responsive genes are up-regulated by exogenous application of JA. Treatment of barley leaves with JA or stress conditions, such as sorbitol treatment, which increase endogenous jasmonates lead to synthesis of abundantly appearing proteins, so-called jasmonate-induced proteins (JIPs) of different molecular masses, such as JIP-6 (Andresen et al., 1992). The JIP-6 was identified as a thionin and thionins are known to function against pathogen attack (Bohlmann, 1994). The most abundant JIP of barley leaves, JIP-23, which exhibits no sequence homology in the database (Andresen et al., 1992), might be related to stress protection of cells. It appears in all leaf cells upon JA treatment or sorbitol stress and is specifically expressed during seedling development in cells stressed osmotically by high solute transport (Hause et al., 1997).

It is thought that SA potentiates reactive oxygen species (ROS) in the hypersensitive response (Klessig et al., 2000). Although it is unclear whether osmotic stress leads to an increased SA level in plants, the observation that osmotic stress and SA activate the same MAPK (mitogen-activated protein kinase) (Hoyos and Zhang, 2000; Mikolajczyk et al., 2000) suggests that the osmotic stress signal transduction and SA signal transduction may employ certain common components. Using transgenic *Arabidopsis* expressing a salicylate hydroxylase (*NahG*) gene, Borsani et al. (2001) demonstrated that these SA-deficient seedlings are more tolerant to

salt and other osmotic stress. They suggested that the increased tolerance might result from decreased SA-mediated ROS generation in the *NahG* expressing plants.

Metallothionein type 1 transcripts were previously found to be up-regulated in response to exogenous ABA (Chatthai et al., 1997; Reynolds and Crawford, 1996) and SA treatment (Zhou et al., 2004). The results presented here suggest that *XvRF17* might also act as a defence gene in the *X. viscosa* plant.

The XvRG6 protein is a novel type of PRP and on the basis of the amino acid sequence repeats within this gene, it is tempting to speculate that XvRG6 belongs to a class of hybrid PRP. The up-regulation of *XvRG6* in response to ABA, JA and SA could be explained by the involvement of this gene in plant defence even thought there is little data available on the expression patterns of PRPs in response to such hormones.

Ethylene is a modulator of plant growth and development (Ecker and Davis, 1987; Ecker, 1995). It is involved in many aspects of plant life cycle, including seed germination, root hair development, root nodulation, flower, senescence, abscission, and fruit ripening (Johnson and Ecker, 1998). The production of ethylene is regulated by internal signals during development and in response to environmental stimuli from biotic and abiotic stresses, such as wounding, hypoxia, ozone, chilling, or freezing (Wang et al., 2002). Ethylene is perceived by a family of five membrane-localised receptors that are homologous to bacterial two-component histidine kinases involved in sensing environmental changes (Wurgler-Murphy and Saito, 1997; Pirrung, 1999).

Showalter et al. (1992) showed that class I and II tomato PRPs mRNA accumulated in response to ethylene treatment. After wounding or pathogen infection, ethylene is synthesised by plants. Maize HRGP mRNA has been shown to accumulate in tissues rich in dividing cells and in response to wounding (Ludevid et al., 1990). The PRPs were first identified as proteins that accumulate in the cell wall in response to physical damage and have subsequently been shown to be temporally regulated during plant development (Chen and Varner, 1985; Tierney et al., 1988). In this study the transcript of *XvRG6* increases (47.5 fold) following 3 and 48 h ethylene treatment. This result suggests that *XvRG6* might be up-regulated in response to ethylene treatment and in turn, suggests a possible role for *XvRG6* in processes such as development and defence response in plants.

The expression of *XvRF17* was down-regulated following ethylene treatment. The results presented here are in contrast with Zhou et al. (2004) who highlighted the up-regulation of rice MT

mRNA after treatment with ethylene. However, since we did not have the upstream promoter region of *XvRF17* we could not determine if it contained the putative ethylene-responsive domains [ACGTGCCC or GCCAAGTCACC] (Ohme and Shinshi, 1995), which are found in many pathogen-responsive genes.

3.6 CONCLUSION

As a step toward functional characterisation of *XvRG6* and *XvRF17*, the response of the two genes under abiotic stress was examined using real-time RT-PCR. Excluding the real-time data obtained for *XvRF17* during dehydration, the gene expression trends obtained cannot be conclusively interpreted due to the significant biological variation between replicates under the same treatment. The may be differences in the uptake of applied hormones by the plants, which may have caused this large variation and might persuade the reader to believing that differences are indeed a hormone effect.

The results indicate that these genes may be differentially expressed in response to abiotic stress. From the data, *XvRG6* mRNA increases during ABA, SA, JA and ethylene treatment but not during dehydration while *XvRF17* mRNA accumulated during dehydration, ABA, SA, and JA but not during ethylene treatment. It is also important to note that no inference on the putative function of these genes in response to abiotic stress may be made in the absence of homology with any plant protein of known function. However, the significant biological variation observed makes it difficult to link the *XvRG6* and *XvRF17* expression patterns directly to adverse environmental conditions. To investigate whether the gene expression patterns observed was reflected at the translational level, western blot and two-dimensional gel electrophoresis analyses were used and are discussed in the next chapter.

Chapter 4

Protein expression of XvRG6 and XvRF17

4.1 INTRODUCTION

In the previous chapter the gene expression patterns obtained for *XvRG6* and *XvRF17* under abiotic treatments could not be interpreted conclusively due to high levels of biological variation. Furthermore it has been shown that gene expression patterns are not always reflected at the translational level. For example, during desiccation in *Tortula ruralis* specific transcript levels remain relatively unchanged and changes occur mainly at the translational level by selection of different mRNAs from the unchanged pool (Wood and Oliver, 1999).

Expression of cloned genes in bacteria is widely used for the production of pharmaceutical proteins and in research. At present, *E. coli* remains the most useful expression host, due to its advantages such as ease of handling and maintenance. The disadvantages of expression in *E. coli* are the possible formation of inclusion bodies due to the overexpression of the recombinant or insolubility of the protein. Each expression system needs specific tailoring to meet the stringent requirements for each protein product to ensure correct activity and desired yield.

Recombinant proteins can be used to investigate a variety of questions related to localisation within a cell or organism, *in vitro* activity, protein expression patterns and structural functional relationships.

In this chapter the cloning of *XvRG6* and *XvRF17* into pCR*T7/NT-TOPO* vector and the expression of the recombinant fusion protein in the bacterial system [*E. coli* BL21 (DE3) plysS] is described. These proteins were used to generate antibodies, so that the expression of XvRG6 protein from *X. viscosa* leaf samples under abiotic stress could be investigated using western blot analyses.

4.2 MATERIALS AND METHODS

4.2.1 Total RNA and soluble protein isolation

Total RNA was extracted from control and stressed *X. viscosa* leaves as described in section 3.3.1. After the aqueous phase had been removed for RNA precipitation, the pellets were used for the protein isolation. Total protein was isolated using Trizol reagent (Invitrogen, USA) with the following modification. Briefly, 0.3 mL of absolute ethanol was added to the pellets, the tubes were inverted and incubated for 2 min at RT. Thereafter the samples were centrifuged for 5 min at 2000 x g. The supernatant (containing the soluble proteins) was transferred into a new tube. Two millilitres of isopropanol was added and incubated for 10 min at RT, followed by centrifugation for 10 min at 12000 x g at 4°C. The pellets were washed three times with 2 mL of 0.1 M ammonium acetate in 100% (w/v) methanol and then washed once with 2 mL cold acetone. The pellets were dried in the fume hood for 1 h and then resuspended in 50 mM Tris-HCl, pH 7.5.

4.2.2 TOPO expression of XvRG6 and XvRF17

The pCR-T7-TOPO TA Expression Kit (Invitrogen, USA) was used according to the manufacturer's instructions for the cloning of *XvRG6* and *XvRF17* into the pCR-T7-TOPO expression vector. The enzyme, *Taq* polymerase, has a non-template-dependent terminal transferase activity that adds a single deoxy-adenosine to the 3' ends of PCR products. Linearised pCR-T7-TOPO has single overhanging 3' deoxy-thymidine residues. This allows PCR inserts to ligate efficiently with the vector. Initially the recombinant vector was stably maintained in TOP10F' cells (Invitrogen, USA) for sequence analysis and subsequently transferred to BL21 (DE3) pLysS cells for protein expression (Invitrogen, USA).

4.2.2.1 Cloning of *XvRG6* and *XvRF17* into pCR-T7-TOPO

Both *XvRG6* and *XvRF17* were amplified using gene specific primers, XvRG6F1 + XvRG6R1 and XvRF17F1 + XvRF17R1 (Appendix B), respectively. The PCR reaction volumes were made up to 25 µl (standard PCR reaction; Appendix A) and run with the following cycling conditions: 94°C for 2 min; 30 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 45 s; and a final extension step of 72°C for 7 min. The PCR products were electrophoresed on a 1% (w/v) agarose gel and stained with ethidium bromide. The band of interest was excised and purified

using the High Pure PCR Product Purification Kit according to the manufacturer's instruction (Appendix A). Ligation of the purified amplimer to the TOPO vector was carried out according to the manufacturer's instruction (Table 4.1).

Table 4.1 Ligation reaction of TOPO vector to purified PCR products

Component	Volume (µl)
Purified amplimer	2.0
TOPO vector (Invitrogen, USA)	0.5
Salt solution*	0.5

*No ligase was required in the ligation reaction

The reagents were added to an Eppendorf tube and incubated overnight at 4°C. Three microlitres of the ligation mix was transformed into TOP10F' *E. coli* cells to select for positive clones. Colony PCR was performed using gene specific primers XvRG6F1 + XvRG6R1 and XvRF17F1 + XvRF17R1; Appendix B), respectively. The TOP10F' cells containing either pTOPO::*XvRG6* or pTOPO::*XvRF17* were inoculated in LB broth supplemented with 100 µg.mL^{-1} ampicillin. Plasmid DNA was isolated using the High Pure Plasmid Extraction Kit according to the manufacturer's instruction (Appendix A). Samples were sequenced to confirm the correct orientation of the insert. Prior to protein expression pTOPO::*XvRG6* and pTOPO::*XvRF17* were transformed into BL21 (DE3) pLysS *E. coli* cells. Colony PCR was performed using gene specific primers. Positive *XvRG6* and *XvRF17* clones were used in the expression studies.

4.2.2.2 Protein expression of XvRG6 and XvF17

For small scale induction, 1 mL of an overnight LB broth containing either pTOPO::*XvRG6* or pTOPO::*XvRF17* supplemented with 100 µg.mL^{-1} ampicillin and 34 µg.mL^{-1} chloramphenicol was used to inoculate 100 mL LB broth supplemented with 100 µg.mL^{-1} ampicillin and 34 µg.mL^{-1} chloramphenicol and incubated with vigorous shaking at 37°C until an OD_{600} of between 0.6-0.8 was obtained. A 1 mL aliquot was removed from the broth culture (un-induced sample) and the remaining culture was induced by the addition of IPTG to a final concentration of 1 mM and incubated for an additional 16 h with vigorous shaking at 30°C. A 1 ml aliquot was collected after 16 h. Cells were pelleted by centrifugation (4000 x g for 20 min) and the pellet redissolved

in 50 mL LB broth supplemented with chloramphenicol (200 $\mu g.mL^{-1}$). The cells were allowed to shake at 37°C for 2 h after which 1 mL aliquot was removed from the broth culture. The un-induced, overnight sample and chloramphenicol sample were centrifuged for 1 min at 8000 x g and the supernatant discarded. Water (80, 160 and 240 µL) was added to the pelleted cells, which were gently resuspended by pipetting. Twenty, 40 and 60 microlitres of 5X SDS loading buffer (0.225 M Tris, pH 6.8; 50% (v/v) glycerol; 5% (w/v) SDS; 0.05% (w/v) bromophenol blue; 0.25 M DTT) was added to the resuspended cells. Samples were electrophoresed on a 12.5% polyacrylamide gel. Protein integrity was determined by staining gels with Coomassie blue [(0.05% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) ethanol, 10% (v/v) glacial acetic acid] and destained with destainer (40% methanol, 10% glacial acetic acid, 50% water).

For large scale induction, 20 mL of an overnight LB broth culture (either pTOPO::*XvRG6* or pTOPO::*XvRF17*) supplemented with 100 $\mu g.mL^{-1}$ ampicillin and 34 $\mu g.mL^{-1}$ chloramphenicol was used to inoculate one litre LB broth supplemented with the same antibiotic concentrations as above and incubated with vigorous shaking at 37°C until an OD_{600} of between 0.6-0.8 was obtained. A 1 mL aliquot was removed from the broth culture (un-induced sample). The one litre culture was induced by addition of IPTG (1 mM final concentration) and incubated for an additional 16 h with vigorous shaking at 30°C. A 1 mL aliquot was collected 16 h post-addition of IPTG (overnight sample). Cells were pelleted by centrifugation for 20 min at 4000 x g and the pellet redissolved in 500 mL LB broth supplemented with a high concentration of chloramphenicol (200 $\mu g.mL^{-1}$). The cells were allowed to shake for 2 h at 37°C after which a 1 mL aliquot was collected (chloramphenicol sample). The un-induced, overnight and chloramphenicol samples were treated as described for small scale induction. The remaining 500 mL culture was cooled for 10 min on ice, split into 4 GSA centrifuge tubes and centrifuged for 30 min at 4000 x g at 4°C. The supernatant was discarded and pelleted cells stored overnight at -20°C. The frozen cells were thawed for 45 min on ice. The pellet was initially resuspended in a small volume (1-2 mL) of lysis buffer [20 mM Tris-HCl; 3 mM EDTA; 0.1% (v/v) β-mercaptoethanol; 1 mM phenylmethylsulfonyl fluoride (PMSF) (added fresh); pH 8] by gentle homogenizing with a glass rod. A small volume of lysis buffer was added to the suspension at short intervals and the cells homogenized with a glass homogenizer until a homogeneous solution was obtained. The final volume of lysis buffer added to the cell solution was between 15-30 mL depending on the size of the pellet and the viscosity of the solution. Thereafter the cells were

sonicated in 8 bursts of 15 s interrupted by seven intervals of 15 s on ice to prevent overheating of the sample. The lysate was centrifuged for 30 min at 17000 x g at 4°C to pellet inclusion bodies. The resulting inclusion body-containing pellet was resuspended in a small volume (1-2 mL) of washing buffer [20 mM Tris-HCl; 3 mM EDTA; 0.1% (v/v) β-mercaptoethanol; 1% (v/v) Triton X-100] by gently homogenizing with a glass rod. A small volume of washing buffer was added to the suspension and the cells homogenized as described above. This washing step was repeated four times and the pellet containing the crude protein was resuspended in 30-40 mL of solubilisation buffer [20 mM Tris-HCl; 3 mM EDTA; 0.1% (v/v) β-mercaptoethanol; 8 M urea]. After homogenising with a glass homogeniser, the lysate was spun for 30 min at 17000 x g at 4°C and the supernatant containing the crude protein extract purified through a Ni-NTA column.

4.2.2.3 Protein purification

To prepare a resin column the bottom of a 20 mL syringe was plugged with glass wool. Homogenised Ni-NTA resin (Qiagen, Germany) was loaded in the syringe over the glass wool and 10 mL of buffer B (8 M urea; 20 mM Tris-HCl; 3 mM EDTA; 0.1% (v/v) β-mercaptoethanol; pH 8) was applied to the top of the resin column and allowed to pass through by gravitational flow. The crude protein extract was loaded onto the column and the recombinant protein allowed to bind to the column due to the interaction of the protein's His-Tag and nickel ions on the Ni-NTA column. A 100 µL aliquot of the flow-through was removed (pre-wash sample). The resin bound protein was washed with 20 mL wash buffer [8 M urea; 20 mM Tris-HCl; 20 mM imidazole; 20% (v/v) glycerol; pH 6.3] and a second 100 µL aliquot of the flow-through was removed (wash sample). The washed protein in the column was treated with 10-20 ml elution buffer [8 M urea; 20 mM Tris-HCl; 250 mM imidazole; pH 5.9] and the eluent collected in 1.5 mL Eppendorf tubes as 1 mL fractions. The OD_{280} of each fraction was determined spectrophotometrically (Beckman, USA) using quartz cuvettes. From these absorbance readings the samples with the highest OD_{280} values were pooled and a 10 µL aliquot removed for SDS-PAGE analysis.

The concentration of the protein was determined using the following formula:

Protein concentration = $E/\varepsilon.d$ where E = absorbance at 280 nm

ε = extinction coefficient (using protparam tool page in expasy) = 8940

d = cuvette diameter = 1 cm

Sample aliquots (pre-wash, wash, eluted and lysed) prepared during the protein purification procedure were prepared for electrophoresis. Samples were electrophoresed on a 12.5% SDS PAGE gel (standard PAGE conditions; Appendix A).

4.2.3 Antibody generation, western blotting and immuno-detection

4.2.3.1 Antibody generation

Purified protein was concentrated using filter columns [3,000MWCO; Millipore Corporation Bedford, USA] according to the manufacturer's instructions. The purified recombinant XvRG6 protein at a concentration of 1 $mg.mL^{-1}$ was used for antibody generation. Polyclonal XvRG6 antibody production was achieved by injecting one New Zealand rabbit with the purified recombinant XvRG6 protein. The injection and bleeding of the rabbit were carried out at the UCT Animal Unit. The animals were injected weekly over a 4 week period each time with a 1 mL antigen emulsion (500 µL Freund's incomplete adjuvant + 500 µL antigen resuspension) (Chart and Rowe, 1992). A pre-immune serum sample was taken, as well as weekly serum samples.

4.2.3.2 Antibody purification

To purify the antibody, two volumes of borate-buffered saline was added to one volume of anti-serum. Thereafter 14% (w/v) of crushed PEG 6000 was added to the diluted serum and gently mixed by inversion. This was centrifuged for 10 min at 12 000 x g at 4°C. The pellet was dissolved in the original serum volume using phosphate buffer (PBS) pH 7.4. Polyethylene glycol (PEG) 6000 was added [14% (w/v)] and dissolved. The sample was centrifuged as before and the pellet dissolved in half the original serum volume using PBS containing 60% (v/v).glycerol

4.2.3.3 Western blotting

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 Coomassie blue (0.05% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) ethanol, 10% (v/v) glacial acetic acid). The proteins were transferred onto nylon membranes (Hybond-P; Amersham Pharmacia Biotech) using the Hoefer® Electro-transfer System (Bio-Rad

Laboratories, USA) according to the manufacturer's instructions. Electrophoretic transfer was conducted for 3 h at 250 mA.

4.2.3.4 Protein analysis by 2D gel electrophoresis

One gram of *X. viscosa* leaf material was excised from a dehydrated plant, ground in liquid nitrogen using a mortar and pestle and transferred equally into 4 Eppendorf tubes each containing 1.5 mL of extraction buffer [0.5 M Tris pH 7.5, 10 mM EDTA, 1% (v/v) Triton X-100 , 2% (v/v) β-mercaptoethanol]. The tubes were centrifuged for 10 min at 12000 x g at 4°C. The supernatants were removed and transfered into new Eppendorf tubes and the protein was extracted by addition of an equal volume of Tris saturated phenol pH 8. Thereafter, protein was precipitated by the addition of 2.5 volumes of 0.1 M ammonium acetate in methanol. The sample was incubated for 16 h at -20°C and thereafter centrifuged for 10 min at 12000 x g at 4°C. The supernatant was discarded. The pellet was washed firstly with 0.1 M ammonium acetate in methanol and secondly with 1 mL cold 80% (v/v) acetone. Thereafter the protein pellet was air-dried for 30 min and resuspended in urea lysis buffer [9 M urea, 2 M thiourea, 4% (w/v) CHAPS].

An aliquot was removed for quantification. The sample for rehydration was prepared as follows: 2 µL of 1% (w/v) DTT, 3.75 µL of 1.2% (w/v) carrier ampholytes (Sigma, Germany), 5 µL of 0.4% (w/v) ASB-14 (Sigma, Germany), 100 µg of the protein and adjusted to 125 µL with urea lysis buffer. This mixture was used to rehydrate immobilized pH gradient strips (pH 3-10, 7 cm; Bio-Rad, Germany).

The PROTEAN IEF Cell System (Bio-Rad, USA) was used for 2D gel electrophoresis. One hundred micrograms of total protein was loaded into the channel of the rehydration trays. The 1-D immobilise Dry Strip was placed over the protein sample and incubated for 1 h. This was in turn overlaid with a thin layer of mineral oil to prevent drying. Isoelectric focusing was performed as follow: 250 V for 20 min, 4000 V for 2 h and a final step of 4000 V for 20.000 Vh.

After isoelectric focusing the 1-D strip was placed in 10 mL equilibration buffer [6 M urea; 30% (v/v) glycerol; 50 mM Tris, pH 6.8; 2% (w/v) SDS; 0.05% (w/v) bromophenol blue] containing 1% (w/v) DTT (prepared fresh) and incubated for 10 min with shaking. Thereafter the 1-D strip was again placed in 10 mL equilibration buffer containing 4.8% (v/v) iodoacetamide and incubated for 10 min with shaking. A 12.5% acrylamide gel was prepared (standard PAGE

conditions, Appendix A). The strip was placed between the glass plates at the surface of the SDS PAGE gel. Agarose (0.5% w/v) containing bromophenol blue was poured over the strip and allowed to solidify for 10 min. Protein samples were electrophoresed at 30 mA and thereafter blotted onto a nitrocellulose membrane (Hybond-P; Amersham Pharmacia Biotech) according to the protocol described previously (section 4.2.4.3). The membrane was probed with XvRG6 antiserum as described in section 4.2.4.5.

4.2.3.5 Chromogenic detection

The membranes were stained with ponceau S solution (2% (w/v) ponceau S, 1.1 M sulfosalicyclic acid, 1.8 M tri-chloroacteic acid) to determine transfer and loading efficacy. Thereafter the membranes were washed twice for 10 min with 1X TBS buffer (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) and incubated for 1 h in blocking solution (3% (w/v) bovine serum albumin (BSA) in 1X TBS (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) with agitation. The appropriate dilution (1:5000) of XvRG6 antiserum was added and the membranes incubated for 1 h at RT. Membranes were subsequently washed twice for 10 min each with TBS-Tween/Triton buffer (20 mM Tris-Cl, 0.5 M NaCl, 0.05 % (v/v) Tween 20, 0.2 % (v/v) Triton X-100, pH 7.5) followed by one wash with TBS. The membranes were incubated for 1 h with secondary antibody (anti-rabbit IgG, peroxidase linked whole antibody from goat) at a dilution of 1:5000 and washes were carried out as before. The membranes were stained with alkaline phosphatase solution [one tablet of NBT/BCIP ready to use tablets (Roche, Germany)]. The chromogenic reaction was terminated by rinsing the membrane in water. The membranes was air-dried and stored between 2 sheets of whatman paper.

4.3 RESULTS

4.3.1 Cloning of *XvRG6* and *XvRF17* into pCR-T7-TOPO

The *XvRG6* and *XvRF17* genes were successfully cloned into pCR-T7-TOPO. This was confirmed by colony PCR screening of the constructs either pTOPO::*XvRG6* or pTOPO::*XvRF17* (Fig. 4.1).

Figure 4.1 Gel electrophoresis of colony amplimer. M, λ *Pst*I DNA marker; Lanes: 1-4, pTOPO::*XvRG6*; 5-8, pTOPO::*XvRF17*. The arrow on the right indicates the pTOPO::*XvRF17* product.

4.3.2 TOPO expression of XvRG6 and XvRF17

The *XvRG6* and *XvRF17* genes were successfully cloned into pCR-T7-TOPO and the recombinant XvRG6 protein expressed in the prokaryotic expression host: *E. coli* BL21 (DE3) pLysS cells. Expression conditions were optimised and good expression yields were obtained for XvRG6 protein. The XvRG6 protein was observed to be induced preferentially 16 h post-IPTG addition at 30°C (Fig. 4.2A). Expression of XvRF17 protein using BL21 (DE3) pLysS cells was not successful (Fig. 4.2B). Therefore further expression analysis for this protein could not be conducted.

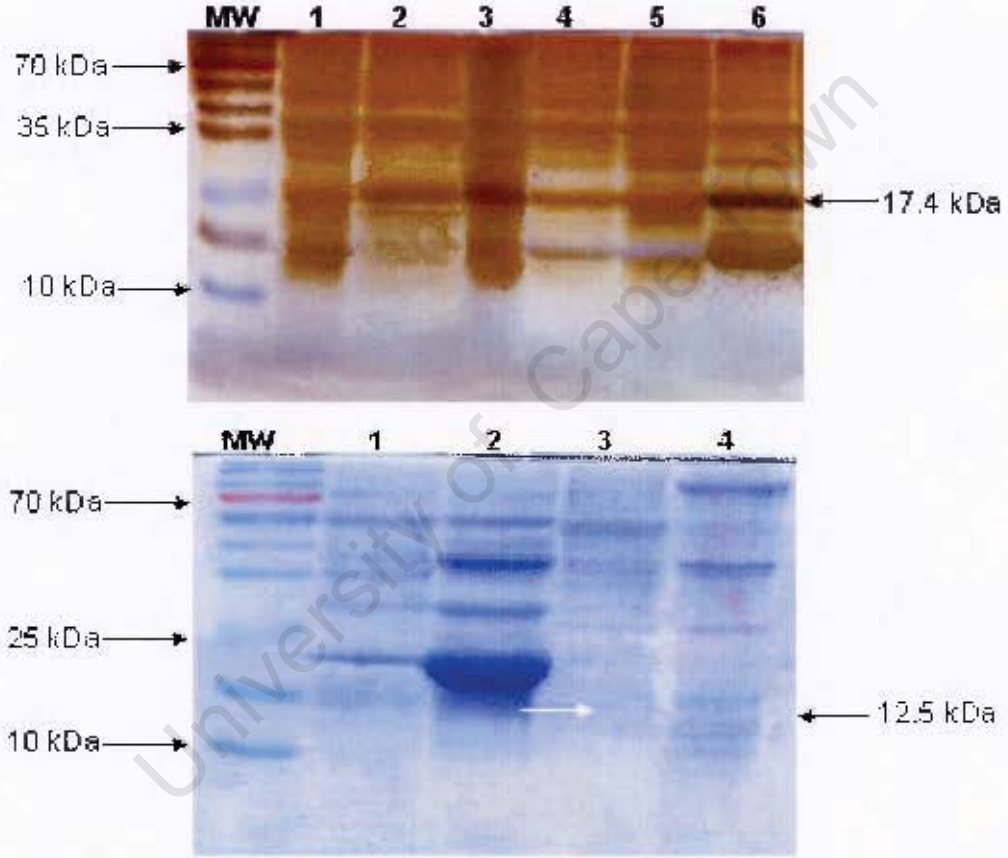

Figure 4.2 A. SDS-PAGE stained with silver showing overexpression of XvRG6. MW: molecular weight marker. Lanes: 1, uninduced samples; 2, overnight induction; 3, 2 hours after addition of chloramphenicol to the overnight sample; 4, pellet wash (induced sample); 5, supernatant after cells lysis; 6, pellet solubilised in urea buffer (induced sample). B. SDS-PAGE stained with Coomassie brilliant blue showing the overexpression of XvRF17. MW: molecular weight marker. Lanes: 1-2, XvRG6 supernatant and pellet solubilised in urea buffer (positive control); 3-4, XvRF17 supernatant and pellet solubilised in urea buffer. Arrows on the right

indicate the expected size of XvRG6 or XvRF17 recombinants proteins as well as the white arrow.

4.3.3 Purification of XvRG6 fusion protein and immuno-blot analysis

A large-scale induction was conducted and the fusion protein was purified under denaturing condition using the Ni-NTA resin. A single protein band of 17.4 kDa (including His-tag) was observed (Fig. 4.3A). Anti-histidine monoclonal antibodies were used to confirm the purity of the recombinant fusion protein (data not shown).

Prior to the antibody generation the proteins were concentrated using filter columns [(ca. 3,000MWCO); Millipore Corporation Bedford, USA] and used as an antigen for XvRG6 antiserum production in rabbits. Polyclonal antibodies were purified from the antiserum. The XvRG6 antibodies were tested against XvRG6 protein and a band of correct size was observed on immuno-blots after chromogenic detection (Fig. 4.3B).

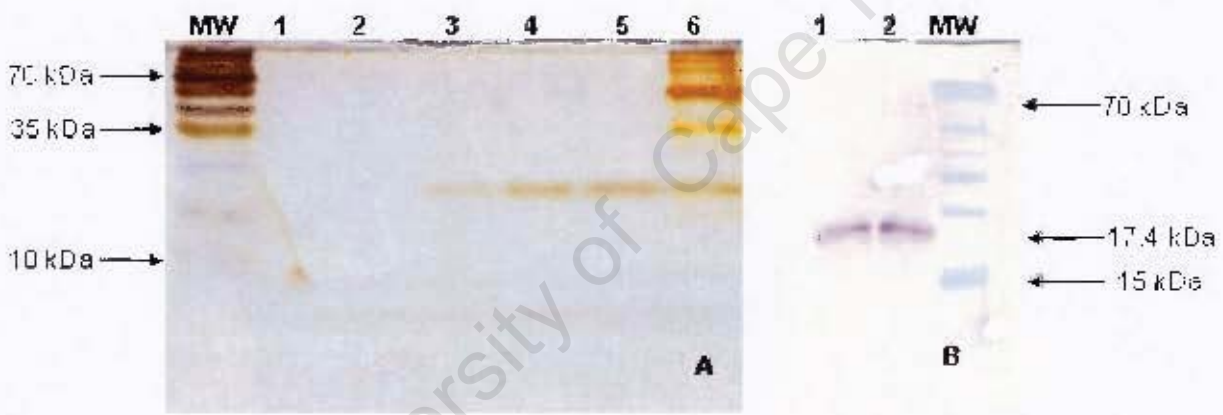

Figure 4.3 A. SDS-PAGE stained with silver showing the purity of XvRG6 recombinant protein; Lanes: 1-2, blank; 3, 4 and 5 purified XvRG6 protein; 6, unpurified XvRG6 protein (control); B. Western blot analysis of the purified samples using XvRG6 antibodies; Lanes: 1-2, purified XvRG6 recombinant protein; MW, molecular weight marker. Arrows indicates molecular sizes determined from molecular weight markers.

4.3.4 Western blot analysis

Equal loading of protein was determined by visualisation of Coomassie stained gels (Fig. 4.4B). Western blot analysis for dehydration stress indicated that XvRG6 protein was present at 62% RWC and the levels increase gradually towards 15% RWC (Fig. 4.4A). The size of XvRG6 protein (ca. 55 kDa) detected was 4 time higher than the expected size (13.4 kDa with the His tag excluded). Similar results were obtained in three independent experiments conducted with three biological replicates (data not shown). This result suggests that the XvRG6 protein could form a complex (tetramers) via cysteine and proline residues present within the sequence.

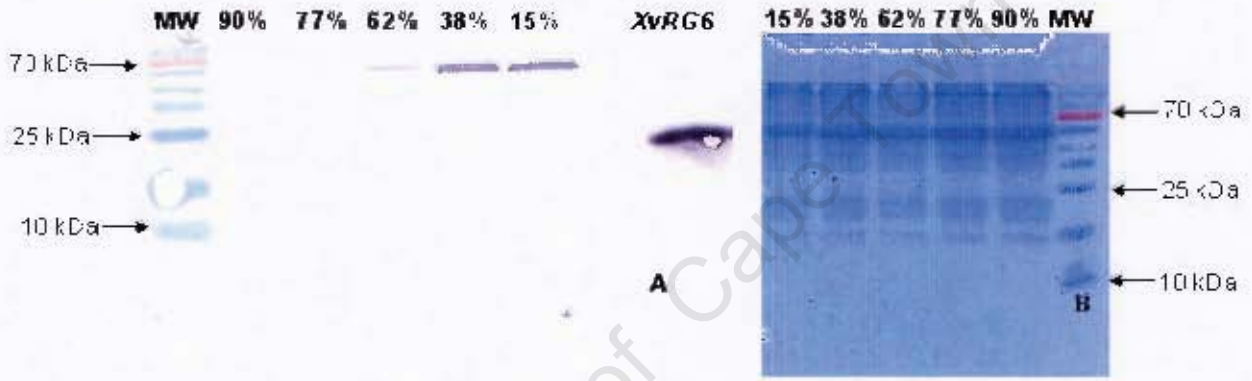

Figure 4.4 Analysis of total protein extracts from dehydrated *X. viscosa* leaves. A. Western blot of total proteins extracted from dehydrating *X. viscosa* leaves. The membranes were probed with anti-XvRG6 raised against XvRG6. B. SDS-PAGE of total protein from leaves from dehydrating *X. viscosa* plant (loading control). MW, molecular weight markers; RWC (relative water content) is indicated on the photograph. For both gels 20 µg of each sample was loaded, electrophoresed and transferred onto nitrocellulose membrane.

4.3.5 Two dimensional gel electrophoresis analysis of XvRG6

In order to verify the discrepancy in size of XvRG6 on SDS-PAGE and immunoblot during dehydration treatment, two dimensional gel electrophoresis was performed. Few spots were observed on the autoradiograph (Fig. 4.5). A distinct spot in the region of 13.5 kDa was also observed. This suggests that the discrepancy in the size of XvRG6 on SDS-PAGE and immunoblot could be attributed to cysteine and proline residues present within the sequence of XvRG6 resulting in the formation of a tetrameric complex.

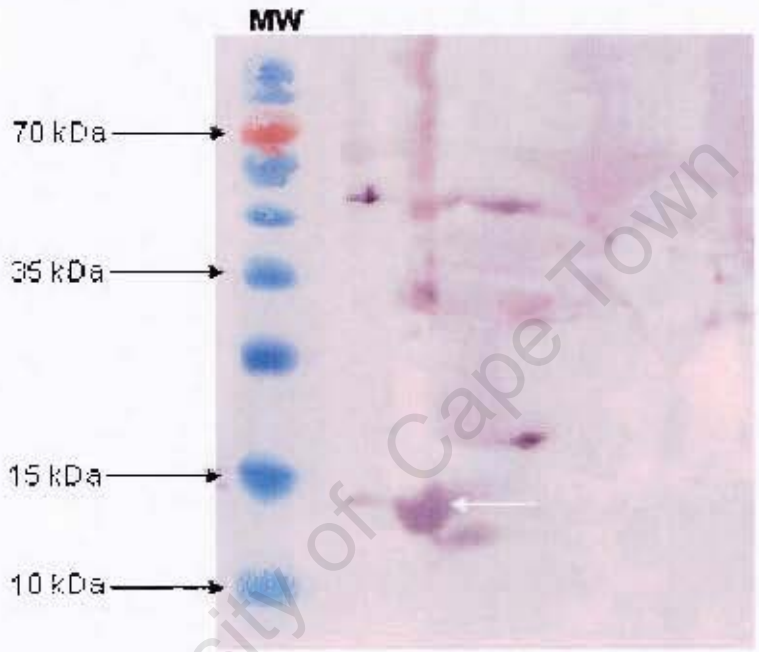

Figure 4.5 Two dimensional gel electrophoresis of *X. viscosa* total protein probed with XvRG6 antiserum. Arrows on the left indicates molecular sizes determined from molecular weight markers. The white arrows indicate XvRG6 protein spot.

The XvRG6 antiserum did not detect any XvRG6 protein in plant material treated with ABA (Fig. 4.6A) and similar results were obtained for JA, SA and ethylene (data not shown) suggesting that the *XvRG6* gene may not be regulated by these phytohormones despite real-time data which point to the induction of *XvRG6* by these hormones.

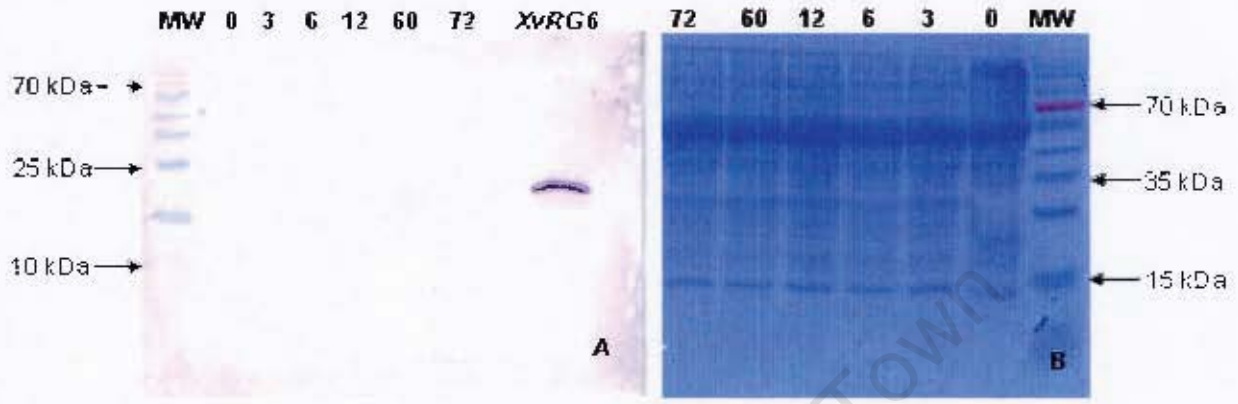

Figure 4.6 Analysis of total protein extracts from *X. viscosa* leaves treated with ABA. A. Western blot of total proteins extracted from *X. viscosa* leaves. MW, molecular weight markers; Time (in hours) is indicated above the lanes. The membranes were probed with anti-XvRG6. B. SDS-PAGE of total protein extracted from of *X. viscosa* leaves (loading control). For both gels 20 µg of each sample was loaded, electrophoresed and transferred onto nitrocellulose membrane. Arrows on the left indicate molecular sizes determined from the molecular weight markers.

4.4 DISCUSSION

The *XvRG6* and *XvRF17* genes were successfully cloned into pCR-T7-TOPO. The recombinant XvRG6 protein was expressed in *E. coli* BL21 (DE3) pLysS cells. Expression conditions were optimised and good expression yields were obtained. The expression of recombinant XvRF17 was attempted in various bacterial expression hosts, and under range of expression conditions with no success. The product of this gene could be toxic to the host cells and future attempts at expressing XvRF17 may involve expression in a eukaryotic host.

To determine the role of *XvRG6* in abiotic stress tolerance in *X. viscosa,* western blot analyses were performed using total proteins isolated from stress-induced plants. The data obtained indicates an increase in XvRG6 protein levels during water deficit. The size of the XvRG6 protein detected (55 kDa) was higher than the expected size (13.4 kDa). However it should be noted that the XvRG6 protein is proline-rich (Chapter 2). Cheung et al. (1993) showed that proline-rich proteins are implicated in the cell wall structure of organs. According to Fowler et al. (1999) the localization of Cys-rich elements with the Pro-rich residue of proline-rich proteins might facilitate the formation of disulfide bonds, ultimately affecting their size. The discrepancy in size of XvRG6 on SDS-PAGE and immunoblot can thus be explained by the localization of Cys-rich elements within the gene, facilitating the formation of disulfide bonds between XvRG6 and/or other proteins.

Two dimensional gel electrophoresis is a unique and powerful approach to resolve proteins and polypeptides in complex protein mixtures. To investigate whether the discrepancy in size of XvRG6 could have been due to its Cys-rich and Pro-rich residues, 2D electrophoresis was performed. Impurities, protein load in the reagents and high substantial streaking in carrier ampholyte-based 2D gels might explain the presence of non specific spots viewed on the autoradiograph (Fig. 4.5). The other spots observed could be XvRG6 protein, which may have been phosphorylated or cleaved. It may also be due to homologues of XvRG6. However, the presence of a distinct spot of the size (13.4 kDa) of XvRG6 protein on the 2D autoradiograph indicates that the protein is able to form a complex, most likely via Cys and Pro residues present within the sequence. The formation of these tetrameric structures in PRPs is thought possibly to play a role in protecting the cell wall membrane by binding these proteins (Fowler et al., 1999).

Abscisic acid is involved in the generation of many stress-inducible genes and is required for changes in gene expression in response to water deficit (Bray, 1997). Most water-stress

inducible genes respond to treatment with exogenous ABA, whereas others do not (Shinozaki and Yamaguchi-Shinozaki 1997). In this study, XvRG6 protein was not detected during ABA treatment. This suggests that this gene may not respond to ABA or may respond to environmental stresses through an ABA-independent pathway. Although transcription does not seem inhibited, translation certainly seems inhibited by ethanol as the working solution contained 0.1% and 1% ethanol. Similar results were obtained for JA, SA and ethylene treatment suggesting that *XvRG6* does not respond to these hormones at the translational level or it is possible that the protein is inhibited by ethanol.

Comparison of *XvRG6* expression at the transcriptional level (Chapter 3) suggested that the *XvRG6* gene is upregulated by this phytohormone. However, *XvRG6* was up-regulated at the translational level by dehydration treatment only. As mentioned earlier, transcript levels are not always reflected at the translational level (Wood and Oliver, 1999). It is possible that the *XvRG6* mRNA transcript is not translated or that it is translated and degraded very rapidly.

4.5 CONCLUSION

A translational study was carried out to determine the expression level of *XvRG6* under various abiotic stresses that included dehydration, ABA, JA, SA and ethylene. The data presented here indicates that *XvRG6* is a drought-associated protein in *X. viscosa,* and is uninduced by ABA, JA, SA or ethylene. It is also important to note that the XvRG6 protein appears to form a tetrameric complex via cysteine and proline residues present within the sequence under dehydration stress.

In light of the findings presented here, it would be of interest to further investigate the role of *XvRG6* in dehydration tolerance.

Chapter 5

General discussion

Software analyses predicted XvRG6 to be a PRP, since it possesses many of the features common to this protein family. These features include the presence of a signal peptide, proline repeats, a cysteine domain and possible phosphorylation sites, all features which are highly conserved in PRPs (José and Puigdomènech, 1993). Another feature of interest identified by software analysis of XvRG6 is the presence of a tyrosine residue present in some repeats of other PRPs. It is thought that the presence of the tyrosine residue in the PRPs plays a role in protecting the plant against environmental stresses. This would occur by cross-linking the pre-existing tyrosine residue of wall proteins. This cross-linking results in rapid hardening of the cell wall (Bradley et al., 1992).

Analysis of the *XvRG6* mRNA transcript expression using RT-PCR showed that the gene was not induced during dehydration, but was induced by ethylene, endogenous ABA application, SA and JA application.

Western blot analysis revealed that the XvRG6 protein levels increased only during dehydration and not during any other imposed stress. It is therefore postulated that *XvRG6* plays an important role in response to water deficit stress at the translational level by protecting proteins during stressful conditions that result in protein denaturation and/or damage.

The XvRF17 protein was predicted by software analysis to be a metallothioneins, a family of metal-binding proteins with the potential to perform distinct roles in the metabolism of different metal ions (Robinson et al., 1993). The presence of CxC domains at the C- and N-terminus is the main characteristic of type 1 MTs and software analysis suggested that the putative protein encoded by XvRF17 possesses these domains.

The *XvRF17* mRNA transcript was shown by RT-PCR to be induced by dehydration stress, endogenous ABA application, SA and JA application but not by ethylene treatment. Despite the non-inducible nature of *XvRF17* to ethylene treatment as revealed by real-time PCR, the differential responses to dehydration, ABA, JA, and SA treatments of *XvF17* provide supportive evidence for the complex mechanisms against abiotic stresses.

The gene expression data obtained for *XvRG6* and *XvRF17* from real-time PCR should be interpreted cautiously as biological variation was very significant. Future work should involve

the abiotic treatments and subsequent real-time PCR with more biological replicates of *X. viscosa* in order to minimize the variation and to obtain a reproducible gene expression pattern.

This study has shown that both *XvRG6* and *XvRF17* are responsive to stress in *X. viscosa*. Since there are no known orthologues of these genes, their respective roles in *X. viscosa* remains speculative. The research presented here serves as an initial step into the elucidation of potential functional roles for these proteins. Further studies that may be undertaken towards this goal could include promoter analyses and immunolocalisation studies.

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Appendix A

General protocol

A1 Standard PCR reaction

The PCR reaction was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, Singapore). For each amplification, 25 µl reactions were set up using component concentrations summarised in Table A1. Supertherm *Taq* DNA polymerase, PCR buffer and $MgCl_2$ used for the amplification process were supplied by SR Products.

Table A1 PCR reagents and final concentrations used in a standard PCR protocol

Component	Final concentration
Primer concentration	200 pM
dNTP mixture (Roche, Germany)	40 µM
$MgCl_2$	1 mM
Taq polymerase buffer	1X
Template DNA	2 ng/µl
Supertherm *Taq* polymerase	0.02 U/µl

A2 Standard ligation protocol

Purified DNA fragments (insert DNA) were ligated to the respective linearised vectors in a reaction comprising 1 µg of insert DNA, 400 ng vector, 2 U of T4 DNA ligase (Roche) and 1X ligation buffer. The reaction volume was made up to 10 µl, mixed well and incubated for 20 h at 16°C.

A3 Standard transformation protocol

Competent *E. coli* cells were allowed to thaw on ice. Ten microlitres of ligation mix or pure plasmid DNA (ca. 10 ng) was added to the competent *E. coli* cells and mixed gently. The transformation mix was incubated for 10 min on ice. The cells were heat shocked by incubation for 5 min at 37°C and immediately thereafter on ice for 2 min. Nine hundred microlitres of LB

broth was added to the transformed cells and incubated for 45 min at 37°C with vigorous shaking. Fifty microlitres of the transformation mix was plated on LB agar plates (supplemented with the appropriate antibiotic) and incubated for 16 h at 37°C.

A4 Plasmid extraction

Plasmid DNA was isolated using the High Pure Plasmid Extraction Kit (Roche). Bacterial cells (ca. 1.5 ml) were centrifuged for 60 s at 6000 x g at RT. The supernatant was discarded and 250 µl of Suspension Buffer added to the pellet. The contents were mixed well and 250 µl of Lysis Buffer was added. The contents were mixed well and thereafter incubated for 5 min at RT. Three hundred and fifty microlitres of chilled Binding Buffer was added to the tube and the contents mixed gently. The tube was incubated for 5 min on ice and thereafter centrifuged for 10 min at 14000 x g at RT. A High Pure filter tube was inserted into one collection tube. The sample was transferred using a pipette to the upper reservoir of the filter tube. The sample was centrifuged for 60 s at 14000 x g in a microcentrifuge. The flow through was discarded and the filter tube combined again with the same collection tube. Five hundred microlitres Wash Buffer I was added to the upper reservoir and centrifuged for 60 s. The flow through was discarded and the filter tube again combined with the collection tube. Seven hundred microlitres of Wash Buffer II was added and the sample centrifuged and recovered as described above. An additional centrifugation step for 60 s at 14000 x g was performed. The flow through solution and collection tube was discarded and the filter tube inserted into a clean 1.5 ml reaction tube. The DNA was eluted using 50 µl elution buffer, which was pipetted onto the filter tube and centrifuged for 60 s at 14000 x g. The purified DNA was stored at 4°C.

A5 PCR Product purification

Amplified DNA was purified using the High Pure PCR Product Purification Kit (Roche). Two hundred and fifty microlitres Binding Buffer was added to a 50 µl PCR reaction and mixed well. The High Pure filter and collection tubes were combined and the sample pipetted into the upper reservoir. The sample was centrifuged for 60 s at 14000 x g in a microcentrifuge. The flow through was discarded and the filter tube combined again with the same collection tube. Five hundred microlitres Wash Buffer was added to the upper reservoir and centrifuged for 60 s. The

flow through was discarded and the filter tube again combined with the collection tube. Two hundred microlitres of Wash Buffer was added and the sample centrifuged and recovered as described above. The collection tube was discarded and the filter tube inserted into a clean 1.5 ml reaction tube. The DNA was eluted using 50 µl elution buffer, which was pipetted onto the filter tube and centrifuged for 60 s at 14000 x g. The purified DNA was stored at 4°C.

A6 Purification of DNA from agarose gels

The DNA fragments excised from agarose gels were purified using the High Pure PCR Product Purification Kit (Roche). The excised agarose gel slice was placed in a sterile 1.5 ml Eppendorf tube and the mass was estimated. For every 100 mg of excised agarose 300 µl of Binding Buffer was added to the Eppendorf tube. The tube was vortexed for 60 s to resuspend the gel slice in Binding Buffer. The suspension was incubated for 10 min at 56°C and vortexed briefly every 2-3 min during this period. For every 100 mg of agarose gel slice in the tube 150 µl of isopropanol was added and vortexed thoroughly. The High Pure filter and collection tubes were combined and the sample pipetted into the upper reservoir. The sample was centrifuged for 60 s at 14000 x g in a microcentrifuge. The flow through was discarded and the filter tube combined again with the same collection tube. Five hundred microlitres Wash Buffer was added to the upper reservoir and centrifuged for 60 s. The flow through was discarded and the filter tube again combined with the collection tube. Two hundred microlitres of Wash Buffer was added and the sample centrifuged and recovered as described above. The collection tube was discarded and the filter tube inserted into a clean 1.5 ml reaction tube. The DNA was eluted using 50 µl elution buffer, which was pipetted onto the filter tube and centrifuged for 60 s at 14000 x g. The purified DNA was stored at 4°C.

A7 Standard PAGE conditions

To each sample, 5X SDS loading buffer (0.225 M Tris, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT) was added. Samples were incubated for 8 min in a boiling water bath and immediately thereafter placed on ice. Ten microlitres of the denatured sample was electrophoresed on a 12.5% polyacrylamide gel (40% BDH acrylamide mix; 1.5 M Tris, pH 8.8; 10% SDS; 10% ammonium persulphate; 10 µl TEMED; made up to 10 ml), which

included a 5% stacking gel (40% BDH acrylamide mix; 0.5 M Tris, pH 6.8; 10% SDS; 10% ammonium persulphate; 5 µl TEMED; made up to 5 ml). The sample was electrophoresed for 3 h in 1X SDS PAGE running buffer (10 g SDS; 30.3 g Tris; 144.1 g glycine) at 30 mA. Gels were stained for 1 h with Coomassie staining solution (2.5 g Coomassie Brilliant Blue R-250; 450 ml methanol; 100 ml glacial acetic acid; made up to 1000 ml with sterile distilled water) and thereafter destained overnight in destaining solution (450 ml methanol; 100 ml glacial acetic acid; made up to 1000 ml with sterile distilled water).

Appendix B

Primer sequences

Table B1 List of primers used for cloning and sequencing

Primer name	Primer sequence
XvRG6F1	5′atggccccgtctaaaaccttcttc 3′
XvRG6R1	5′ctacggtgttgctggaggaattc 3′
XvRF17F1	5′atggcgtcttgtggaggaaactg 3′
XvRF17R1	5′gggagagatagatgacgcctatttg 3′
M13F	5′gtaaaacgacggccagt 3′
M13R	5′ctaccagtatcgacaa 3′

Table B2 List of primers used for real time PCR

Primer name	Primer sequence
XvRG6F2	5′ ggaggcagcacagaggtctattc 3′
XvRG6R2	5′atcggtctggctgggttgtgg 3′
XvRF17F2	5′caacccctacaactgcaaatgagc 3′
XvRF17R2	5′gtccataacatacaaataagtccctgatc 3′
18SF	5′caggcgcgcaaattacccaatcc 3′
18SR	5′cctaccgtcccgtcccaaggtc 3′