Molecular characterisation of the lateral organ boundaries gene (*XvLOB*) from the resurrection plant *Xerophyta viscosa*

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Go placidly amid the noise and the haste,
and remember what peace there may be in silence.
As far as possible, without slander,
be on good terms with all persons.
Speak your truth quietly and clearly;
and listen to the others, even to the dull
and the ignorant; they too have their story.

Avoid loud and aggressive persons,
they are vexations to the spirit.
If you compare yourself with others,
you may become vain or bitter;
for always there will greater and lesser persons than yourself.
Enjoy your achievements as well as your plans.

Keep interested in your own career, however humble;
it is a real possession in the changing fortunes of time.
Exercise caution in your business affairs;
for the world is full of trickery.
But let this not blind you to what virtue there is;
many persons strive for high ideals;
and everywhere life is full of heroism.

Be yourself.
Especially, do not feign affection.
Neither be cynical about love;
for in the face of all aridity and disenchantment
it is as perennial as the grass.

Take kindly the counsel of the years,
gracefully surrendering the things of youth.
Nurture strength of spirit to shield you in sudden misfortune.
But do not distress yourself with dark imaginings.
Many fears are born of fatigue and loneliness.
Beyond a wholesome discipline,
be gentle with yourself.

You are a child of the universe,
no less than the trees and the stars;
you have a right to be here.
And whether or not it is clear to you,
no doubt the universe is unfolding as it should.

Therefore be at peace with God,
whatever you conceive him to be,
and whatever your labors and aspirations,
in the noisy confusion of life keep peace with your soul.

With all its sham, drudgery, and broken dreams,
it is still a beautiful world.
Be cheerful.
Strive to be happy.

MAX EHRMANN, DESIDERATA

DECLARATION

I hereby declare that this thesis entitled:

Molecular characterisation of the lateral organ boundaries gene (*XvLOB*) from the resurrection plant *Xerophyta viscosa*

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

I know the meaning of Plagarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own.

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Signature

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Date

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First and foremost I would like to express my sincere gratitude towards My Creator Allah (the most Merciful, most Gracious) for granting me the opportunity, the health, the emotional strength and a loving supportive family for which without I would not have been able to complete this research.

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List of Abbreviations

µl	microlitre
µm	micrometre
2, 4-D	dichlorophenoxyacetic acid
ABA	abscisic acid
ARF	auxin responsive factors
AuxRE	auxin response element
BD	binding domain
bp	base pair(s)
CaMV	cauliflower mosaic virus
cDNA	copy DNA
cys	cysteine
CZ	central zone
dicot	dicotyledonous
DNA	deoxyribonucleic acid
DTT	dithiothrietol
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
FDU	fastdigest unit
Gal4	a nuclear protein
GFP	green fluorescent protein
Gln	glutamine
GST	glutathione-S-transferase
GUS	beta-glucuronidase
his	histodine
kb	kilobase
KNOX	KNOTTED-like homeobox
LB	luria broth
LBDs	LOB domain-containing proteins
LEAs	late embryogenesis abundant proteins
leu	leucine
LOB	lateral organ boundaries
M	molar
mg	milligram
ml	millilitre
monocot	monocotyledonous
mM	millimolar
mRNA	messenger RNA
N	nitrogen
N =	number of sample size
N =	number of technical repeats
NAA	naphthalene acetic acid
ng	nanogram
NH_4^+	ammonium
NLS	nuclear localisation signal
NO_3^-	nitrate
NTC	no template control

ORF	open reading frame
PAGE	polyacrylamide gel elctrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
pI	isoelectric point
PZ	peripheral zone
RAM	root apical meristem
RFOs	raffinose family of oligosaccharides
RHS	right hand side
RT	room temperature
RT-PCR	realtime PCR
RWC	relative water content
RZ	rib zone
SAAB	selection and amplification binding assay
SAM	shoot apical meristem
SDS	sodium dodecyl phosphate
trp	tryptophan
TrxA	thioredoxin tag

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ABSTRACT

Resurrection plants possess unique mechanisms that allow them to be able to withstand severe desiccation of their vegetative tissue, survive in this dried state for prolonged periods and resume full metabolic function within hours of rehydration. They have become valuable models in investigating the coping mechanisms employed by plants to alleviate the effects of environmental stresses. They are also a rich source of genes that could be used to improve stress tolerance in other susceptible crop plants. The aim of this study is to preliminary characterise *XvLOB*, a LOB domain containing gene, which has been isolated from a dehydration stress library in *X. viscosa*. In this study the gene was isolated and cloned, the DNA and amino acid sequences were analysed by *in silico* analysis, the recombinant XvLOB protein expressed, subcellular localisation examined and both the transcript and protein expression levels in response to dehydration was investigated. The XvLOB cDNA length is 648bp and the gDNA length is 746bp. The amino acid sequence contains the highly conserved C motif and GAS block found within the LATERAL ORGAN BOUNDARIES (LOB) domain. Based on BLAST and sequence alignment analysis, there was a high sequence similarity to the LOB domain containing (LBD) gene in *Arabidopsis, AtLBD39*. Sequence alignment results between XvLOB and 43 *Arabidopsis* LBD genes as well as the lack of a predicted coiled coil structure within XvLOB suggest that it may belong to the Class II LBD genes group. Recombinant XvLOB protein was expressed in *E. coli* BL21 using the pET32b expression vector. *In silico* analysis indicated nuclear localisation of the XvLOB protein albeit the lack of any known plant nuclear localisation signals. Expression analysis of XvLOB::EYFP fusion protein in onion epidermal cells indicated XvLOB is localised to the nucleus. Western blot analysis indicated that the XvLOB protein is not expressed in response to a dehydration and rehydration treatment. Quantitative realtime PCR analysis showed a significant increase in XvLOB transcript abundance during dehydration between RWC range 48 – 26 % and RWC range 2 -1 %. This project provides the preliminary molecular characterization of XvLOB for further study.

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CHAPTER 1

Literature Review

1.1 RESURRECTION PLANTS

1.1.1 General characteristics of angiosperm resurrection plants

Resurrection plants possess unique mechanisms that allow them to be able to withstand desiccation of their vegetative tissue and remain in an air-dry state until water becomes available (Gaff, 1971; Gaff, 1987; Scott, 2000). These plants can lose 90% of their relative water content, survive in a dried state for prolonged periods and resume full metabolic function within hours of rehydration (Sherwin and Farrant, 1996; Sherwin and Farrant, 1998; Mundree *et al*., 2002). Resurrection plants grow in shallow, sandy soils on rocky outcrops where water supply is limited and rainfall patterns are sporadic (Sherwin and Farrant, 1995). These extremophiles are widely distributed across most continents and are especially found within southern Africa, southern America and Western Australia (Gaff, 1977; Gaff, 1987). There are both monocotyledonous resurrection plants such as *Xerophyta viscosa* (*X. viscosa*), *Xerophyta humilis* (*X. humilis*) and *Sporobolus stapfianus* as well dicotyledonous species such as *Myrothamnus flabelifolia*, *Chamaegigas intrepidus* and *Craterostigma plantagineum* (Gaff, 1971). These resurrection plants have become valuable models to investigate the effects of environmental stresses and the coping mechanisms employed by plants to mitigate these stresses. They have also become a rich source of genes and stress inducible promoters that could be used to improve stress tolerance in other susceptible crop plants (Ramanjulu and Bartels, 2002; Garwe *et al*., 2006).

1.1.2 Protective mechanisms in resurrection plants

Based on the time scale of the response of resurrection plants to water loss as tolerance mechanisms are acquired, there is increasing evidence which groups genes upregulated in response to desiccation into two classes. There are genes, which respond to water deficit within seconds or minutes and may therefore play a role in the initial protection and amplification of stress induced signaling pathways. Genes that respond later as in hours, days or even weeks are most likely to be involved in the adaptation process (Ramanjulu and Bartels, 2002). Some of the protective mechanisms and putative genes involved in this adaptive process are examined below.

Reactive Oxygen species (ROS) which form as a natural consequence of metabolic processes involving electron transport are exacerbated under water deficit conditions (Halliwell and Gutteridge, 1999; Apel and Hirt, 2004; Bailly, 2004). Photosynthesis, in particular, is very sensitive to water deficit as electron leakage during photosynthetic electron transport and the formation of singlet oxygen are significantly increased when cells of photosynthetic tissues suffer water loss and this has frequently been cited as a primary cause of damage and resultant plant death in most species (Seel *et al*. 1992; Smirnoff 1998; Kranner and Birtić, 2005). Angiosperm resurrection plants firstly minimize amount of photosynthetically produced ROS formed during drying and effectively quench remaining ROS by upregulation of antioxidants. Minimization of ROS is achieved in one of two ways. Homiochlorophyllous plants retain chlorophyll and chloroplast structure during desiccation, but chlorophyll is hidden from light by leaf movements to induce shading, or the production of pigments such as carotenoids and anthocyanins. Poikilochlorophyllous plants however destroy their chlorophyll and the thylakoid membranes are dismantled (Sherwin and Farrant, 1996; Sherwin and Farrant, 1998; reviewed in Farrant, 2007). Free radical quenching molecules include antioxidant enzymes such as ascorbate peroxidases, catalases, glutathione-S-tranferase (GST), superoxide dismutases and glutathione peroxidases, whose activity is upregulated during drying (Sherwin and Farrant 1998; Farrant, 2000; Illing *et al*., 2005; Farrant *et al*., 2007). As well as increased levels of water and lipid soluble antioxidants such as ascorbate, glutathione and tocopherol (Ramanjulu and Bartels, 2002; Kranner and Birtic, 2005). An example of a gene upregulated during these processes is the glutathione-S-transferase gene which was isolated from *Glycine soja* (*GsGST*), transformed and overexpressed in tobacco (Ji *et al*., 2010). The overexpression of *GsGST* exhibited a sixfold higher GST activity than in wildtype plants. These transgenic tobacco plants also showed an enhanced tolerance to dehydration.

Late Embryogenesis-Abundant proteins (LEAs) generally accumulate during the last stages of seed development when desiccation tolerance is acquired or in response to dehydration, low temperature, salinity or exogenous ABA treatment (Wise and Tunnacliffe, 2004; Illing *et al*., 2005). LEA proteins are characterized by their high hydrophilicity and solubility in water. They have been divided into groups based on their predicted biochemical properties and the motifs found within their sequences. During dehydration, these proteins have been proposed

to play a role in protecting the cytoplasmic structures by acting as 1) water replacement molecules and/or hydration buffers; 2) ion sequesters; 3) chaperonins and/or heat shields; 4) protein/membrane anti-aggregants and 5) promoters of vitrification (Bray, 1997; Hoekstra *et al*., 2001; Wise and Tunnacliffe 2004; Bartels, 2005; Goyal *et al*., 2005; Mtwisha *et al*., 2006; Berjak *et al*., 2007; Chakrabortee *et al*., 2007; Tunnacliffe and Wise 2007). Studies done by Olvera-Carrillo et al (2010) in *Arabidopsis*, has shown that the deficiency of a LEA protein from group 4 resulted in a reduced number of floral and axillary buds when compared to wild type plants as well as a decrease in seed production. Data revealed susceptible phenotypes during seed germination, or in mature plants when recovering from water limited conditions (Olvera-Carillo *et al*., 2010). According to quantitative RT-PCR analysis, the late *EMBRYOGENESIS ABUNDANT 14* (*LEA14*) in *Ipomoea batatas* (sweet potato) showed an increased expression in response to dehydration and abscisic treatment. In transgenic sweetpotato calli, the overexpression of *IbLEA14* under the control of the CaMV 35*S* promoter showed enhanced tolerance to drought and salt stress (Park *et al*., 2011).

Other protective mechanisms that resurrection plants employ during desiccation include the synthesis and accumulation of carbohydrates (sugars or sugar derivatives) such as sucrose and raffinose family oligosaccharides (RFOs) (Reviewed in Farrant *et al*., 2011). In *X. viscosa*, concentrations of these molecules has been shown to increase during the dehydration and therefore must play a crucial role in the protection of leaf cells as well as act as a source of carbon (Peters *et al*., 2007). As water is lost from the cells, the cytoplasmic content becomes highly viscous and molecular interactions, which may cause protein denaturation and membrane fusion, are likely to happen. The accumulation of sugars such as RFO's and sucrose prevents interactions and hence denaturation from occurring by acting as a substitute for water as they can bind the proteins and lipids by hydrogen interactions thereby stabilizing the protein structure and cell integrity (reviewed in Hoekstra *et al*., 2001).

Mechanical stress is defined as the tension that develops between and within the plasma membrane and the cell wall due to a loss of turgor pressure, which thereby results in plasmolysis (Walters *et al*., 2002). Reversible cell wall folding, often in combination with water replacement in vacuoles appear to be a common protective mechanism amongst resurrection plants (reviewed in Farrant, 2007; Moore *et al*., 2009). Wall folding has been

reported to involve architectural changes to xyloglucans (Vicrě *et al*., 2004) or, more commonly, the cell walls contain constitutively high proportions of arabinose, associated with pectins in *Myrothanmus flabellifolia* (Moore *et al*., 2006) and xyloglucans in the *Eragrostis nindensis* (Plancot *et al*., 2009).

In recent studies, secretory cavities have been identified on the adaxial leaf surface in *Xerophyta viscosa* (Naidoo *et al*., 2009). These cavities secrete a sticky lipophilic substance that is composed of diterpenes, phenols and stearic acid that probably serves an important defense against herbivores, insects and pathogens whilst in a dehydrated state.

1.1.3 Significance and use of *X. viscosa* as a model for studying stress tolerance

The resurrection plant, *X. viscosa* (Velloziaceae), is a plant species that is indigenous to southern Africa and grows around the Cathedral Peak in the Drakensberg Mountains (Figure 1.1). *X. viscosa* is a poikilochlorophyllous species, which dismantles its chloroplasts during the dehydration process and therefore need several days to reconstitute its thylakoid membranes and resynthesize chlorophyll on rehydration (Bhatt *et al*., 2009).

Figure 1.1 *X. viscosa* plants located within its niche found in rocky outcrops at Cathedral Peak Nature Reserve in the Drakensberg mountains. Note that both the hydrated (blue arrow) and dehydrated (red arrow) states are represented.

The Plant Stress Lab (University of Cape Town) is studying the mechanism of desiccation tolerance in *X. viscosa* with the ultimate aim of enhancing drought tolerance in agricultural crops such as maize. Accordingly, *X. viscosa* is being investigated as a source of genes that are involved in abiotic stress. A dehydration cDNA library was constructed with the aim of identifying and characterizing genes that showed significant upregulation in response to dehydration (Mundree *et al*., 2002; Mundree *et al*., 2006). To date, functional characterization has been performed on a number of *X. viscosa* gene products from this library and shown to be upregulated in response to dehydration (Mundree *et al*., 2000; Mowla *et al*., 2002; Garwe *et al*., 2003; Marais *et al*., 2004; Govender, 2006; Peters *et al*., 2007; Bresler, 2010). Several of these genes have been shown to play a role in inferring desiccation tolerance in *Arabidopsis* and tobacco (Garwe *et al*., 2006; Maredza, 2007). Preliminary screening of the cDNA dehydration library also revealed a gene sequence with similarity to genes containing the LOB (Lateral Organ Boundaries) domain.

1.2 LATERAL ORGAN BOUNDARIES GENE

1.2.1 Shoot apical meristem (SAM) regulation and maintenance

The shoot apical meristem (SAM) is a reservoir of self-renewing pluripotent stem cells that are located at the very apex of the stem and are the key to development of higher plants and continuous aerial growth. These cells are initially formed during embryogenesis and throughout its life cycle it produces stem tissues and lateral organs. The other meristem which arise during embryogenesis is the root apical meristem (RAM) which generates the underground part of the plant (Carles and Fletcher, 2003; Williams and Fletcher, 2005).

There are three major zones found within the SAM, with characteristic cytoplasmic densities and cell division rates for each zone (Figure 1.2). The peripheral zone (PZ) contains cells that produce the lateral primordia whereas the rib zone (RZ) directs cells towards the stem tissue. The central zone (CZ) acts as a reservoir of stem cells that replenish both the peripheral and rib zones. Based on these zones the SAM performs three main functions namely; produces lateral organs, formation of the stem tissue and the maintenance of stem cells required for further growth. Proper SAM function requires the maintenance of a delicate balance between the production of lateral organs from the PZ and indeterminate growth at the CZ. During primordial (lateral organ) initiation, cells in the peripheral zone become the founder cells of

the new plant tissue. To sustain this homeostasis, shifts in gene expression must occur in the cells as their positions within the meristem changes. The study of phytohormones have been become the focus in unraveling meristem homeostasis (Bowman and Esthed, 2000; Shani *et al*., 2006). Hormones such as auxins, cytokinins, gibberellin and ethylene have been shown to display dynamic concentration gradients in the SAM. A number of transcription factors have also been shown to participate in SAM maintenance (Ha *et al*., 2003; Hibara *et al*., 2003; Vroemen *et al*., 2003; Hepworth *et al*., 2005; Cole *et al*., 2006; Kieffer *et al*., 2006). The relationship between these transcription factors and hormones appear to involve hormone ratios and the interactions between the hormones and transcription factors maintain the homeostasis of the SAM (Shani *et al*., 2006).

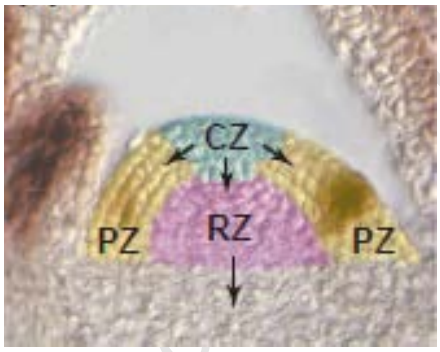


Figure 1.2 Histology displaying shoot apical meristem (SAM). The peripheral zone (PZ) contains cells that are recruited to become lateral organs whereas the rib zone (RZ) contains cells that become the stem cell. The central zone (CZ) acts as a reservoir of cells needed to replenish the PZ and RZ. (Adapted from Bowman and Eshed, 2000)

There are two important pathways that are pivotal in maintaining meristem homeostasis; these pathways are the WUSCHEL (WUS) function and CLAVATA (CLV) signaling. The *WUS* gene encodes a transcription factor that has homology to the homeodomain-containing transcription factors found in plants and animals. This gene is expressed in a defined group of cells below the CZ, which implies that it plays a role in the maintenance of the meristem indeterminacy. WUS cell activities are non-cell autonomous, as this protein is expressed below the CZ but is required to maintain the CZ (Shani *et al*., 2006). The CLV signal transduction pathway is comprised of three main component molecules, namely CLV1, CLV2 and CLV3. The CLV3 is a small secreted polypeptide that is produced by the stem cells and is postulated to interact with the CLV1-CLV2 receptor complex in the underlying

cells. Activation of this pathway initiates downstream signaling events that limit the size of the *WUS* expression domain. In turn the *WUS* activity specifies the neighboring stem cells to induce the expression of *CLV3* (Figure 1.3). This negative regulatory feedback loop results in meristem homeostasis in which the output of stem cells is balanced by the input of new stem cell derivatives (Carles and Fletcher, 2003; Williams and Fletcher, 2005).

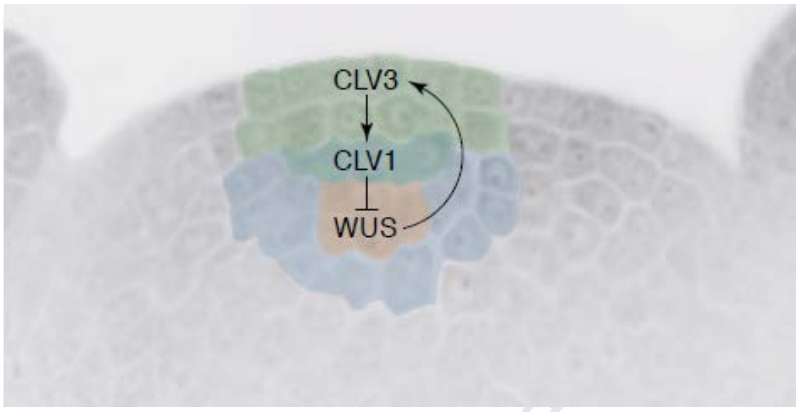


Figure 1.3 Stem cell regulation in the SAM via the CLAVATA-WUSCHEL feedback loop. CLV 3 acts as a signaling molecule that binds to the CLV1 receptor and activates a downstream event that limits the size of *WUS* expression. WUS in turns results in the activation of CLV1 expression. (Adapted from Carles and Fletcher, 2003)

1.2.2 Transcription factors involved in SAM maintenance and formation of lateral primordia

There are a number of transcription factors that have been shown to take part in SAM maintenance. The class 1 *KNOX* homeobox genes have been found to play an important role in SAM function. These genes are specifically expressed in the SAM and are downregulated in the lateral primordia in plants species such as maize, *Arabidopsis*, tobacco and rice (Jackson *et al*., 1994; Long *et al*., 1996; Nishimura *et al*., 1999; Sentoku *et al*., 1999; Vollbrecht *et al*., 2000; Scofield and Murray, 2006) The *knotted1* gene (*kn1*) was the first homeodomain transcription factor to be in identified in plants. It was isolated from a maize gain of function mutant that produced outgrowths (‘knots’) of undifferentiated tissue on leaves (Freeling and Hake, 1985). According to Vollbrecht et al (2000), the loss of function mutation in the *kn1* gene in *Arabidopsis* has demonstrated that class 1 *KNOX* genes are important for SAM formation and maintenance. Repression of *KNOX* gene facilitates the initiation of lateral primordia from the SAM (Jackson *et al*., 1994; Long *et al*., 1996). A

review by Hay and Tsiantis (2010) discusses how KNOX proteins influence plant growth and development in a versatile context-dependant manner.

In *Arabidopsis*, the *shoot meristemless* gene (*STM*) which is an ortholog of the *kn1* gene in maize was isolated from a loss of function mutant that failed to establish and maintain the SAM such that no lateral organs are produced once the cotyledons have formed (Barton and Poethig, 1993). *STM* is required to prevent cell differentiation and maintain the indeterminate cell fate in the meristem. This role has been shown to be linked to wuschel expression in which these genes fulfill complementary functions in the regulation of the meristem (Lenhard *et al*., 2002). A more evident role of *STM* is its ability to negatively regulate the expression of the *asymmetric leaves1* (*AS1*) gene in the meristem (Byrne *et al*., 2000).

AS1 and *AS2* have been identified as transcription factors which regulate the development of asymmetrical leaves and the establishment of a leaf venation system (Byrne *et al*., 2000; Semiarti *et al*., 2001; Sun *et al*., 2002; Xu *et al*., 2003; Iwakawa *et al*., 2007). The phenotypical characteristics of *Arabidopsis AS1* and *AS2* mutants have been extensively studied. These mutants display malformed vein systems, have asymmetric lobes and have leaves that exhibit downward curling that is bilaterally asymmetric. Other abnormalities that occur due to these mutations include an accumulation of the class 1 *KNOX* gene transcripts such as *BREVIPEDICELLUS* (*BP*), *KNAT1*, *KNAT2* and *KNAT6* in the leaves (Byrne *et al*., 2000; Semiarti *et al*., 2001; Sun *et al*., 2002). The accumulation of these transcripts indicates that AS1 and AS2 regulate the expression of the class 1 *KNOX* genes. Therefore, *KNOX* genes restrict the expression of the *AS1* and *AS2* genes to organ primordia and thus prevent inappropriate leaf development at the shoot apex. In turn, AS1 and AS2 regulate *KNOX* genes, which promote stem cell activity, by excluding them from being expressed in organ primordia (Ori *et al*., 2000; Semiarti *et al*., 2001; Lin *et al*., 2003; Guo *et al*., 2008). A study done by Iwakawa et al (2002) discovered that the AS2 gene appears to encode a novel protein containing leucine zipper-like sequence at the amino-terminal and cysteine repeats (designated C-motif) designated the Lateral Organ Boundaries domain (LOB). Expression patterns of *AS1* and *AS2* indicated accumulation of transcripts in leaf primordia at early stages of development. During the later stages of development, the *AS1* and *AS2* transcripts were detected between the adaxial and abaxial of the inner region areas and in the adaxial domain

of young leaves, respectively. These results indicate *AS2* may play a role in establishing polarity of leaf primordia (Iwakawa *et al*., 2007).

1.2.3 Lateral organ boundaries (LOB) gene and LOB-domain containing (LBD) genes

The *LATERAL ORGANS BOUNDARIES (LOB)* gene was identified in *Arabidopsis* based on its expression pattern during the initiation of lateral primordia. A study done by Shuai et al (2002) showed the expression and localization of *LOB* by creating a *LOB*-promoter::GUS reporter fusion construct. This construct was introduced into *Arabidopsis* ecotype *Landsberg erecta* and GUS expression patterns were examined throughout development. The results showed that the expression of LOB localized to the shoot and root apices during the late stages of embryogenesis (Figure 1.4A and B), between the primary root and lateral root primordia (Figure 1.4C) and during inflorescence at the base of lateral primordia (Figure 1.4D). These expression patterns may reflect roles in plant developmental processes.

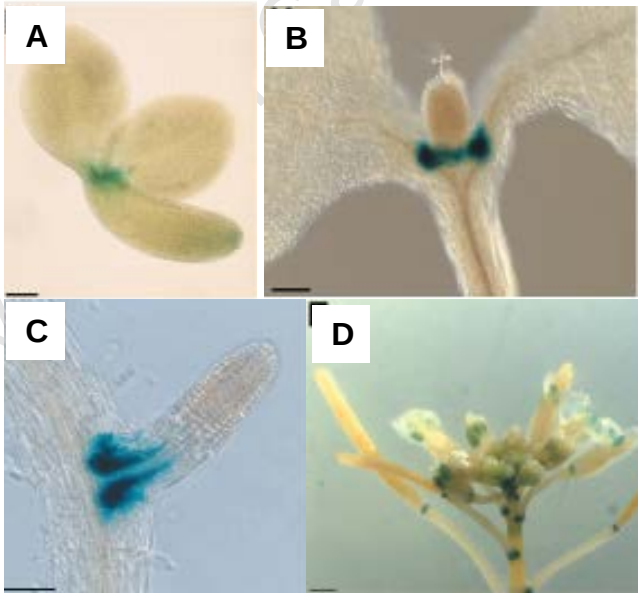


Figure 1.4 Analysis of GUS activity in Arabidopsis containing LOB-promoter::GUS fusion constructs. Images taken during development of seedling namely; **A** – mature embryo, **B** – seven-day old seedling, **C** – Lateral root and **D** – Inflorescence. Scale bar in **A** = 50 µm, **B** and **C** = 100 µm and **D** = 1mm. (Adapted from Shuai *et al*., 2002)

The LOB domain is approximately 100 base pairs (bp) in length and encodes a novel plant specific protein which has no known functional motifs and can be found in a number of plant species including *Arabidopsis*, *Oryza sativa* (rice), *Zea mays* (maize), and *Brassica napus* (canola), amongst others (Shuai *et al*., 2002; Dong *et al*., 2004; Inukai *et al*., 2005; Bortiri *et al*., 2006; Evans, 2007; Husbands *et al*., 2007; Li *et al*., 2008; Soyana *et al*., 2008).

To date there has been no proteins found outside of plant databases that have similarity to the LOB domain. This suggests that proteins containing the LOB domain may play an important role within plant specific processes. In *Arabidopsis*, there are 43 proteins which contain this conserved LOB domain (AtLOB and 42 AtLBDs). According to Shuai et al (2002), these LBD proteins have been grouped into two classes. The class I group include 36 *Arabidopsis* LBD proteins and have between 25 and 82 % sequence similarity to the LOB domain. Class II however consists of six LBD proteins and is less similar to the LOB domain (between 28 and 33 % sequence similarity). The LOB domain is defined by the presence of two conserved blocks namely: the C block and the GAS block. The C block is 22 amino acids in length and contains four conserved Cysteine (Cys) residues in a $CX_2CX_6CX_3C$ motif (Shuai *et al*., 2002). According to Shuai et al (2002), this C motif may form a zinc finger transcription factor, although the spacing between Cys residues may not be typical to the C_2/C_2 type zinc finger (Takatsuji, 1998). The GAS block is 49 amino acids in length and begins with a FX_2VH motif and ends with a DP(V/I)YG motif (Appendix C5 for alignments). The possible secondary structure of LBD amino acid sequences was also examined; results predicted the formation of a coiled coil structure containing four leucines (Leu) in a $LX_6LX_3LX_6L$ pattern within the Class I proteins and not within the Class II proteins (Shuai *et al*., 2002). This pattern is reminiscent of a Leu-zipper protein (Landschultz *et al*., 1988).

A comparison study performed by Yang et al (2006) identified thirty-five LBD genes within both rice subspecies *Oryza japonica* and *O. indica*. As with the *Arabidopsis* LBDs, the rice LBD genes are classified into two classes. Both the *O. japonica* and *O. indica* subspecies have twenty-nine Class I genes and five Class II genes. Within both subspecies there is an LBD gene which contains two LOB domains namely; *OsJLBD1-12* and *OsILBD1-12* (gene number 12 on chromosome 1). These *LBD* genes were assigned to a third class within each subspecies.

The biochemical function of the LOB domain has not yet been fully defined, although proteins containing the LOB domain have been assumed to be transcription factors (Gong *et al*., 2004). To investigate whether AtLOB acts as a DNA binding protein, subcellular localization was performed by creating a *35S::GFP-AtLOB* construct and transformed into *Arabidopsis* plants. These results showed GFP fluorescence predominately in the nucleus in contrast to plants containing the *35S::GFP* control construct, which showed fluorescence in both the cytoplasm and nucleus (Husbands *et al*., 2007). A selection and amplification binding assay (SAAB) was also performed to identify a recognizable DNA motif to which the LOB domain may bind. These results revealed a six base pair consensus sequence, 5' – (G)**CGGC**(G) – 3', which was identified in 24 clones obtained. Within this consensus sequence, the four nucleotide core sequence (in bold) was absolutely conserved with guanine (G) being the most common nucleotide flanking either end of the core sequence. An electrophoretic mobililty shift assay (EMSA) confirmed the SAAB results. A modified yeast one hybrid approach was used to establish whether the LOB protein can act as a transcriptional activator in a heterologous yeast system (Husbands *et al*., 2007). The full length LOB was fused inframe to a Gal4 DNA-binding domain to create a LOB-BD construct that was transformed into the yeast reporter strain Ah109. As a control, an empty plasmid only carrying the Gal4 DNA-binding domain was used (empty-BD). Yeast strains containing either the LOB-BD or empty-BD constructs showed vigorous growth on –Trp media which selected for the presence of the plasmid. When grown on –Trp–His media, yeast carrying the LOB-BD construct exhibited vigorous growth when compared to yeast carrying an empty-BD construct. The growth of the transformed yeast on –His media will indicated that AtLOB can act as a transcriptional activator and drive the expression of the *HIS3* reporter gene. This data together with the subcellular localization of AtLOB to the nucleus and its ability to bind to a six base pair DNA sequence indicates that the LBD family of proteins may represent a new class of transcription factors within plant species (Husbands *et al*., 2007).

Dong et al (2004) performed a differential gene screening on *Brassica napus* seeds during its early stages of development (10 days after pollination). Fifty four highly expressed mRNAs were identified which shared similarities to a broad range of gene families encoding structural proteins, metabolic enzymes, transport and protective proteins. These cDNAs were categorized into two main groups namely, seed specific genes and seed abundant genes.

Amongst the seed specific group one clone, 15D17A, was identified; based on its intensity and temporal expression patterns; as being similar to three storage proteins genes (napin, cruciferin and oleosin). The expression pattern of 15D17A showed expression in the seed coat as well as in embryos. Seven putative homologues of 15D17A were found in *Arabidopsis*, including the AtLBD41 protein (Dong *et al*., 2004) as well as a homologue in maize (Alexandrov *et al*., 2009). The role that LBD genes may play during seed development has not yet been established and further studies need to be done to elucidate its function.

In rice (*Oryza sativa*), a LOB-domain-like protein called DH1 was identified as playing a role in glume formation. In this study, a T-DNA insertion pool was screened and a mutant (*dh1*) with a phenotype of degenerate hull and malformed pistils and stamens in the florets was identified. DH1 (OsDH1) was predicted to encode a 216 amino acid protein and BLAST analysis indicated its high similarity to the *Arabidopsis* LBD (AtLBD16) protein. Alignment between OsDH1 and other LBDs revealed the presence of a LOB domain represented by the C-motif and GAS block. Wildtype rice plants transformed with an extra *DH1* gene displayed a super-dwarf phenotype, downward leaves as well as a shorter pedicle and panicle axis. This may suggest that *DH1* and other *LBD* genes participate in the establishment of polarity in lateral organs (Li *et al*., 2008).

There has been other rice *LBD* genes which have been isolated and characterized such as the *crown rootless1* (*crl1*) and the *adventitious rootless1* (*arl1*) genes. In 2001, Inukai et al isolated a *crl1* mutant that was defective in the formation of crown roots. The *crl1* mutant displayed severe defects in root formation and therefore suggests that the *crl1* gene may be involved in auxin related root formation. Auxin is a phtytohormone, which is essential for root development especially the formation of lateral and adventitious roots (Schielfelbein, 2003). In 2005, Inukai et al characterized the biological function of the *crl1* gene in terms of auxin signaling. Bioinformatic analysis performed on the CRL1 amino acid sequence revealed a protein that contains an AS2/LOB domain. When wildtype and *crl1* mutant seedlings were exogenously exposed to increasing concentrations of indole-3-acetic acid, 2,4–D and naphtalene acetic acid (NAA), the wildtype seedlings exhibited a dose dependant increase in both crown and lateral root formation whereas the *crl1* mutants exhibited no such changes. These results indicate the positive effect that auxin has on crown and lateral root

formation is defective in *crl1* mutants. The *crl1* expression pattern was investigated by the detection of β–glucuronidase (GUS) activity under the control of the *crl1* promoter. The results showed GUS activity only at the base of the shoot and preferentially localized in tissues from which lateral roots are initiated. The *crl1* promoter region contained two TGTCTC motifs (AuxRE1 and AuxRE2 with its inverted sequence, GAGACA). These motifs are needed for the binding of auxin responsive factors (ARFs) to the promoter region. An EMSA was performed with the rice ARF (OsARF1) and the *crl1* promoter sequence. The results indicated that the OsARF1 bound to the AuxRE2 within the promoter suggesting the AuxRE2 sequence may act as a *cis*-acting factor for transcriptional regulation of the *crl1* gene. In this study, Inukai et al (2005) demonstrated that *crl1* functions as a mediator linking the control of auxin to the initiation of crown and lateral root formation. It was also demonstrated that ARFs interact with the AuxREs in the *Crl1* promoter region to activate the *crl1* gene. A homologue of the Crl1 protein is the ARL1 protein which has also been shown to contain a LOB domain as well as being localized to the nucleus when fused to GFP, which suggest its role as transcription factor. Further studies revealed that the ARL1 protein may also act as an auxin responsive factor (Liu *et al*., 2005).

Recently, a novel member of the lateral organ boundaries domain family of transcription factors was isolated from a *Populus tremula* X *Populus alba* mutant (Yordanov *et al*., 2010). Sequence analysis of this uncharacterized gene showed highest sequence similarity (80 %) to the *Arabidopsis* LBD1 protein and was annotated as PtaLBD1. The expression pattern of *PtaLBD1* transcripts within various organs and tissues including xylem, phloem and stems undergoing primary and secondary growth was analyzed. Results indicated the highest levels of *PtaLBD* were located in the cambial zone of phloem tissue and the stems undergoing secondary growth. Analysis of gain- or loss of function mutants displayed mainly phloem-related phenotypical changes. Transgenic plants expressing PtaLBD fused to the SRDX repressor protein, a short 12–amino acid motif that converts transcription factors to dominant repressors was also analysed. Results showed highly suppressed and irregular phloem differentiation. These results suggest a possible role for PtaLBD in phloem development (Yordanov *et al*., 2010).

In a study performed in *Arabidopsis* by Rubin et al (2009), results indicated that other than the proposed function of initiating the development of lateral organ primordia; *AtLBD37*, *AtLBD38* and *AtLBD39* is induced by nitrogen (N) and nitrate (NO_3^-) and serves to repress the anthocyanin synthesis process. N and NO_3^- regulate many aspects of plant metabolism, growth and development. There is clear evidence indicating the role of NO_3^- in regulating pathways involving flavonoid, phenylpropanoid and anthocyanin metabolism. Other important sources of nitrogen such as ammonium (NH_4^+) and glutamine (Gln) have also shown to induce the expression of *AtLBD37*, *AtLBD38* and *AtLBD39* but to a lesser extent (Rubin *et al*., 2009). In transgenic *Arabidopsis*, the overexpression of *AtLBD37*, *AtLBD38* and *AtLBD39* displayed unusual pigmentation phenotypes indicating a possible role in flavonoid metabolism. Further analysis revealed a strong repression of the *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*) and *PAP2* in *AtLBD37*, *AtLBD38* and *AtLBD39* overexpressed transgenic plants when compared to wildtype plants. PAP1 and PAP2 are key regulators of the flavonoid/anthocyanin synthesis process. These results suggests that *LBD37*, *LBD38* and *LBD39* are N/NO_3^- induced repressors acting upstream of the *PAP1* and *PAP2*. Whether the LBDs directly bind to *PAP1* and *PAP2* promoter regions, remains to be investigated. LBD37 was fused to the C-terminus of a green fluorescent protein (GFP) and expressed under the control of the cauliflower mosaic virus 35S promoter (P35S). This P35S::GFP::LBD37 construct was transformed into *Arabidopsis*, fluorescence examined in the leaves and results showed GFP::LBD37 fusion protein targeted to the nucleus. These results are consistent with its predicted role as a transcription factor (Rubin *et al*., 2009). Evidence thus indicates that these three *LBD* genes are induced by the presence of N, act as regulators of anthocyanin production and are thus important molecular components in plant N/NO_3^- signaling. This is the first reported study involving characterization of class II LBD proteins (Rubin *et al*., 2009).

In a recent review, a number of *LBD* genes have been discussed and grouped based on their proposed function in the regulation of metabolism, their expression in response to phytohormones and environmental stimuli as well as their role in leaf, flower and root development (Majer and Hochholdinger, 2011). A phylogenetic tree was created containing *LBD* genes from the *Arabidopsis*, maize and rice genomes, identifying similarities of *LBD* genes between species. Seventeen pairwise orthologs were identified between rice and maize

whereas only 7 were identified between *Arabidopsis* and maize or rice. The roles of many LBD genes have yet to be investigated. A major task in understanding how these *LBD* genes play a role in organ boundary definition is to identify the regulatory elements and upstream genes involved in the regulation of *LBD* expression as well as the downstream molecular components that trigger developmental responses (Majer and Hochholdinger, 2011).

1.3 AIM OF THIS STUDY

To date there has been no reported link between dehydration stress and the expression or regulation of *Lateral Organ Boundaries* (LOB) domain containing genes (*LBD*s). Preliminary screening of the *X. viscosa* dehydration cDNA library revealed a gene (*XvLOB*) with similarity to *LBD* genes and suggested that *XvLOB* is upregulated in response to dehydration. Based on the literature provided there are speculations that the *XvLOB* gene may play a role in lateral organ development and/or metabolic processes which occur during dehydration.

Our main aim is to determine if *XvLOB* is expressed in response to dehydration in *X. viscosa* which could suggest a link (direct or indirect) between *XvLOB* and desiccation.

In order to investigate the expression of *XvLOB* in response to dehydration a molecular characterization of *XvLOB* was attempted. This involved *in silico* analysis, measurement of *XvLOB* expression in response to dehydration at the mRNA and protein levels and determination of subcellular localization of the XvLOB protein.

CHAPTER 2

Molecular characterisation and bioinformatic analysis of *XvLOB* DNA and amino acid sequences

2.1 INTRODUCTION

Abiotic stresses such as drought, cold, extreme temperatures, salinity and oxidative stress cause major adverse affects on the productivity and quality of agriculture as well as on the natural status of the environment. These stresses are the primary cause of crop loss, reducing the average yield of most of the major crop plants with drought and salinity becoming particularly widespread in many regions (Wang *et al*., 2003; Rodriguez *et al*., 2005). Resurrection plants such as *X. viscosa* can be dehydrated to 5 % relative water content (RWC) and upon rewatering the desiccated plant rehydrates completely within 80 hours, resuming full physiological activities (Sherwin and Farrant, 1996). Understanding the molecular mechanisms resurrection plants use to tolerate environmental stresses will aid in genetic engineering approaches to improve crop performance.

To investigate which genes are activated during the dehydration process, RNA was extracted from dehydrated leaves (35 % RWC) of *X. viscosa* and used to construct a full length cDNA library (B. Baker, pers. comm.). According to Ingle et al (2007) there are three specific stages of dehydration at which there is a significant amount of gene regulation regarding protein expression. There are genes which are regulated during the early stages of dehydration (65 % RWC), those that are regulated during the later stages of dehydration (35 % RWC) and those that regulated once the plant has fully dehydrated. Proteomic analysis of these regulated genes indicated that the proteins which are expressed during the late dehydration stage are of greatest interest as the expression of these proteins are likely to be unique to resurrection plants (Ingle R. *et al*., 2007). A number of individual clones from the dehydration (35 % RWC) library was amplified and identified as upregulated by more than two fold in response to dehydration including the *XvLOB* gene. These genes were cloned into the cDNA library donor vector pDNR-LIB. To investigate the possible biological role *XvLOB* plays within the molecular mechanism of desiccation tolerance, analysis of both the DNA and amino acid sequence was performed.

In this chapter, the genomic *XvLOB* sequence was isolated from *X. viscosa* genomic DNA and analysed to obtain the genomic organisation of the *XvLOB* gene. Bioinformatic characterisation of both the DNA and amino acid sequences were investigated. These included multiple sequence alignments with other LBD containing genes as well as predicting possible localisation and post translational modifications. Predicting possible localisation and modifications may infer possible protein function.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of *XvLOB* genomic DNA and cloning into pJET1.2

2.2.1.1 Amplification of genomic *XvLOB*

Genomic *XvLOB* was amplified from genomic *X. viscosa* which was given as a gift from Dr K. Govender (UCT) using gene specific primers XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10). A 25 µl PCR reaction was set up (standard PCR reaction, Appendix A1) with the following cycling conditions: 94ºC for 5 min; 5 cycles of 94ºC for 30 s, 45ºC for 40 s, 72ºC for 2 min; 25 cycles of 94ºC for 30 s, 54ºC for 40 s, 72ºC for 2 min; and a final elongation step of 72ºC for 5 min was performed. A high fidelity Taq polymerase (Expand High Fidelity PCR System, Roche) which has proofreading activity was used. PCR products were electrophoresed on a 1.2 % agarose/EtBr gel after which the band of interest was excised and purified using the Biospin Gel Extraction Kit (BioFlux, Japan) according manufacturer's instructions (Appendix A, A7).

2.2.1.2 Ligation of genomic *XvLOB* into pJET1.2 cloning vector

Purified genomic XvLOB was cloned into pJET1.2 cloning vector (Appendix D, D1a and b) using the CloneJET™ PCR Cloning Kit (Fermentas, USA) according to manufacturer's instructions (Appendix A, A3). The pJET1.2 cloning is a linearised cloning vector and allows for blunt end cloning strategies as well as site specific cohesive end ligations. The procedure for blunt end cloning was followed because Expand High Fidelity Taq polymerase used to amplify genomic *XvLOB* inserts dA overhangs at the 3' ends of PCR fragments. The thermostable DNA blunting enzyme provided with the kit therefore removes the 3' dA overhangs prior to ligation.

2.2.1.3 Transformation of pJET1.2::*XvLOB*, plasmid screening and extraction

Fifteen microlitres of ligation reaction was transformed into 100 µl competent *E. coli* DH5α strain cells (standard transformation conditions; Appendix A, A4). Colony PCR was performed using gene specific primers XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10) to screen for positive clones. Bacterial cells from positive clones were inoculated into 5 ml Luria Broth [LB (Appendix B, B1)] supplemented with 100 µg/ml ampicillin and incubated overnight at 37ºC with shaking. The pJET1.2::*XvLOB* constructs from 3 different clones were isolated using Qiagen Plasmid Midi Kit (USA)

according manufacturer's instructions (Appendix A, A5). The 3 individual pJET1.2::*XvLOB* clones were sent for DNA sequencing analysis (section 2.2.3) and stored at -20ºC.

2.2.2 Nucleotide sequencing and BLAST analysis

Plasmid DNA isolated containing both *XvLOB* cDNA (pDNR-LIB::*XvLOB*) as well as genomic *XvLOB* (pJET1.2::*XvLOB*) was sent for sequencing via the DNA Sequencing Unit (Department of Molecular and Cell Biology, UCT, South Africa). Nucleotide sequencing was performed using the DYEnamic ET DYE Terminator Cycle sequencing Kit from MegaBACE (Molecular Dynamics, USA) according to manufacturer's instructions. Sequencing reaction products were separated using MegaBACE 500 Sequence system (Amersham Pharmacia Biotech). Analysis of sequences such as translation and alignment procedures was performed using DNAMAN software packages Version 5.2.10 (Lynnon Biosoft Copyright©1994-2001). Nucleotide and amino acid homology searches were performed using BLAST algorithms from NCBI databases. The gapped BLAST and PSI-BLAST were used for protein database searches (Altschul *et al*., 1997).

2.2.3 Bioinformatic analysis on *XvLOB* DNA and amino acid sequences

2.2.3.1 Identifying intron regions within genomic *XvLOB* sequence

Both cDNA and genomic *XvLOB* sequences were obtained from sequencing results. A sequence alignment was performed to identify the presence and location of intron regions. A dot matrix comparison was performed using DNAMAN software packages Version 5.2.10 (Lynnon Biosoft Copyright©1994-2001).

2.2.3.2 Bioinformatic analysis of XvLOB amino acid sequence

The inferred amino acid sequence was determined by translating and identifying the open reading frame of XvLOB cDNA sequence. Conserved motifs were identified using the ScanProsite tool by EXPASY (Gattiker *et al*., 2002; de Castro *et al*., 2006). XvLOB protein subcellular localisation was predicted using ProtComp 9.0 linked to the Softberry software tools website (Softberry ProtComp 9.0), Subnuclear Compartments Prediction System Version 2.0 (Lei and Dai, 2005; Lei and Dai, 2006), PredictNLS Online (Cokol *et al*., 2000) and the Plant-mPLoc Server (Chou and Shen, 2008). The SignalP 3.0 Server (Emanuelsson *et al*., 2007) as well as the SIG-Pred server (Bradford, 2001) was used to predict the presence

and location of signal peptide cleavage sites within the XvLOB protein sequence. For the prediction of possible phosphorylated amino acids the NetPhos 2.0 Server was used (Blom *et al*., 1999). The prediction of any coiled coil structures within the XvLOB sequence was analysed by the use of the Coils Server (Lupas *et al*., 1991; Lupas, 1996). The 2ZIP-Server was used to predict potential leucine zipper-like structures within the XvLOB protein (Bornberg-Bauer *et al*., 1998)

A PSI-BLAST analysis was preformed to find similar proteins in a variety of organisms (Altschul *et al*., 1997). Several sequences were downloaded and an amino acid multiple sequence alignment was constructed using the DNAMAN software packages Version 5.2.10 (Lynnon Biosoft Copyright©1994-2001), ClustalW2 Server (Chenna *et al*., 2003) as well as the T-coffee Server (Notredame *et al*, 2000). A multiple sequence alignment was used to create a phylogenetic tree with the evolutionary distances mapped onto the tree. The tree was created by selecting the maximum likelihood option and 5000 bootstrap trials with the Jones, Taylor and Thornton protein model [DNAMAN software packages Version 5.2.10 (Lynnon Biosoft Copyright©1994-2001)].

To find homologs within the *Arabidopsis* species, the XvLOB protein sequence was used to BLAST against the *Arabidopsis* genome with The Arabidopsis Information Resources (TAIR) online software (http://www.arabidopsis.org/Blast/index.jsp). The AGI ID of homologs with 70 % or more identities to the XvLOB sequence was used in the Botany Array Resource (BAR), *Arabidopsis* eFP Browser online tool (Winter *et al*., 2007). This browser was used to establish whether *LBD* genes have been identified in the response to hormone and/or environment stimuli as well as verify *LBD* expression during developmental stages.

2.3 RESULTS AND DISCUSSION

2.3.1 Isolation of *XvLOB* genomic DNA, cloning into pJET1.2 and sequencing analysis

XvLOB genomic DNA was successfully amplified from genomic *X. viscosa* DNA using gene specific primers XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10). When electrophoresed a DNA fragment size of ca. 750 bp was observed (Figure 2.1, Lane 3). As a control, *XvLOB* cDNA was amplified from pDNR-LIB::*XvLOB* using the above mentioned primers to amplify an ca. 650 bp fragment (Figure 2.1, Lane 2). According to the gel electrophoresis of both genomic and cDNA *XvLOB* fragments there is an ca. 100 bp size difference, indicating the presence of 1 or more intron regions.

Sequence analysis indicated that isolated genomic *XvLOB* has a length of 746 bp with an open reading frame (ORF) of 648 bp which codes for a protein of 215 amino acids.

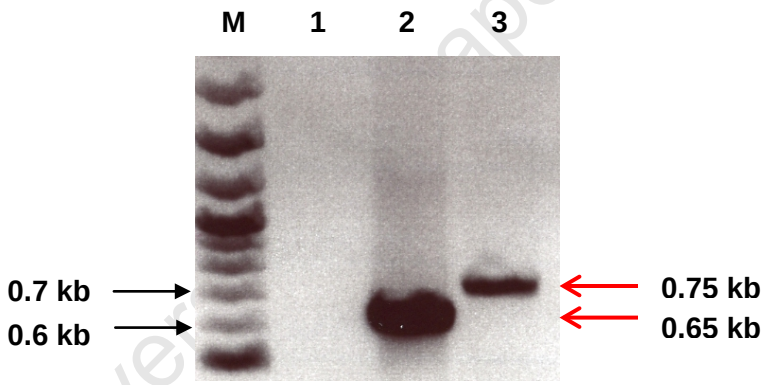


Figure 2.1 1.2 % (w/v) Agarose/EtBr gel electrophoresis of amplified genomic and cDNA *XvLOB*. Lane 1 - PCR reaction NTC control. Lane 2 - amplified cDNA *XvLOB* fragment. Lane 3 - amplified genomic *XvLOB* fragment. M - 1 kb DNA ladder (NEB). Red arrows on the RHS indicate sizes of *XvLOB* fragments.

To locate the position of any intron regions a sequence alignment (data not shown) and a dot matrix (Figure 2.2) was carried out which indicated the presence of one intron located between the 183rd and 281st bp region of the genomic *XvLOB* sequence. The intron is 98 base pairs in length.

2.3.2 BLAST, sequence alignment and bioinformatic analysis on *XvLOB* DNA and protein sequences

BLAST searches were performed on the *XvLOB* cDNA nucleotide sequence using BLAST algorithms from NCBI databases. The *A. thaliana* LOB domain-containing protein 38 (AtLBD38) and asymmetric leaves2-like 40 (AtASL2-like 40) were amongst the blast analysis hits with both maximum identities of 78 %. These two proteins are however the same gene found within *Arabidopsis*. AtLBD38 is classified as a Class II LBD protein and shares between 28-34 % identity to AtLOB (Shuai *et al*., 2002).

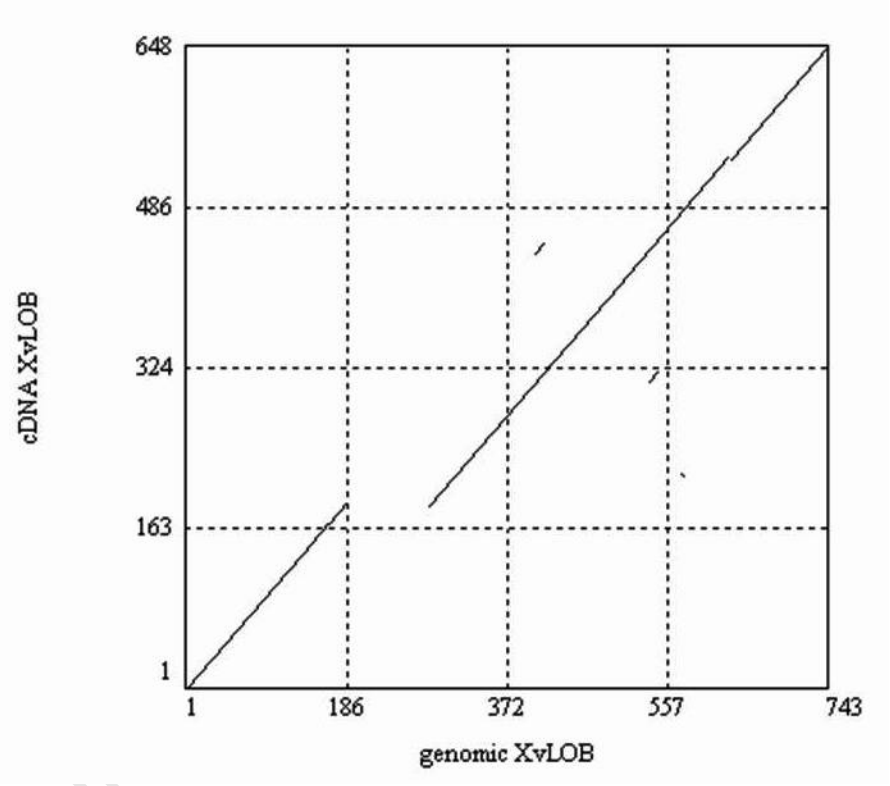


Figure 2.2 Dot matrix alignment of the genomic and cDNA *XvLOB* sequence using DNAMAN software and indicating the location of one intron region between the 183rd and 281st basepairs.

The inferred amino acid sequence was obtained by translation of the *XvLOB* cDNA sequence using the DNAMAN software. The longest open reading frame encoded a 215 amino acid protein with a theoretical molecular weight of 22,808 kDa (Figure 2.3) and a pI value of 5.08. The XvLOB amino acid sequence contains the conserved C-motif ($CysX_2CysX_6CysX_3Cys$) found in all LOB domain containing proteins, this can be seen in figure 2.3 and is highlighted in pink. The ScanProsite Server was also used to identify any domains found within the

XvLOB amino acid sequence. Results obtained from this website indicated the presence of the LOB domain between the 1st and 107th amino acid (data not shown). The location of this LOB domain within the amino acid sequence can be seen in figure 2.3 (highlighted in light blue). The LOB domain is divided into two parts; the C block (blocked in) and the GAS block (underlined). The C block contains the C-motif that is 22 amino acids in length and located at the N - terminus. The GAS block is 49 amino acids in length, located at the C - terminus and has not been thoroughly characterised (Shuai *et al*., 2002).

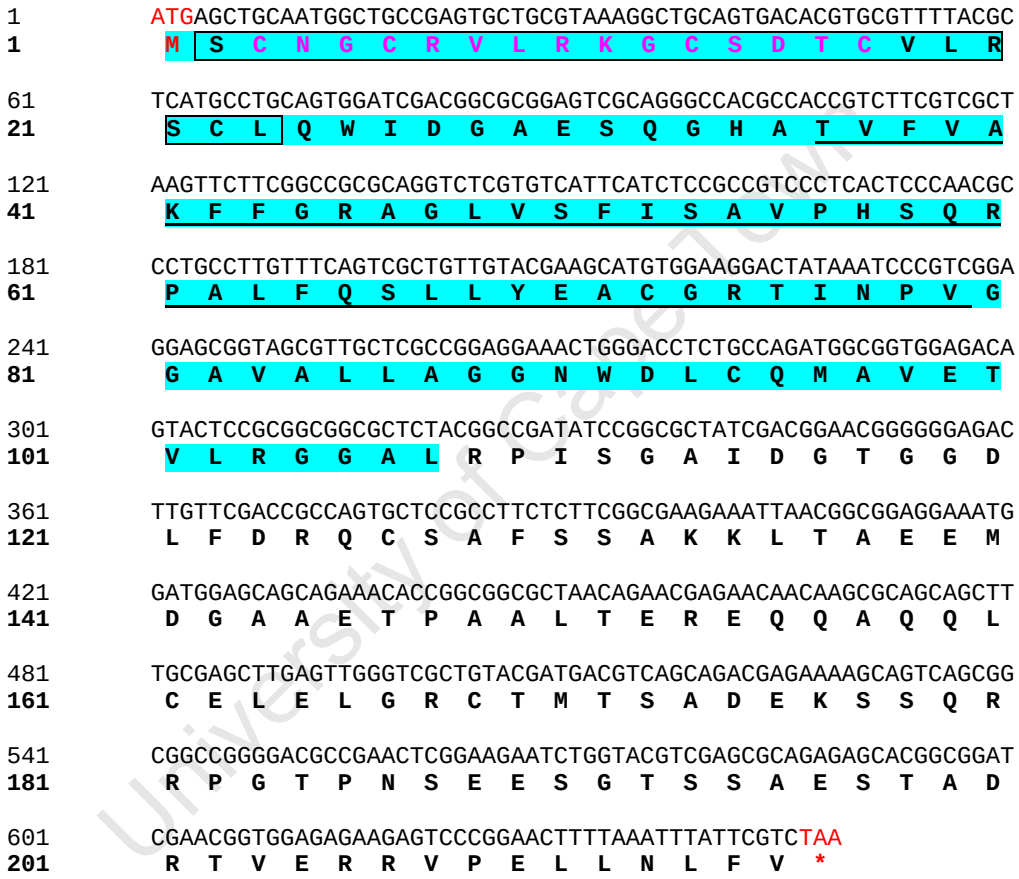


Figure 2.3 Amino acid sequence (bold) translated from the longest open reading frame identified in the cDNA sequence. The start and stop codons have been highlighted in red. The LOB domain is highlighted in light blue and contains both the C and GAS blocks. The C block is blocked in and contains the conserved C-motif ($CX_2CX_6CX_3$) which is indicated in pink. The GAS block is underlined.

According to the NetPhos2.0 online bioinformatics tool, there are 9 serine (amino acid positions 31, 58, 172, 178, 187, 190, 193, 194 and 197), 5 threonine (amino acid position

146, 151, 171, 184 and 198) and 0 tyrosine predicted phosphorylation sites. These possible phosphorylation potentials are above the threshold value of 0.5 (Figure 2.3). Protein phosphorylation and dephosphorylation forms part of the regulation of almost all cellular metabolic processes by controlling protein activity (Graves and Krebs, 1999). The phosphorylation of these specific sites found within the XvLOB protein sequence and the influence it has on protein activity needs to be investigated.

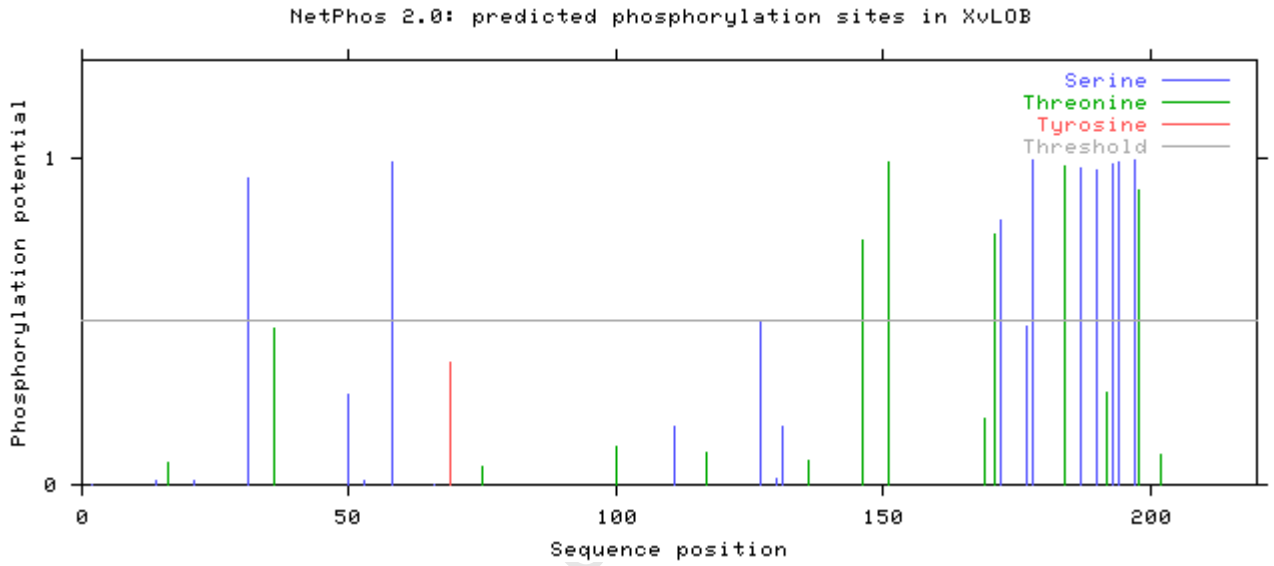


Figure 2.4 NetPhos2.0 diagram predicting possible phosphorylation sites within the XvLOB amino acid sequence.

To identify the presence of any signal peptides the SignalP 3.0 as well as the SIG-Pred Server was used. These servers predicted no signal peptides located within the XvLOB amino acid sequence. The presence of any signal peptides will predict possible extracellular secretion of the XvLOB protein. It can therefore be proposed that the XvLOB protein is intracellularly located. Analysis of the *Arabidopsis* LOB (AtLOB), *Oryza sativa* Crown-rootless1 (OsCrl1) and *Sorghum bicolour* ramosa2 (Sb_ramosa2) proteins also revealed the lack of any signal peptides within the respective sequences (data not shown). The identification and function of signal peptides within LBD protein sequences has not been investigated. Further *in silico* localisation analysis was performed on the XvLOB protein sequence to identify possible localisation.

A number of different subcellular localisation prediction servers were used to infer possible localisation of the XvLOB protein, these results are summarised in Table 2.1. The Plant mPLoc server is a plant specific online bioinformatic tool which uses a large dataset consisting of 1055 plant protein sequences which have been classified into 11 different plant subcellular locations (Chou and Shen, 2008). The Softberry ProtComp server is also a programme used to identify subcellular localisation of eukaryotic plant proteins. It combines several methods of protein localisation some of which include a neural networks-based prediction, direct comparison with updated base of homologous proteins of known localisation and prediction of certain functional peptide sequences (Softberry ProtComp 9.0). Results obtained from the Plant-mPLoc as well as the Softberry ProtComp servers both infer that the XvLOB protein will be localised to the nucleus. Subnuclear localisation of XvLOB was predicted using the Subnuclear Prediction System server. Results obtained from this server indicated nuclear lamina localisation.

According to the subcellular localisation results obtained from all bioinformatics analysis, XvLOB is predicted to be a nuclear localised protein (Table 2.1).

Table 2.1 List of all subcellular localisation prediction servers and results obtained for the XvLOB amino acid sequence.

Subcellular localisation prediction Server	**XvLOB predicted localisation**
SoftBerry ProtComp Version 8	nucleus
Subnuclear Prediction System Version 2	nuclear lamina
Plant-mPLoc Version 2	nucleus

The web based server PredictNLS was used to predict the presence of a nuclear localisation signal (NLS) within the XvLOB amino acid sequence. The results obtained from this server indicated that XvLOB does not contain any known signals. According to PredictNLS, the AtLOB and OsARL1 proteins do not contain any known NLS signals although these proteins have been experimentally localised to the nucleus (Liu *et al*., 2005; Husbands *et al*., 2007).

At the amino acid level, multiple sequence alignments between XvLOB and other LBD genes were carried out using the DNAMAN Software (Figure 2.6 and 2.7). Sequences used were obtained from NCBI protein database, list of accession numbers can be seen in Appendix C, Table C2. Multiple sequence alignments using the ClustalW2 and Tcoffee Servers was also obtained (Appendix C, C3 and C4).

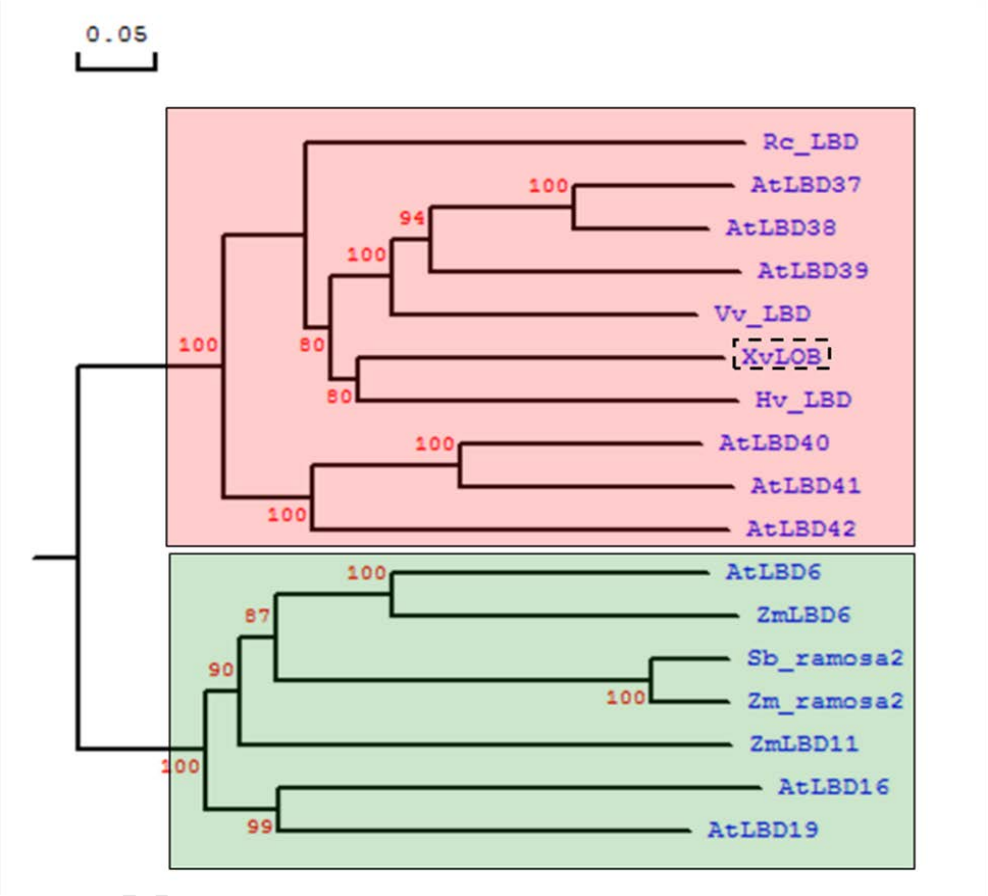


Figure 2.5: A phylogenetic tree showing the evolutionary relationship between homologs of LBD related proteins from various organisms. The tree was constructed using DNAMAN software selecting the maximum likelihood with 5000 bootstrap trials using the Jones, Taylor & Thornton protein model. LBD protein sequence were used from a number of different organisms including *Arabidopsis* (AtLBD6, AtLBD16, AtLBD19, AtLBD37, AtLBD38, AtLBD39, AtLBD40, AtLBD41, AtLBD42), *Hordeum vulgare* [barley (Hv_LBD)], *Zea mays* [maize (Zm_ramosa2, ZmLBD6, ZmLBD11)], *Vitis Vinifera* [grape vine (Vv_LBD)], *Ricinus communis* [castor oil plant (Rc_LBD)], *Sorghum bicolor* (Sb_ramosa2) and *X. viscosa* (XvLOB). The XvLOB protein is represented by a dashed box. Class I group is boxed in blue and Class II group in blue.

A phylogenetic tree was constructed showing the divergence of proteins most related to XvLOB from various organisms (Figure 2.5). Any proteins which have been identified as containing the LOB domain are all plant specific proteins. Thus, the proteins within the phylogenetic tree all belong to various plant species including *Arabidopsis* (AtLBD6, AtLBD16, AtLBD19, AtLBD37, AtLBD38, AtLBD39, AtLBD40, AtLBD41, AtLBD42), *Hordeum vulgare* [barley (Hv_LBD)], *Zea mays* [maize (Zm_ramosa2, ZmLBD11, ZmLBD6)], *Vitis Vinifera* [grape vine (Vv_LBD)], *Ricinus communis* [castor oil plant (Rc_LBD)], *Sorghum bicolor* (Sb_ramosa2) and *X. viscosa* (XvLOB). As would be expected the Class I proteins formed one group (boxed in blue) and the Class II proteins (boxed in blue). Phylogenetic relationships between genes may help to elucidate a possible shared function between genes.

In figure 2.6, the XvLOB amino acid sequence is aligned to a number of different LBD proteins including Zm_ramosa2 (*Zea mays*), ZmLBD6 (*Zea mays*), ZmLBD11 (*Zea mays*), Sb_ramosa2 (*Sorghum bicolour*), OsCrl1 (*Oryza sativa*), AtLBD16 and AtLBD19 (*Arabidopsis*). The conserved $CysX_2CysX_6CysX_3Cys$ motif (represented by solid line in Fig 2.5) is 100% conserved across the alignment and the GAS block (represented by dashed line in Figure 2.6) is 75% similar. A multiple sequence alignment was also constructed using the ClustalW2 and T-coffee Servers (Appendix C, C3 and C4 respectively).

In order to determine whether XvLOB is a Class I or Class II LBD protein, XvLOB was aligned with *Arabidopsis* LBD proteins from both Class I (data not shown) and Class II. The alignment with *Arabidopsis* Class II proteins (AtLBD38, AtLBD39, AtLBD40, AtLBD41 and AtLBD42) can be seen in figure 2.7. Results indicated a higher sequence similarity between XvLOB and the Class II LBD proteins than with Class I proteins. Class I proteins are predicted to form coiled coil structures and contain the four conserved leucine amino acids in a $LX_6LX_3LX_6$ spacing which is a characteristic of a Leu-zipper (Landschultz *et al*., 1988). Whereas the Class II proteins do not form coiled coil structures (Yang *et al*., 2006).

```
XvLOB                                                                0
AtLBD16                                                 MASSGNGTTA  10
AtLBD19                                                 MMTGNLNGGGR  11
OsCrl1                                                        MT     2
Sb_ramosa2                          MASPSSTSNNSALPPVAAS....GATTP    24
Zm_ramosa2                          MASPSSTSNNSAFSPVAASA...GTTMP    25
ZmLBD6                              MASSVPAPSGSVITVASSSSSAAAAAVC    28
ZmLBD11     MDDYGNASAGTAAPQPQQYYCRSVSPPSRVSSSCSPPPPPPPAVLQVMVG     50
Consensus
                                    ____________                ----.

XvLOB                    MS NG RV  KG SDT VLRSCLQWIDGAESQGHATVFVAK    41
AtLBD16     GTG......SP GA KF  RK ASD IFAPYFS....SEQGAARFAAIHK     50
AtLBD19     GGE......GP GA KF  RK VKG VFAPYFD....AEQGTARFAAVHK     51
OsCrl1      GFG......SP GA KF  RK VRG VFAPYFC....HEQGAAHFAAIHK     42
Sb_ramosa2  GAG......AP AA KF  RK LPG VFAPYFP....PEE.PQKFANVHK     63
Zm_ramosa2  GAG......AP AA KF  RK LPG VFAPYFP....PEE.PQKFANVHK     64
ZmLBD6      GTG......SP AA KF  RK QPD VFAPYFP....PDN.PQKFVHVHR     67
ZmLBD11     NVPPTTVVLSP AA KI  RR ADG VLAPYFP....PTE.PAKFTTAHR     95
Consensus                 c  c  lr  c   c
            - - - - - - - - - - - - - - - - - -

XvLOB       F  RAGLVSFISAVPHSQ PALFQSLL   CG TIN VG AVALLAGGNW     91
AtLBD16     V  ASNVSKLLLNVPIHD CEAVVTIA   QA LHD VY CVSHIFALQQ    100
AtLBD19     V  ASNASKMLLRLPLHK LDAVVTLC   MA IRD VY SVGHLFSLQH    101
OsCrl1      V  ASNVSKLLAHLPLAD PEAAVTIS   QA LRD IY CVAHIFALQQ     92
Sb_ramosa2  V  ASNVTKLLNELLPHQ EDAVSSLA   EA VKD VY CVGAISVLQR    113
Zm_ramosa2  V  ASNVTKLLNELLPHQ EDAVSSLA   EA VKD VY CVGAISVLQR    114
ZmLBD6      V  ASNVTKLLNELHPFQ EDAVNSLA   DM LRD VY CVGVISILQH    117
ZmLBD11     V  ASNIIKLLQDLPESS ADAVSSMV   EA LRD VY CAGAVCRLQK    145
Consensus    fg             r        yea  r   p  g

XvLOB       DLCQMAVETVLRGGALRP..............ISGAIDGTG........    118
AtLBD16     QVAFLQSQVMQMKAQIAG...HQ....TSAAGDLRHSSESTN.......Q    136
AtLBD19     QVMNLQAELAHVQARLST..............IQRFPLQSPQ........    129
OsCrl1      QVMTLQAQLASLKAAAAQGIHHQDVGATTKGGYMSAAATAADDQLG.YGG    141
Sb_ramosa2  QVHRLQKELDAAHAELLR..........YACGDVAAGI.PTALPVSVSVS    152
Zm_ramosa2  QVHRLQKELDAAHAELLR..........YACGDVAAGI.PTALPV...VS    150
ZmLBD6      NLRQLQQDLARAKYELSK..........YQAAAAAS...ASTAPTG.PQA    153
ZmLBD11     QANELKVQLARAQADLLN..........AQADLLNAQAKHANLFALFCVE    185
Consensus

XvLOB       .GDLFDRQCSAFSSAKKLTA.................EEMD AAETPAA    149
AtLBD16     FMTWQQTS..VSPIGSAYSTP.............YNHHQPYY HVNPNNP    171
AtLBD19     ..QMQPPS.FDPAHNNEYAME.............PSNLDSVW EEHLLQD    163
OsCrl1      YNQWCGSNGGGAPAASQPGAY.............SSNGGAGH HDSITAL    178
Sb_ramosa2  AAPRLSTA..MTTSPGQFAAAAATAHATAGMYGSSRRLGVLD VVAAPPP    200
Zm_ramosa2  AAPRLSTAPMTTTSPGQFAA.........GMYGSGRRLGVVD LAAPPLP    191
ZmLBD6      MAEFIGNA..MPNGAHNFIN...........IGHSAALGSLG .SASVFG    189
ZmLBD11     MANRRSNQ....QHPSPLTT..................MMD .......    205
Consensus                                              g

XvLOB       LTEREQQAQQLCELELGRCTMTS.ADEKSSQRRPGTPNSEESGTSSAEST    198
AtLBD16     VSPQSS.LEESFSNTSSDVTTTANVRETHHQTGG..GVYGHDGIGFHEGY    218
AtLBD19     GTGDGD.FQELASQFVSRYLPAVKLPACT                          191
OsCrl1      LAAGSDYMQHSLYHAFEHSEGAGAVDDGHAAAAAAFEAAAESSSCGMAASF    228
Sb_ramosa2  PPPAGG.CYFMRNHSN..VISSP..PGADVAPVLPYASMANWAVNAISAS    245
Zm_ramosa2  PSGGGGGCYFMRNHHNNVVISSS..PGADVAPVLPYASVANWAVNAISAS    239
ZmLBD6      QEQFGNAQMLSRSYDGGEPIARLGINGGGYEFGYSTAMGGSGAVSGLGTL    239
ZmLBD11     GGGFGAAAYYQQTTLYDSDLDSATWPDHEAQLWT                     239
Consensus

XvLOB       ADRTVERRVPELLNLFV                                      215
AtLBD16     PNKK...RSVSYCSSDLGELQALALRMMKN                         245
AtLBD19
OsCrl1      AADESVWRSSSSGYQDCEDLQSVAYAYLNR                         258
Sb_ramosa2  TTTTSGSESIGGLDHKEGGDSSM                                268
Zm_ramosa2  TTTTSGSESIG.LDHKEGGDSSM                                261
ZmLBD6      GISPFLKSGTAGGDEKPNGGQ                                  260
ZmLBD11
Consensus
```

Figure 2.6 Multiple sequence alignment between XvLOB and other LBD genes. The proteins aligned include: Zm_ramosa2 (*Zea mays*), ZmLBD6 (*Zea mays*), ZmLBD11 (*Zea mays*), Sb_ramosa2 (*Sorghum bicolour*), OsCrl1 (*Oryza sativa*), AtLBD16 and AtLBD19 (*Arabidopsis*). The black, pink, blue and yellow shading represents 100, ≥75, ≥50 and ≥33 % homology respectively. The alignment was produced using the DNAMAN software. The conserved C-motif and GAS block is represented by a solid and dash line, respectively. See Appendix C Table C2 for accession numbers of proteins used.

According to the 2ZIP and Coils Server, the XvLOB protein sequence will most likely not form any leucine zipper-like structures or any coiled coil structures (data not shown).

A distinguishing feature of the Class II proteins is that they are more cys rich than the Class I proteins (Shuai *et al*., 2002). According to Shuai et al (2002), the *Arabidopsis* Class I proteins contained between 4 and 7 cys amino acids whereas the Class II proteins contained between 9 and 13. The XvLOB amino acid sequence contains 10 cys residues.

Blast results obtained from the TAIR website identified 42 hits all of which were related to *LBD* genes. The top 5 hits included LBD37, LBD39, LBD38, LBD40 and LBD41 with sequence identities to XvLOB of 71, 73, 70, 53 and 54 %, respectively. These results together with the alignment results suggest that XvLOB may belong to a Class II group of *LBD* genes.

```
                 ----------------                    ---------
XvLOB       M           SDT VL S LQ  DGAES GH  V V   F          48
AtLBD37     M           SEN IL P IQ  ETADA GH  V V   F          48
AtLBD38     M           SEN IL P IQ  ESPEA GH  V V   F          48
AtLBD39     M           SET IL P LQ  ESAES GH  V V   F          48
AtLBD40   MRM           SEN SI P LQ  KSAES AN  V L   Y          50
AtLBD41   MRM           SED SI P LA  KSPEA AN  V L   Y          50
AtLBD42   MRI           NQD TI P LQ  KSADS AN  L L   Y          50
Consensus    scngcrvlrkgc   c  r c  wi     q  at f akf gragl
             ------------------
XvLOB     VSFISAV HSQR AL Q   Y     TI  VG AVALLAGG  DL  M      98
AtLBD37   MSFISAV DSQR AL Q   Y     TV  VN AIGMLWTG  NI  A      98
AtLBD38   MSFISAV ESQC AL Q   Y     TV  VN AVGLLWTG  NV  A      98
AtLBD39   MSFISSV ELQR AL Q   F     TV  VN AVGMLWTR  HV  A      98
AtLBD40   MNLLNTG DHLR AI R   Y     IV  IY SVGLLWSG  HL  A     100
AtLBD41   MNLINAG NHLR GI R   H     IV  IY SVGLLWSG  QL  D     100
AtLBD42   LNLIESG DHLR AI R   Y     IV  VD SVGLMWSG  AQ  A     100
Consensus        p    p  f sll eacgr  np  g        nw  cq av

XvLOB     ET LR GALRPISGAID.....................GTGGDLFDRQ...   125
AtLBD37   ET LR GSLRPIPELLTHGGGFAGFPSPTSEEASEICTEMLNLQQND.ST   147
AtLBD38   ET LR GSLKPIPELLN.GGGFAGFPSPTSDEASEICTEMLNLRKADDSG   147
AtLBD39   ET LR GTLRPISDLLE.........SPSLMIS...CDESSEIWHQD..V   134
AtLBD40   EA MR SPVTPIACDAA...........VTGQAPPFNNKLCDIRHVS...   136
AtLBD41   EA MK EPVKEIATDAA...........TIGQGPPL..KIYDIRHIS...   134
AtLBD42   DA LN LPITHTPLPSA...........SASHQIIPPHRTYDIRHVA...   136
Consensus   v  g

XvLOB     CSAFSSAK............KLTAEEMDGAAETPAALTEREQQAQQLCEL   163
AtLBD37   DRNIYHHS............RFSSSRSRSTMDSSSPTKRKRLSSEDQPSS   185
AtLBD38   DRNIYHHC............RFSSSRSRSRS.TASPPKRKRLSSEQQPSS   184
AtLBD39   SRNQTHHC............RFSTSRSTTEM.KDSLVNRKRLKSDSDLDL   171
AtLBD40   SRDE..................NVKRRSRGACKEERNVRS..LSHESSLSH   167
AtLBD41   KDDNSAAAATGSTDLKLAKTRRAKRVSTVAIQAESEGKSDEASHDSSLSH   184
AtLBD42   KDPTTGGD............SSENLATRVNANKSKTQTGRFKRES.VNQ   172
Consensus

XvLOB     ELGRCT...MTSADEKSSQ................RRPGTP..NSEES.G   191
AtLBD37   ELDLSL...IPNFPIKQATPS...........STRRRSVTPSMNSEDSGT   221
AtLBD38   ELDLSL...IPIYPIKTLP................FKEDTPSMYSEES.V   214
AtLBD39   QVNHGLTLTAPAVPVPFLPPSSF.........CKVVKGDRPGSPSEES.V   211
AtLBD40   ESP.VSSEETTTEEPKT.................WIGLELTLGLEPLARG   199
AtLBD41   QSEIVAAHEGESKESESNVSEVLAFSPPAVKGSGEIKLDLTLRLEPVSRA   234
AtLBD42   LGE..CSHDMWQLPSSS................ATHGYGHFTLENVESR   203
Consensus

XvLOB     TSSAESTADRTVER.........RVPELLN FV                   215
AtLBD37   TTTTTAFCDKGDVY....GNGGGETTKLLN FV                   250
AtLBD38   TTVSFQNNNAGDRYVRCGGGGGGATTKLLN FA                   247
AtLBD39   TTSCWENGMRGDNK....QKRNKGEKKLLN FV                   240
AtLBD40   NHVVVPMKKRKLER...CGTSEDEDTCKIE GLVCS                232
AtLBD41   YH.VVPVKKRRIGV...FGTCQKESTCKTE ML                   263
AtLBD42   REAPFNQSSPNLG....FDDQVDINEVGLE RLG                  233
Consensus                                l
```

Figure 2.7 Multiple sequence alignment between XvLOB and Class II LBD genes (AtLBD37, AtLDB38, AtLBD39, AtLBD40, AtLBD41, AtLBD42) from *Arabidopsis*. The alignment was produced using the DNAMAN software. The conserved C-motif and GAS block is represented by a solid and dash line, respectively. The black shaded areas denote identities and the pink shaded areas denote 75 % similarities. See Appendix C Table C2 for accession numbers of proteins used.

Using the Arabidopsis eFP Browser website, the expression profiles of *AtLBD39* was analysed with regards to its response to abiotic stress, hormone treatments as well as during developmental stages. The experimental result and design can be seen in Appendix E, E2. During the development stages of *Arabidopsis,* there is an increase (as indicated by a red colour) in expression of the *AtLBD39* gene during the hypocotyl development of the *Arabidopsis* seed. In the vegetative shoot apex there is a slight increase (indicated by orange colour) in expression. These results are consistent with the proposed role of *LBD* genes in the development of new lateral organ primordia as well as root formation as discussed above. In response to hormone treatments such as methyl jasmonate, ABA and brassinosteroids amongst others indicated no significant change in expression in comparison to control treatments. During abiotic stress treatment, affymetrix data revealed a slight increase in expression after 1 hour of exposure to cold (4 °C) and high osmotic (300 mM mannitol) stress. To date, very little evidence is available regarding a link in which the effect hormones, cold and osmotic stress might have on *LBD* genes.

2.5 CONCLUSION

In this chapter, the *X. viscosa* LOB (*XvLOB*) gene was isolated, cloned, sequenced and *in silico* analysed. According to sequencing results, the full length *XvLOB* DNA sequence contains one intron of approximately 98 base pairs. In terms of the amino acid composition, motifs and subcellular localisation, the XvLOB amino acid sequence has shown to be very similar to the LOB homologs in species such as *Arabidopsis*, *Oryza sativa*, *Sorghum bicolor*, *Zea mays* and *Lycoris longituba*. It was determined that XvLOB protein has 14 phosphorylation sites and no NLS and no signal peptides were identified. The exact function of the phosphorylation sites within the XvLOB protein needs to be further investigated. These preliminary *in silico* results provided the background needed to experimentally determine function and localisation of the XvLOB protein.

CHAPTER 3

Recombinant XvLOB protein expression, purification, western blotting, antibody generation and purification

3.1 INTRODUCTION

The use of heterologous recombinant proteins has rapidly increased within the field of proteomics and has fast become a valuable tool in understanding structural and functional biology (Hammarstrom *et al*., 2001; Terpe, 2003). The production of recombinant proteins involves a number of processes from large scale expression, intricate extraction methods to simplified purification steps. There is a large array of biological molecular tools for production of recombinant proteins with gene fusion technology being the most advanced. Of the many host systems available for protein production, *Escherichia coli* remains one of the most frequently used prokaryotic systems for high level production.

The prokaryotic *E. coli* expression system has a number of advantages including high growth and production rates as well as being easy to use and cost effective. Amongst all these advantages there is still no guarantee that a recombinant gene will be expressed at high levels and in a biologically active state as each gene is unique and expression thereof requires optimization. The prokaryotic expression vectors are well designed constructs which contain configured genetic elements to optimally affect both transcription and translation of recombinant proteins. Such elements include promoters which are either chemically or thermally induced to drive high levels of transcription; elements affecting translation initiation and termination as well as the addition of an antibiotic-resistance gene which facilitates phenotypic selection of the vector (Hannig *et al*., 1998; Baneyx, 1999).

According to Hannig et al (1998) there are three cellular compartments for protein accumulation namely; cytoplasm, periplasm and secretion into the extracellular medium. Cytosolic accumulation favors higher protein yields and the need for much simpler plasmid constructs. A consequence of cytosolic accumulation however includes the misfolding of proteins and the segregation into insoluble aggregates known as inclusion bodies. Many strategies have been developed to overcome this difficulty and facilitate an increase in solubility of the expressed protein. The affinity-tag system has become one of the most

popular means of increasing recombinant protein yield and solubility as well as facilitating easy purification of recombinant proteins. The choice of tags used during recombinant protein expression is based on the advantages and disadvantages of the various tags as no single tag will be ideal with respect to all expression parameters (Waugh, 2005).

In this study, the pET32b expression vector from the pET vector series (Novagen, USA) has been selected for recombinant XvLOB protein expression. This vector facilitates the usage of an 109 amino acid thioredoxin (Trx-Tag™) tag to be fused to the N-terminal end of the protein of interest (XvLOB). The *trxA* tag has been shown to increase solubility (LaVallie *et al*., 1993). A disadvantage however is that TrxA proteins cannot be purified using a specific matrix and therefore needs to be used in combination with a small affinity tag such as a polyhistidine tag (His-tag). The pET32b vector contains two His-tags fused which will also be fused to the N-terminal end of the XvLOB protein. These tags allow for easy purification of the recombinant fusion protein through binding of His-tags onto an immobilized metal affinity resin. The bound recombinant fusion protein can then be released and eluted by adjusting the pH of the column. The use of the pET32b vector was selected based on the presence of these tags to increase recombinant protein yield as well as solubility.

3.2 MATERIALS AND METHODS

3.2.1 Cloning of *XvLOB* into expression vector pET32b

3.2.1.1 Amplification and restriction enzyme digest of *XvLOB*

XvLOB was amplified using pDNR-LIB::*XvLOB* (see Chapter 2, 2.2.1) as a template with gene specific primers XvLOB-*Eco*RI F (Appendix C, C1.1) and XvLOB-*Hin*dIII R (Appendix C, C1.2). These primers were designed to amplify *XvLOB* to be translated in frame with the pET32b vector tags. Four 25 µl PCR reactions (standard PCR reaction; Appendix A1) with the following cycling conditions: 94ºC for 3 min; 30 cycles of 94ºC for 30 s, 53ºC for 40 s and 72ºC for 3 min; and a final extension step of 5 min were performed. High fidelity Taq polymerase (Expand High Fidelity PCR System, Roche) was used as this enzyme has proofreading activity which decreases the amount of errors incorporated during PCR amplification. PCR products were electrophoresed on a 0.8 % agarose/EtBr gel after which the band of interest was excised and purified using the Biospin Gel Extraction Kit (BioFlux, Japan) according manufacturer's instructions (Appendix A7).

Purified *XvLOB* fragments were digested in a total reaction volume of 30 µl using *Eco*RI and *Hin*dIII restriction enzymes (FastDigest, Fermentas). The reaction mixture contained approximately 900 ng *XvLOB* PCR product, 3 µl FastDigest Buffer (10X), 1 FDU *Eco*RI and 1 FDU *Hin*dIII. Digest reaction was incubated for 20 min at 37ºC in a water bath. Digested fragments were purified using the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Canada) according to manufacturer's instructions (Appendix A6).

3.2.1.2 Simultaneous digest and dephosphorylation of expression vector pET32b

One microgram of pET32b was simultaneously digested and dephosphorylated in a total reaction volume of 20 µl with *Eco*RI, *Hin*dIII (FastDigest, Fermentas) and Thermosensitive Alkaline Phosphatase (FastAP, Fermentas). Reaction components included 1 FDU *Eco*RI, 1 FDU *Hind*III, 1 FDU AP and 2 µl of FastDigest Buffer (10X). Components were mixed well and incubated for 10 min at 37ºC in a water bath. Digested vector was cleaned up using the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Canada) according to manufacturer's instructions (Appendix A6) and analysed by electrophoresis on a 0.8 % agarose/EtBr gel to confirm successful digestion.

3.2.1.3 Ligation reaction of *XvLOB* into expression vector pET32b

Standard ligation reactions (Appendix A2) were set up for a vector to insert ratio of 1:10 using 50 ng of vector. Reagents were mixed well and incubated for approximately 3 hours at room temperature (RT).

3.2.1.4 Transformation of pET32b::*XvLOB,* plasmid screening and extraction

Ten microlitres of ligation reaction was transformed into 50 µl competent *E. coli* DH5α strain cells (standard transformation conditions; Appendix A4). Colony PCR was performed using gene specific primers XvLOB-*Eco*RI F (Appendix C, C1.1) and XvLOB-*Hin*dIII R (Appendix C, C1.2) to select for positive clones. Bacterial cells from positive clones were inoculated into 5 ml Luria Broth [LB (Appendix B1)] supplemented with 100 µg/ml ampicillin and incubated overnight at 37ºC with shaking. The recombinant pET32b::*XvLOB* construct was isolated using Qiagen Plasmid Midi Kit (USA) according manufacturer's instructions (Appendix A5). The pET32b::*XvLOB* plasmid was then verified by PCR using gene specific primers XvLOB-*Eco*RI F (Appendix C, C1.1) and XvLOB-*Hin*dIII R (Appendix C, C1.2), restriction digestion, and DNA sequencing analysis. Plasmid samples were stored at -20ºC until further use.

3.2.2 XvLOB fusion protein expression and purification

3.2.2.1 Transformation of pET32b::*XvLOB* into expression cells and expression

Two hundred nanograms of each pET32b::*XvLOB* and pET32b expression vectors were separately transformed (standard transformation conditions, Appendix A4) into competent expression *E. coli* BL21 (DE3) cells. For each plasmid, single colonies were inoculated into 2 x 20 ml overnight LB culture made according to manufacturer's instructions (Overnight Express Instant TB Medium, Novagen) and supplemented with 100 µg/ml ampicillin. Cultures were incubated for 20 hours at 30ºC with vigorous shaking. Each 20 ml culture was decanted into GSA SS34 centrifuged tubes and cells were harvested by centrifugation at 10,000 x g for 10 min at 4ºC. The supernatant was discarded and pelleted cells stored at -20ºC overnight and -80ºC for longer periods.

3.2.2.2 Recombinant XvLOB protein extraction

All extractions were done according to manufacturer's instructions (The QIAexpressionist Handbook, QIAGEN). For each plasmid (either pET32b::*XvLOB* or pET32b), one 20 ml overnight culture was used for extraction under denaturing conditions to extract insoluble protein. The other 20 ml overnight culture was used initially for extraction under native conditions for soluble protein and then carried through to extraction under denaturing conditions for insoluble protein. A schematic diagram can be seen in figure E.1 (Appendix E1).

For native conditions; pelleted cells containing plasmids were thawed on ice and then gently resuspended in a total of 5 ml lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; pH 8) by gentle pipetting. The resuspended cell culture was then supplemented with 1 mg/ml lysozyme (Bio Basic Inc., Canada). The centrifuge tubes were placed on its side in a bucket of ice and allowed to shake for 2 hours to facilitate cell lysis. The lysed cells were then placed into sterile McConkey bottles and sonicated for a total of 5 min with 30 s bursts at power output 3 interrupted by 20 s intervals. The sonicated lysate was transferred back into sterile GSA SS34 centrifuge tubes and centrifuged at 10,000 x g 30 min at 4ºC. The supernatant was collected and stored at -20ºC until further analysis.

For denaturing conditions; pelleted cells were thawed on ice and then gently resuspended in 5 ml lysis buffer B (100 mM NaH_2PO_4; 10 mM Tris-Cl; 8 M urea; pH 8). While in GSA SS34 centrifuge tubes, lysate was placed on shaker for 1 hour at RT to assist cell lysis. The lysate was centrifuged at 10,000 x g for 30 min at 4ºC, supernatant saved and stored at -20ºC until further analysis.

3.2.2.3 Protein electrophoresis analysis

Crude protein samples were electrophoresed on a 12 % polyacrylamide gel (standard PAGE conditions; Appendix A8) and analysed before protein purification. A total of up to 25 µl of protein sample was loaded containing crude protein sample, 1.25 µl 1M dithiothrietol (DTT) and 5 µl 5 x SDS loading buffer (Appendix B2). Samples were boiled at 90ºC for 10 min before loading.

3.2.2.4 Recombinant XvLOB protein purification

Trial (mini-prep) purification was performed on soluble and insoluble protein fractions according to manufacturer's instructions (HIS-Select Nickel Affinity Gel Purification, Sigma-Aldrich). Large scale column purification and all buffers used during both the trial and large scale purifications were performed and made according to the QIAexpressionist Handbook (Qiagen).

Trial scale purification; 25 µl of homogenised resin (HIS-Select Nickel Affinity Gel, Sigma-Aldrich) was aliquoted into 1.5 ml microcentrifuge tubes and centrifuged at 8,000 x g for 30 s. The supernatant was carefully removed and discarded. A 200 µl aliquot of equilibration buffer (Buffer B: 100 mM NaH_2PO_4; 10 mM Tris-Cl; 8 M urea; pH 8) was added to the resin and mixed well by inverting. The resin was centrifuged at 8,000 x g for 30 s and supernatant carefully removed and discarded. Two hundred microliters crude protein extract was added to the resin and gently mixed for 1 min to allow His-Tag proteins to interact with the nickel ions attached to the resin. The resin bound protein was collected by centrifugation at 8,000 x g for 30 s and supernatant was carefully removed and saved (pre-wash sample). Thereafter resin was washed with 500 µl aliquots of wash buffer (Buffer C: 100 mM NaH_2PO_4; 10 mM Tris-Cl; 8 M urea; pH 6.3), mixed well, centrifuged at 8,000 x g for 30 s and the supernatant saved for analysis. The above wash step was repeated. The protein was eluted off the resin by adding 50 µl elution buffer (Buffer E: 100 mM NaH_2PO_4; 10 mM Tris-Cl; 8 M urea; pH 4.5) to the resin and mixed well by inverting. The resin was centrifuged at 8,000 x g for 30 s and supernatant saved. This elution step was repeated 3 times. All supernatant samples saved above were analysed by SDS PAGE analysis (Appendix A8).

For large scale purification; 2 ml of homogenised affinity gel (HIS-Select Nickel Affinity Gel, Sigma-Aldrich) was added to a column and allowed to settle. Eight milliliters of equilibration buffer (Buffer B) was loaded onto the top of the column and allowed to pass through by gravitational flow. A 2 ml aliquot of crude protein was added onto the column allowing the recombinant protein to bind to the resin. The flow through was collected and saved. An 8 ml aliquot of equilibration buffer (Buffer B) was added to the column and the flow through (pre-wash sample) was collected and saved. The resin was washed with 8 ml of wash buffer (Buffer C) and the flow through collected and saved. The recombinant protein

was then eluted off the affinity gel column by adding 8 ml of elution buffer (Buffer E). The flow through was collected in 1 ml aliquots and analysed by SDS PAGE analysis (Appendix A8). All protein samples were boiled at 90ºC for 10 min before loading and electrophoresed.

3.2.3 Western blotting, antibody generation and purification

3.2.3.1 Western blotting

Two duplicate SDS protein gels containing above saved supernatant samples were prepared according to standard SDS PAGE conditions (Appendix A8). Western blotting and immunodetection was performed following the QIAexpress Ni-NTA Fast Start Handbook (Qiagen). The one gel was used for the transfer process and the other for Coomassie blue (Appendix B3) staining. The gel for transfer was soaked in transfer buffer (25 mM Tris base; 150 mM glycine; 20 % methanol; pH 8.3). The TankSub electroblotting system by Cleaver Scientific Ltd (United Kingdom) was used. The transfer set up was assembled as follows and according to manufacturer's instructions whilst submerged in transfer buffer. Two soaked packing sponges were placed on top of the cathode module and then 4 pieces of moistened filter paper on top of the sponges. The gel was positioned on top of the filter papers and then the nitrocellulose membrane (PROTRAN Pure Nitrocellulose Transfer Membrane, Whatman) with pore size 0.2 µm placed on top of the gel. Then another 4 pieces of filter paper was placed on top followed by 2 soaked packing sponges. The transfer assembly was closed with the anode module and placed in module holder. Electrophoresis was performed at 300 mA for 2 hours at 4ºC.

After transfer the membrane was incubated in Ponceau S (0.5 % Ponceau S in glacial acetic acid) staining solution with gentle agitation for 2 min to confirm transfer and equal loading. The membrane was then destained using distilled water until clear bands are visible.

3.2.3.2 Chromogenic detection

The blotting membrane was washed twice at RT for 10 min each time in TBS buffer (10 mM Tris-Cl pH 7.5; 500 mM NaCl). Blocking was performed by incubating membrane in blocking buffer (10 % skim milk in TBS buffer) overnight at 4ºC with gentle agitation. The membrane was washed twice for 10 min each time at RT in TBS Tween/Triton buffer (20 mM Tris-Cl pH 7.5; 500 mM NaCl; 0.05 % v/v Tween 20; 0.2 % Triton 100) and then

once for 10 min at RT in TBS buffer . The membrane was incubated for 1.5 hours in primary antibody [1:2000 dilution of His-Tag antibody (Cell Signaling Technology)] diluted in blocking buffer at 4ºC with gentle agitation and thereafter washed twice for 10 min each time at RT in TBS Tween/Triton buffer. The membrane was incubated for 1.5 hours in secondary antibody [1:5000 dilution of anti-rabbit IgG alkaline phosphatase conjugate antibody developed in goat (Sigma, Germany)] diluted in blocking buffer at 4ºC with gentle agitation and thereafter washed 4 times for 10 min each time at RT in TBS Tween/Triton buffer. Detection was achieved by incubating membrane in alkaline solution [1 NBT/BCIP tablet in 10 ml distilled water (Roche, Germany)] for ca. 10 min in the dark until bands became clearly visible. The chromogenic reaction was stopped by rinsing membrane twice in distilled water and then left to air dry.

3.2.3.3 Antibody generation

Purified XvLOB protein was quantified using BIO-RAD protein assay dye reagent [Appendix A9 (Bio-Rad Laboratories GmbH, Germany)] and then concentrated using a 30 kDa cut off centrifugal filter which allows all proteins smaller than 30 kDa to pass through saving and concentrating larger sized proteins. Concentration of protein was performed according to manufacturer's instructions (Ampicon Ultra filters, Millipore) and then suspended in phosphate buffered saline [PBS (Appendix B4)]. Concentrated protein was quantified using BIO-RAD protein assay dye as well as SDS-PAGE analysis alongside a bovine serum albumin standard [BSA (Sigma)]. Ethical clearance for the generation of antibodies was obtained from the UCT Animals Research Ethics Committee, Faculty of Health Sciences, UCT (Reference number: REC REF 009/011). The immunization protocol was performed as previously described by Rybicki (1979). All antibody production procedures were performed by and at the Animal Unit, Faculty of Health Sciences, UCT. In brief; antibody production was generated using female New Zealand rabbits ca. 3 months old. A 10 ml collection of pre-immune sera was obtained prior to administration of the antigen. Each rabbit was injected subcutaneously on the back with ca. 500 µg of recombinant XvLOB in the presence of Freund's incomplete adjuvant. A total of 4 injections were administered each being 0.25 ml in volume with an additive total of 2 mg of recombinant XvLOB per inoculation time point. Subsequent booster injections were administered (as above) at weekly intervals. Thereafter 10 ml whole blood was collected every two weeks with a total number

of 5 bleeds being obtained plus the pre-bleed. Rabbits were then exanguinated and physically killed by opening the chest cavity and sectioning the aorta and posterior vena cava to ensure death by circulatory failure.

3.2.3.4 Enzyme-linked immunosorbent assay (ELISA)

An ELISA was performed using recombinant XvLOB as the antigen against all serum bleeds including the pre-bleed serum. This is a biochemical technique used to determine the presence and concentration of serum antibody and may also be used to select the most appropriate bleed which shows the highest specificity against any background signal. The ELISA was performed using 96 well polysorb microtitre plates (Nunc, Denmark). Undiluted and diluted (10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}) serum from bleed time points 3, 4 and 5 as well as the pre-bleed was tested against recombinant XvLOB. The wells were coated with ca. 300 ng of antigen in a total of 100 µl PBS (Appendix B4) and allowed to incubate at 37ºC for 90 min. The excess antigen was tipped out and the wells were then washed thrice with TBS tween (100 mM NaCl; 10 mM Tris-Cl pH 7.5 and 0.1% tween). The blocking step was performed by incubating wells with 200 µl 5 % skim milk (5g skim milk powder in 100ml TBS tween) for 1 hour at RT. The wells were then washed three times with TBS tween and then incubated with 100 µl serum containing primary antibody as either undiluted or appropriately diluted for 1 hour at RT. The excess antibody was tipped out and wells were then washed three times with TBS tween. A 100 µl volume of 1:5000 dilution secondary antibody [Anti-rabbit IgG alkaline phosphatase conjugate antibody developed in goat (Sigma, Germany)] in TBS tween was added to each well and allowed to incubate for 30 min at RT. The excess secondary antibody was tipped out and wells were then washed three times with TBS tween. A final wash step was performed with 10 % diethanolamine (Merck, Germany) supplemented with 0.5mM $MgCl_2$ (pH 9.6) for equilibration. The wells were then incubated with 4-nitrophenyl disodium orthophosphate substrate (Appendix B5) and allowed to incubate for 30 min at RT. Absorbance readings were obtained for each well using the Titertek Multiskan® PLUS (ICN Biomedicals, USA) microplate reader.

3.2.3.5 Antibody purification

Purification of polyclonal antibodies from antibody serum was obtained using the polyethylene glycol (PEG) method adapted from Polson *et al* (1964). One volume of serum

was mixed with 2 volumes of borate buffered saline (Appendix B6). PEG 6000 (Sigma-Aldrich, Germany) was crushed using a clean mortar and pestle and then added (14 % w/v) to the antiserum and dissolved by gentle inversion. The mixture was then centrifuged at 12, 000 x g for 10 min at 4ºC, supernatant discarded and the pellet redissolved in the original volume of antiserum with PBS. PEG 6000 was again added (14 % w/v) and gently dissolved by inversion. The mixture was centrifuged as before at 20, 000 x g for 10 min at 4ºC, supernatant discarded and pellet redissolved in a half of the original antiserum volume with PBS containing 60 % glycerol. The purified antibodies were stored in aliquots at -20ºC and ready to be used for western blot analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 Cloning of *XvLOB* into expression vector pET32b

3.3.1.1 Amplification and restriction enzyme digest of *XvLOB*

XvLOB was successfully amplified from pDNR-LIB::*XvLOB* (Figure 3.1). A DNA fragment size of ca. 650 bp was observed in lanes 1 to 4 when PCR products were electrophoresed. The band of interest was distinct with no non-specific amplification visible.

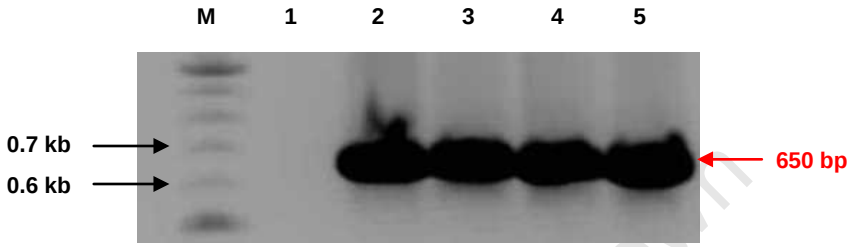


Figure 3.1 0.8 % (w/v) Agarose/EtBr gel electrophoresis of amplified *XvLOB* PCR products. Lane 1 - PCR reaction NTC control. Lanes 2 to 5 - amplified *XvLOB* PCR product. M - 100 bp DNA ladder (NEB).

3.3.1.2 Simultaneous digest and dephosphorylation of expression vector pET32b

The expression vector pET32b was successfully digested with *Eco*RI and *Hin*dIII and a single linear band of ca. 6 kb was visible when electrophoresed (Figure 3.2). To prevent false positives during colony PCR screening, pET32b vector was dephosphorylated to prevent religation of vector that had been digested with only one of the enzymes used in the double digest.

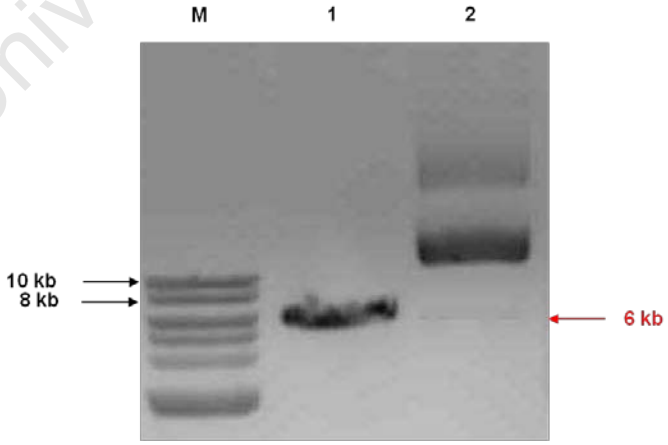


Figure 3.2 0.8 % (w/v) Agarose/EtBr gel electrophoresis of digested and undigested expression vector pET32b. Lane 1 - pET32b (290 ng) digested with *Eco*RI and *Hin*dIII. Lane 2 - undigested pET32b (460 ng). M - 1 kb DNA ladder (NEB)

3.3.1.3 Expression vector pET32b::*XvLOB* constructs screening

After the *XvLOB* digested fragment was ligated into pET32b, the ligation reaction was transformed into competent *E. coli* DH5α cells. PCR screening was performed to isolate positive clones and of the 30 colonies selected for screening all of them contained the *XvLOB* insert. Out of all 30 colonies screened, one of the positive clones was selected for further analysis. Digestion of and PCR amplification from the pET32b::*XvLOB* construct extracted from the selected positive clone preliminarily indicated that *XvLOB* was successfully cloned into expression vector pET32b (Figure 3.3). Sequencing results confirmed XvLOB was cloned in frame with pET32b expression vector and its tags (data not shown).

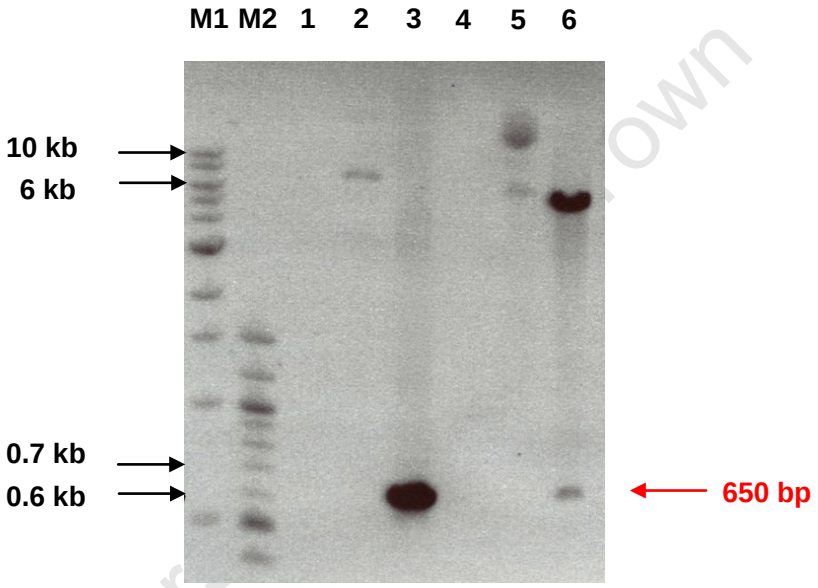


Figure 3.3 0.8 % (w/v) Agarose/EtBr gel electrophoresis of PCR products amplified from different vectors as well as digested and undigested pET32b::*XvLOB* constructs. Lane 1 - PCR products amplified from pET32b digested with *Eco*RI and *Hin*dIII. Lane 2 - PCR products amplified from undigested pET32b. Lane 3 - *XvLOB* PCR products amplified from pDNR-LIB::*XvLOB*. Lane 4 - PCR reaction NTC control. Lane 5 - undigested pET32b::*XvLOB*. Lane 6 - pET32b::*XvLOB* digested with *Eco*RI and *Hin*dIII. M1 - 1 kb DNA ladder (NEB). M2 - 100 bp DNA ladder (NEB).

As a positive control; *XvLOB* was amplified from pDNR-LIB::*XvLOB* with gene specific primers XvLOB-*Eco*RI F (Appendix C, C1.1) and XvLOB-*Hin*dIII R (Appendix C, C1.2) and the PCR product electrophoresed (Figure 3.3, lane 3). An amplicon of ca. 650 bp was amplified representing the open reading frame of *XvLOB*. For negative controls; PCR

amplification was performed using the digested expression vector pET32b (lane 1) and undigested pET32b (lane 2). The faint band seen in lane 2 is undigested plasmid pET32b DNA. No amplification was observed in these lanes as well as in the NTC PCR reaction (lane 4). Both undigested (lane 5) and digested pET32b::*XvLOB* (lane 6) was electrophoresed with the digested pET32b::*XvLOB* showing the release of an ca. 650 bp fragment. PCR analysis as well as digestion results indicated that *XvLOB* was successfully cloned into pET32b expression vector. The sequencing results obtained confirmed that *XvLOB* was cloned in frame with pET32b and its tags (data not shown). These positive results allowed for recombinant XvLOB protein expression to be carried out.

3.3.2 XvLOB fusion protein expression and purification

3.3.2.1 Transformation of pET32b::*XvLOB* into expression cells and recombinant protein expression

Transformation into competent expression *E. coli* BL21 (DE3) cells was successful and positive screening was performed on selected colonies. Colonies containing the pET32b::*XvLOB* constructs were subsequently inoculated into overnight LB media. Extraction of XvLOB under native (Culture A) and denaturing conditions (Culture B) was achieved and the crude protein extracts were electrophoresed (Figure 3.4).

For recombinant XvLOB protein expression in *E. coli* BL21 (DE3) cells, good expression yields were obtained although most of the protein formed inclusion bodies and was located within the insoluble fraction. The recombinant XvLOB is theoretically calculated to be 22.8 kDa in size. When fused with the Trx-Tag™, S tag as well as 2 His-tags (consisting of 6 histidine amino acids each) which are associated with the expression vector pET32b, the recombinant XvLOB protein is expected to be an ca. 40 kDa protein. In the soluble and insoluble fractions a band representing recombinant XvLOB was observed between 43 and 55 kDa. This can be seen in figure 3.4 lanes 7 and 8 (red arrow on the RHS). The expression patterns of pET32b::*XvLOB* (Figure 3.4, lanes 4, 5, 7 and 8) shows a greater band intensity in the vicinity where recombinant XvLOB should be located when compared to the expression pattern of pET32b (Figure 3.4, lanes 3 and 6). The difference in XvLOB band intensity between the pET32b::*XvLOB* and pET32b expression profiles suggests successful expression of recombinant XvLOB.

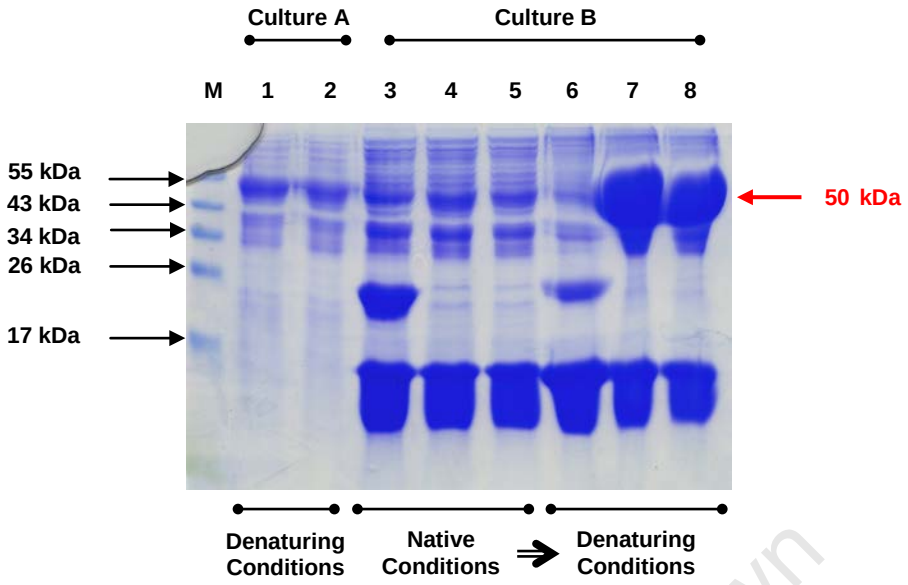


Figure 3.4 12 % Polyacrylamide SDS-PAGE analyses of crude protein extract samples extracted under native and denaturing conditions. Lanes 1 and 2 represent protein samples extracted from culture A. Lane 1 and 2 - insoluble crude protein extract from pET32b::*XvLOB* clone 1 and 2, respectively. Lanes 3 to 8 represent protein samples extracted from culture B. Lane 3 - soluble crude protein extract from pET32b expression. Lane 4 and 5 - soluble crude protein extract from pET32b::*XvLOB* clone 1 and 2, respectively. Lane 6 - insoluble crude protein extract from pET32b expression after soluble extraction. Lane 7 and 8 - insoluble crude protein extract from pET32b::*XvLOB* clone 1 and 2, respectively, after soluble extraction. M - 5 µl prestained protein ladder (Fermentas). The gel was stained with Coomassie blue. Red arrow on the RHS indicates recombinant XvLOB protein.

According to SDS PAGE analysis (Figure 3.4, Lanes 7 and 8), it is observed that recombinant XvLOB protein is ca. 10 kDa larger than its theoretically calculated size. The Trx-S-His tag is calculated to be ca. 17 kDa, however it appears to be ca. larger than 17 kDa and can be seen between 17 and 26 kDa. Therefore the Trx-S-His tag is also ca. 10 kDa larger than its theoretical size. This can be seen in both the soluble and insoluble fractions (Figure 3.4, lanes 3 and 6 respectively). This could explain why recombinant XvLOB protein migration is slower than expected.

There is a greater amount of XvLOB being expressed and found within the insoluble fraction (Figure 3.4, lanes 7 and 8) than the soluble fraction (Figure. 3.4, lanes 4 and 5). Initial

recombinant protein extraction of culture B under native conditions which was then followed by extraction under denaturing conditions (Figure 3.4, lanes 7 and 8) generated a greater yield of insoluble recombinant XvLOB protein than extraction of insoluble protein from culture A extracted under denaturing conditions (Figure 3.4, lanes 1 and 2). Using Recombinant Solubility Predictions programme (http://www.biotech.ou.edu/); recombinant XvLOB has a 67.4 % chance of insolubility when overexpressed in *E. coli* cells. Therefore the recombinant XvLOB protein was fused to a Trx-Tag™ tag so as to help increase solubility. It appears that even with the use of this tag most of recombinant XvLOB was located within the insoluble fractions. Future studies should include further optimizing expression conditions, different expression vector systems as well as perhaps cell free translation systems (Endo and Sawasaki, 2006; Esposito and Chatterjee, 2006). Other than the Trx-Tag™ tag other commercially available expression vectors containing tags such as the *E. coli* maltose-binding protein (MBP) and the N-utilization substance A (NusA) has been shown to increase solubility of recombinant expressed proteins (Davis *et al*., 1999; Kapust and Waugh, 1999).

Extraction under denaturing conditions resulted in a greater yield of protein and consequently the insoluble fraction of recombinant XvLOB was used for further purification and antibody generation.

3.3.2.2 Recombinant XvLOB protein purification

Protein purification was performed on the insoluble crude protein extract and analysed by electrophoresis. All wash and flow through samples were analysed (Figure 3.5). The recombinant XvLOB was released and eluted from the second (Figure 3.5, lane 6) elution onwards with most of the protein being eluted within the first 3 ml of eluants. A distinct single band was observed indicating that only recombinant XvLOB bound to the column and no non-specific binding occurred (Figure 3.5, lanes 6, 7, 8 and 9). All other native *E. coli* proteins passed through the column and can be seen in lanes 2 and 3 and the wash buffer flow through (Figure 3.5, lane 4).

The recombinant XvLOB was passed through a filter to concentrate the protein and resuspended in a PBS buffer. During filtering and buffer exchange a large amount of recombinant XvLOB was lost due to its insolubility in PBS buffer. The concentration of

recombinant XvLOB was calculated to be 0.45 mg/ml according to the Bradford assay (Bradford, 1976) and BSA standard curve analysis. Based on these results western blot analysis was performed to confirm recombinant XvLOB expression.

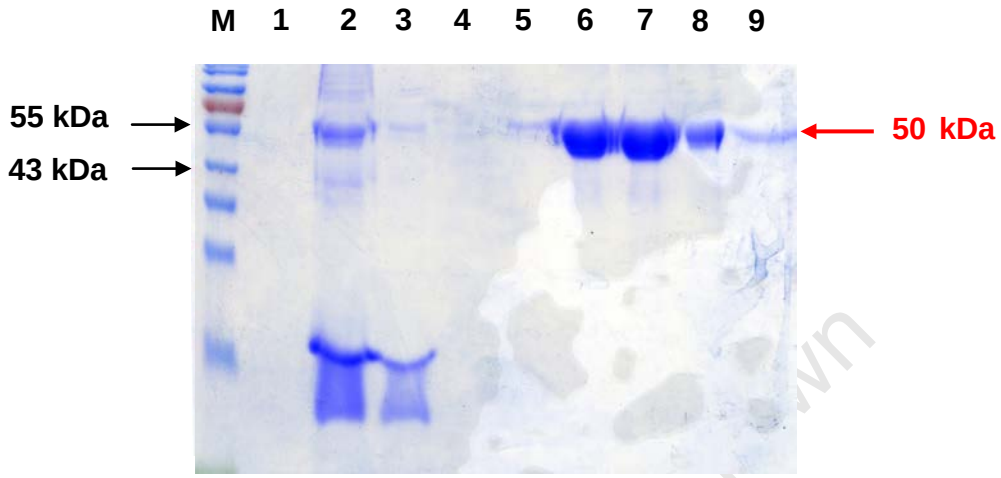


Figure 3.5 12 % Polyacrylamide SDS PAGE analysis of purified XvLOB insoluble protein samples. Lane 1 - equilibration buffer passed through settled resin. Lane 2 - crude extract flow through. Lane 3 - equilibration buffer (pre-wash) flow through. Lanes 4 - wash buffer flow through. Lanes 5 to 9 - aliquots of eluted protein from consecutive 1 ml elution eluantes. M - 5 µl prestained protein ladder (Fermentas). The gel was stained with Coomassie blue. Red arrow on the RHS indicates recombinant XvLOB protein.

3.3.3 Western blotting and antibody production and purification

3.3.3.1 Western blotting

To confirm recombinant XvLOB expression; western blot analysis was performed on the recombinant XvLOB protein using anti-histidine antibodies. SDS PAGE gel and western blot membrane images can be seen in figure 3.6.

As a control, recombinant XvGolS protein was used. This fusion protein was expressed using the pET32b vector and is therefore also fused to the Trx-S-His tag. Recombinant XvGolS has previously been shown to be positively detected during western analysis using chromogenic detection (Alexis Neumann 2009, pers. comm.) and is ca. 55kDa is size (Figure 3.6, lane 1). The anti-histidine primary antibody detected the pET32b expressed tags which can be seen in figure 3.6B, lanes 2 and 3. This Trx-S-His tag is observed at about 27 kDa. Detection of

recombinant XvLOB fused to the pET32b tag is also observed at 50 kDa in both the insoluble crude extract as well as in the purified sample (Figure 3.6, lane 6 and 7 respectively). There was no non-specific binding to unrelated proteins observed during western blot analysis. The western blot analysis confirmed the successful expression and purification of recombinant XvLOB which was later used for antibody generation.

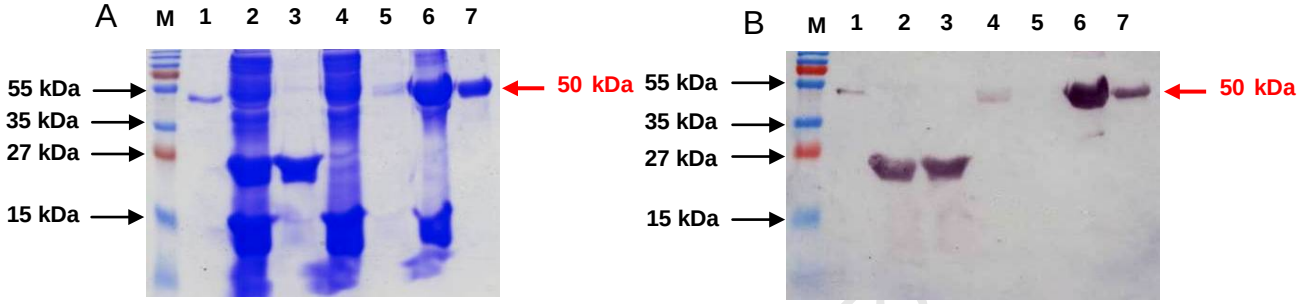


Figure 3.6: Western blot analysis on *E. coli* expressed pET32b Trx-S-His tag and recombinant XvLOB protein. A - 12 % Polyacrylamide SDS PAGE gel containing protein samples and stained with Coomassie blue. B - Chromogenic detected nitrocellulose membrane containing protein samples. Lane 1 - positive control; recombinant XvGolS protein. Lane 2 - crude protein extract from pET32b expression. Lane 3 - purified protein extract from pET32b expression containing the Trx-S-His tag. Lane 4 - soluble crude protein extract from pET32b::*XvLOB* expression. Lane 5 - purified soluble protein extract from pET32b::*XvLOB* expression containing purified recombinant XvLOB. Lane 6 - insoluble crude protein extract from pET32b::*XvLOB* expression. Lane 7 - purified insoluble protein extract from pET32b::*XvLOB* containing recombinant XvLOB. M - 5 µl prestained protein ladder (Fermentas). Red arrow on the RHS indicates recombinant XvLOB protein.

3.3.3.2 Enzyme-linked immunosorbent assay (ELISA)

An ELISA assay was performed to ascertain which bleed and dilution would be most appropriate to use during protein expression profiling analysis.

Based on the results obtained in figure 3.7, bleed 5 was selected for purification and subsequent use in protein expression profiling analysis as this bleed indicates the highest specific binding when compared to non specific background occurring in the pre-bleed. The

PEG method (Polson *et al*., 1964) of antibody purification was performed and antibodies were stored until further use.

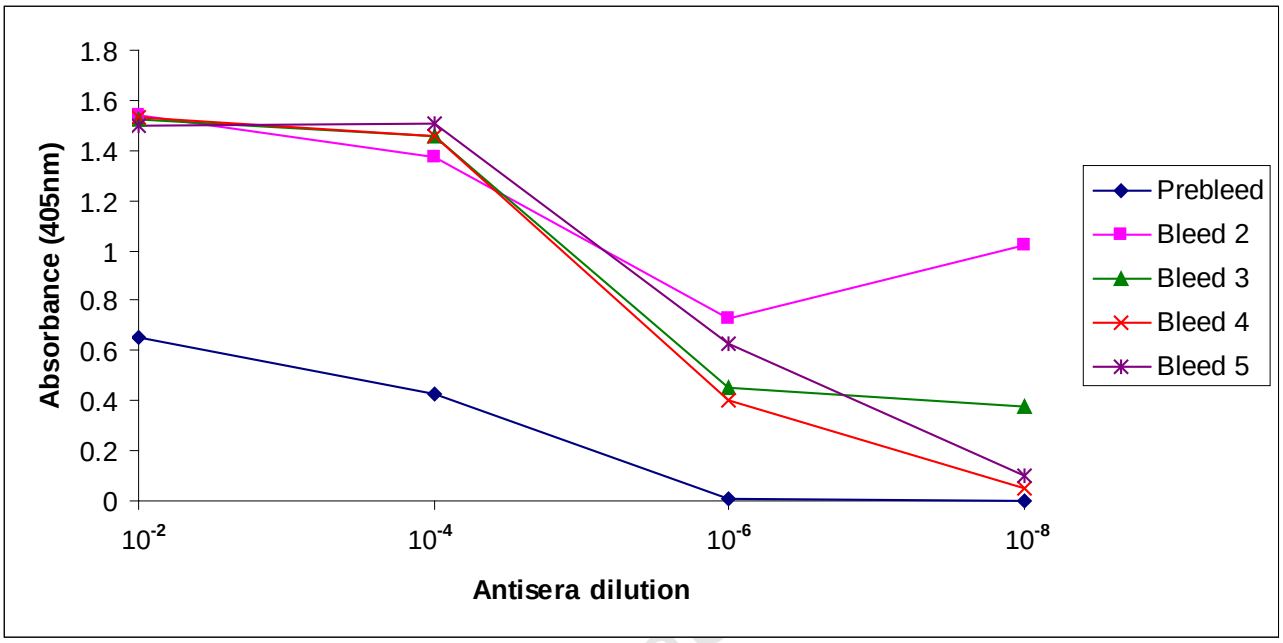


Figure 3.7 Graph depicting ELISA assay results for pre-bleed and bleeds 2 to 5 at different antisera dilutions.

3.4 CONCLUSION

In this chapter, the recombinant XvLOB was successfully expressed and purified from the bacterial *E. coli* expression system. Expression of soluble recombinant XvLOB was difficult to achieve albeit the use of the Trx-Tag™ which has previously been shown to increase solubility during expression (LaVallie *et al*., 1993; Uegaki *et al*., 1996; Davis *et al*., 1999; Tenno *et al*., 2004; Xu *et al*., 2007). However, a good enough yield of insoluble recombinant XvLOB was obtained for antibody production. The insoluble protein used generated a sufficient immunological response to obtain anti-XvLOB antibodies. These antibodies were used for the western analysis studies (see chapter 4).

CHAPTER 4

XvLOB gene and protein expression analysis

4.1 INTRODUCTION

The plant's response to an abiotic stress such as dehydration is controlled by an array of genes with the products of these genes functioning within the stress response or tolerance. The characterisation of a stress related molecular response depends on the overall gene expression patterns as well as the control thereof at the transcriptional, posttranscriptional and translational levels. Stress regulated gene response research has in part mainly been focused on non-desiccation tolerant plants such as *A. thaliana* (Shinozaki and Yamaguchi-Shinozaki, 1997; Desikan *et al*., 2001; Shinozaki *et al*. 2003). Recently this focus in stress response at the gene and protein level has also been carried out in resurrection plants such as *X. viscosa* (Mundree *et al*., 2000; Mowla *et al*., 2002; Garwe *et al*., 2003; Ingle *et al*., 2007; Abdalla *et al*., 2009), *Craterostigma plantagineum* (Bartels and Salamini, 2001) and *Sporobolus stapfianus* (Neale *et al*., 2000) amongst others. The characterisation of stress induced genes is a complex task as not all genes upregulated are required for cellular and stress tolerance. During abiotic stress, genes which give rise to stress tolerance include those that serve as a protective function in the cytoplasm, control ion accumulation and alter cellular water potential to promote water uptake whereas others may be upregulated due to injury or damage occurred during the stress (Bray, 1993). Genes induced during abiotic stress function not only in the direct protection of cells from water deficit via the production of metabolic proteins but also with the regulation and control of gene expression and signal transduction. These genes have been classified into 3 major groups: (i) those that encode products that directly protect cell membrane and proteins against stress; (ii) those that are involved in transcriptional control and the signalling cascade; and (iii) those that involved in the water and ion uptake and transport thereof (Shinozaki *et al*., 2003; Wang *et al*., 2003; Rodriguez *et al*., 2005).

RNA dot blots and cDNA microarray technology has previously been used in a number of plant studies to analyse gene expression profiling (Kawasaki *et al*., 2001; Seki *et al*., 2001). Today, methods such as real time PCR has become widely used and provides a precise, sensitive and reproducible technique to quantify specific mRNA transcripts for the analysis of

gene expression patterns. The sensitivity of real time PCR allows for the detection of rare mRNA transcripts, detection of mRNA from small amounts of tissue as well as providing a means to detect a single copy of a particular gene (Schmittgen and Zakrajsek, 2000; Palmer *et al*., 2003). RNA acquisition and purification mark the first step in quantitative real time PCR and therefore template RNA needs to be of high quality, should be free of DNA (if the target is a gene which does not contain introns) and nucleases when extended storage of RNA is intended.

Gene expression is controlled at many levels including post transcriptional regulation which includes mRNA splicing, mRNA stability, translation as well as post translational control such as protein stability and modification. As a result of this, protein expression levels in response to abiotic stress needs to be examined as an increase in mRNA levels may not correlate to the translation of functional proteins.

In this chapter, the expression profiling of both the *XvLOB* mRNA and protein levels in *X. viscosa* under hydrated and dehydrated conditions are investigated with the use of real time PCR and western blot analysis.

4.2 MATERIALS AND METHODS

4.2.1 Plant dehydration rehydration treatment and RNA isolation

4.2.1.1 Plant material

X. viscosa plants used in this study was previously collected from the Cathedral Peak Nature Reserve in the Drakensberg Mountains (KwaZulu-Natal, South Africa). These plants were kept and grown under green house conditions as described by Sherwin and Farrant (1996). Before performing stress treatments; plants were transferred to growth rooms under controlled environmental conditions and allowed to acclimatise to these conditions for 4 weeks. Growth room conditions were set at 50 – 70% humidity, 22 - 24ºC temperature, light intensity of 150 - 200 μmol/m^{-2}/s^{-1} and a photoperiod of 16 hour light phase and 8 hour dark phase. Once acclimatised; any dead leaf material at the tips of leaves was removed and plants were allowed to restore for an additional 2 weeks. In this study, 3 biological repeats isolated from the same parental line were used. The 3 individual *X. viscosa* plants chosen were of similar size and phenotype so as to minimise plant to plant variation during the stress treatment.

4.2.1.2 Dehydration and rehydration treatment

Dehydration treatment was performed on whole plants and carried out by withholding water for a period of 16 days. Leaf samples were collected just before the start of dehydration and then on days 3, 5, 7, 9, 11, 13 and 15 of the dehydration stress treatment. On the 16th day plants were watered using a spray watering jug to simulate rainfall after which the soil was kept damp throughout the remainder of the experiment. Leaf samples were collected at 16, 30, 42 and 64 hours after rehydration. At each time point 4 leaves were collected, one from each quadrant from each plant. Each leaf sampled was split vertically along the central vein; one section was dissected into smaller fragments, wrapped in aluminium foil, flash frozen in liquid nitrogen and then stored at -80ºC whilst the other section containing the central vein was used to calculate relative water content (RWC). The fresh weight (FW) of each leaf section was determined immediately after sampling. Leaf sections were then incubated at 70ºC for 48 hours, weighed and recorded as dry weight (DW). Absolute water content (AWC) was calculated using the formula:

$$AWC = (FW-DW)/DW$$

AWC was calculated for each leaf from each plant. For each plant; 3 leaf AWC values were selected, averaged and then recorded as the whole plant AWC. The full turgor weight (FTW_i) for each plant was determined from only one time point, which is the initial sampling point performed before starting dehydration stress. FTW_i value is calculated using the AWC equation. RWC values were calculated for each plant at each time point (n) using the formula:

$$RWC_n = (AWC_n/FTW_i) \text{ X } 100$$

4.2.1.3 RNA isolation

All plastic and glassware used were double autoclaved and all solutions made with 0.001% diethylpyrocarbonate (DEPC; Sigma-Aldrich, Germany) treated water. The stored leaf sections were individually ground to a fine powder with liquid nitrogen using a mortar and pestle. To aid grinding, small amounts (ca. spatula tip) of acid purified sand (Merck, Germany) and polyvinyl-polypyrrolidone (PVPP; Sigma-Aldrich, Germany) was added together with the leaf material. The ground material was kept at low temperatures (4ºC) to prevent RNA degradation. Total RNA was isolated by adding ca. 100 mg of ground material to 1 ml Trizol reagent (Invitrogen Life Technologies, USA). The mixture was homogenised by vortexing for 10 min and thereafter incubating for 5 min at RT. After incubation, 200 µl of chloroform was added and mixed gently by inversion for ca. 1 min. The homogenate was incubated for 3 min at RT and then centrifuged at 12,000 x g for 15 min at 4ºC. The upper aqueous phase containing the RNA was then carefully removed and added to 500 µl of isopropanol and incubated for 10 min at RT to allow for RNA precipitation. The organic phase was saved and stored at -80ºC for protein extraction at a later stage. The RNA was pelleted by centrifugation at 12,000 x g for 10 min at 4ºC. The supernatant was discarded and the pellet washed with 1 ml of cold 75 % ethanol (EtOH). The EtOH was carefully removed with a pipette and the pellet was allowed to air dry for ca. 10 min. The RNA pellet was dissolved in 50 µl of DEPC water and incubated for 10 min at 55ºC to aid resuspension.

4.2.1.4 DNAse treatment and Phenol:Chloroform:Iso-amyl alcohol clean up

Each RNA extraction was treated with Deoxyribonuclease I (DNAse I; Sigma-Aldrich, Germany) to digest and remove any genomic DNA contamination. The DNAse

reaction mixture consisted of; 44 µl of isolated RNA, 6 µl of 10X DNAse buffer and 2 units of DNAse I made up to a total volume of 60 µl with DEPC water. The reaction was mixed gently and incubated for 10 min at 37ºC. An equal volume (60 µl) of phenol:chloroform:iso-amyl alcohol (ration of 25:24:1) was added to the reaction, mixed by inversion and then centrifuged at 14,000 x g for 10 min at 4ºC. The top aqueous phase was carefully removed, added to 8 µl 3M sodium acetate pH 5.2 (Appendix B7) and mixed by inversion to precipitate the RNA. Two hundred and fifty microlitres of 100% EtOH was then added, gently mixed by inversion and centrifuged at 14,000 x g for 20 min at 4ºC. The supernatant was discarded and the pellet was allowed to air dry for ca. 10 min. The RNA pellet was resuspended in 40 µl of DEPC water.

4.2.1.5 Removal of Trizol and phenol contaminants

To remove Trizol and phenol contaminants, water saturated butanol and diethyl ether were used in RNA wash steps as described by Krebs et al (2009). These washes have been indicated to improve 260/280 and 230/260 ratios as well as the 18S/28S RNA ratio. Five hundred microlitres of 1:1 water saturated butanol was added to the 40 µl RNA suspension and mixed thoroughly by inversion. The mixture was then centrifuged at 10,000 rpm for 30 s. The upper organic water saturated butanol phase was carefully removed so as not to lose any of the RNA sample and then discarded. Five hundred microlitres of 1:1 water saturated diethyl ether was added to the RNA sample, mixed thorough by inversion and then briefly centrifuged at 10,000 x g for 30 s. The upper organic diethyl ether phase was carefully removed and discarded. This diethyl ether wash step was repeated. Microfuge tubes were left open in the fume hood to allow any diethyl ether left behind to evaporate. RNA samples were quantified using a Nanodrop® ND-1000 spectrophotometer (Nanodrop® Technologies Inc., USA), analysed by electrophoresis and then stored at -80ºC until further use.

4.2.2 cDNA synthesis and quantitative real time reverse transcription PCR

4.2.2.1 cDNA synthesis

The purified RNA extracted from *X. viscosa* leaf samples were used for cDNA synthesis. For each biological repeat at each dehydration and rehydration time point, the RNA extracted from two leaves was pooled and approximately 500 ng was used per cDNA synthesis reaction. This reaction was performed in duplicate to act as technical cDNA

synthesis repeats. For cDNA synthesis the Promega ImpromII™ Reverse Transcription System (Promega Corporation, USA) was used according to manufacturer's instructions. In brief, two different master mix reactions were set up namely the target RNA-primer mix and the reverse transcription (RT) mix. The target RNA-primer mix consisted of ca. 500 ng of RNA, 0.05 µg oligo $(dT)_{15}$ primers and 0.45 µg random primers made up to a total volume of 5 µl with nuclease free water. A ratio of 1:10 of random primer to oligo $(dT)_{15}$ was selected for cDNA synthesis reaction. The target RNA-primer mix was preheated for 5 min at 70ºC and then immediately chilled on ice for 5 min. The RT mix consisted of final concentration of 1X ImpromII™ reaction buffer, final concentration of 8 mM $MgCl_2$, 20 units of Recombinant RNasin® Ribonuclease Inhibitor, 1 µl of ImpromII™ Reverse Transcriptase and 0.5 mM of each dNTP. The components were added to together and made up to a total volume of 15 µl with nuclease free water. Once target RNA-primer mix was preheated and cooled it was combined with the RT mix and briefly centrifuged. The PCR cycle conditions consisted of incubation at 25ºC for 5 min, 42ºC for 1 hour, 70ºC for 15 min followed by a final step at 4ºC for 2 min. An aliquot of cDNA synthesis was electrophoresed on a 1.2 % agarose/EtBr gel to analyse cDNA quality. The cDNA was stored at -20ºC until further use.

4.2.2.2 Quantitative real time primer design

The DINAMelt server [http://dinamelt.bioinfo.rpi.edu/download.php; (Markham and Zuker, 2005)] was used to predict the possible folding structure of single stranded *XvLOB* mRNA. Real time primers were designed to bind to areas where there are little to no secondary structures present as well as to bind near the 3' end of genomic *XvLOB* sequence. A number of different real time primer sets were designed and analysed by end point PCR to check specific and non specific amplification. The *XvLOB* gene fragment was amplified using cDNA (see section 4.2.2.1) as a template with gene specific primers XvLOB-RT F (Appendix C, C1.3) and XvLOB-RT R (Appendix C, C1.4).

4.2.2.3 Quantitative real time reverse transcription PCR

The relative expression of *XvLOB* in *X. viscosa* was investigated by real time PCR using the Rotor Gene 6000 2 plex HRM (Corbett Life Science Research, Australia). The SensiMix™ dT Kit (Qantace, Australia) master mix containing reaction buffer, heat activated DNA polymerase, dNTPs, working concentration of 3mM $MgCl_2$, internal reference and

stabilizers were used for PCR reaction. Each PCR reaction was performed in a reaction volume of 12.5 µl containing; 0.25 µl 50X SYBR® Green I solution, 0.25 µl of 10 µM gene specific primers, 6.25 µl of 2X SensiMix dT and 1 µl of cDNA. Gene specific sense and anti sense primer sets for both *XvLOB* (XvLOB RT-F; Appendix C, C1.3 and XvLOB RT-R; Appendix C. C1.4) and a house keeping gene (HKG) *Xv18S* (Xv18S RT-F; Appendix C, C1.7 and Xv18S-R; Appendix C, C1.8) were used in separate reactions such that PCR products of ca 135 bp and ca 220 were amplified, respectively. *XvVTC2* (XvVTC2 RT-F; Appendix C, C1.5 and XvVTC2 RT-R; Appendix C, C1.6) was used as a positive control. *XvVTC2* primers were designed and tested by Andries Petrus Bresler (Bresler, 2010). A no reverse transcription control (pooled RNA from either dehydrated or rehydrated samples) as well as a no template control (NTC) was carried out for each biological repeat during each real time run. The thermal cycle conditions consisted of an initial enzyme activation step at 95ºC for 10 min followed by 40 cycles of 95ºC for 5 sec, 57ºC for 8 sec and elongation at 72ºC for 10 sec. The fluorescence for each reaction was obtained at the end of the elongation step.

PCR efficiencies were determined by generating standard curves for each gene analysed. A standard curve for *XvLOB* was created by using an *XvLOB* amplified fragment consisting of the full coding sequence as a template. This fragment was prepared by amplifying *XvLOB* from the pDNR-LIB::*XvLOB* construct using gene specific primers XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10). Standard curves for *XvVTC2* and *Xv18S* were created with serial dilutions of pooled cDNA of either dehydrated or rehydrated samples. The dilutions that fell within the range of *XvLOB*, *XvVTC2* and *Xv18S* expression were used to generate the standard curves. These standard curves were imported into every run to be able to calculate concentrations of the gene of interest (GOI) and the house keeping gene (HKG). The calculated concentration of GOI was divided by the calculated concentration of HKG (i.e. GOI/HKG). The values calculated for each biological replicate was averaged and used for relative quantification of transcripts. To evaluate whether there is a significant change in expression of genes during dehydration treatment, the transcript levels in non-stressed plants (time zero of dehydration stress) were used as a calibrator ie. 100% RWC. Relative quantification of the expression was done using the REST method via the Pfaffl equation (Pfaffl, 2001) as described in figure 4.1.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control – sample)}}}{(E_{\text{reference}})^{\Delta Ct \text{ reference (control – sample)}}}$$

Figure 4.1 Equation representing the Pfaffl mathematical equation for calculating relative expression ratios in qPCR. The ratio of the gene of interest (target) expressed in a sample versus a calibrator sample (control) in comparison to a reference gene (reference). E_{target} and $E_{reference}$ represent the amplification efficiency of the target and reference gene, respectively. Δ^{Ct} target and Δ^{Ct} reference is the difference in Ct values of target gene in the control and sample and reference gene in the control and sample, respectively. PCR amplification efficiencies (E) were calculated according to $E = 10^{[-1/slope]}$ (Pfaffl, 2001).

The PCR products were checked for specificity by obtaining a melt curve using the Rotor-Gene™ 6000 Series software (Corbett Life Science Research, Australia). An aliquot of the end product was analysed by electrophoresis on a 2 % agarose/EtBr gel to verify that one amplicon was obtained.

4.2.2.4 Statistical analysis

All statistical tests and graphs was calculated and generated using the GraphPad Prism software package Version 5 (GraphPad Software Inc., 1992-2007). The two way ANOVA statistical test was used to analyze the significance of the relative expression of *XvLOB* in response to dehydration during a time course.

4.2.3 Total crude protein extraction from plant material

Protein extraction was performed individually from each leaf sampled per plant for western blot analysis. The total protein was extracted from organic phase retained during RNA extraction (see section 5.2.1.3). Three hundred microlitres of cold absolute EtOH, inverted ca. 25 times and incubated at RT for 5 min. The protein extract was centrifuged at 2,500 x g for 10 min at RT and the supernatant containing soluble protein was transferred to a

fresh 2 ml microfuge tube containing 1.5 ml isopropanol. The soluble protein extract was incubated at RT for 10 min to aid precipitation of protein and thereafter centrifuged at 10,250 x g for 10 min at 4ºC. The protein pellet was washed thrice with 2 ml of 0.1M ammonium acetate (Appendix B8), washed once with 2 ml cold acetone and thereafter air dried for ca. 10 min. The pellet was resuspended in a total volume of 100 µl of urea lysis buffer (Appendix B9) supplemented with 125 mM of DTT. The protein suspension was vortexed at RT for 30 min to aid resuspension. The total protein extraction was then quantified using the Bradford Reagent (Biorad, Germany) method (Appendix A8) and based on these concentrations, aliquots of extracted protein was electrophoresed according on a 12% polyacrylamide gel (standard SDS-PAGE conditions; Appendix A7) to check protein integrity as well as attempt equal loading.

4.2.4 Western blotting and chemiluminescence detection

4.2.4.1 Western blotting

As a positive control, purified recombinant XvLOB (see section 3.2.2.4) was used. Various dilutions of recombinant XvLOB was electrophoresed and detected to determine the appropriate concentration to be used during western analysis. Two duplicated SDS protein gels containing extracted crude protein samples were prepared according to standard SDS PAGE conditions (Appendix A7). Western blotting and immunodetection was performed following the QIAexpress Ni-NTA Fast Start Handbook (see section 3.2.3.1).

4.2.4.2 Chemiluminescence detection.

Chemiluminescence detection was performed as previously described (see section 3.2.3.2) with the following modifications; blotting membrane was incubated overnight in primary antibody [purified anti-XvLOB IgG developed in rabbit (see section 3.2.3.3)]. The primary antibody dilutions tested ranged from 1:10 000 as indicated by ELISA results (see section 3.3.3.2) to 1:500 after optimization. The membrane was then incubated for 1 hour in secondary primary [1:20 000 dilution of anti-rabbit IgG peroxidase conjugate antibody developed in goat (Sigma, Germany)]. The SuperSignal West Pico Rabbit IgG Detection Kit (Pierce, USA) was used according to manufacturer's instructions to detect chemiluminescence. In brief; equal volumes of Luminol/Enhancer solution was mixed with Stable Peroxidase solution and then placed on top of membrane. To visualize and capture

chemiluminescence from the membrane, the Molecular Imager® ChemiDoc™ XRS+ System (BioRad, USA) was used.

4.3 RESULTS AND DISCUSSION

4.3.1 Plant dehydration rehydration treatment

4.3.1.1 Morphological changes during dehydration rehydration treatment

Dehydration stress was carried out on 3 individual whole plants. Images taken of biological plant 3 during the course of the dehydration and rehydration treatment can be seen in figure 4.2. The images in figure 4.2 show the morphological changes such as leaf movements and colour changes which occurred during the dehydration and rehydration stress treatment.

During the stress treatment the leaves became yellow and started folding inwards along the main rib of the leaf (Figure 4.2B and 4.2C) leaving the abaxial surface exposed. Leaf folding is a protective mechanism to decrease the leaf surface area exposed to direct sunlight although may not be sufficient enough to protect from photo-oxidative stress. The yellowing of leaves is because *X. viscosa* is a poikilochlorophyllous plant that loses its chlorophyll and dismantles its thylakoid membranes during dehydration, which further protects against photo-oxidative stress (Sherwin and Farrant, 1996; Sherwin and Farrant, 1998; Farrant *et al*., 2003; Vicrě *et al*., 2004). Loss of chlorophyll and thylakoid membranes decreases the formation of reactive oxygen species (ROS) which if left unquenched may cause considerable amounts of cellular damage (Smirnoff, 1998).

Other changes that occurred during the later stages of dehydration include darkening of leaves to a purplish colour due to an increase in anthocyanin (Figure 4.2C and 4.2D). Anthocyanin is a protective pigment molecule thought to mask chlorophyll and act as a filter to prevent excess light entering leaves guarding against photo-oxidative stress (Hopkins, 1992; Sherwin and Farrant, 1998). As the leaves became more dehydrated the abaxial surfaces became coated with a reflective sticky substance, which may serve to reduce light absorbed by the leaf.

During rehydration (Figure 4.2E and 4.2F) leaves started unfolding from the proximal end, the chlorophyll are resynthesised and thylakoid membranes are assembled as leaves become yellowish green in colour (Sherwin and Farrant, 1998; Farrant, 2000).

Resurrection plants employ a number of various strategies to stabilise their cellular milieu and protect the cell tissue during dehydration and rehydration stresses (Shinozaki and Yamaguchi-Shinozaki, 1997; Bartels and Salamini, 2001; Farrant, 2000; Farrant, 2007; Moore *et al*., 2007; Moore *et al*., 2009).

Figure 4.2 *X.viscosa* plants (biological plant A) under green house conditions during dehydration stress treatment. Image **A**, before start of dehydration stress (100% RWC). Image **B**, Day 5 of dehydration stress (79.3% RWC). Image **C**, Day 9 of dehydration stress (33.7% RWC). Image **D**, Day 15 of dehydration stress (1.5% RWC). Image **E**, 42 hours after rehydration (55.8% RWC). Image **D**, 64 hours after rehydration (70.9% RWC).

4.3.1.2 Relative water content measurements of each biological plant

The RWC measurements obtained from all 3 biological plants decreased from 100 % to 2 % during dehydration and increased back to 71 % approximately 64 hours after rehydration (Figure 4.3). RWCs calculated for each time point for each biological was averaged and RWC dehydration-rehydration curves were created for each biological. The rate of dehydration and rehydration for each biological plant exhibited similar trends of a decrease in RWC during dehydration phase and an increase during the rehydration phase.

The trends indicated in % RWC values during dehydration and rehydration in figure 4.3 are similar to those shown in previous studies where *X. viscosa* plants were also subjected to a dehydration-rehydration stress (Mowla *et al*., 2002; Garwe *et al*, 2003; Kiyaei, 2008; Bresler, 2010).

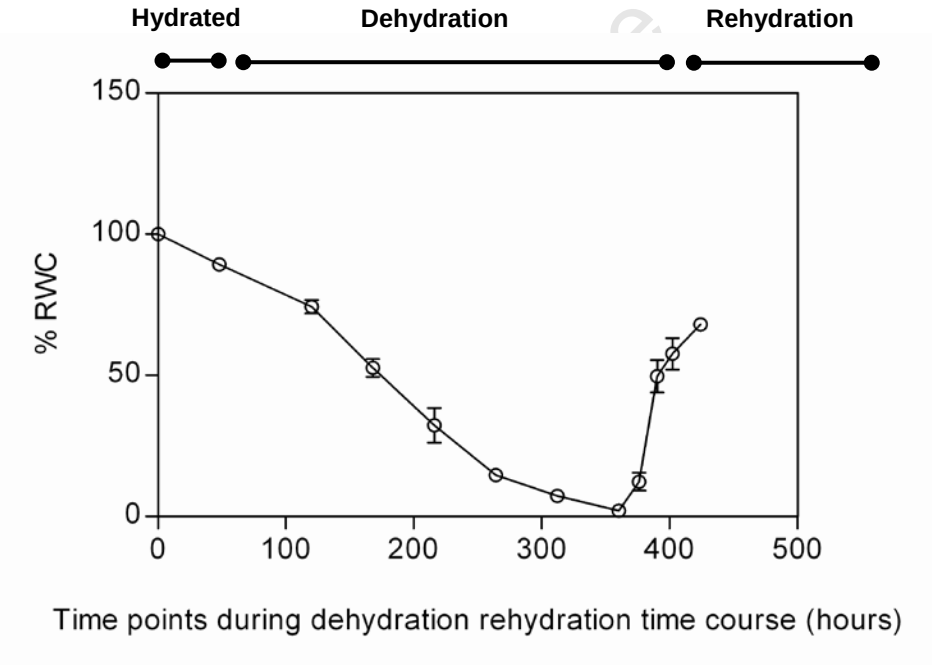


Figure 4.3 Graph showing the % RWC of all *X. viscosa* biological plants A, B and C during the dehydration - rehydration treatment (N = 3). Each sample point indicates the average % RWC calculated for each time point. Errors bars represent the SEM across all 3 biologicals. The graph and SEM values were generated using the GraphPad Prism software (Version 5).

4.3.1.3 RNA isolation and cDNA synthesis

Good quality RNA was extracted from leaf samples taken at each time point for each biological plant. The concentration of RNA extracted from each individual leaf sample ranged from 5.2 µg to 10 µg per leaf sample and little to no degradation was observed when RNA was electrophoresed on a 1 % (w/v) non-denaturing agarose/EtBr gel (data not shown). RNA integrity was also analysed by quantifying and gel electrophoresis before and after the DNAse treatment which was carried out to remove any genomic DNA contamination. The results indicated that even though the RNA concentration was less after DNAse treatment, the quality of RNA appeared to be better. The 25S and 18S ribosomal RNA (rRNA) bands were clearly visible on the gel electrophoresis image (data not shown).

Approximately 500 ng of purified RNA was used for each cDNA synthesis reaction. cDNA intergrity was analysed on a 1 % (w/v) agarose/EtBr gel. The gel electrophoresis results showed a DNA smear indicating successful cDNA synthesis (data not shown).

4.3.2 Quantitative real time PCR analysis

To study changes in *XvLOB* transcription patterns, mRNA levels were monitored using real time PCR. The expression levels of 18S rRNA, in the same cDNA samples, were used for normalisation to correct for sample-to-sample and PCR efficiency variation. The XvLOB expression levels were expressed as relative fold changes against 18S rRNA levels (GOI/HKG) using the Pfaffl method. It has previously been reported that 18S has been successfully used as the standard reference gene for qRT-PCR dehydration stress studies (Yamada H., 1997; Maredza, 2007; Mba Medie, 2007; Bresler, 2010).

The 3 individual plants used for this experiment dried down at relatively the same rate (Figure 4.3) and the change in relative expression levels remained reasonably the same across all 3 individual plants (Figure 4.4). Initially at the RWC range of 94 to 48 % there was very little change in relative expression levels. At the RWC range of 48 to 26 % there was an approximate 2.5 fold increase in transcript abundance levels ($P \leq 0.001$) which then decreased once plants reached between 19 and 13 % RWC range. However, there was an approximately 1.5 fold increase in transcript abundance levels observed between the RWC range of 2 – 1 % RWC ($P \leq 0.01$). During the rehydration phase (6 to 70 % RWC) there was

a further decrease in relative expression levels to below a 0.5 fold change in expression, similar to basal expression levels at the initial stages of dehydration.

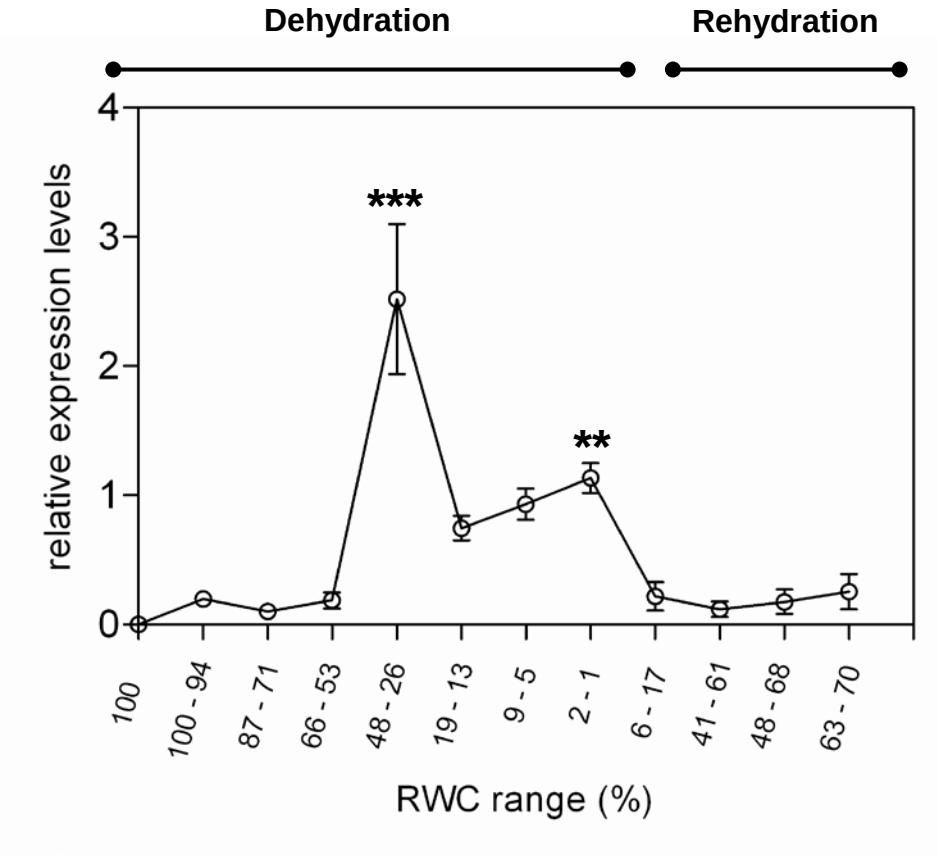


Figure 4.4 A graph showing the relative gene expression levels of *XvLOB* during the dehydration and rehydration process of *X. viscosa*. Data points represent the mean and standard error margins (SEM) of relative expression levels of *XvLOB* (N = 3, n = 9) compared to *18s* ribosomal RNA calculated by the Pfaffl method (Pfaffl MW., 2001). The change in expression levels are represented at the Y axis. The X-axis represents the ranges of % RWC across all samples. Statistical analysis was conducted using a two way ANOVA test. Statistical significance is indicated by asterisks (*). The data represented here is statistically significant at RWC ranges 48 – 26 % ($P \leq 0.001$) and 2 – 1 % ($P \leq 0.01$). Errors bars represent the SEM across all 3 biologicals. The graph and SEM values were generated using the GraphPad Prism software (Version 5).

As a positive control, *XvVTC2* primers were used on the same dehydration rehydration time course. The *XvVTC2* primer set has previously been used by Andries Bresler (Bresler, 2010)

in a similar dehydration rehydration time course and has been shown to be upregulated by at least a 1000 fold increase in expression levels. Results obtained from the *XvVTC2* primers was similar to that established by Andries Bresler (Appendix F1) suggesting the dehydration-rehydration time course, RNA extraction, cDNA conversion and real time reactions were preformed correctly.

To date there has been no evidence indicating a direct link between the expressions of *LBD* genes in response to abiotic stress such as drought. Although according to the Arabidopsis eFP browser; abiotic stress such as cold (4 °C) and high osmoticum (300 mM mannitol) has an effect on the fold change in expression of *AtLBD39* (the closet homologue to *XvLOB*) of 1.73 and 1.3, respectively. The high osmoticum stress can be seen as a dehydration stress as the amount of available water molecules becomes limited once saturated with mannitol.

Rehydration is the period in which the repair of any physical damage incurred during dehydration takes place (Sherwin and Farrant, 1996). It may be that during the dehydration process, the plant increases *XvLOB* transcript levels such that in case of severe vegetative tissue damage there is an abundance of transcripts ready to be translated into protein which will activate the initiation of cell division in existing meristems and/or new lateral primordia during rehydration. The accumulation of transcripts during dehydration and translated during rehydration has been observed for other resurrection plants such as *Xerophyta humilis* (Dace *et al.*, 1998)

4.3.3 Western blot analysis

Western blot analysis was performed to determine whether the regulation of *XvLOB* mRNA transcripts resulted in XvLOB protein accumulation. Total crude protein was extracted from leave tissue for each time point from each biological plant and separated by SDS-PAGE. Western blot analysis was carried out using XvLOB specific polyclonal antibodies generated in rabbits to probe for XvLOB (see section 3.2.3.3).

Recombinant XvLOB was loaded as a positive control and can be seen in figure 4.5 and 4.6, lane 1 (indicated with red arrow). To establish the appropriate XvLOB antibody dilution and recombinant XvLOB protein concentrations to use; western blot optimisation was carried out

using different antibody dilutions ranging from 1:10 000 to 1:500 and recombinant XvLOB concentrations ranging from 450 ng to 45 ng (data not shown). Based on optimisation a 1:500 XvLOB antibody dilution and 400 ng recombinant XvLOB concentrations was selected for further western analysis on dehydration-rehydration samples.

Equal loading of total crude protein for each time point was successfully achieved and can be seen in figures 4.5b and 4.6b. These membranes were stained with ponceau S to temporarily stain all protein that has been transferred onto the membrane.

The chemiluminescence was detected anti-rabbit IgG peroxidase conjugate antibodies. In figures 4.5 and 4.6 there was no XvLOB detected neither in any of the dehydrated samples (Figure 4.5c, lanes 2 to 9) nor in the rehydrated samples (Figure 4.6c, lanes 2 to 9) except the positive control (Figure 4.5c and 4.6c, lanes 1). These results indicate that no XvLOB protein was detectable by western blotting during the dehydration or rehydration time course of *X. viscosa* biological plants A, B and C.

The discrepancy between results obtained from realtime PCR and western blot analysis is not uncommon as studies involving yeast and a number of higher eukaryotic species including alfalfa, mammalians, *Plasmodium falciparum* and *Arabidopsis* have showed that in some cases there is no direct correlation between mRNA and protein levels (Jonak *et al*., 1996; Anderson and Seilhamer, 1997; Gygi *et al*., 1999; Connolly *et al*., 2002; Greenbaum *et al*., 2003; Le Roch *et al*., 2004;). An indirect correlation between mRNA and protein levels may occur due to a number of different post transcriptional regulation points such as mRNA turn over, translation initiation, elongation and termination as well as protein degradation (de Sousa Abreu *et al*., 2009).

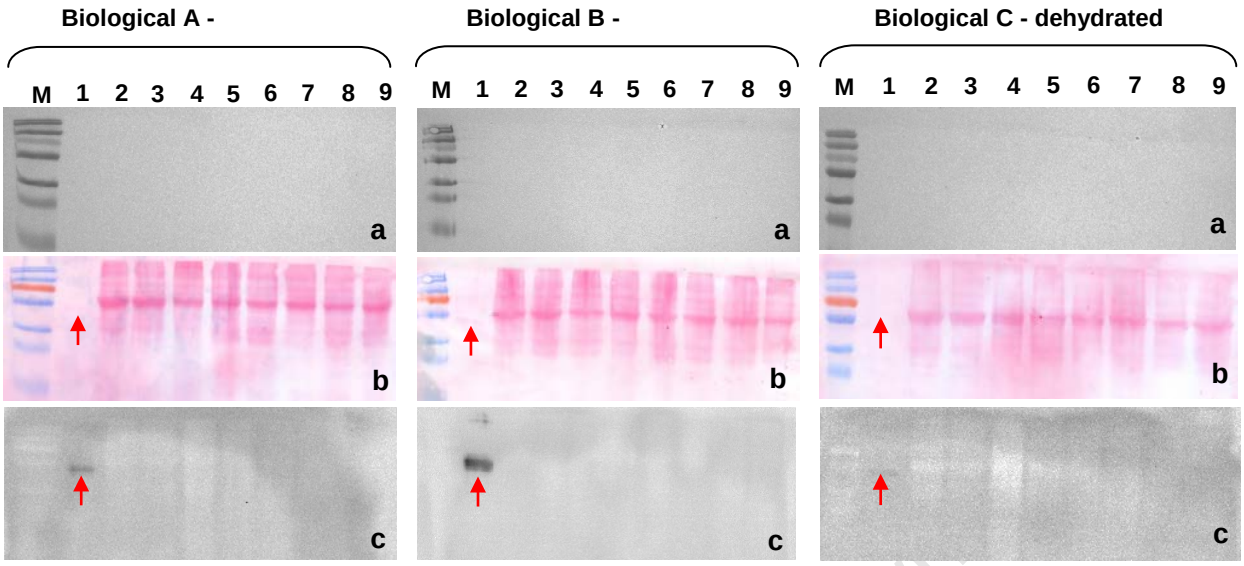


Figure 4.5 Western blot analysis of XvLOB accumulation during dehydration of *X. viscosa* biological plants A, B and C. Image **a**: nitrocellulose membrane after transfer of 40 µg total crude proteins from SDS-PAGE gel onto membrane, successful transfer is indicated by the presence of the marker on left hand side of image. Image **b**: ponceau S stained nitrocellulose membrane. Image **c**: chemiluminescence image taken from nitrocellulose membrane using the ChemiDoc molecular imager where polyclonal XvLOB antibodies were used to probe for XvLOB. Lane **1**, 400 ng recombinant XvLOB protein. Lanes 2 to 9 each contains 40 µg of total crude protein extract for different time points. Lane **2**, day 0. Lane **3**, day 3. Lane **4**, day 5. Lane **5**, day 7. Lane **6**, day 9. Lane **7**, day 11. Lane **8**, day 13. Lane **9**, day 15. Lane **M**, 5 µl prestained protein ladder (Fermentas). Red arrows indicate recombinant XvLOB protein.

An understanding of how protein abundance is related to mRNA transcript levels is essential for interpreting gene expression (Hatzimanikatis *et al*., 1999). It could be speculated that perhaps accumulation of XvLOB protein levels only occurs once the plant has progressed further along the rehydration pathway and re-establishment of the meristem activity and production of new primordia is required. Furthermore, it is possible that the region in which translation occurred was localised only in the meristem tissues, these being so small that it could not be detected in a total protein extract from an entire leaf blade. Another possibility may be that there was a delay between transcription and translation. Future investigations should include western blot analysis on isolated meristems of samples and these be extracted further along during the rehydration process to establish whether XvLOB protein levels do in

fact accumulate at a later stage. Immunogold localisation of proteins using transmission electron microscopy could also facilitate determination of whether proteins are present, where they occur and the timing of expression.

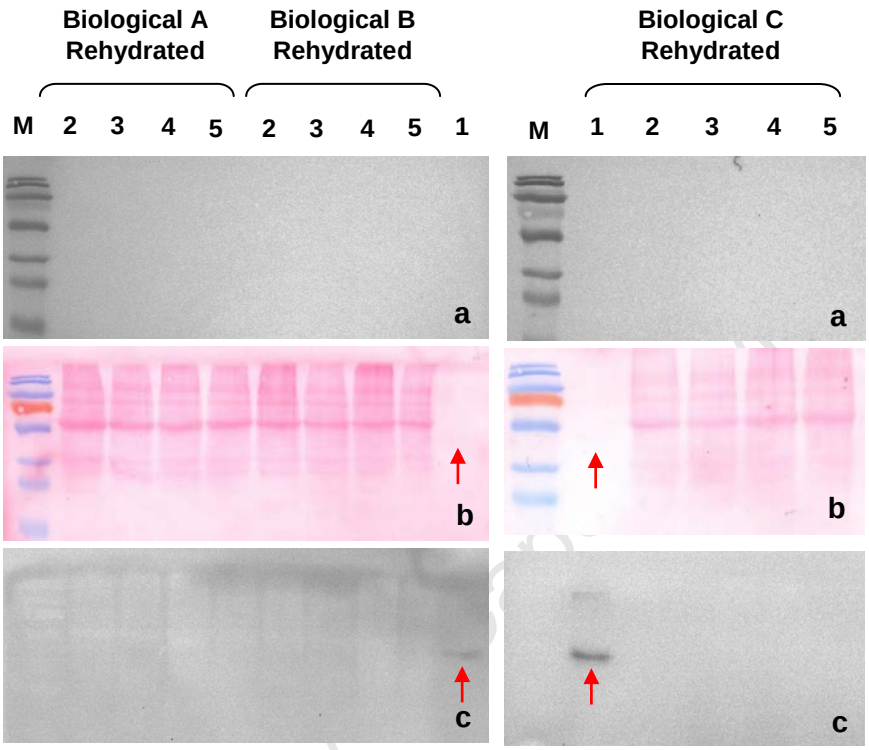


Figure 4.6 Western blot analysis of XvLOB accumulation during rehydration of *X. viscosa* biological plants A, B and C. Image **a**: nitrocellulose membrane after transfer of 40 µg total crude proteins from SDS-PAGE gel onto membrane, successful transfer is indicated by the presence of the marker on left hand side of image. Image **b**: ponceau S stained nitrocellulose membrane. Image **c**: chemiluminescence image taken from nitrocellulose membrane using the ChemiDoc molecular imager where polyclonal XvLOB antibodies were used to probe for XvLOB. Lane **1**, 400 ng recombinant XvLOB protein. Lanes 2 to 9 each contains 40 µg of total crude protein extract for different time points. Lane **2**, day 0. Lane **3**, day 3. Lane **4**, day 5. Lane **5**, day 7. Lane **6**, day 9. Lane **7**, day 11. Lane **8**, day 13. Lane **9**, day 15. Lane **M**, 5 µl prestained protein ladder (Fermentas). Red arrows indicate recombinant XvLOB protein.

4.4 CONCLUSION

In this chapter, the response of *XvLOB* to a dehydration and rehydration treatment was investigated by analysing both the transcript and protein levels. Results showed a transient increase in transcript levels during the later stages (48 - 26 % RWC) of the dehydration process. There was no detection of XvLOB protein across dehydration and rehydration treatment and thus no direct correlation between transcript levels and that of protein accumulation could be made. There are a number of regulatory points between transcription and translation which needs to be investigated further in order to understand the reasons for abundance in transcript levels and not in protein levels.

CHAPTER 5

XvLOB subcellular localisation

5.1 INTRODUCTION

The highly dynamic processes which regulate cell function are carried out by a diverse number of proteins each located within specific subcellular compartments, during specific times and under certain environmental conditions. It is therefore imperative to establish protein properties within the temporal and spatial context of a cell. An important step in understanding protein function includes the prediction and determination of its intracellular localisation. Predicting localisation based on DNA sequence alone can be useful but not very conclusive. Fluorescence microscopy together with the advent of green fluorescent proteins (GFP) has become one of the most widely used technologies for establishing protein localisation (Bastiaens and Pepperkok, 2000; Blancaflor and Gilroy, 2000; Gonzalez and Bejarano, 2000).

GFP from the jellyfish *Aequorea victoria*, along with its spectral variants namely; cyan (CFP), yellow (YFP) and blue (BFP) fluorescent proteins are fully genetically encoded proteins and can be used as *in vivo* fluorescent markers without the need for exogenous co-factors. A red fluorescent protein known as DsRED (drFP583) from the corallimorpharian *Discosoma* sp. has also been isolated and has become the subject of various biochemical studies in plants (Matz *et al*., 1999; Jach *et al*., 2001). One of the many advantages of this technology is the ability to fuse the GFP coding sequence with that of a target gene cDNA allowing for the monitoring of transiently expressed fusion proteins within intact living cells. Other advantages of GFP fusion constructs include the ability to track protein mobility intracellularly, observing protein-protein interactions, promoter analysis as well as the ability to simultaneously examine localisation of more than one protein with the usage of different spectral variants (Haselhoff, 1999; Hanson *et al*., 2001; Haraguchi, 2002; Chudakov *et al*., 2005). For this study YFP which is a brighter spectrum shifted variant of GFP was used. It has excitation maximum and emission peaks at 513 nm and 527 nm (in the yellow-green region), respectively (Tsien, 1998).

Biolistic technology has become one of the most powerful techniques used to analyse the transient expression of foreign genes in fully developed intact plant cells. It is a simple mechanical operation which bypasses host specificity limitations which other DNA delivery methods impose such as *Agrobacterium*-mediated transformation (Gray and Finer, 1993; Buckley *et al*., 1995). Implanting DNA through particle bombardment was first developed and described by Sanford et al (1987) and involves the acceleration of DNA coated heavy metal particles (either tungsten or gold) at high velocities through cell membranes. Here we will use gold particles as they are biologically inert and therefore do not degrade DNA nor harm cells. A commercially available apparatus namely the Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Germany) which uses pressurized helium together with rupture discs and macrocarriers was used (Kikkert, 1993).

In this chapter the localisation of XvLOB was achieved by bombarding the p35S::*XvLOB*::YFP::NosT construct into onion (*Allium cepa*) epidermal cells using the Biolistic® PDS-1000/He system which has previously been described by Scott et al (1999). The onion epidermis is a monolayer of large, transparent cells which makes them perfect for visualizing fluorescence localisation (Scott *et al*., 1999).

5.2 MATERIALS AND METHODS

The localisation vector used in this study was created and kindly donated by Seidal et al (2005). In brief, the construct was created using pEYFP (Clontech, USA) as the backbone. The pEYFP is an enhanced variant of GFP which contains four amino acid substitutions and when excited at 513 nm, E_M of EYFP is 36,500 $cm^{-}1.M^{-1}$ and the fluorescent quantum yield is 0.63 (Ormö *et al*., 1996). The cauliflower mosaic virus promoter (CaMV 35S) region was used to drive expression of fusion proteins. Expression driven by this promoter region results in high levels of expression as well as a stronger fluorescence signal without affecting the intracellular localisation of the protein of interest as abnormally high levels of expression may lead to artefactual localisation (Tian *et al*., 2004). This promoter region was released from the rsGFP-vector and ligated into the 3' multiple cloning site of pEYFP using restriction sites *Bam*HI and *Hind*III. The Nos-terminator was amplified using a forward primer with restriction site *Not*I incorporated into it and the reverse primer carrying an endogenous site *Eco*RI of Nos-terminator. The pEYFP vector and insert were both digested with *Not*I and *Eco*RI and the insert ligated into the 5' multiple cloning site of pEYFP to generate the p35S::EYFP::NosT construct (Appendix D2). As a control, p35S::EYFP::NosT was used to determine independent YFP subcellular localisation.

5.2.1 Cloning of *XvLOB* into p35S::EYFP::NosT construct

5.2.1.1 Amplification and restriction digest of *XvLOB* fragment

XvLOB was amplified from pDNR-LIB::*XvLOB* (see Chapter 2, 2.2.1) using gene specific primers XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10). The forward and reverse primers have restriction enzyme sites *Bam*HI and *Kpn*I respectively incorporated into the 5' end of the primer. A 25 µl PCR reaction was set up (standard PCR reaction, Appendix A1) with the following cycling conditions: 94ºC for 5 min; 25 cycles of 94ºC for 30 s, 53ºC for 40 s, 72ºC for 2 min; and a final elongation step of 72ºC for 5 min was performed. High fidelity Taq polymerase (Expand High Fidelity PCR System, Roche) was used as this enzyme has proofreading activity which decreases the amount of errors incorporated during PCR amplification. PCR products were electrophoresed on a 1.2 % agarose/EtBr gel after which the band of interest was excised and purified using the Biospin Gel Extraction Kit (BioFlux, Japan) according manufacturer's instructions (Appendix A7).

5.2.1.2 p35S::EYFP::NosT and *XvLOB* digest and ligation

One microgram of vector and insert were digested in a total reaction volume of 30 µl. Restriction was performed with 20 U of each *Bam*HI and *Kpn*I restriction enzymes (New England Biolabs, UK) and 3 µl of 10X BamHI Buffer (Fermentas). Reaction mixture was gently mixed, briefly centrifuged and then incubated overnight at 37ºC in a water bath. Vector and insert purification was performed using the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Canada) according to manufacturer's instructions (Appendix A6). A standard ligation reaction (Appendix A2) was set up using a vector to insert ratio of 1:5 with 50 ng of digested p35S::EYFP::NosT and 36 ng of *XvLOB*. Ligation reagents were mixed well and centrifuged briefly and incubated at 4ºC overnight.

5.2.1.3 Transformation of p35S::*XvLOB*::EYFP::NosT, plasmid screening and extraction

Ten microlitres of ligation reaction was added to 50 µl competent *E. coli* DH5α cells using standard transformation conditions (Appendix A4). Colony PCR was performed using gene specific primers XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10) to select for positive clones. The bacterial cells from positive clones were inoculated into 5 ml Luria Broth (LB) supplemented with 100 µg/ml ampicillin for plasmid extraction. Recombinant p35S::*XvLOB*::EYFP::NosT was isolated using Qiagen Plasmid Midi Kit (USA) according manufacturer's instructions (Appendix A5). The reconstructed vector was then verified by PCR using XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10), restriction digestion with *Bam*HI and *Kpn*I, and DNA sequencing. Plasmid samples were stored at -20ºC until further use.

5.2.2 p35S::*XvLOB*::EYFP::NosT transformation into onion epidermal cells

5.2.2.1 Gold particle preparation and precipitation

All procedures were performed within a laminar flow and sterile techniques were observed at all times. Gold particle preparation and precipitation was performed as described by Kikkert JR (1993) as well as the Biolistic® PDS-1000/He Particle Delivery System manual (Bio-Rad Laboratories, Germany) with modifications. One millilitre of absolute ethanol was added to 60 mg (1 µm) of gold particles, vortexed for 1 min and then pelleted by brief centrifugation for 10 s. The supernatant was discarded and the above sterilization step repeated 4 times. The pelleted gold particles were then washed with 1 ml sterile distilled

water by vortexing for 1 min and particles pelleted as described in the ethanol wash steps. This wash step was repeated 8 times and then gold particles resuspended in 1 ml of 50 % glycerol. The resuspended gold particles were kept shaking on a nutator so as to prevent coagulation whilst 50 µl aliquots were prepared and stored at room temperature.

The tubes containing 3 mg of previously prepared gold particles were placed on a nutator and kept shaking while 5 µg of either p35S::EYFP::NosT or p35S::*XvLOB*::EYFP::NosT construct was added and continued shaking. Fifty microlitres of 2.5M $CaCl_2$ was added and allowed to mix for 30 s. While shaking, 20 µl of 0.1M spermidine was added and vortexed at the highest speed for 3 min. Three quick centrifuge pulses were used to pellet gold particles and the supernatant was carefully removed so as not to disturb the pellet and discarded. A final wash step was done by adding 250 µl of absolute ethanol, mixed for 1 min and then pelleted by pulsing briefly 3 times. The supernatant was discarded and gold particles resuspended in 75 µl of absolute ethanol.

Macrocarrier discs were placed into macrocarrier holders and the holders placed into Petri dishes. Nine and a half of microlitres of DNA coated gold particles was added to the centre of the macrocarrier discs and allowed to air dry for 30 min. The rupture discs of (1100 psi) were dipped into isopropanol and allowed to air dry for 20-30 minutes.

5.2.2.2 Particle bombardment of onion cells

The onions (*Allium cepa*) were peeled and individual layers separated from each other. The layers were then cut into small rectangular segments and each segment placed into a Petri dish. For each construct, 3 segments were used with each segment receiving 2 bombardments. The bombardment procedure was performed as described by Scott et al (1999) using the Biolistics® PDS-1000/He (Bio-Rad Laboratories, Germany) system with 1100 psi rupture discs and 27 inches Hg. Onion segments were incubated at 25ºC for 24 hours before viewing.

5.2.2.3 Preparation of cells and fluorescence microscopic analysis

After bombardment and incubation for 24 hours, the top epidermal layer of each onion segment was cut in a rectangular shape, carefully removed and placed onto a slide which contained a drop or sterile distilled water. One hundred microlitres of an Aniline Blue-

Hoechst combination stain (Appendix B10) was added on top of the epidermal layer and the slide was left to incubate in the dark for 20 min before viewing. The Hoechst stain is used as a DNA fluorochrome (Thakur *et al.*, 2003) whereas Aniline Blue stains the cell membranes (Majewska-Sawka *et al*., 2002). The transformed cells were viewed using a confocal laser scanning microscope (Zeiss LSM 510 META, Germany) with a 514 nm (yellow spectrum) Argon laser. To view the Aniline Blue-Hoechst stained organelles the conventional HBO light (epifluorescence) was used. To view epidermal monolayer cells images were captured under brightfield light. Images were captured using the Zeiss LSM core software (Zeiss, Germany) available and for post-acquisition image processing the Axiovision 4.7, Photoshop (Zeiss, Germany) was used. Images containing either epiflourescence or laser fluorescence were individually captured as well as an overlap of all different images. The Zeiss LSM software was used to include the appropriate scale bars to all images.

5.3 RESULTS AND DISCUSSION

5.3.1 Cloning of *XvLOB* into p35S::EYFP::NosT construct

Amplification of *XvLOB* from pDNR-LIB::*XvLOB* was successful and can be seen in figure 5.1 lane 2. A clear band of ca. 650 bp can be seen without non-specific amplification. As a positive PCR control; PCR reaction using gene specific primers from the protein expression study (see section 3.2.1.1) was used and appeared to be successful (Figure 5.1, lane 3). As a negative control (no template control – NTC); a PCR reaction was set up including all PCR components except the pDNR-LIB::*XvLOB* template (Figure 5.1, lane 1).

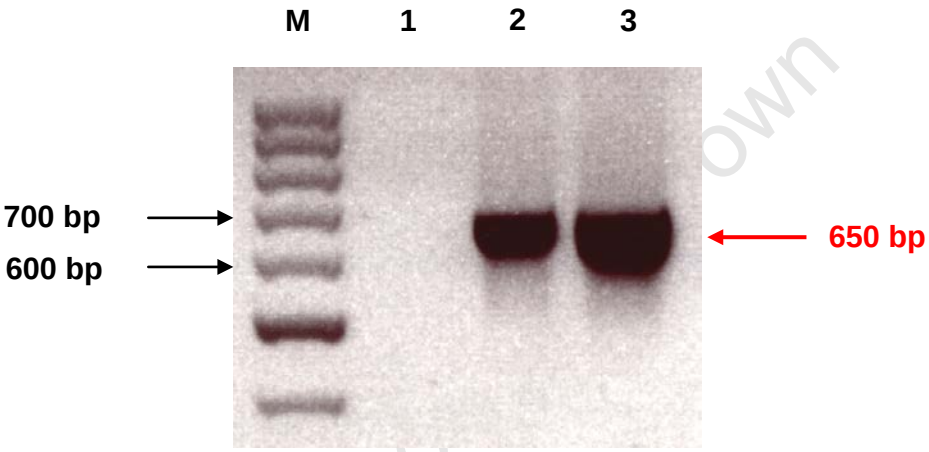


Figure 5.1 1.2 % (w/v) Agarose/EtBr gel electrophoresis of *XvLOB* PCR reactions. Lane 1 - negative PCR control (NTC). Lane 2 - XvLOB fragment amplified from pDNR-LIB::*XvLOB* using protein expression primers (positive PCR control). Lane 3 - *XvLOB* fragment amplified from pDNR-LIB::*XvLOB* using localisation primers. M - 100 bp DNA ladder (NEB). Red arrow on the RHS indicates *XvLOB* amplicon.

The band of interest was successfully excised and ligated into the linearised localisation construct p35S::EYFP::NosT. Colony PCR confirmed that 8 out of every 10 colonies screened were positive for the p35S::*XvLOB*::EYFP:NosT construct (data not shown).

An enzyme digest confirmation of 3 isolated p35S::*XvLOB*::EYFP::NosT clones (1, 2 and 3) can be seen in figure 5.2. Agarose gel electrophoresis of digest reactions indicated the release of ca. 650 bp (*XvLOB*) fragment. This can be seen in lanes 2, 4 and 6 (Figure 5.2). Colony PCR, digest results and sequencing results (data not shown) indicate successful ligation of

XvLOB into p35S::EYFP::NosT and reconstruction of the appropriate restriction sites (*Bam*HI and *Kpn*I).

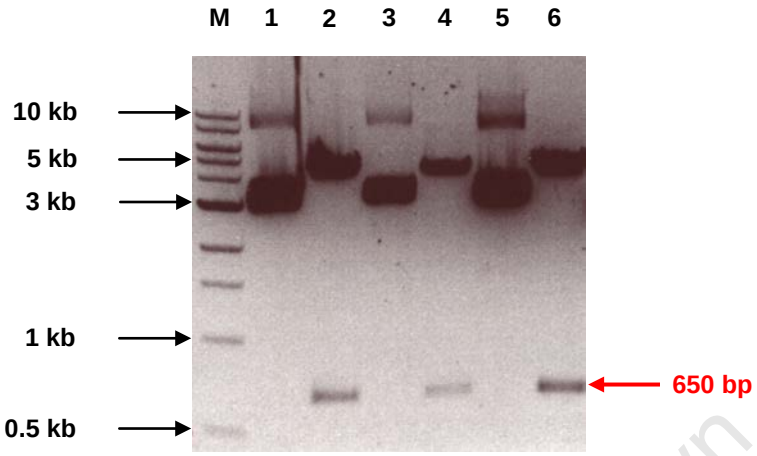


Figure 5.2 1.2 % (w/v) Agarose/EtBr gel electrophoresis of 3 undigested and digested p35S::*XvLOB*::EYFP:NosT clones. Lanes 1, 3 and 5 - undigested p35S::*XvLOB*::EYFP::NosT clones 1, 2 and 3 respectively. Lanes 2, 4 and 6 - p35S::*XvLOB*::EYFP:NosT clones 2, 4 and 6 respectively digested with *Bam*HI and *Kpn*I. M - 1 kb DNA ladder (NEB). Red arrow on the RHS indicates the released *XvLOB* fragment.

The amplification of XvLOB using specific primers XvLOB-*Bam*HI F (Appendix C, C1.9) and XvLOB-*Kpn*I R (Appendix C, C1.10) from p35S::*XvLOB*::EYFP:NosT clones 1, 2 and 3 also indicated an amplification of an approximate 650 bp fragment (data not shown). Plasmid DNA isolation of the 3 clones was successful with high yields of 24, 10 and 37 µg/µl and $A_{260/280}$ ratios of 1.91, 1.83 and 1.92, respectively.

5.3.2 Localisation of p35S::*XvLOB*::EYFP::NosT in onion epidermal cells

The p35S::*XvLOB*::EYFP::NosT and p35S::EYFP::NosT constructs were transformed into onion epidermal cells rather than *X. viscosa* leaf tissue because *X. viscosa* leaf tissue is tough, hardy and not flexible for manipulation. The p35S::EYFP::NosT construct was used as a control and transformed into the onion epidermal cells to check unfused YFP fluorescence and independent localisation (Figure 5.3). The *XvLOB* cDNA was fused to the 5' end of EYFP gene sequence, transformed into onion epidermal cells and then viewed using confocal laser scanning microscopy (Figure 5.4). Computational localisation prediction analysis revealed that the XvLOB protein sequence did not contain any localisation signals (see

section 2.3.2). Unfused YFP has a molecular weight of 25 kDa. The theoretically calculated molecular weight of XvLOB-YFP fusion protein is 47.8 kDa.

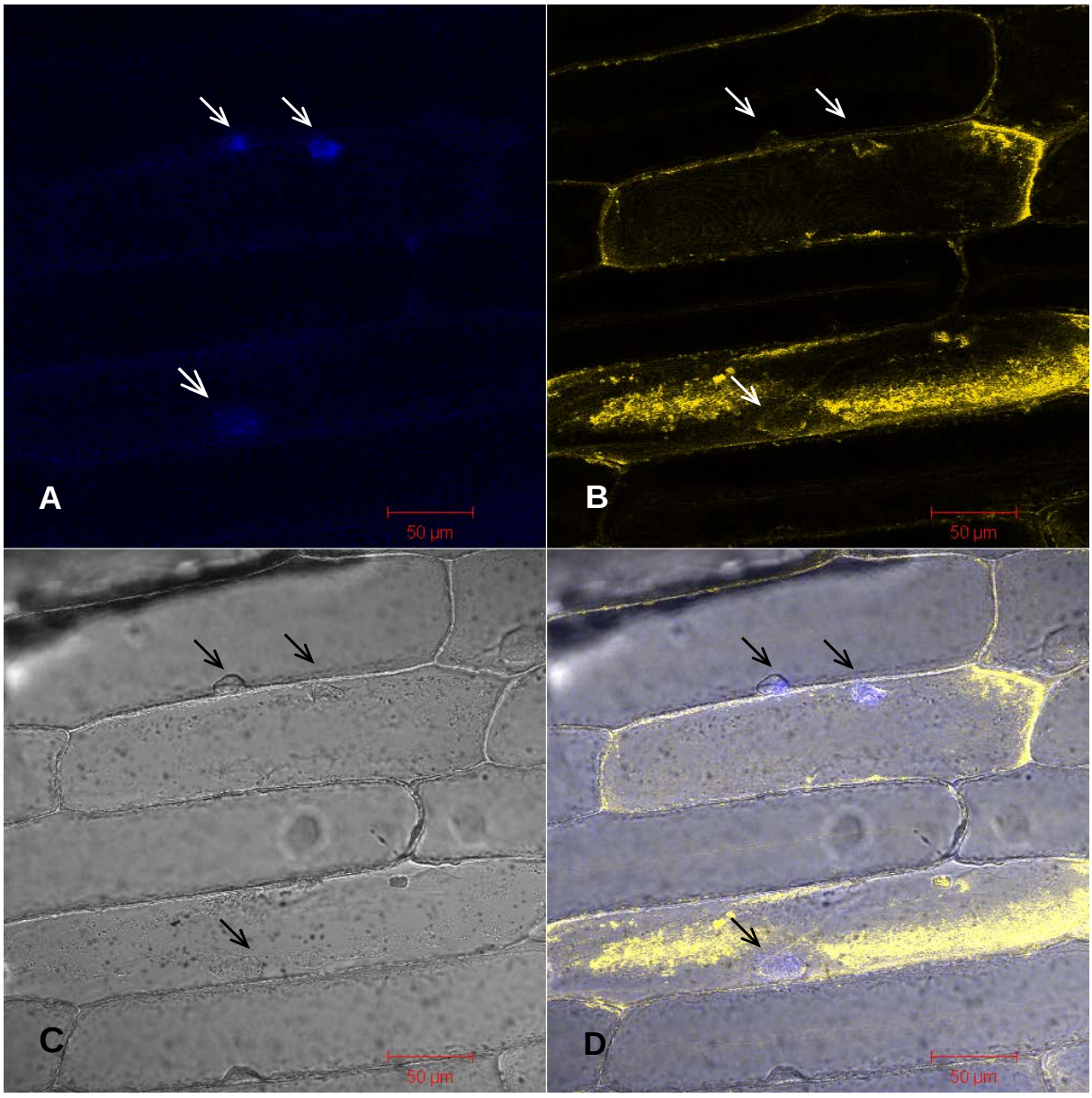


Figure 5.3 Subcellular localisation of unfused YFP protein viewed in onion epidermal cells. **A** - cells indicating Aniline Blue-Hoechst stained nucleus. **B** - subcellular distribution of unfused YFP. **C** - view of cells under brightfield light. **D** - overlay image combining A, B and C. The cells were incubated at 27°C for 24 hours, stained and then viewed and captured using the ZEISS LSM 520 META confocal laser scanning microscope. Arrows indicate nucleus. Scale bars represent 50µm.

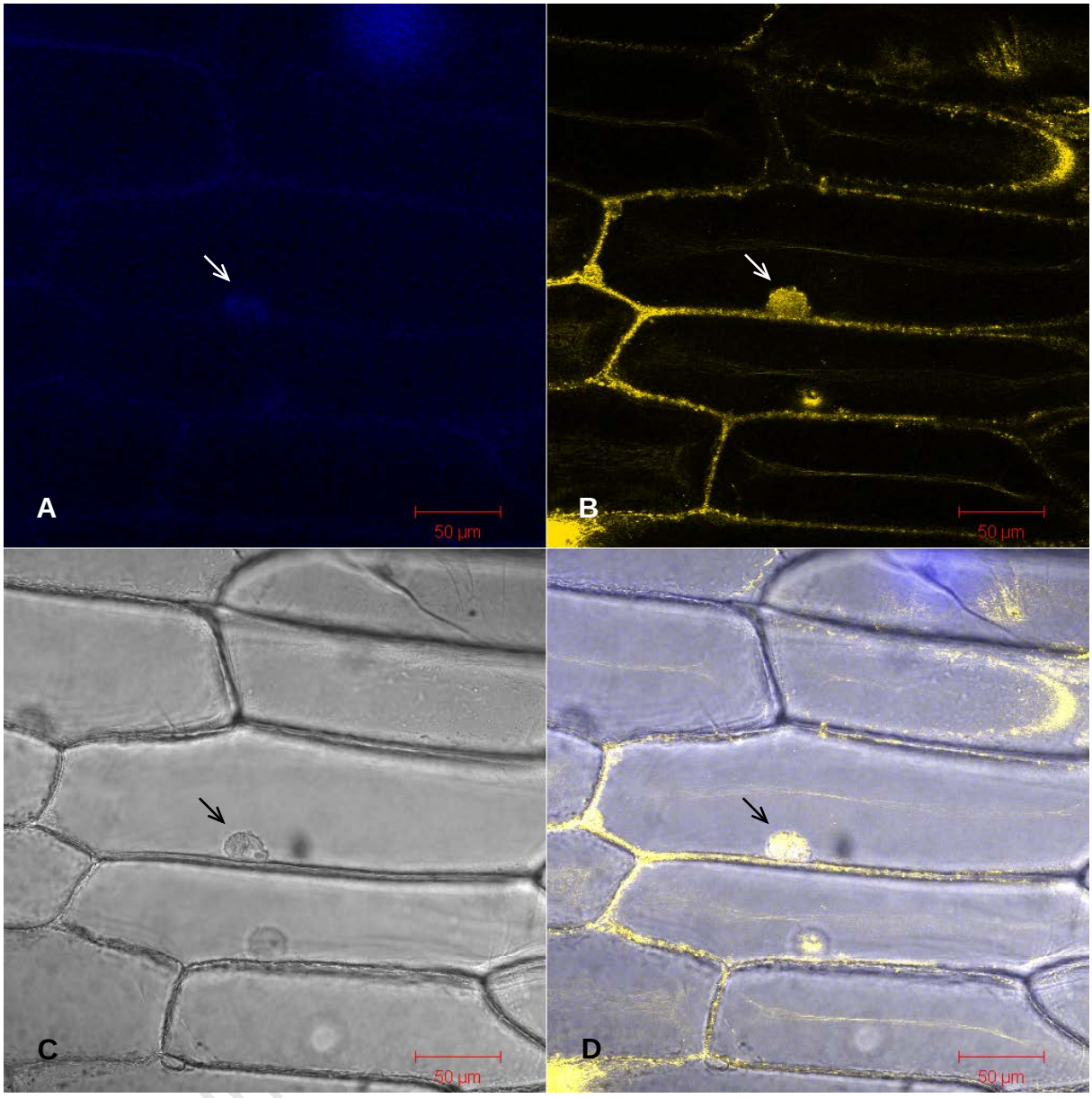


Figure 5.4 Subcellular localisation of *XvLOB*::YFP fusion protein viewed in onion epidermal cells. **A** - cells indicating Aniline Blue-Hoechst stained nucleus. **B** - subcellular distribution of *XvLOB*::YFP fusion protein. **C** - view of cells under brightfield light. **D** - overlay image combining A, B and C. The cells were incubated at 27°C for 24 hours, stained and then viewed and captured using the ZEISS LSM 520 META confocal laser scanning microscope. Arrows indicate nucleus. Scale bars represent 50µm.

According to Allen et al (2000) proteins smaller than 60 kDa are able to passively diffuse through the nuclear pore complex and enter the nucleus. Therefore, in theory both unfused

YFP as well as XvLOB-YFP fusion protein are able to enter the nucleus passively without any nuclear localisation signals (NLS).

During experimental analysis, unfused YFP was shown to be localised to the cytoplasm with no localisation in the nucleus (Figure 5.3, B and D). However when YFP is fused to XvLOB the fusion protein is targeted to the nucleus (Figure 5.4, B and D). These results indicate that the XvLOB protein is actively being targeted to the nucleus. To investigate whether nuclear localisation was indeed observed, all onion epidermal cells were stained with Aniline Blue-Hoechst stain to identify the nucleus (Figure 5.3 and 5.4, A and D). The *XvLOB*::EYFP fusion protein showed nuclear localisation when expressed in onion epidermal cells (Figure 5.4 B).

In *Arabidopsis*, subcellular localisation of the Arabidopsis LOB (AtLOB) protein was determined by Husbands et al in 2007. The AtLOB was fused to the GFP protein and placed under the control of the ubiquitously expressed Cauliflower Mosaic Virus 35S promoter. This construct was then transformed into wild-type *Arabidopsis* plants and fluorescence was observed in the roots of 28 transgenic plants. Results obtained from these experiments revealed fluorescence of the AtLOB-GFP fusion protein in the nucleus.

The *adventitious rootless 1* gene (*ARL1*) in rice (*Oryza sativa* L.) encodes a protein which contains a LOB domain. Subcellular localisation studies of the ARL1 protein involved the transformation of onion epidermal cells with an ARL1-GFP fusion construct. Fluorescence of the ARL1-GFP fusion protein also indicated nuclear localisation (Liu *et al*., 2005).

Bioinformatic analysis of the XvLOB amino acid sequence suggests that XvLOB does not contain a NLS (see chapter 2.3.2) yet localisation studies indicate otherwise. According to Fagotto et al (1998), the β-catenin protein found in *Xenopus* is able to locate to the nucleus without the presence of a NLS. A review by Boilikas (1993) involving characterisation of transcription factors across all eukaryotic species including yeast, plants, mammals and flies suggests that transcription factors lacking a NLS may enter the nucleus via strong cross complex formation with other nuclear proteins containing a NLS. According to bioinformatic analysis (see section 2.3.2) of the above mentioned proteins (AtLOB in *Arabidopsis* and

ARL1 in *rice*) which have been shown to localise to the nucleus, do not contain any known NLS signals.

5.3 CONCLUSION

Fusion of XvLOB to the YFP protein was successful and indicated that subcellular localisation of XvLOB is to the nucleus. The results obtained in this chapter are similar to the studies done by Husbands et al (2007) and Liu et al (2005) which also found LBD containing proteins localised to the nucleus and suggests a predictive role of XvLOB as a transcription factor as shown for these LBD proteins. However in order to prove that XvLOB is a transcription factor, studies involving identification of a DNA sequence motif bound by LOB proteins need to be carried such as selection and amplification binding assays (SAAB) and electrophoretic mobility shift assays.

CHAPTER 6

Conclusion

The aim of this dissertation is to investigate the expression of the *XvLOB* gene in response to a dehydration stress treatment with the intention of elucidating its possible role in desiccation tolerance in *X. viscosa*. Molecular characterization of the *XvLOB* gene involved *in silico* analysis, recombinant expression and subcellular localization of the XvLOB protein as well as examining its response to dehydration at both the transcript (mRNA) and protein levels.

DNA sequence analysis indicated that the full length *XvLOB* gene sequence contained an intron of approximately 98 bp. The XvLOB amino acid sequence contained a highly conserved domain identified as the Lateral Organ Boundaries (LOB) domain, a plant specific domain, which suggests it may be involved in a process that is unique to plants. The XvLOB LOB domain contained both the C and the GAS motifs that are characteristic features of the LOB domain (Shuai *et al*., 2002). According to bioinformatic and sequence alignment results, the XvLOB amino acid sequence was found to be significantly similar to the *Arabidopsis* Class II homologous group of LBD genes especially AtLBD39. *In silico* analysis identified 14 predicted phosphorylation sites within the XvLOB amino acid sequence. Online prediction software predicted nuclear localization of the XvLOB protein even though it did not contain any known plant nuclear localization signals.

Recombinant XvLOB was successfully expressed in *E.coli* BL21 (DE3) cells. The expression of an adequate amount of soluble recombinant XvLOB was not achieved even though a vector containing the Trx-S-His tag was used. A greater yield of protein was expressed and purified from the insoluble fractions than the soluble fractions. Consequently the insoluble protein was used for antibody generation. Future studies regarding XvLOB protein expression requires significant optimization of the soluble protein expression and extraction methods, including the use of other expression vectors with alternate tags that aid in solubilisation.

Subcellular localization of the XvLOB::EYFP fusion protein indicated localization to the nucleus albeit the lack of an NLS. This result was predicted by *in silico* analysis as well as by previous studies involving the localization of other LBD proteins to the nucleus (Liu *et al*., 2005; Husbands *et al*., 2007; Rubin *et al*., 2009). The role which LBD proteins may play within plant nuclei is yet to be established. However; nuclear localization, identification of a consensus DNA sequence which binds LBD proteins as well as bioinformatic analysis predicting the formation of leucine zipper like structures provides evidence that supports a hypothesized role of LBD proteins acting as transcription factors (Shuai *et al*., 2002; Xu *et al*., 2003; Husbands *et al*., 2007; Okushima *et al*., 2007; Lee *et al*., 2009). For future studies regarding localization, it would be interesting to determine precisely where in the SAM, XvLOB is expressed during the formation of new lateral primordia. This could be achieved by examining the expression of XvLOB fused to either GUS or luciferase in tissue specific regions. The results would allow us to establish whether the expression of XvLOB is involved in establishing the polarity of leave primordia similar to the LBD genes AS1 and AS2 (Iwakawa *et al*., 2007; Xu *et al*., 2003). The generation of transgenic lines expressing an XvLOB::EGFP fusion protein in *Arabidopsis* and tobacco would provide further evidence of nuclear localization of XvLOB. The use of realtime quantitative PCR can also be performed on mRNA extracted from different areas of SAM tissue to establish precise location of *XvLOB* transcript expression.

During the dehydration treatment, quantitative analysis of the *XvLOB* mRNA levels showed a significant increase between 48 – 26 % RWC ($P \leq 0.001$) and between 2 – 1 % RWC ($P \leq 0.01$). This response is observed relatively late during the dehydration process with transcript levels dropping back to basal levels upon rehydration. Western blot analysis showed that there was no increase in the corresponding protein levels throughout the dehydration and rehydration stress treatment. Thus there was no correlation between the protein expression levels and the expression of *XvLOB* transcript abundance. A previous study analyzing both mRNA and protein levels during dehydration in the resurrection plant *Xerophyta humilis* has been investigated (Dace *et al*., 1998). In this study it was suggested that during the dehydration phase protective mechanisms are activated, some of which includes the accumulation of transcripts without them being translated. These transcripts are accumulated such that during rehydration, the recovery process is virtually independent of *de novo*

transcription of nuclear genes. It could be speculated that during rehydration, *X. viscosa* translates the *XvLOB* gene if there is a need for more vegetative tissue.

It is well known that nitrogen status is sensed by plants to regulate development, physiology, growth and metabolism. Nitrogen forms part of a number of different amino acids and proteins. In 2009, Rubin et al identified 3 *LBD* genes in *Arabidopsis* (*LBD37*, *LBD38* and *LBD39*) which were shown to be upregulated in the presence of nitrogen. The addition of 3mM KNO_3 to nitrogen depleted *Arabidopsis* seedlings showed a significant (≥ 20 fold) increase in expression levels of these genes but not with the addition of 3mM KCl. A phenotypical pigmentation change was observed in both overexpressed and knock out LBD37, LBD38 and LBD39 mutant transgenic *Arabidopsis* plants. This result encouraged an investigation into the regulatory control *LBD* genes may have on the anthocyanin biosynthesis pathway. Anthocyanin production metabolites were examined in both LBD overexpression and *LBD* mutant transgenic seedlings. Results obtained indicated the repression of *PRODUCTION OF ANTHOCYANIN PIGMENT1* and *2* genes (*PAP1* and *PAP2*). These results suggest that *LBD37*, *LBD38* and *LBD39* are induced by the presence of nitrogen and act as negative regulators of the anthocyanin production pathway (Rubin *et al*., 2009).

A study by Pinheiro et al (2001) investigated the effect dehydration has on the *Lupinus albus* L species. In response to water deficit stress, Pinheiro et al (2001) observed a drastic decrease in nitrogen levels in leaf tissue and leaf petioles and an increase in nitrogen levels in the stem cortex and stele. It is presumed that this increase of nitrogen in the stem region is a result of translocation from senescing leaves before being discarded by the plant. Although *L. albus* is not considered a resurrection plant, it can survive up to 13 days without watering, experience massive loss of leaf biomass due to water deficit and still capable of regrowing once watered. A decrease in protein levels that is induced by water deficit has been implicated as being part of the general response strategy employed by plants to cope with such stress. This evidence suggests that if translocation of nitrogen from senescing leaves to the stem region occurs in *X. viscosa* during dehydration this would activate the expression of *LBD* genes (such as *XvLOB*) as shown by Rubin et al (2009) and initiate lateral primordia development. The translocation of nitrogen may act as a signaling molecule indicating the

loss of leaf biomass and the need to develop new lateral primordia. Further studies involving both quantity and translocation of nitrogen in senescing leaves as well as new lateral primordia should be investigated. Very little is known about nitrogen metabolism during dehydration therefore we cannot speculate the exact role XvLOB may play in metabolism.

Because there is no detection of protein during western analysis, the speculation that XvLOB may play a role in repressing anthocyanin production may not be plausible. A repeated western analysis on both dehydrated and rehydrated samples should be performed which would aid in validating the results obtained in this study. A detailed morphological description of leaf senescence and new leaf development should also be looked at especially during the rehydration phase.

Without further analysis, the hypothesized role that XvLOB plays in the dehydration and rehydration process is highly speculative based on limited literature and background on the function of LBD genes in response to dehydration stress. Future studies should include the generation of transgenic gain of function mutants in both *Arabidopsis* and tobacco. Because a number of homologues of *LBD* genes have been found in *Arabidopsis* and rice, the possibility of *LBD* homologues is *X. viscosa* is possible. Gain of function mutants would therefore provide tangible evidence due to the possibility of functional redundancy experienced in loss of function mutants. This result might prove to establish whether XvLOB is in fact involved in the generation of new leaf primordia.

In conclusion, the goals set out to achieve a better understanding of *XvLOB* and its expression has been achieved. This study provides the basic characterization of *XvLOB* in terms of in silico analysis, expression profiles and subcellular localization. These results provide the preliminary evidence which might prove to establish a possible role XvLOB plays in inferring drought tolerance in *X. viscosa*.

Appendices

Appendix A

General protocols

A1 Standard PCR reaction

A2 Standard ligation protocol

A3 CloneJET™ Cloning Kit ligation protocol

A4 Standard transformation protocol

A5 Plasmid isolation

A6 PCR product purification

A7 DNA purification from agarose gels

A8 Standard PAGE conditions

A9 Bradford Reagent protein quantification method

Appendix B

Media and solutions

B1 LB media (LB)

B2 5X SDS PAGE loading buffer

B3 Coomassie blue stain

B4 Phosphate buffered saline

B5 4-Nitrophenyl disodium orthophosphate substrate

B6 Borate buffered saline

B7 3M sodium acetate pH 5.2

B8 0.1M Ammonium acetate

B9 Urea lysis buffer

B10 Aniline Blue-Hoechst stain

B11 LB agar media

B12 Buffer P1

B13 Buffer P2

B14 Buffer P3

B15 12 % Polyacrylamide gel

Appendix C

List of primers

Table C1: Primers used in this study

List of accession numbers

Table C2: Accession numbers of LBD genes

Multiple sequence alignments

Appendix D

Vector maps

Appendix E

Schematic diagrams and tables

Appendix F

Realtime analysis

F2 Statistically analysis of *XvLOB* relative expression using ANOVA

Appendix A

General protocols

A1 Standard PCR reaction

Each PCR amplification reaction was set up in a total of 25 µl volume. Component concentration are summarised in Table A1. Unless otherwise stated within text; DNA polymerase, standard *Taq* buffer and dNTP mixture New England Biolabs (USA) was used.

Table A1 PCR components and final concentrations used in standard PCR protocol

Component	Final concentration
Primer concentration	0.4 µM
dNTP mixture	0.2 mM
Standard *Taq* buffer	1X
Taq polymerase	0.04 U/µl
Template DNA	4 ng/µl

A2 Standard ligation protocol

Purified DNA fragments (insert DNA) were ligated to linearised vector in a reaction volume of 20 µl. Each ligation reaction contained 10 U of T4 DNA ligase and Ligase Buffer (New England Biolabs, USA) at a working concentration of 1X. Mixture was mixed gently, briefly centrifuged and then incubated for 2 hours at room temperature.

A3 CloneJET™ Cloning Kit ligation protocol

A blunting ligation reaction was set up for a vector to insert ratio of 1:3 using 50 ng of vector. Each ligation reaction contained 10 µl 2X reaction buffer, ca. 50 ng purified genomic *XvLOB* PCR fragments and 1 µl blunting enzyme made up to a total volume of 18 µl with nuclease free water. The reagents were vortexed, briefly centrifuged and incubated for 5 min at 70ºC. The reaction mix was then allowed to cool on ice. A ligation reaction was then set up by which the following components were added to the blunting reaction mixture; 1 µl

pJET1.2 (50 ng) and 1 µl T4 DNA ligase (5 U/µl). The ligation reaction was vortexed, briefly centrifuged and incubated for 30 min at room temperature (RT).

A4 Standard transformation protocol

Ten microlitres of ligation reaction or approximately 100 ng of pure plasmid DNA was added to 50 µl of thawed competent *E. coli* cells and mixed briefly. Competent cells were then incubated on ice for 10 min and then heat shocked for 5 min at 37ºC. The transformation mix was then placed back on ice for 2 min after which 450 µl of LB media (Appendix B1) was added to it. Cells were then incubated for 1 hour at 37ºC with shaking. Fifty microlitre aliquots of transformed cells were spread onto LB-Agar (Appendix B11) plate (supplemented with the appropriate antibiotics) and incubated for 16 hours at 37ºC.

A5 Plasmid isolation

Plasmid isolation was modified and performed according to the Plasmid Midi Kit (Qiagen, USA). Five millilitres of bacterial cells were harvested by centrifugation at 7000 x g for 1 min at RT. The supernatant was discard and pellet was resuspended in 500 µl of Buffer P1 (Appendix B12) supplemented with freshly added 100 µg/ml RNAse A (Bio Basic Inc., Canada). Five hundred microlitres of Buffer P2 (Appendix B13) was then added to and mixture was inverted vigorously 4 to 6 times and allowed to incubate for 5 min at RT. Five hundred microlitres of Buffer P3 (Appendix B14) was added, mixed immediately and then incubated for 15 min on ice. Bacterial suspension was centrifuged at 7000 x g for 5 min at 4ºC. Nine hundred microlitres of supernatant was added to 600 µl of isopropanol, mixed gently and incubated for 2 min at RT to allow precipitation of DNA. Precipitated DNA was pelleted by centrifuging at 7000 x g for 9 min at 4ºC. The supernatant was discarded and 1 ml of 70 % ethanol was added to the pellet. Pellet was washed by centrifugation at 7000 x g for 30 s. The ethanol was carefully poured off and pellet was allowed to air dry on bench for 10 min at RT. DNA was resuspended in 50 µl of sterile distilled water. Purified DNA was stored at 4ºC until further use.

A6 PCR product purification

PCR product purification was performed using the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Canada). PCR reaction mixture was transferred to a 1.5 ml

microfuge tube and 3 volumes of binding buffer I was added. The mixture was then added to a column and allowed to incubate for 2 min at RT. Column was then centrifuged at 5000 rpm for 1 min. The flow through was discarded and column placed back into microfuge tube. Five hundred microlitres of wash solution was added and centrifuged at 10 000 rpm for 1 min. The flow through was discarded and wash step was repeated. The column was then transferred to a clean microfuge tube, 30 µl of prewarmed elution buffer was added to the centre part of the column and incubated for 2 min at RT. DNA was eluted off the column by centrifuging at 10 000 rpm for 1 min. Purified PCR products were stored at 4ºC until further use.

A7 DNA purification from agarose gels

Purification of DNA from agarose gels was performed using the Biospin Gel Extraction Kit (BioFlux, Japan). DNA fragment was excised from agarose gel using a clean, sharp scalpel. The excised gel was placed into a sterile microfuge tube and the mass was estimated. For every 100 mg of excised gel 300 µl of Extraction Buffer was added and incubated at 50ºC in a heating block until gel melts, microfuge tube was vortexed every 2 to 3 min. Melted agarose was added to a spin column and centrifuged at 6000 x g for 1 min. The flow through was discarded and spin column placed by into microfuge tube. Five hundred microlitres of Extraction Buffer was added to the column, centrifuged at 12000 x g for 1 min and the flow through was discarded. Seven hundred and fifty microlitres of Wash Buffer was then added to the column and centrifuged at 12000 x g for 1 min. An additional centrifugation at 12 000 x g for 1 min was performed and column was then transferred to a clean, sterile microfuge tube. Thirty microlitres of prewarmed Elution Buffer was adder to the centre part of the column, incubated for 1 min at 37ºC and centrifuged at 12000 x g for 1 min. Purified DNA was stored at 4ºC.

A8 Standard PAGE conditions

To each protein sample, 5X SDS loading buffer (Appendix B2) was added, samples were then boiled for 10 min at 90 ºC before loading. The samples were electrophoresed on a 12 % polyacrylamide gel (Appendix B15) which included a 5 % stacking gel (Appendix B16) for a total of 3 hours in 1X SDS running buffer (Appendix B17) at 30mA. The Bio-Rad Mini-Protean Tetra Cell (Bio-Rad Laboratories GmbH, Germany) apparatus was used. Gels were

stained in Coomassie staining solution (Appendix B3) and then destained in destaining solution (Appendix B18).

A9 Bradford's protein quantification

Bovin serum albumin (BSA) in urea lysis buffer [ULB (Appendix B9)] was used as a standard for XvLOB protein quantification. The standard contained known BSA concentrations which included 0, 5, 10, 20, 40 and 50 µg of BSA. The Bradford reagent (Bio-Rad Laboratories GmbH, Germany) used was diluted at a ratio of 1:5 with distilled water before adding to samples. All BSA samples were carried out in triplicate. BSA standard components are listed in Table A2. A BSA standard curve equation was calculated based on BSA absorbance readings.

Each recombinant XvLOB sample contained 5 µl recombinant XvLOB, 80 µl 0.1 M HCl, 900 µl Bradford Reagent and 85 µl of distilled water. Each sample was mixed thoroughly, allowed to stand for 5 minutes after which absorbance readings were taken at 595 nm. The Beckman DU® 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter; USA) was used. All recombinant XvLOB samples were carried out in triplicate. The Recombinant XvLOB concentration was calculated using the BSA standard curve equation.

Table A2 BSA standard curve sample components

BSA conc. (µg)	BSA (5 mg/ml) (µl)	ULB (µl)	Distilled water (µl)	0.1 M HCl (µl)	Bradford Reagent (µl)
0	0	10	80	80	900
5	1	9	80	80	900
10	2	8	80	80	900
20	4	6	80	80	900
40	8	2	80	80	900
50	10	0	80	80	900

Appendix B

Media and solutions

B1 Luria broth (LB)

5 g Yeast Extract

10 g Tryptone

5 g NaCl

Made up to 1 litre with distilled water

B2 5x SDS-PAGE loading buffer

Components per 10 ml

2.25 ml 1M Tris-Cl, pH 6.8

5 ml glycerol

0.5 g SDS

5 mg bromophenol blue

2.5 ml 1M dithiothreitol (DTT)

B3 Coomassie blue stain

0.5% (w/v) Coomassie brilliant blue R250 (Merck, Germany)

40% methanol

10% acetic acid

50% distilled water

B4 Phosphate buffered saline (PBS)

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

Dissolve in 800 ml distilled water

Adjust pH to 7.4

Made up to 1L

Autoclave

B5 4-Nitrophenyl phosphate substrate

10 mg 4-nitrophenyl disodium orthophosphate (BMD chemicals, England) in 10 ml 10% diethanolamine supplemented with 0.5 mM Mg_2Cl

B6 Borate buffered saline (BBS)

2.16 g Boric acid

2.19 g NaCl

0.7 g NaOH

620 µl 32% HCl

Dissolve in 800 ml distilled water

Adjust pH to 8.6

Made up to 1L

B7 3M Sodium acetate pH 5.2

122.43 g sodium acetate

Dissolved in 250 ml DEPC treated water

Adjust pH to 5.2 with glacial acetic acid

Made up to 300 ml with DEPC treated water

B8 0.1M Ammonium acetate

2.3127g ammonium acetate

Made up in 300 ml methanol

B9 Urea lysis buffer

8M urea

3M thiourea

4 % (w/v) CHAPS (Melford Laboratories Ltd., United Kingdom)

Dissolve in distilled water at 35ºC in a water bath

Aliquot into small volumes and store at -80ºC for a maximum 1 month period

B10 Aniline blue-hoechst stain

0.01 % w/v Aniline blue (Sigma-Aldrich, USA)

20 μg/ml Hoechst 33342 (Sigma-Aldrich, USA)

All components were made up in 0.07M K_2HPO_4

B11 LB agar media

5 g Yeast Extract

10 g Tryptone

5 g NaCl

15 g Bacterial agar

Made up to 1 litre with distilled water

Autoclave

B12 Buffer P1

50 mM Tris-Cl pH8

10 mM EDTA pH8

100 μg/ml RNAse A (NEB, RSA)

B13 Buffer P2

200 mM Sodium Hydroxide (NaOH)

1 % Sodium dodecyl sulphate [SDS (Sigma-Aldrich, USA)]

B14 Buffer P3

3 M Potassium Acetate pH5.5

B15 12 % Polyacrylamide gel

12 % (v/v) 40 % Acrlyamide/BisAcrylmaide (Sigma-Aldrich, USA)

0.375 M Tris-Cl pH 8.8

0.1 % Sodium dodecyl sulphate [SDS (Sigma-Aldrich, USA)]

0.1 % Ammonium persulphate

0.2 % Tetramethylethylenediamine [TEMED (Sigma-Aldrich, USA)]

All components made up in distilled water

B16 5 % Stacking gel

5 % (v/v) 40 % Acrlyamide/BisAcrylmaide (Sigma-Aldrich, USA)

0.125 M Tris-Cl pH 6.8

0.1 % Sodium dodecyl sulphate [SDS (Sigma-Aldrich, USA)]

0.1 % Ammonium persulphate

0.4 % Tetramethylethylenediamine [TEMED (Sigma-Aldrich, USA)]

All components made up in distilled water

B17 1X SDS running buffer

15 g Glycine (Merck)

3 g Tris-Cl

1 g Sodium dodecyl sulphate [SDS (Sigma-Aldrich, USA)]

All components dissolved in 1 litre distilled water

B18 Destain solution

40 % Methanol

10 % Acetic acid

50 % distilled water

Appendix C

List of Primers

Table C1 **Primers used in this study:** Restriction enzyme recognition sites are highlighted in bold and underlined.

Number	Name	Sequence	Amplicon length (base pairs)
C1.1	XvLOB-*Eco*RI F	5’ - CAG**GAATTC**TATGAGTTGTAATGGCTG - 3’	
C1.2	XvLOB-*Hind*III R	5’ - GAC**AAGCTT**GACGAATAAATTTAAAAGTTCC - 3’	650bp
C1.3	XvLOB RT-F	5’ - GACAACAACAAGCGCAGCAGCTTTGC - 3’	
C1.4	XvLOB RT-R	5’ - TGCTCTCTGCGCTCGACGTACCA - 3’	135bp
C1.5	XvVTC2 RT-F	5’ - CTGTATGGGAGATAAGCGGTCACATGG - 3’	
C1.6	XvVTC2 RT-R	5’ - CCAGTGGCCTCGAATATGGATGCTTTC - 3’	-
C1.7	Xv18S RT-F	5’ - CAGGCGCGCAAATTACCCAATCC - 3’	
C1.8	Xv18S RT-R	5’ - CCTACCGTCCCGTCCCAAGGTC - 3’	220bp
C1.9	XvLOB-*Bam*HI F	5’ - TA**GGATCC**TATGAGTTGTAATGGCTG - 3’	
C1.10	XvLOB-*Kpn*I R	5’ - AC**GGTACC**TGGACGAATAAATTTAAAAGTT - 3’	650bp

List of accession numbers

Table C2 List of LBD proteins and accession numbers used for multiple sequence alignments

Accession Number	Gene	Species	Reference
NM_129804	*AtLBD16*	*Arabidopsis*	Okushima Y. *et al*
NP_182065	*AtLBD19*	*Arabidopsis*	Matsumura Y. *et al*
NP_201543	*AtLBD37*	*Arabidopsis*	Matsumura Y. *et al*
NP_190563	*AtLBD38*	*Arabidopsis*	Matsumura Y. *et al*
NP_195470	*AtLBD39*	*Arabidopsis*	Matsumura Y. *et al*
NP_176881	*AtLBD40*	*Arabidopsis*	Zentella R. *et al*
NP_566175	*AtLBD41*	*Arabidopsis*	Matsumura Y. *et al*
ABC54562	*Sb_ramosa2*	*Sorghum bicolour*	Bortiri E. *et al*
ABC54560	*Zm_ramosa2*	*Zea mays*	Bortiri E. *et al*
AAY24684	*ZmAS2*	*Zea mays*	Phelps-Durr TL. *et al*
BAD88523	*OsCrl1*	*Oryza sativa*	Inukai Y. *et al*
NP_001105838	*ZmLBD6*	*Zea mays*	Evans MMS. *et al*
NP_001147202	*ZmLBD11*	*Zea mays*	Alexandrov NN. *et al*

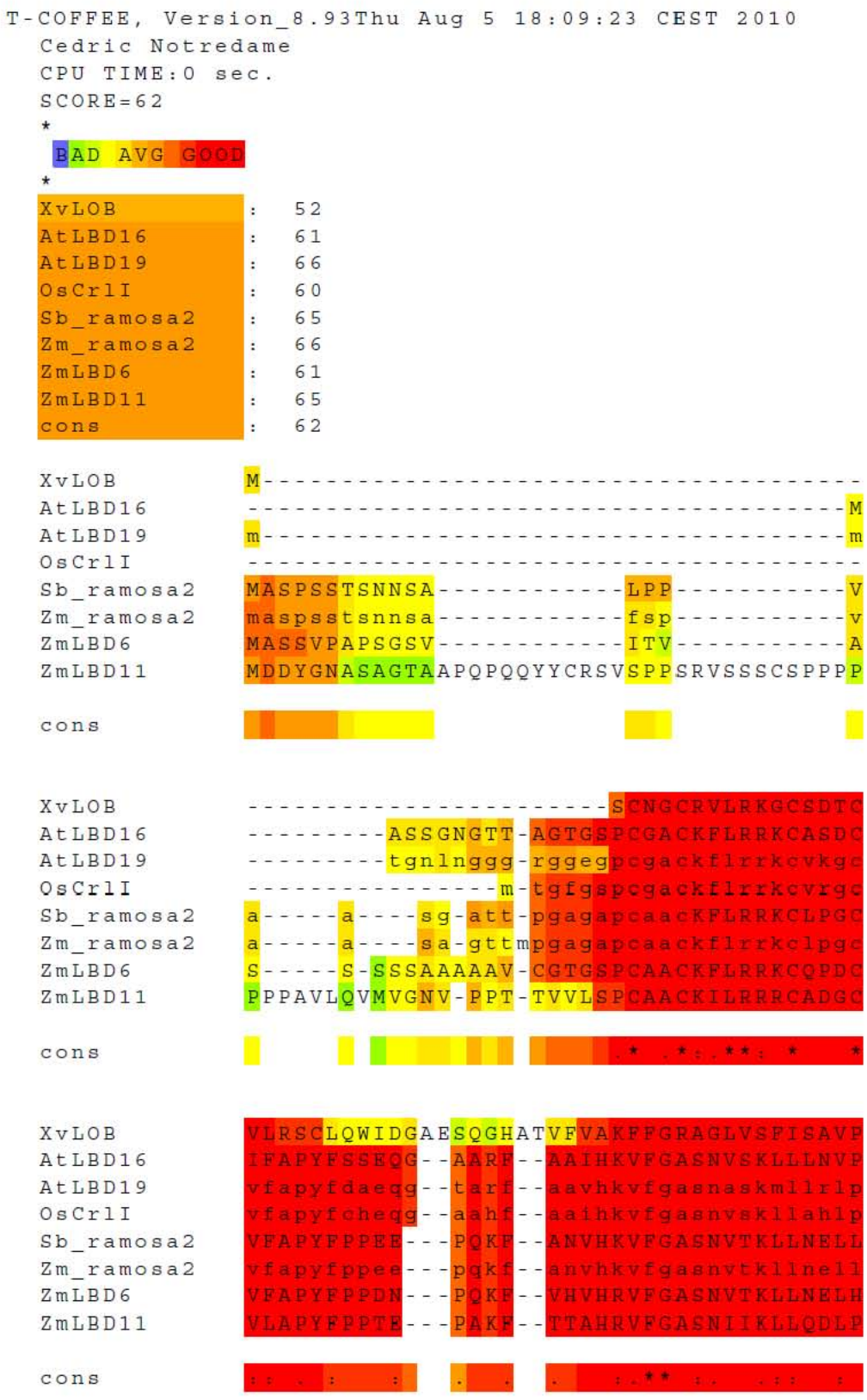
C3 ClustalW2 multiple sequence alignment

```
Sb_ramosa2      ----------------------------MASPSSTSNNSALPPVAAS----GATTPGAGA 28
Zm_ramosa2      ----------------------------MASPSSTSNNSAFSPVAASA---GTTMPGAGA 29
ZmLBD6          ----------------------------MASSVPAPSGSVITVASSSSSAAAAAVCGTGS 32
AtLBD16         ---------------------------------------------MASSG---NGTTAGTGS 14
OsCrlI          -------------------------------------------------------MTGFGS 6
AtLBD19         ------------------------------------------MMTGNL---NGGGRGGEG 15
ZmLBD11         MDDYGNASAGTAAPQPQQYYCRSVSPPSRVSSSCSPPPPPPPAVLQVMVGNVPPTTVVLS 60
XvLOB           ------------------------------------------------------------M 1

Sb_ramosa2      PCAACKFLRRKCLPGCVFAPYFPPEE-PQKFAN----VHKVFGASNVTKLLNELLPHQRE 83
Zm_ramosa2      PCAACKFLRRKCLPGCVFAPYFPPEE-PQKFAN----VHKVFGASNVTKLLNELLPHQRE 84
ZmLBD6          PCAACKFLRRKCQPDCVFAPYFPPDN-PQKFVH----VHRVFGASNVTKLLNELHPFQRE 87
AtLBD16         PCGACKFLRRKCASDCIFAPYFSSEQGAARFAA----IHKVFGASNVSKLLLNVPIHDRC 70
OsCrlI          PCGACKFLRRKCVRGCVFAPYFCHEQGAAHFAA----IHKVFGASNVSKLLAHLPLADRP 62
AtLBD19         PCGACKFLRRKCVKGCVFAPYFDAEQGTARFAA----VHKVFGASNASKMLLRLPLHKRL 71
ZmLBD11         PCAACKILRRRCADGCVLAPYFPPTE-PAKFTT----AHRVFGASNIIKLLQDLPESSRA 115
XvLOB           SCNGCRVLRKGCSDTCVLRSCLQWIDGAESQGHATVFVAKFFGRAGLVSFISAVPHSQRP 61
                .* .*:.**: *   *:: . :   : .     :.** :.  .::  :   .*

Sb_ramosa2      DAVSSLAYEAEARVKDPVYGCVGAISVLQRQVHRLQKELDAAHAELLR----------YA 133
Zm_ramosa2      DAVSSLAYEAEARVKDPVYGCVGAISVLQRQVHRLQKELDAAHAELLR----------YA 134
ZmLBD6          DAVNSLAYEADMRLRDPVYGCVGVISILQHNLRQLQQDLARAKYELSK----------YQ 137
AtLBD16         EAVVTIAYEAQARLHDPV-GCVSHIFALQQQVAFLQSQVMQMKAQIAG---HQ----TSA 122
OsCrlI          EAAVTISYEAQARLRDPIYGCVAHIFALQQQVMTLQAQLASLKAAAAQGIHHQDVGATTK 122
AtLBD19         DAVVTLCYEAMARIRDPVYGSVGHLFSLQHQVMNLQAELAHVQARLST------------ 119
ZmLBD11         DAVSSMVYEAEARLRDPVYGCAGAVCRLQKQANELKVQLARAQADLLN------------ 163
XvLOB           ALFQSLLYEACGRTINPVGGAVALLAGGNWDLCQMAVETVLRGGALRP------------ 109
                    :: ***  *  :*:.*... :   : :   :  :

Sb_ramosa2      CGDVAAGIPTALPVSVSVSAAPRLSTA--MTTSPGQFAAAAATAHATAGMYGSSRRLGVL 191
Zm_ramosa2      CGDVAAGIPTALPV---VSAAPRLSTAPMTTTSPGQFAA---------GMYGSGRRLGVV 182
ZmLBD6          AAAAAS--ASTAPTG-PQAMAEFIGNA--MPNGAHNFIN-----------IGHSAALGSL 181
AtLBD16         AGDLRHSSESTN------QFMTWQQTS--VSPIGSAYSTP---------YNHHQPYYGHV 165
OsCrlI          GGYMSAAATAADDQLGYGGYNQWCGSNGGGAPAASQPGAY---------SSNGGAGHGHD 173
AtLBD19         ---IQR-----------------------FPLQSPQQMQ---------PPSFDPAHNNE 143
ZmLBD11         ------------------AQADLLNAQAKHANLFALFCVE---------MANRRSNQQHP 196
XvLOB           ---ISGAIDGTGGDLFDRQCSAFSSAKKLTAEEMDGAAET---------PAALTEREQQA 157

Sb_ramosa2      DGVVAAPPPPPPAGG-CYFMRNHSN--VISSPPGADVAPVLPYASMAN--WAVNAISAST 246
Zm_ramosa2      DGLAAPPLPPSGGGGGCYFMRNHHNNVVISSSPGADVAPVLPYASVAN--WAVNAISAST 240
ZmLBD6          GG-SASVFGQEQFGNAQMLSRSYDGGEPIARLGINGGGYEFGYSTAMGGSGAVSGLGTLG 240
AtLBD16         NPNNPVSPQSS-LEESFSNTSSDVTTTANVRETHHQTGG--GVYGHDGIGFHEGYPNKK- 221
OsCrlI          SITALLAAGSDYMQHSLYHAFEHSEGAGAVDDGHAAAAAFEAAAESSSCGMAASFAADES 233
AtLBD19         YAMEPSNLDSVWGEEHL---LQDGTGDGDFQELASQFVS-------------------- 179
ZmLBD11         SPLTTMMDGGGGFGAAAYYQQTTLYDSDLDSATWPDHEAQLWT----------------- 239
XvLOB           QQLCELELGRCTMTSADEKSSQRRPGTPNSEESGTSSAESTADRTVER----------- 205

Sb_ramosa2      TTTSGSESIGGLDHKEGGDSSM---- 268
Zm_ramosa2      TTTSGSESIG-LDHKEGGDSSM---- 261
ZmLBD6          ISPFLKSGTAGGDEKPNGGQ------ 260
```

```
AtLBD16          --RSVSYCSSDLGELQALALRMMKN- 244
OsCrlI          VWRSSSSGYQDCEDLQSVAYAYLNRS 259
AtLBD19          ------------RYLPAVKLPACT-- 191
ZmLBD11          -------------------------- 
XvLOB            -------------RVPELLNLFV--- 215
```

Figure C3 Multiple sequence alignment between XvLOB and other LBD genes using the ClustalW2 Server. The proteins aligned include: Zm_ramosa2 (*Zea mays*), ZmLBD6 (*Zea mays*), ZmLBD11 (*Zea mays*), Sb_ramosa2 (*Sorghum bicolour*), OsCrl1 (*Oryza sativa*), AtLBD16 and AtLBD19 (*Arabidopsis*). The C-motif has been blocked in. A star (*) indicates 100% homology in amino acids across all species. A colon (:) represents amino acids that have residues that are approximately the same size and hydropathy. A full stop (.) represents amino acids which have residues that are either similar in size or hydropathy.

C4 T-coffee multiple sequence alignment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OsCrlI : 60
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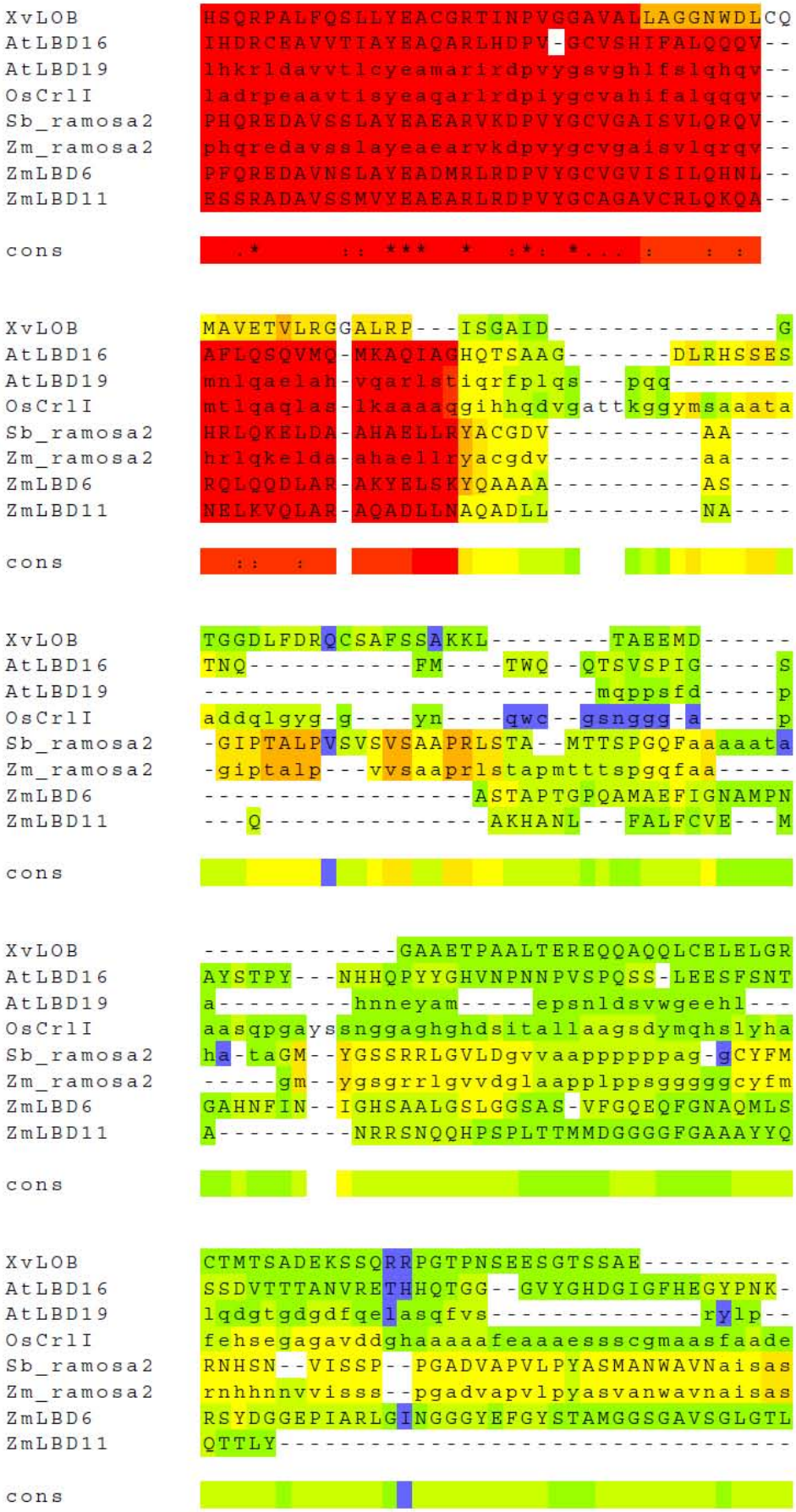
ZmLBD6 : 61
ZmLBD11 : 65
cons : 62
XvLOB M
AtLBD16 M
AtLBD19 m m
OsCrlI
Sb_ramosa2 MASPSSTSNNSA LPP V
Zm_ramosa2 maspsstsnnsa fsp v
ZmLBD6 MASSVPAPSGSV ITV A
ZmLBD11 MDDYGNASAGTAAPQPQQYYCRSVSPPSRVSSSCSPPPP
cons
XvLOB SCNGCRVLRKGCSDTC
AtLBD16 ASSGNGTT-AGTGSPCGACKFLRRKCASDC
AtLBD19 tgnlnggg-rggegpcgackflrrkcvkgc
OsCrlI m-tgfgspcgackflrrkcvrgc
Sb_ramosa2 a a sg-att-pgagapcaacKFLRRKCLPGC
Zm_ramosa2 a a sa-gttmpgagapcaackflrrkclpgc
ZmLBD6 S S-SSSAAAAAV-CGTGSPCAACKFLRRKCQPDC
ZmLBD11 PPPAVLQVMVGNV-PPT-TVVLSPCAACKILRRRCADGC
cons
XvLOB VLRSCLQWIDGAESQGHATVFVAKFFGRAGLVSFISAVP
AtLBD16 IFAPYFSSEQG--AARF--AAIHKVFGASNVSKLLLNVP
AtLBD19 vfapyfdaeqg--tarf--aavhkvfgasnaskmllrlp
OsCrlI vfapyfcheqg--aahf--aaihkvfgasnvskllahlp
Sb_ramosa2 VFAPYFPPEE---PQKF--ANVHKVFGASNVTKLLNELL
Zm_ramosa2 vfapyfppee---pqkf--anvhkvfgasnvtkllnell
ZmLBD6 VFAPYFPPDN---PQKF--VHVHRVFGASNVTKLLNELH
ZmLBD11 VLAPYFPPTE---PAKF--TTAHRVFGASNIIKLLQDLP
cons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AtLBD16 IHDRCEAVVTIAYEAQARLHDPV-GCVSHIFALQQQV--
AtLBD19 lhkrldavvtlcyeamarirdpvygsvghlfslqhqv--
OsCrlI ladrpeaavtisyeaqarlrdpiygcvahifalqqqv--
Sb_ramosa2 PHQREDAVSSLAYEAEARVKDPVYGCVGAISVLQRQV--
Zm_ramosa2 phqredavsslayeaearvkdpvygcvgaisvlqrqv--
ZmLBD6 PFQREDAVNSLAYEADMRLRDPVYGCVGVISILQHNL--
ZmLBD11 ESSRADAVSSMVYEAEARLRDPVYGCAGAVCRLQKQA--
cons
XvLOB MAVETVLRGGALRP---ISGAID----------------G
AtLBD16 AFLQSQVMQ-MKAQIAGHQTSAAG-------DLRHSSES
AtLBD19 mnlqaelah-vqarlstiqrfplqs---pqq--------
OsCrlI mtlqaqlas-lkaaaaqgihhqdvgattkggymsaaata
Sb_ramosa2 HRLQKELDA-AHAELLRYACGDV----------AA----
Zm_ramosa2 hrlqkelda-ahaellryacgdv----------aa----
ZmLBD6 RQLQQDLAR-AKYELSKYQAAAA----------AS----
ZmLBD11 NELKVQLAR-AQADLLNAQADLL----------NA----
cons
XvLOB TGGDLFDRQCSAFSSAKKL--------TAEEMD------
AtLBD16 TNQ----------FM----TWQ--QTSVSPIG-----S
AtLBD19 ----------------------------mqppsfd-----p
OsCrlI addqlgyg-g----yn----qwc--gsnggg-a-----p
Sb_ramosa2 -GIPTALPVSVSVSAAPRLSTA--MTTSPGQFaaaaata
Zm_ramosa2 -giptalp---vvsaaprlstapmtttspgqfaa-----
ZmLBD6 ------------------ASTAPTGPQAMAEFIGNAMPN
ZmLBD11 ---Q----------------AKHANL---FALFCVE---M
cons
XvLOB -------------GAAETPAALTEREQQAQQLCELELGR
AtLBD16 AYSTPY---NHHQPYYGHVNPNNPVSPQSS-LEESFSNT
AtLBD19 a---------hnneyam-----epsnldsvwgeehl---
OsCrlI aasqpgaysnggaghghdsitallaagsdymqhslyha
Sb_ramosa2 ha-taGM--YGSSRRLGVLDgvvaapppppag-gCYFM
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ZmLBD6 GAHNFIN--IGHSAALGSLGGSAS-VFGQEQFGNAQMLS
ZmLBD11 A---------NRRSNQQHPSPLTTMMDGGGGFGAAAYYQ
cons
XvLOB CTMTSADEKSSQRRPGTPNSEESGTSSAE----------
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AtLBD19 lqdgtgdgdfqelasqfvs-------------rylp--
OsCrlI fehsegagavddghaaaaafeaaaessscgmaasfaade
Sb_ramosa2 RNHSN--VISSP--PGADVAPVLPYASMANWAVNaisas
Zm_ramosa2 rnhhnnvvisss--pgadvapvlpyasvanwavnaisas
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ZmLBD11 QTTLY----------
cons

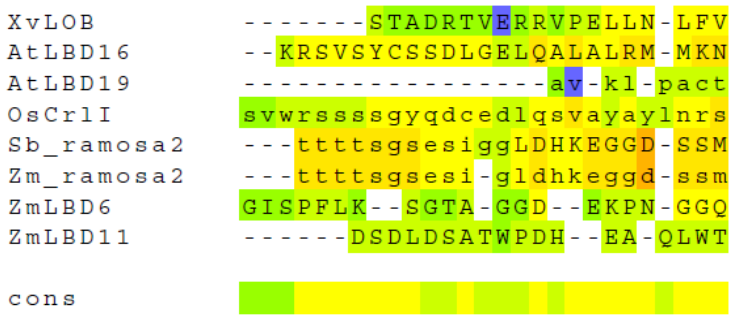
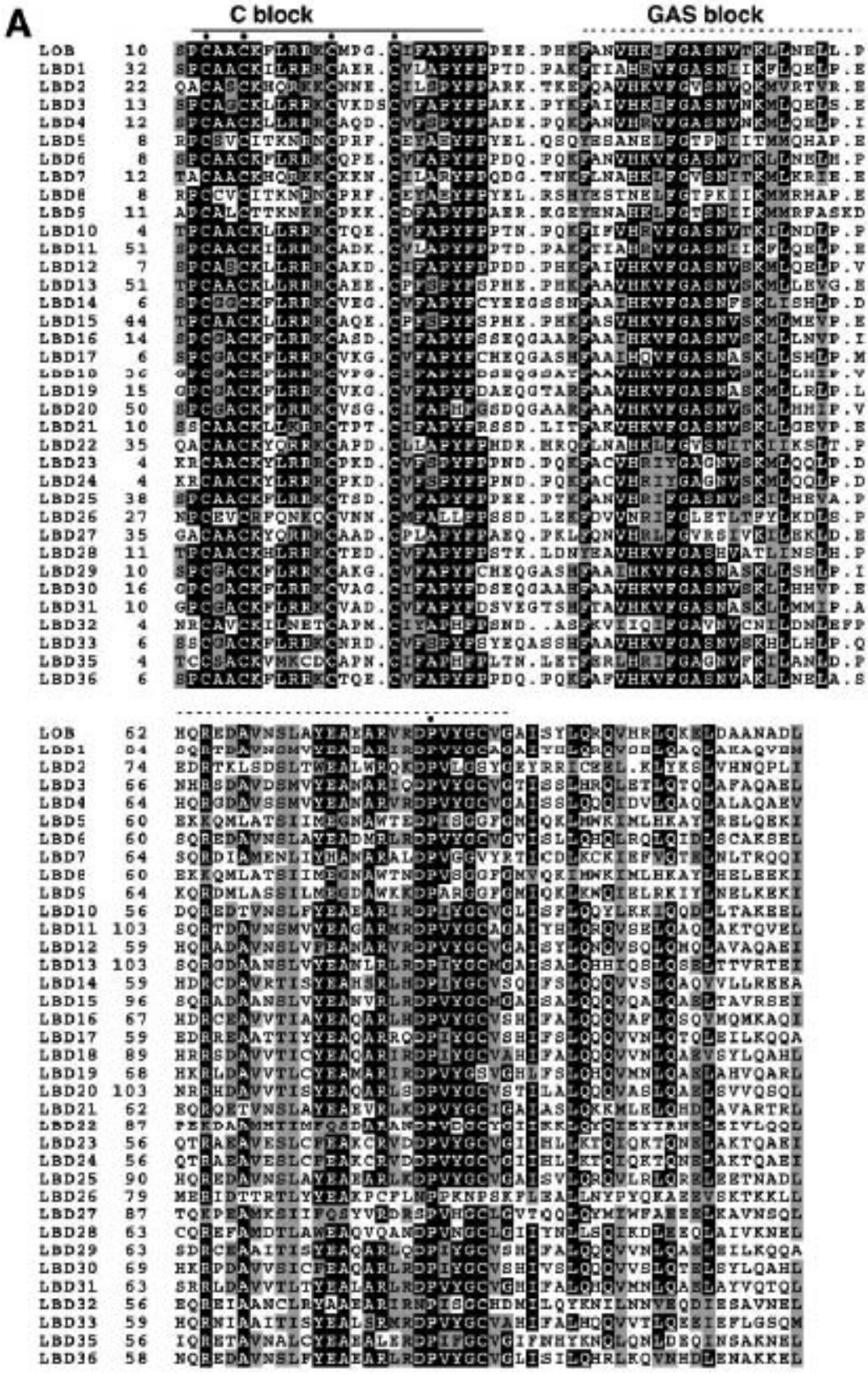


Figure C4 Multiple sequence alignment between XvLOB and other LBD genes using the Tcoffee Server. The proteins aligned include: Zm_ramosa2 (*Zea mays*), ZmLBD6 (*Zea mays*), ZmLBD11 (*Zea mays*), Sb_ramosa2 (*Sorghum bicolour*), OsCrl1 (*Oryza sativa*), AtLBD16 and AtLBD19 (*Arabidopsis*). Key for colour coding homology is indicated within the figure above the multiple sequence alignment. A star (*) indicates 100% homology in amino acids across all species. A colon (:) represents amino acids that have residues that are approximately the same size and hydropathy. A full stop (.) represents amino acids which have residues that are either similar in size or hydropathy.

C5 Alignment of Class I and Class II *Arabidopsis* LBD proteins



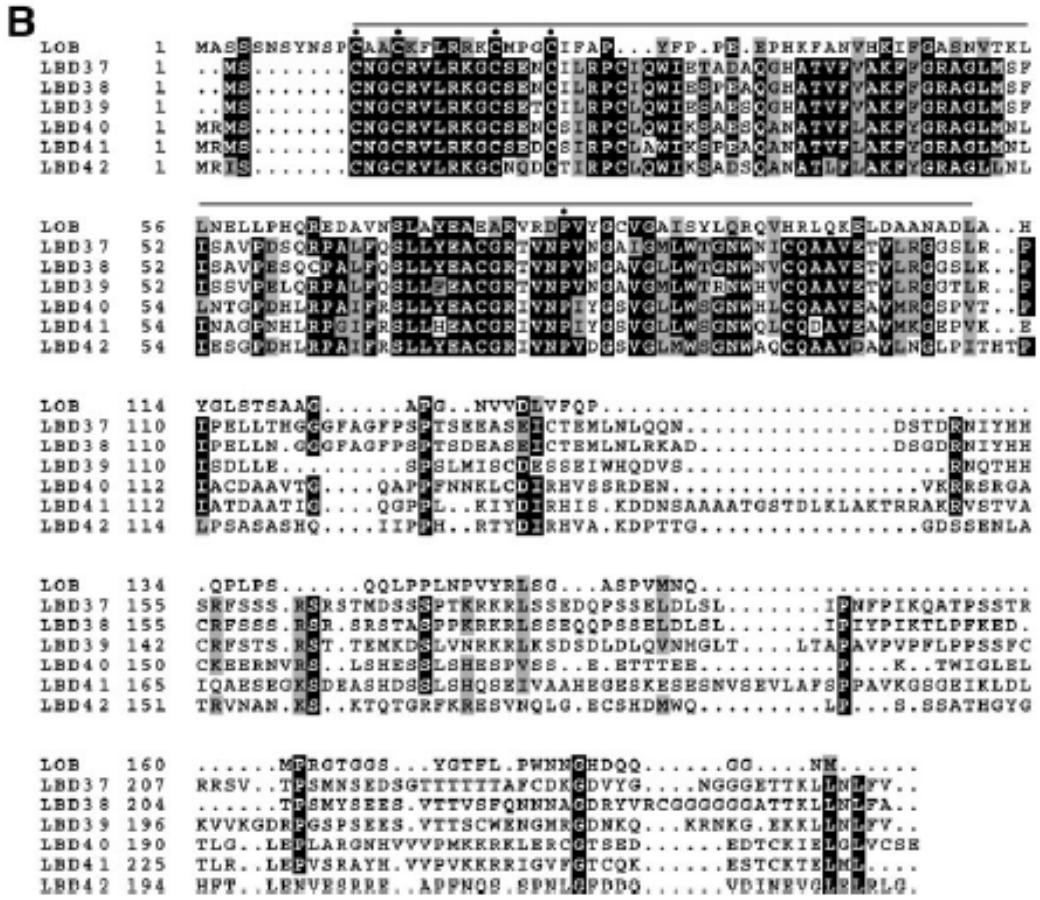


Figure C5 Alignment of *Arabidopsis* LOB and LBD proteins with the Vector NTI program, which uses ClustalW algorithm. **A** - alignment of Class I LBDs except LBD34. **B** - alignment of Class II LBDs . The conserved amino acids are highlighted in black and the similar amino acids are highlighted in grey. The C block is represented by a solid line with the conserved amino acids identified by a dot (•). The GAS block is represented by a dashed line (Adapted from Shuai *et al*., 2002).

Appendix D

Vector maps

D1a pJET1.2 cloning vector

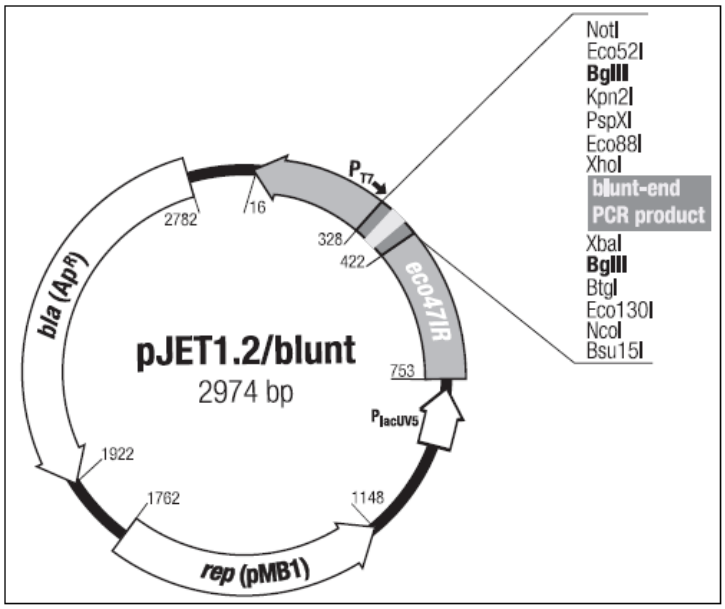


Figure D1a pJET1.2 plasmid used to clone genomic XvLOB using blunt end technologies

D1b pJET1.2 multiple cloning site

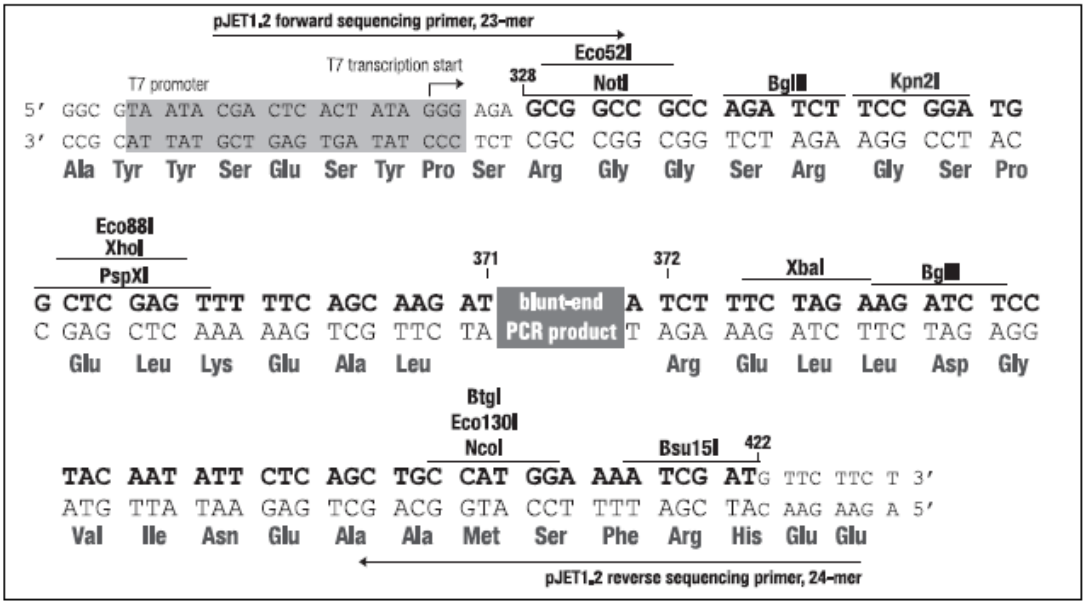


Figure D1b Multiple cloning site of the pJET1.2 plasmid indicating the location of insert when using blunt end technologies

D2 p35S::EYFP::NosT

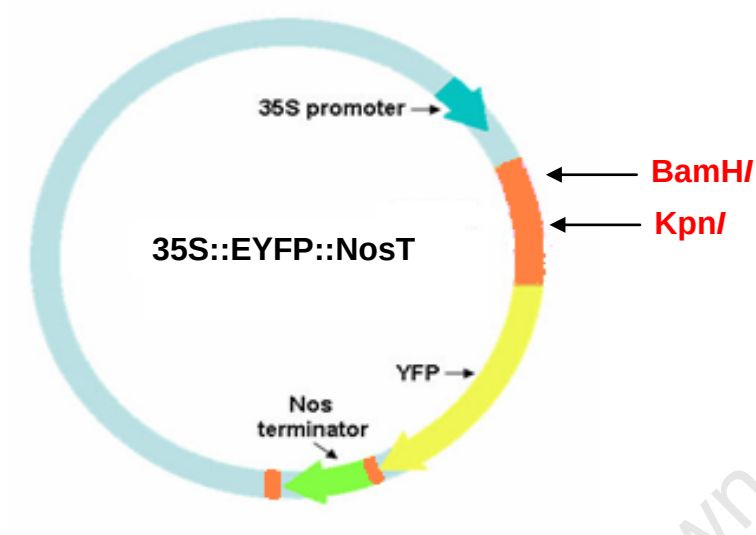


Figure D2 35S::EYFP::Nost construct used for XvLOB localisation studies (see section 5.2.1). The *XvLOB* cDNA sequence was cloned upstream of the EYFP gene within the multiple cloning site using *Bam*HI and *Kpn*I restriction sites (indicated in red). The 35S constitutive promoter (in dark blue) was used to drive the expression of the *XvLOB* coding sequence. The NosT terminator (in green) is used to terminate transcription of *XvLOB* sequence.

Appendix E

Schematic diagrams

E1 Recombinant protein extraction under denaturing and native conditions

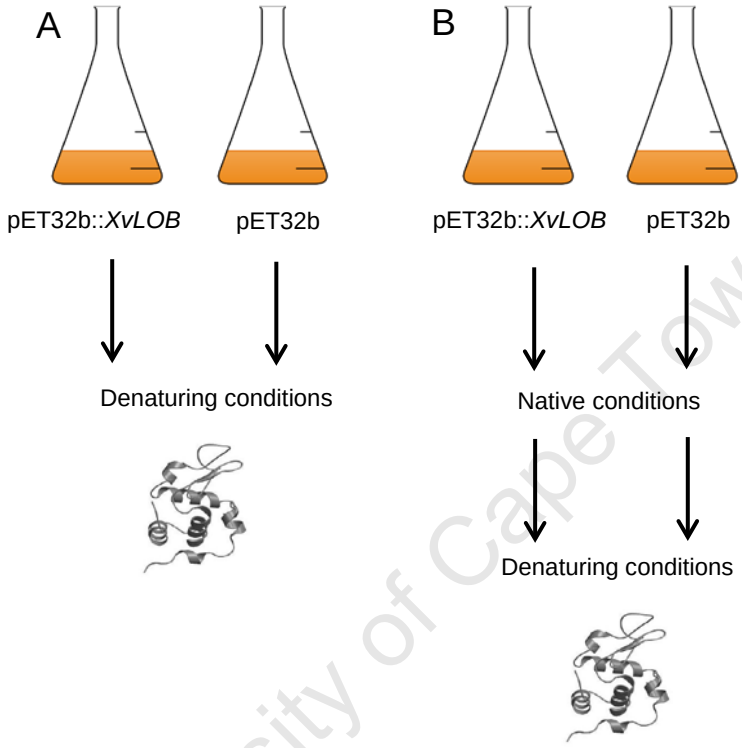
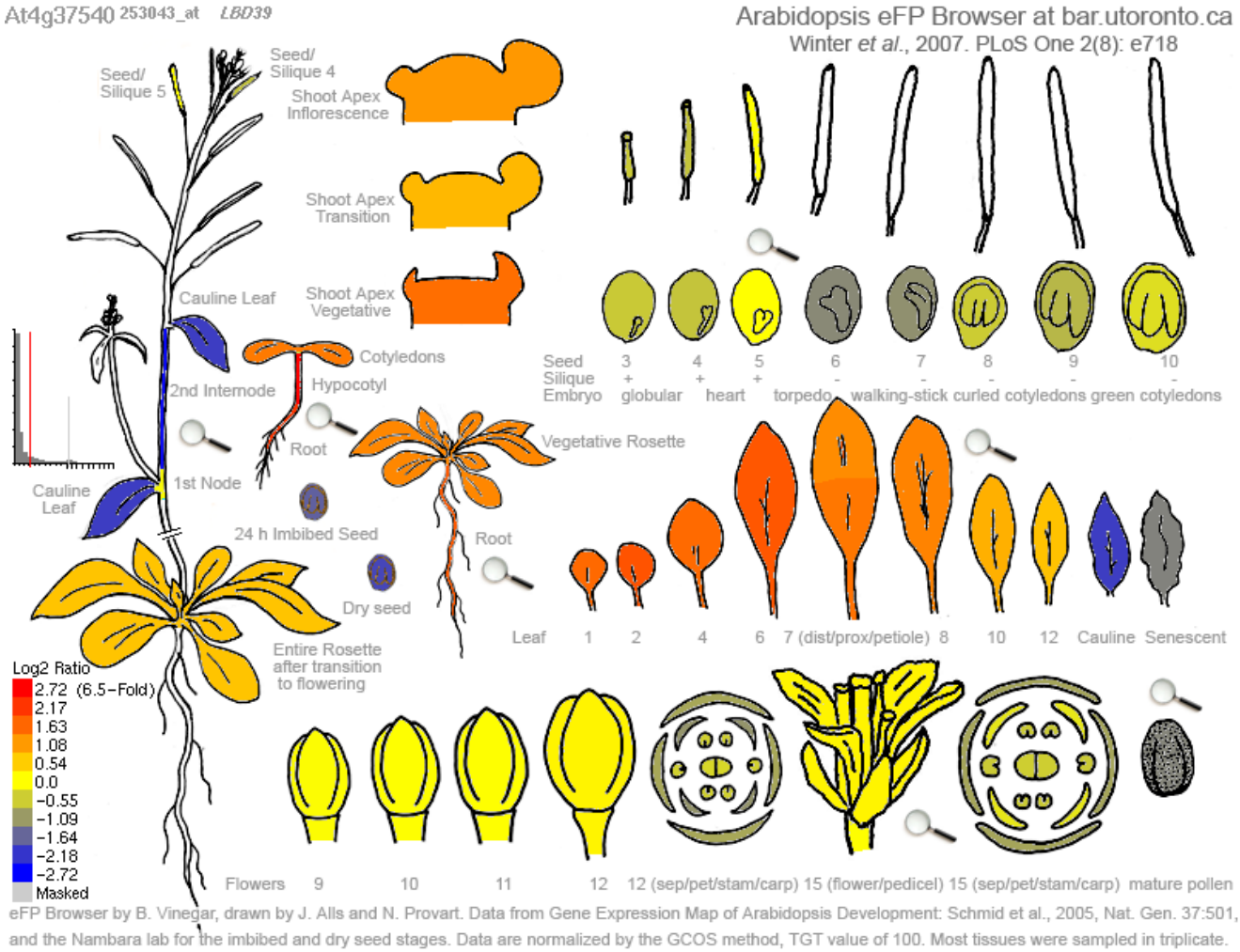


Figure E1 Schematic diagram illustrating protein extraction under denaturing and native conditions for both pET32b::*XvLOB* and pET32b. Plan A represents extraction only under native conditions. Plan B represents extraction under native conditions and then carried through to extract protein under denaturing conditions.

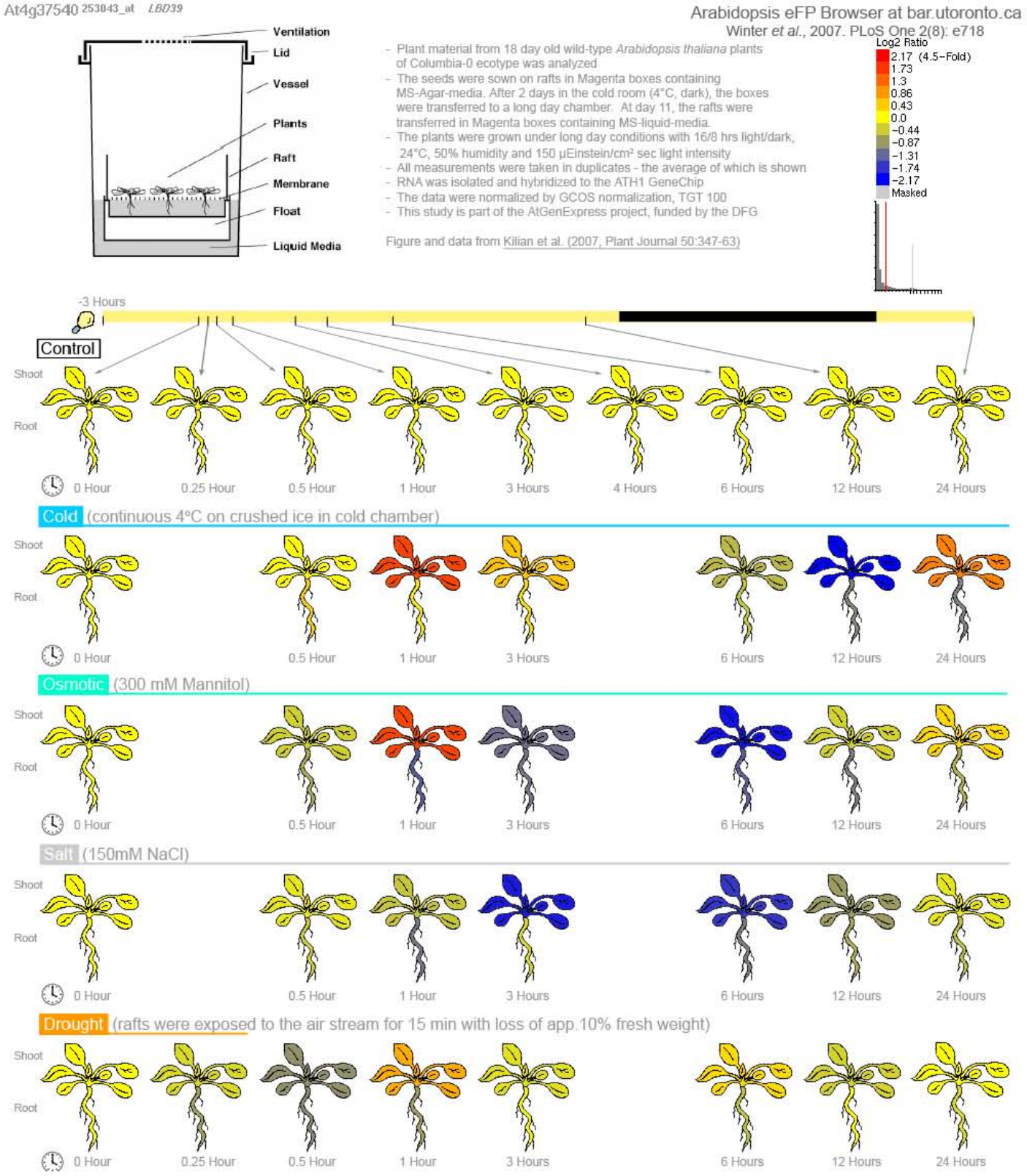
E2 *Arabidopsis* eFP Browser expression profiles for LBD39

DEVELOPMENTAL STAGES



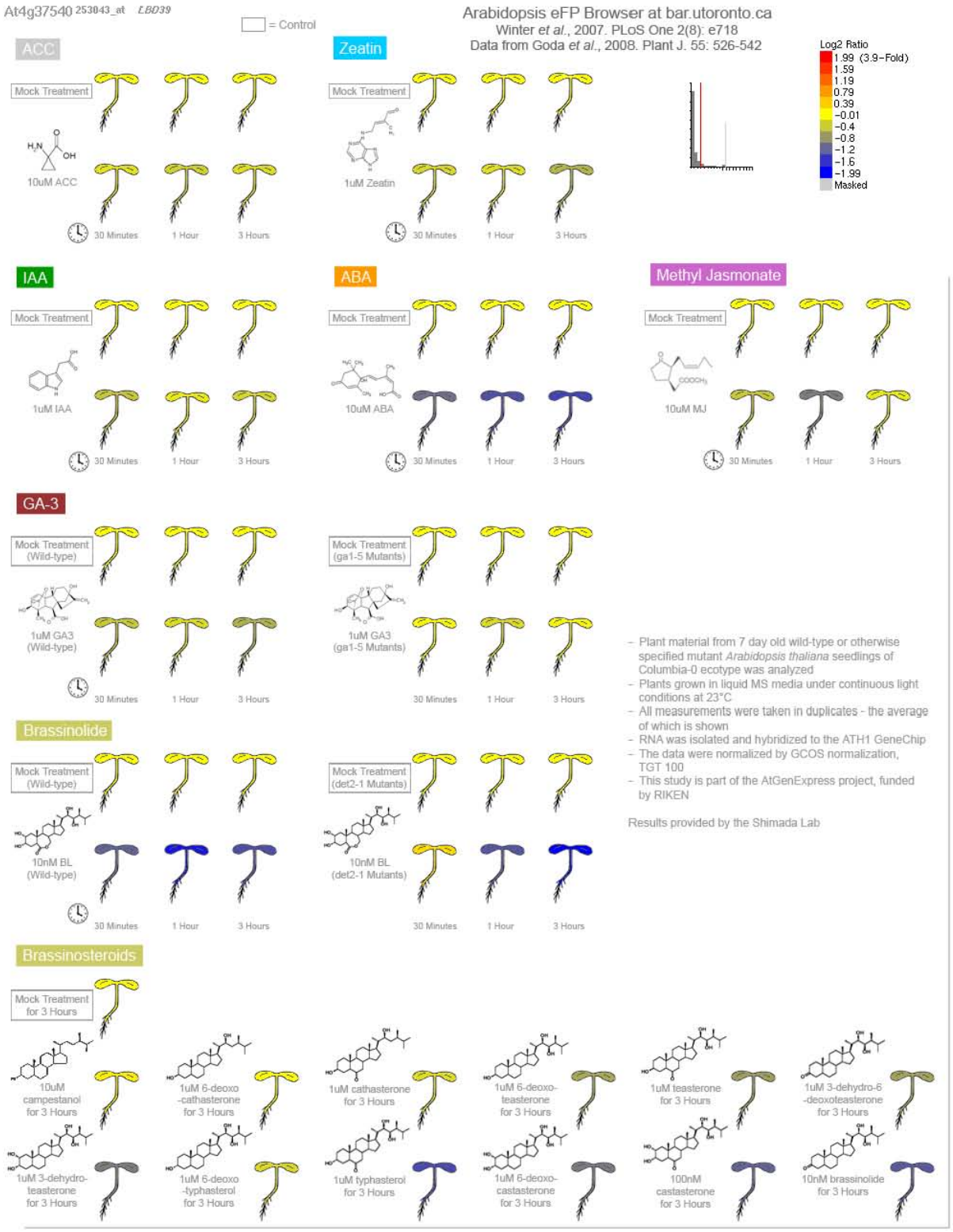
eFP Browser by B. Vinegar, drawn by J. Alls and N. Provart. Data from Gene Expression Map of Arabidopsis Development: Schmid et al., 2005, Nat. Gen. 37:501, and the Nambara lab for the imbibed and dry seed stages. Data are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate.

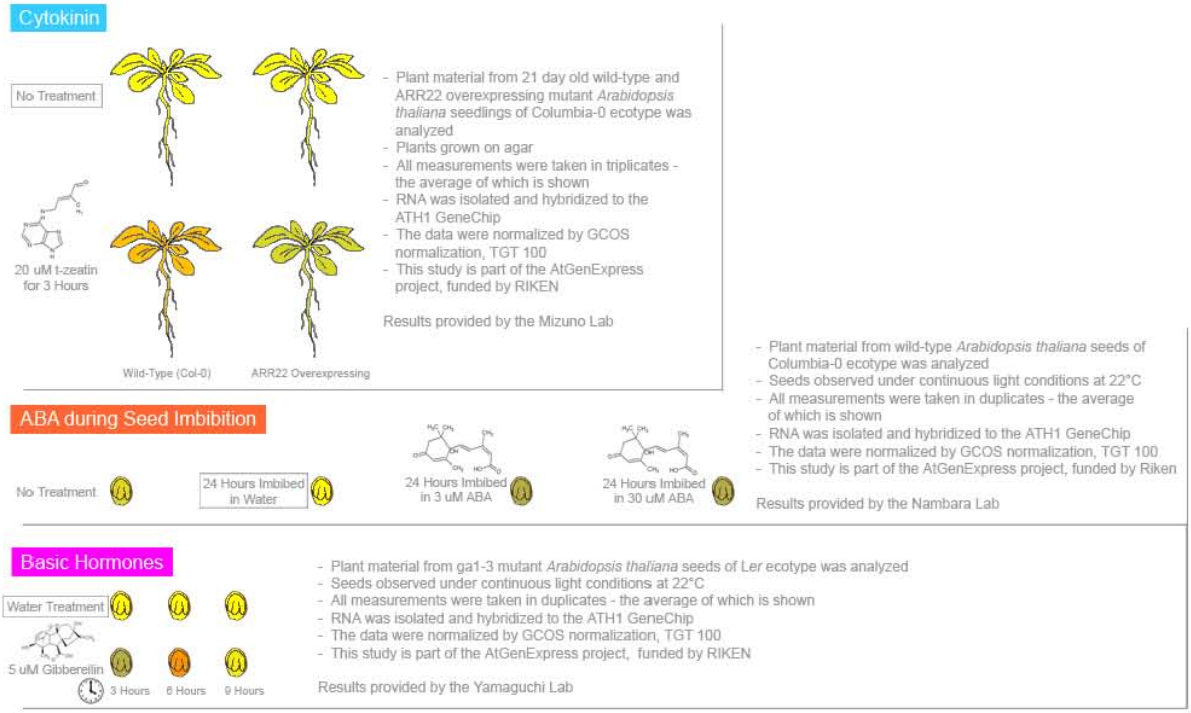
ABIOTIC STRESS



eFP Browser Stress Series by B. Vinegar and D. Winter, drawn by D. Winter. Data from AtGenExpress Abiotic Stress Series from Kilian et al. (2007, Plant J. 50:347-63)

HORMONE TREATMENT





eFP Browser Hormone Series by B. Vinegar and D. Winter. Data from AtGenExpress Hormone Series.

Appendix F

Realtime analysis

F1 Relative expression profile of *XvVTC2*

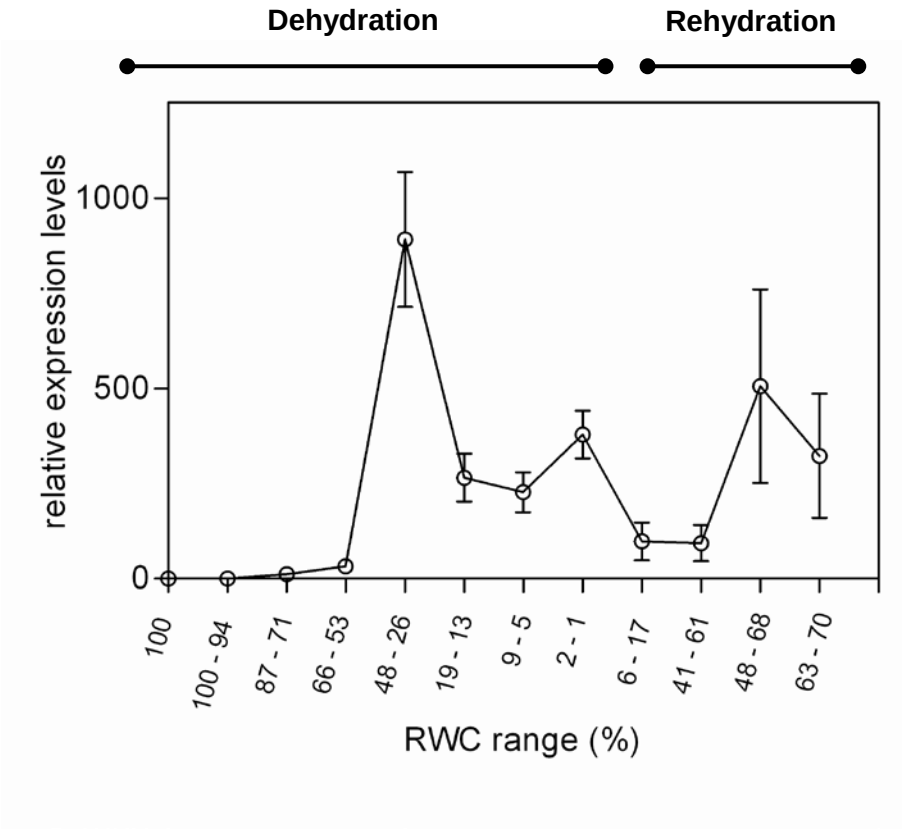


Figure F1 A graph showing the relative gene expression levels of *XvVTC2* during the dehydration and rehydration process of *X. viscosa*. Data points represent the mean and standard error margins (SEM) of relative expression levels of *XvVTC2* (N = 3, n = 9) compared to *18s* ribosomal RNA calculated by the Pfaffl method (Pfaffl MW., 2001). The change in expression levels are represented at the Y axis. The X-axis represents the ranges of % RWC across all samples. Statistical analysis was conducted using a two way ANOVA test. The data represented here is statistically significant ($P < 0.0001$). Graph was generated using the GraphPad Prism software.

F2 Statistically analysis of *XvLOB* relative expression using ANOVA

Table Analyzed	Two-way ANOVA , not RM			
Two-way RM ANOVA	Matching by cols			
Source of Variation	% of total variation	P value		
Interaction	33.1	P<0.0001		
Time	33.1	P<0.0001		
Hydrated	17.24	P<0.0001		
Subjects (matching)	0.1654	0.978		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Time	***	Yes		
Hydrated	***	Yes		
Subjects (matching)	ns	No		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	11	10.01	0.9098	8.075
Time	11	10.01	0.9098	8.075
Hydrated	1	5.211	5.211	416.7
Subjects (matching)	4	0.05002	0.01251	0.111
Residual	44	4.958	0.1127	
Number of missing values	0			
Bonferroni posttests				
Hydrated vs Dehydrated				
% RWC	Hydrated	Dehydrated	Difference	95% CI of diff.
100	0	0	0	-0.7935 to 0.7935
100 – 94	0	0.426	0.426	-0.3675 to 1.220
87 – 71	0	0.09767	0.09767	-0.6959 to 0.8912
66 – 53	0	0.1847	0.1847	-0.6089 to 0.9782
48 – 26	0	2.762	2.762	1.968 to 3.555
19 – 13	0	0.742	0.742	-0.05155 to 1.536
9 – 5	0	0.623	0.623	-0.1705 to 1.417
2 – 1	0	1.127	1.127	0.3331 to 1.920
6 – 17	0	0.2164	0.2164	-0.5771 to 1.010
41 – 61	0	0.1175	0.1175	-0.6761 to 0.9110
48 – 68	0	0.1598	0.1598	-0.6337 to 0.9534
63 – 71	0	0.001246	0.001246	-0.7923 to 0.7948
% RWC	Difference	t	P value	Summary
100	0	0	P > 0.05	ns
100 – 94	0.426	1.615	P > 0.05	ns

87 – 71	0.09767	0.3703	P > 0.05	ns
66 – 53	0.1847	0.7002	P > 0.05	ns
48 – 26	**2.762**	**10.47**	**P<0.001**	***
19 – 13	0.742	2.814	P > 0.05	ns
9 – 5	0.623	2.362	P > 0.05	ns
2 – 1	**1.127**	**4.272**	**P<0.01**	**
6 – 17	0.2164	0.8207	P > 0.05	ns
41 – 61	0.1175	0.4455	P > 0.05	ns
48 – 68	0.1598	0.6061	P > 0.05	ns
63 – 71	0.001246	0.004724	P > 0.05	ns

Figure F2: A table representing the ANOVA calculations indicating the significance of XvLOB relative expression across different RWCs. A significant relative change in XvLOB expression was observed at RWC ranges 48 – 26 % ($P<0.001$) and 2 – 1 % ($P<0.01$). Calculations were performed using the GraphPad Prism software.

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