The functional analysis of XhLEA3-2 – a LEA_4 from the resurrection plant, *Xerophyta humilis*

by

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Declaration

I, Timothy James Dennis, declare that this Master's thesis has been submitted according to the guidelines laid out in the Faculty of Science Postgraduate Student Information Booklet – 2017. I know the meaning of plagiarism and declare that all of the work in this thesis, save for that which is properly acknowledged, is my own.

Abstract

Climate change is a pressing reality in the current era. Changing environmental conditions and limited water availability are associated with the loss of arable land in areas where farming has traditionally thrived. Thus, linked to climate change, is the risk of a global food shortage. Resurrection plants are phenomenal in that they are able to survive extended periods of drought in a state of anhydrobiosis and then resume full metabolism upon rehydration. These plants serve as models to scientists and genetic engineers who hope to replicate, to a degree, the 'resurrection phenomenon' in drought sensitive crop species. The ability of resurrection plants to survive drought needs to be studied on a molecular level if it is to be implemented in transgenic crops. Currently, the molecular mechanisms of desiccation tolerance are only somewhat understood, and considerable investigation is still required. *Xerophyta humilis* is a monocotyledonous resurrection plant in which one of the responses to extreme water loss is the upregulation of several Late Embryogenesis Abundant (LEA) genes. The protein products of these genes, called LEA proteins, are known to be correlated with abiotic stress tolerance in plants, invertebrates and microorganisms. However, the precise molecular mode(s) of action of LEA proteins are still poorly understood. In this study, a group LEA_4, LEA protein, which we have termed XhLEA3-2, shown to be transcriptionally upregulated during desiccation of the resurrection plant *X. humilis*, has been characterized. A bioinformatic, predictive analysis was performed to detect any LEA-like characteristics of XhLEA3-2. Recombinant XhLEA3-2 was produced in *Escherichia coli*, purified, and used to generate XhLEA3-2 specific antibodies for expression analyses. The ability of XhLEA3-2 to function as a molecular chaperone was assessed using a lactate dehydrogenase (LDH) enzyme stability assay. Transgenic expression of XhLEA3-2 in *E. coli* and tobacco was also investigated. In summary, this thesis demonstrates

that XhLEA3-2: has typical LEA protein properties according to bioinformatic analyses, has two close homologs in *X. viscosa,* is present in dry *X. humilis* leaf tissue, has homologs present in dry *X. viscosa* leaf tissue, has some molecular chaperone activity, can protect *E. coli* from desiccation but not from osmotic stress, and can be transiently expressed in tobacco.

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Abbreviations

Amp	Ampicillin
AWC	Absolute Water Content
AWCft	Absolute Water Content at Full Turgor
BLAST	Basic Local Alignment Search Tool
BRU	Biopharming Research Unit
BSA	Bovine Serum Albumin
CASP	Critical Assessment of Techniques for Protein Structure Prediction
CD	Circular Dichroism
Chlr	Chloramphenicol
CS	Citrate Synthase
DHN	Dehydrin
DTT	Dithiothreitol
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAST	Fast Agrobacterium-Mediated Seedling Transformation
FW	Fresh Weight
GO	Gene Ontology
GRAVY	Grand Average of Hydropathy
HMM	Hidden Markov Model
HRP	Horse Radish Peroxidase
HSP	Heat Shock Protein
IDP	Intrinsically Disordered Protein
IFA	Incomplete Freund's Adjuvant
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kan	Kanamycin
LB	Luria Broth
LDH	Lactate Dehydrogenase
LEA	Late Embryogenesis Abundant

MCS	Multiple Cloning Site
MoRF	Molecular Recognition Feature
MS	Murashige-Skoog
MSA	Multiple Sequence Alignment
NMR	Nuclear Magnetic Resonance
O/N	Overnight
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
Pfam	Protein Family
PMSF	Phenylmethylsulfonyl Fluoride
PTM	Post-Translational Modification
qPCR	Quantitative Polymerase Chain Reaction
rif	Rifampicin
RuBisCo	Ribulose-1,5-bisphosphate Carboxylase/Oxygenase
RWC	Relative Water Content
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SMP	Seed Maturation Protein
TBS	Tris-Buffered Saline
TDP	Tardigrade-specific Intrinsically Disordered Protein
UCT	University of Cape Town
UV	Ultraviolet

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Chapter 1 - Resurrection Plants, Desiccation Tolerance and Late Embryogenesis Abundant Proteins – An Introduction

1.1 Drought, Plant Response to Drought and Resurrection Plants

Water is essential to plant survival. It makes up approximately 90% of herbaceous plant weight; functions as the fundamental cellular solvent; is involved chemically in essential metabolism; and maintains cell turgor/shape (Kramer 1995).

Approximately 35% of the world's land is classified as arid or semi-arid (Meigs 1952) and due to climate change, this is predicted to become exacerbated (Dai 2011). Water limitation is a major factor that influences the distribution of plant species both naturally-occurring and farmed. Water limitation negatively influences crop-plant productivity and can result in total crop loss as has recently been experienced in South Africa (AgriSA 2016). Long-term water availability is thus one of the most important environmental factors for farmers when choosing appropriate land for cultivation.

All land experiences drought – sometimes as a part of a predictable cycle but other times, unpredictable (Wood 2005). Cyclic droughts, although challenging, can be managed more effectively, by farmers, than unpredictable spikes in water limitation. Research shows that unpredictable, intense periods of drought are increasing globally, prompting fear for arable lands that may become lost due to 'desertification' (Dai 2011). The development of crops that are resistant to drought but still maintain productivity is crucial for food security in the future. Engineering drought resistant crops requires a comprehensive understanding of the physiological, cellular and molecular responses to drought.

Most plants are homoiohydric – they need to maintain steady water potentials regardless of the environmental conditions. For survival, these plants rely on strategies like succulence,

deep root penetration or a rapid life cycle that allows them to produce seeds that can survive dehydration, ahead of a dry season (Wood 2005). Most commercial food crops are homoiohydric and sensitive to drought.

Poikilohydric plants, on the other hand, are able to survive, even at low cellular water concentrations. A few plants and animals are able to tolerate an extreme loss of water for long periods – this ability is called 'desiccation tolerance' (Oliver et al. 2000). 'Desiccation' refers to total or near-total loss of all water in the protoplasm hence, 'partial desiccation' does not exist (Moore et al. 2009). 'Dehydration' on the other hand refers to the loss or partial loss of cellular water and can be incremental (Wood 2005). Thus, while most plants are dehydration tolerant to a degree, very few are desiccation tolerant – usually a trait reserved for their seeds (termed orthodox seeds).

However, some plants display tolerance to desiccation in vegetative tissues. These species are termed 'resurrection plants' (Gaff 1971; Gaff 1977). They can survive to a relative water content (RWC) of less than 5%, this being equivalent to a concentration of ≤ 0.1 g H_2O g^{-1} dry mass (Moore et al. 2009; Farrant et al. 2012). In this state of anhydrobiosis they are metabolically quiescent. After exposure to water, these plants rapidly recover, back to fully functional metabolism, with minimal deleterious effects. Approximately 330 resurrection plants exist, of which 135 species are angiosperms (Wood 2005; Farrant et al. 2012; Gaff & Oliver 2013). Desiccation tolerant angiosperms are of interest to plant researchers in the field of drought-tolerant crop development as these plants present a unique model that could be mimicked by genetic-engineering in crops. This has been shown to be a promising approach (Liu et al. 2009; Li et al. 2015). The molecular mechanisms presently known to be involved in the 'resurrection phenomenon' include: the degradation or masking of chlorophyll, the upregulation

of antioxidants, osmolytes, heat shock proteins, non-reducing sugars and, the focus of this study, Late Embryogenesis Abundant (LEA) proteins (Illing et al. 2005; Farrant et al. 2012; Farrant et al. 2015).

1.2 Late Embryogenesis Abundant Proteins

LEA proteins are a heterogeneous group of proteins shown to be involved in the abiotic stress response of many plants, as well as some invertebrates and microorganisms (reviewed in Tunnacliffe & Wise 2007; Hand et al. 2011; Amara et al. 2014). They were first discovered to be expressed in *Gossypium hirsutum* (cotton) seeds at the late stages of embryogenesis during maturation drying, concomitant with the acquisition of desiccation tolerance (Galau & Dure 1981; Goldberg et al. 1989; Hughes & Galau 1991). This observation led LEAs to be considered functional in the seed's ability to survive desiccation (Galau & Dure 1981). Furthermore, LEA proteins and their encoding mRNAs are maintained in cotton seeds until germination, after which they both decline (Goldberg et al. 1989), in sync with the new seedling's loss of desiccation tolerance. Many studies have since supported the relationship between water-deficit stress and LEA-expression although other abiotic stresses, like cold-stress and osmotic-stress, have also been linked to the upregulation of LEAs (Welin et al. 1994; Close 1997; Liu et al. 2013).

1.3 LEA Protein Classification

In the absence of the ability to classify LEAs in terms of functional significance, several attempts have been made to organize LEA proteins into groups based on common features in their amino acid sequences. The abundance of resulting classification systems is confusing, each having separate or modified classification criteria (Battaglia et al. 2008). Causing further puzzlement is the superfamily name: "Late Embryogenesis Abundant" – a name no longer appropriate for a group of proteins that is constrained neither temporally nor spatially to seed

tissues during late-stage embryogenesis. In some instances, LEA proteins have been called "dehydrins" (short for "dehydration inducible"), a name that now is held to describe a (large) subset of LEAs which are conventionally referred to as group LEA_2, LEAs.

Bioinformatics has thus recently played a key role in the classification of LEAs. In bioinformatic terminology, a profile is a description of the consensus of a multiple sequence alignment (MSA). Profile analysis (also called 'motif-finding') is commonly used to group proteins based on profile similarity and to identify sequences of known function in novel proteins. Unlike pairwise sequence alignment methods (like BLAST – https://blast.ncbi.nlm.nih.gov/Blast.cgi), profiles use a scoring system that determines the degree of conservation at specific sites in a sequence. In other words, instead of simply matching amino acids, profile analysis makes allowance for mismatches at specific sites without automatically marking them as non-conserved. Profile analysis is thus a powerful tool for the identification of similar motifs in a heterogeneous group of proteins, like the LEAs.

Traditionally, LEA proteins were grouped according to the presence of short, repeating amino acid motifs. These motifs are classified primarily by the electrostatic charge of each residue in the sequence as opposed to conserved, specific amino acid residues (Dure 1993; Battaglia et al. 2008). For example, positions one, two, five and nine of the conserved 11-mer amino acid motif of the LEA_4 group can contain any apolar residue, including threonine, amounting to nine potential amino acids. Positions 6 and 8 are positively charged, and positions 3, 7 and 11 are negatively charged (Dure 1993). Taking this variability into account as well as potential sequence errors (mismatches), profile-based-grouping of LEAs is a sensible choice out of the many classification systems that have been proposed (Amara et al. 2014).

Thus, consistent with recent trends in LEA-literature, the profile-based classification system first described in Hundertmark & Hincha (2008) will be assumed in this thesis. This system is based on protein family (Pfam) profiles generated by Hidden Markov Models (HMMs) and named according to the Pfam-database (http://pfam.xfam.org/). Each LEA group is represented by one of the following Pfam IDs for a total of 8 groups: LEA_1; LEA_2; LEA_3; LEA_4; LEA_5; LEA_6; DHN; SMP.

1.4 LEA Sequence Characteristics

LEA proteins are made up mostly of hydrophilic amino acids (Hong-Bo et al. 2005). As a consequence of this amino acid bias, LEA proteins have an overall hydrophilicity. This is demonstrated by an overrepresentation of glycine, alanine, glutamic acid, lysine, arginine and threonine residues (Battaglia et al. 2008). Furthermore, LEAs typically have a lack or low proportion of cysteine and tryptophan residues (Battaglia et al. 2008). Most LEAs have a low molecular weight of 10-30 kDa (Hong-Bo et al. 2005).

1.5 LEA Physical Characteristics

Typically, hydrophobic regions of proteins are folded inside an outer hydrophilic shell, in aqueous solution, resulting in a rigid secondary structure (Fersht 1999). However, because LEA proteins lack hydrophobic regions, the entire length of the peptide chain favors interaction with the surrounding solution. This means that LEAs have no ordered secondary structure in aqueous solution, and instead, they exist predominantly as random coils. Thus, LEAs have been termed ‘intrinsically disordered proteins’ (IDPs) (Pazos et al. 2013) – a group of proteins which lack a fixed or ordered three-dimensional structure and instead fluctuate between a variety of different conformations (Wright & Dyson 2015). Bioinformatic software can be used to predict the likelihood of a protein to be disordered based on its amino acid sequence and physical properties.

This *in silico* approach is often used to characterize putative LEAs and the predictions made by the software can then be confirmed *in vitro* using Circular Dichroism (CD) and/or Nuclear Magnetic Resonance (NMR) spectroscopy. Interestingly, many LEAs have been shown to adopt a rigid, largely alpha-helix secondary structure under fully dehydrated conditions (Boudet et al. 2006; Tolleter et al. 2007; Li & He 2009; Ginbot 2011; Waters 2015).

As a consequence of their hydrophilicity, LEA proteins have been observed to resist aggregation and remain soluble at high temperatures up to 100 ^{o}C (Russouw et al. 1995). Typically, heat-denaturation of globular (ordered) proteins, in aqueous solution, results in the unfolding of their secondary structure, and consequently their hydrophobic regions become exposed. Aggregation with the surrounding proteins follows due to an electrostatic repulsion, of the exposed hydrophobic regions, away from polar H_2O molecules (Fersht 1999). The abovementioned does not occur in LEAs: firstly because they are already, in a sense, 'denatured' because of their tendency not to adopt an ordered structure, and secondly, they lack hydrophobic regions and therefore do not aggregate like globular proteins.

1.6 Temporal and Spatial LEA Protein Expression

Temporally, LEA protein expression has been observed to occur in response to water-limitation stress (Ingram & Bartels 1996; Bray 1997; Waters 2015), osmotic stress and low-temperature stress (Wise & Tunnacliffe 2004). Thus, they are thought to play a role in abiotic stress resistance (Hand et al. 2011). Moreover, transgenic plants and bacteria transformed with LEA-expressing constructs have shown an increased ability to resist various abiotic stresses better than their wild-type counterparts (Liu et al. 2009; Gao & Lan 2016).

Spatially, LEA proteins as a whole do not appear to locate to a single or specified group of intracellular targets but rather are spread throughout various cellular compartments

(Tunnacliffe & Wise 2007; Gao & Lan 2016). However, most LEA_4s appear to localize in the cytoplasm as opposed to at a particular organelle (Gao & Lan 2016).

1.7 Putative LEA Protein Functions

Although the correlation of LEAs and abiotic stress resistance is clear, the precise, functional, molecular mechanism(s) of LEA proteins remain somewhat arcane. A few functions have been hypothesized and carry some evidence. These functions include: molecular chaperoning (Goyal et al. 2005; Reyes et al. 2005; Reyes et al. 2008), membrane protection (Furuki & Sakurai 2014), ion chelation (Dure 1993; Wise 2003) and vitrification of the cytoplasm (Wolkers et al. 2001; Berjak 2006). Additionally, evidence for the multi-functionality of individual LEAs ('moonlighting') exists (reviewed by Hara, 2010). Perhaps, LEAs have evolved multi-functionality as a means of conserving energy in already energy-depleted stress conditions – the notion being that the production and homeostasis of one, small, multifunctional protein requires less metabolic energy than several individual proteins.

1.8 This Study

In 2004, several LEAs were identified as desiccation-induced in the resurrection plant *Xerophyta humilis* by Collett et al. (2004). One of these, a LEA_4 called XhLEA3-2, was chosen as the subject of this study. The overall aim of this study was to try to gather information about the potential function, characteristics and abiotic stress resistance ability of XhLEA3-2. A bioinformatic analysis was performed to predict typical LEA-characteristics of XhLEA3-2. The genome of *Xerophyta viscosa*, recently published in Costa et al. (2017) and made available by those authors, was scanned for any high-similarity XhLEA3-2 homologs. Recombinant XhLEA3-2 was expressed in *Escherichia coli*, purified and tested for phosphorylation. A polyclonal, XhLEA3-2-specific antibody was produced, purified and used as an XhLEA3-2

specific probe. *X. humilis* and *X. viscosa* leaf tissue at different stages of dehydration were probed for XhLEA3-2 or XhLEA3-2 homolog expression. Recombinant XhLEA3-2 was tested for potential molecular chaperone activity using a lactate dehydrogenase (LDH) enzyme assay as outlined by Goyal et al. (2005) and Reyes et al. (2005). Lastly, transgenic tests, where *E. coli* and tobacco (*Nicotiana benthamiana*) had been transformed with XhLEA3-2-expressing constructs, were performed to test for the expression of XhLEA3-2 and its protective ability against abiotic stress.

Chapter 2 - A Bioinformatic Analysis of XhLEA3-2

2.1 Introduction

Bioinformatic analyses continue to increase in power and accuracy. Many free tools are available online and this allows for large-scale, *in silico* protein characterization that can be done anywhere in the world with few limitations. Due to the relatively recent discovery of LEAs, 20-30 years ago (Galau et al. 1986), coincident with an explosion in computing power and accessibility, bioinformatics has played an important role in grouping and functionally characterizing LEAs. At least two LEA classification systems are based on bioinformatic profile-analysis of LEA sequences (Wise 2003; Hundertmark & Hincha 2008) while the other systems are based on the presence of group-specific, repeated sequence motifs (Amara et al. 2014) which are easily detected by software. Furthermore, many putative LEA-functions (Tunnacliffe & Wise 2007; Amara et al. 2014) were first identified through bioinformatics. For example, the *in silico* prediction by Wise & Tunnacliffe (2004) that LEA_4s act as molecular chaperones, led to the canonical study of *in vitro* LEA chaperone activity a year later (Goyal et al. 2005). Bioinformatics is readily and easily available and requires relatively little time and thus, when characterizing a novel LEA, it is sensible to begin with what has largely become the standard first-step in novel-protein characterization.

2.1.1 LEA Profile-Based Classification

HMM-profile-based classification using Pfam motifs was used in this thesis to assign XhLEA3-2 to a specific LEA sub-group. The advantages of this system were outlined in 1.3. In short, this system is a robust means of dealing with sequence variability as well as insertions/deletions (Sonnhammer et al. 1998). Furthermore, it has been the standard used for

two resurrection plant genome-sequencing projects (Xiao et al. 2015; Costa et al. 2017). Online software can be used to group amino acid sequences according to their HMM-profiles.

2.1.2 LEA Sequence Characteristics

The characteristics of LEA amino acid sequences have been outlined in 1.4. Certain bioinformatic tools can be used to identify these LEA-characteristics *in silico*. For example, the proportion of individual amino acids in a sequence as well as the molecular weight and hydropathic character (hydrophilicity or hydrophobicity) can all be predicted.

2.1.3 Predicting LEA Protein Disorder and Folded Structure

LEA proteins form part of the intrinsically disordered protein (IDP) group. Several computational methods exist to predict the probability of a protein being intrinsically disordered in the native (functional) state (reviewed in Pazos et al. 2013). Typically, disorder prediction tools trained on sequence databases of IDPs (e.g. DisProt – http://www.disprot.org/) (Romero et al. 1997; Ward et al. 2004) are used in conjunction with those based on the biophysics that underlie amino acid sequences (Prilusky et al. 2005; Dosztányi et al. 2005).

The tendency of LEA proteins to form ordered, mostly alpha-helix secondary structures when dehydrated is another useful marker to take into consideration when characterizing novel LEAs. Many algorithms exist online that can be used to predict protein folding. However, to my knowledge, none have been specifically designed to take into account the unconventional biophysical properties of IDPs. Nevertheless, structural predictions of LEAs *in silico* seem to correspond to data measured *in vitro* using CD spectroscopy (Tolleter et al. 2007; Ginbot 2011).

2.1.4 Predicting LEA Protein Molecular Recognition Features

Molecular Recognition Features (MoRFs) are sequences within IDPs that form transient secondary structures when bound to a partner (Mohan et al. 2006). They are thought to act as a

type of ‘active site’ in IDPs, to determine partner-recognition specificity of IDPs, and to play a role in intermolecular signaling (Fuxreiter et al. 2004). The identification of characterized MoRFs in a novel IDP can provide insight into protein function. Several bioinformatic predictors of MoRFs exist as listed in Sharma et al. (2016).

2.1.5 Predicting LEA Protein Function, Localization and Post-Translation Modifications

Several online servers are available for the prediction of novel-protein function. Protein function is defined as the molecular processes acting to or through a protein. As discussed in 1.6, several putative functions, mostly based on conserved physical properties (e.g. hydrophilicity, intrinsic disorder), have been put forward for LEAs. *In silico* functional predictions as well as LEA functional-trends in the literature give clues towards which potential studies could elucidate novel-LEA function.

The subcellular-localization of LEAs can assist in drawing conclusions about LEA function and can be predicted using bioinformatic software. For example, if a LEA is located to the cell membrane it might then be hypothesized to play a role specific to that location such as cross-membrane transport or osmolyte activity.

Finally, post-translational modifications (PTMs) can help to elucidate LEA function. Phosphorylation suggests putative cellular signaling function (Day et al. 2016) while glycosylation could play a role in LEA-folding or partner-binding (Zerze & Mittal 2015). PTMs could also play a role in the reduced mobility of LEAs that has been observed in SDS-PAGE gels (Liu et al. 2009). Several different online programs can be used to predict phosphorylation and glycosylation given an amino acid sequence (Blom et al. 2004; Cozzetto et al. 2016).

2.2 Methods and Materials

The amino acid sequence of the putative *X. humilis* LEA protein, XhLEA3-2, was put through a number of bioinformatic tools, itemized below, in order to predict LEA-like features and functions:

- XhLEA3-2 was first grouped according to the Pfam, profile-based method of Hundertmark & Hincha (2008) using the freely available software, HMMER (http://hmmer.org/) (Finn et al. 2015).
- The proportions of amino acids in XhLEA3-2 were tallied using ProtParam (http://web.expasy.org/protparam/).
- The hydropathic character of XhLEA3-2, in the form of a Kyte-Doolittle plot, was assessed using GRAVY Calculator (http://www.gravy-calculator.de/).
- The potential of structural disorder was predicted using MetaDisorder (http://iimcb.genesilico.pl/metadisorder/) – a freely available web-server that combines the output of 13 separately developed protein-intrinsic-disorder-predictors weighted by accuracy to produce a single output (Kozlowski & Bujnicki 2012).
- Putative MoRFs were identified using MoRFchibi (http://morf.chibi.ubc.ca:8080/mcw/index.xhtml) and predicted MoRF sequences were blasted against the MoRF database, mpMoRFsDB (http://bioinformatics.biol.uoa.gr/mpMoRFsDB/).
- Secondary structure was predicted using FFPred (http://bioinf.cs.ucl.ac.uk/psipred/). Subcellular-localization and post-translational modifications (PTMs) were both predicted using PSIPred (http://bioinf.cs.ucl.ac.uk/psipred/).

- The XhLEA3-2 amino acid sequence was queried against the recently-published *X. viscosa* (a close *X. humilis* relative) genome (Costa et al. 2017) and two LEA-homologs were aligned using the UniProt Align tool (http://www.uniprot.org/align/). The *X. viscosa* database and expression-data were generously provided by Dr M. C. Costa.

2.3 Results and Discussion

2.3.1 The Pfam Classification of XhLEA3-2

Using HMMER, XhLEA3-2 was predicted to have the HMM profile of the LEA_4 Pfam group PF02987.15. Furthermore, according to the rules laid out by Dure (1993), XhLEA3-2 was shown to contain two iterations of the 11-mer motif originally used to define LEA_4s (Figure 2.1). Both of these observations confirm XhLEA3-2's identity as a LEA_4. The absence or low occurrence of Dure's 11-mer LEA_4 motif is not uncommon (Grelet et al. 2005; Menze et al. 2009). This further motivates for a profile-based system, like Pfam, to be the means by which LEAs or LEA-like proteins are classified as opposed to a purely sequence-motif based system.

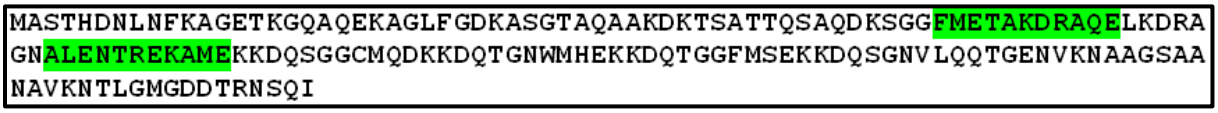

Figure 2.1 XhLEA3-2 amino acid sequence with predicted 11-mer LEA_4 motifs (Dure 1993) **highlighted in green**

2.3.2 XhLEA3-2 Sequence Analysis

ProtParam calculates the proportion of individual amino acids in a given sequence. The proportions of amino acids in XhLE3-2 were characteristic of LEAs (Battaglia et al. 2008). High proportions of alanine, lysine and glycine (12.7%, 12.7% and 11.4% respectively) were predicted as well as a high proportion of hydrophilic amino acids (67%) and a low proportion of cysteine and tryptophan (0.6% together) (Figure 2.2). XhLEA3-2 was predicted to be 16.8 kDa in size.

An amino acid sequence can be used to calculate the Grand Average of Hydropathy (GRAVY) and generate a Kyte-Doolittle hydropathy plot based on the proportion of hydrophilic and hydrophobic residues. In Figure 2.3 below, negative scores indicate hydrophilicity, while positive scores represent hydrophobicity on a 9-point scale ranging from -4.5 to 4.5. XhLEA3-2 was predicted to have a net hydropathy of -1.22 indicated by the red line in Figure 2.3. This score (indicating overall hydrophilicity) is consistent with the hydrophilic character of LEAs (Battaglia et al. 2008).

Residue	Percentage
Ala	12.7
Lys	12.7
Gly	11.4
Gln	8.9
Asp	8.2
Thr	8.2
Asn	7
Glu	6.3
Ser	6.3
Met	4.4
Leu	3.8
Arg	2.5
Phe	2.5
Val	1.9
His	1.3
Cys	0.6
Ile	0.6
Trp	0.6
Pro	0
Tyr	0

Nature	Residue	Percentage
Hydrophilic	Lys	12.7
	Gln	8.9
	Asp	8.2
	Thr	8.2
	Asn	7
	Glu	6.3
	Ser	6.3
	Met	4.4
	Arg	2.5
	His	1.3
	Cys	0.6
	Trp	0.6
	Tyr	0
	Total	**67**
Hydrophobic	Ala	12.7
	Gly	11.4
	Leu	3.8
	Phe	2.5
	Val	1.9
	Ile	0.6
	Pro	0
	Total	**33**

Figure 2.2 Proportions of all amino acids and the cumulative proportions of hydrophilic and hydrophobic amino acids in the XhLEA3-2 amino acid sequence as determined by ProtParam

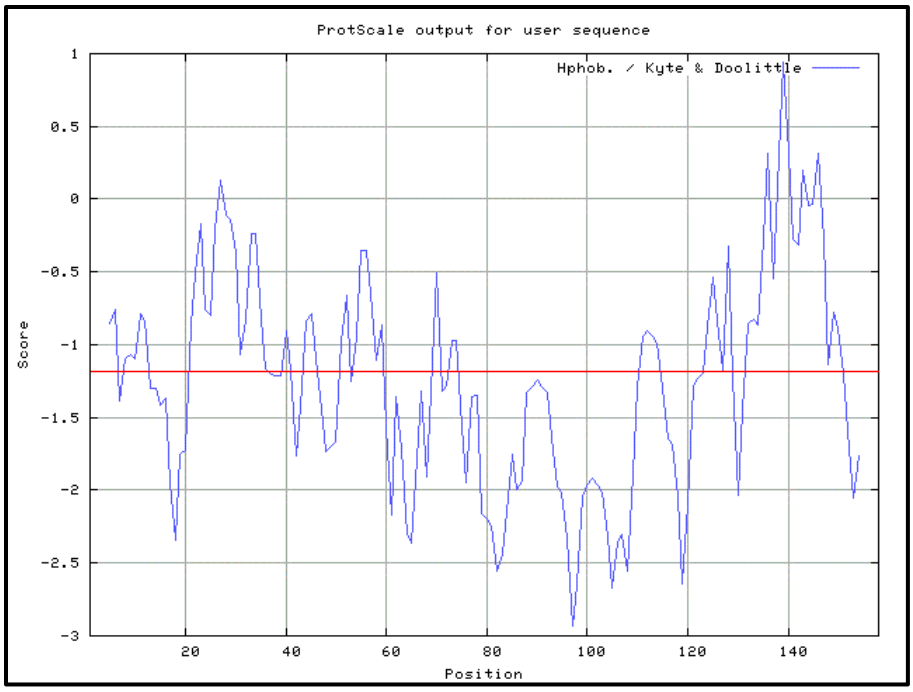

Figure 2.3 A Kyte-Doolittle Hydropathy Plot that shows the relative hydropathy at each residue along the XhLEA3-2 amino acid sequence as generated by GRAVY Calculator. The red line at -1.22 indicates the net hydropathy of XhLEA3-2

2.3.3 XhLEA3-2 Disorder Prediction

In 2010, the MetaDisorder algorithm, MetaDisorderMD2, was judged to be the most accurate disorder predictor at the 9th Critical Assessment of Techniques for Protein Structure Prediction (CASP) experiment (Kozlowski & Bujnicki 2012) thus justifying it's use as opposed to one of the many other disorder predictors available. This software takes an amino acid sequence input and produces a graph where amino acids that lie above 0.5 are predicted to be disordered and those below 0.5, ordered. MetaDisorder predicted XhLEA3-2 to be wholly disordered as indicated by the purple line in Figure 2.4 which lies entirely above 0.5. This predicted disorder is characteristic of LEAs.

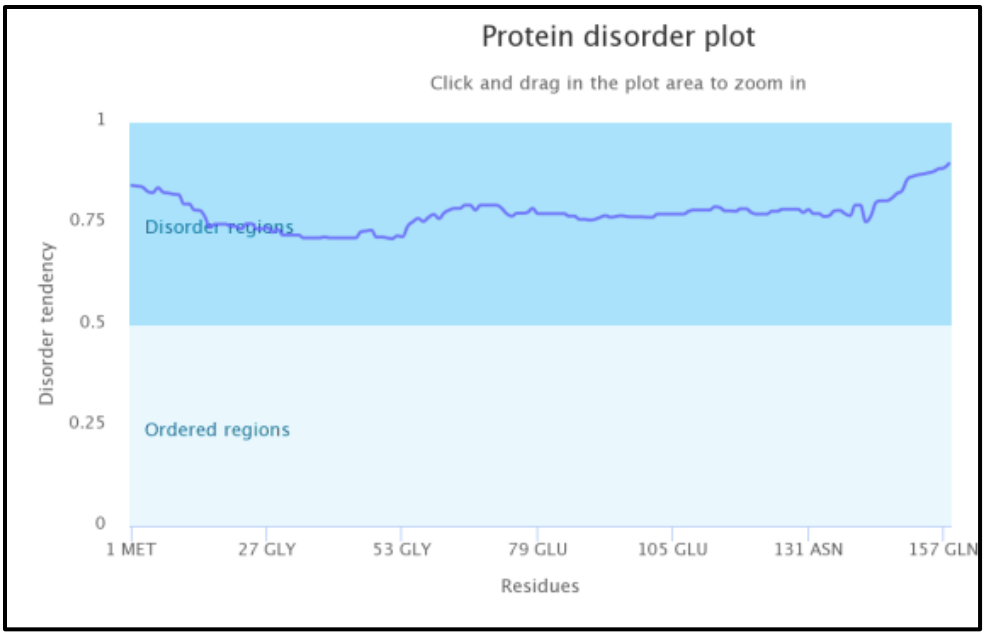

Figure 2.4 Predicted regions of disorder in the XhLEA3-2 amino acid sequence as determined by MetaDisorder. Disordered regions lie above 0.5 on the graph and are highlighted in blue

2.3.4 Identification of XhLEA3-2 Molecular Recognition Features

Identifying MoRF sequences in novel proteins can be used to infer function if they correspond to previously described MoRFs in the MoRF database, mpMoRFsDB. XhLEA3-2 was predicted to have putative MoRFs at positions 10-46 and 54-158 (Figure 2.5 – highlighted in blue). These sequences were blasted against the mpMoRFsDB but returned no hits. This is not unexpected as the mpMoRFsDB is still in its infancy containing only 172 entries. As the characterization of MoRFs grows, this field will become more useful in determining novel IDP function.

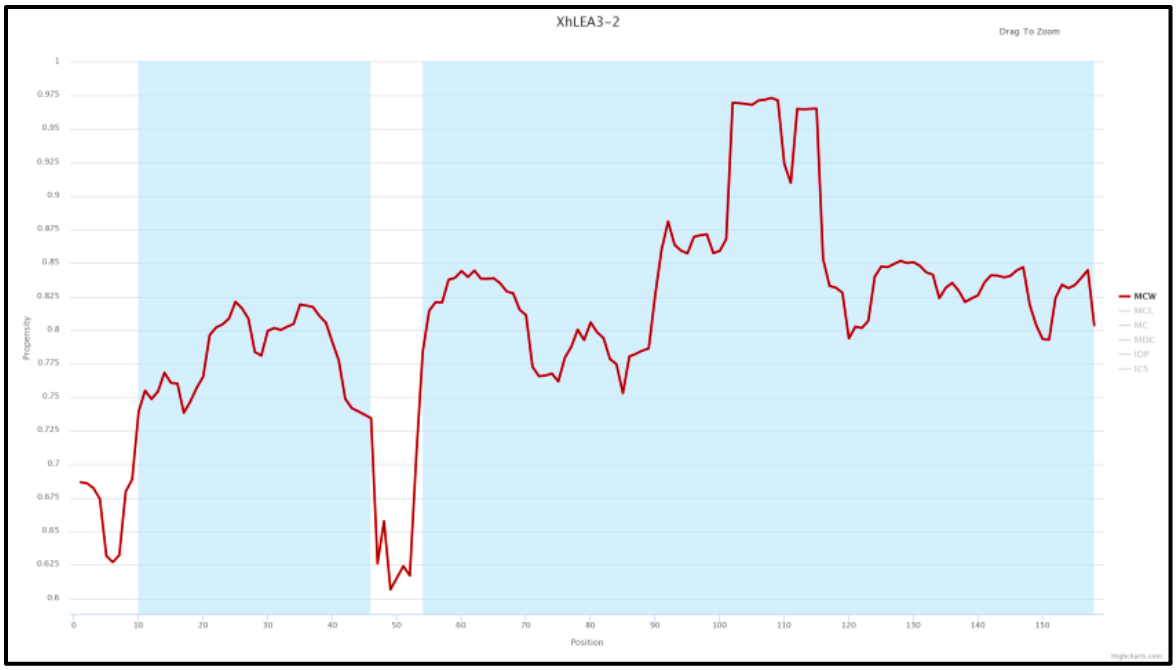

Figure 2.5 Predicted Molecular Recognition Features (MoRFs) in the XhLEA3-2 amino acid sequence as determined by MoRFchibi. Predicted MoRFs are highlighted in blue

2.3.5 Predicted XhLEA3-2 Function, Localization and Post-Translational Modifications

The PSIPred server (Buchan et al. 2013) developed by the Bloomsbury Centre for Bioinformatics (BCB) at University College London (UCL) is a well-established server for novel-protein function prediction. FFPred is an online tool hosted by the PSIPred server which predicts protein biological and molecular function based on Gene Ontology (GO) terms. FFPred predicted XhLEA3-2 to play a role in cross-membrane transport and to act as a cytoskeletal binding protein with probabilities greater than 0.8.

FFPred is also capable of predicting subcellular-localization based on GO terms. Furthermore, it can predict PTMs of novel proteins. FFPred predicted XhLEA3-2 to be localized at the cell membrane and to have several sites of glycosylation and phosphorylation as seen in Figure 2.6. Many studies (Goyal et al. 2005; Reyes et al. 2005; Reyes et al. 2008 *inter alia*) have

shown LEAs to have a molecular chaperone activity yet this prediction suggests somewhat otherwise. The role of XhLEA3-2 at the cell membrane deserves further study.

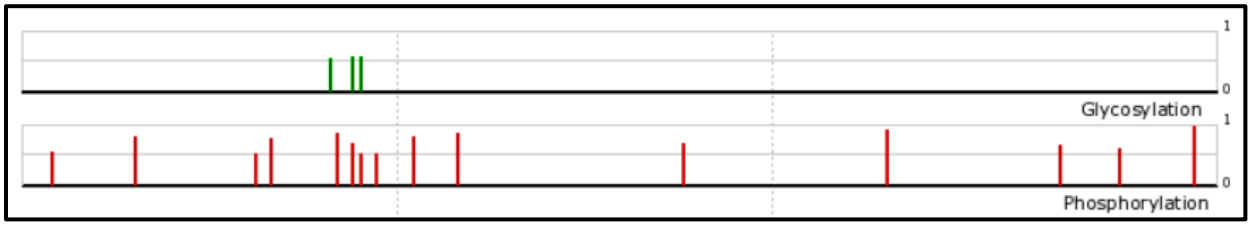

Figure 2.6 Predicted sites of phosphorylation and glycosylation in the XhLEA3-2 amino acid sequence as determined by FFPred

2.3.6 Predicted XhLEA3-2 Secondary Structure

When folded, PSIPred predicted XhLEA3-2 to have a largely alpha-helix secondary structure (Figure 2.7). This is consistent with the features typical of LEA proteins – when folded; they tend to form alpha-helices.

Figure 2.7 Predicted secondary structure of the XhLEA3-2 amino acid sequence as determined by PSIPred. Blue represents predicted helix. Black represents predicted random coil

2.3.7 XhLEA3-2 Homologs in *X. Viscosa*

The amino acid sequence of XhLEA3-2 was blasted against the *X. viscosa* genome to search for LEA-homologs (Costa, personal communication; Costa et al. 2017). Two proteins – Xvis02_15976 and Xvis02_11331 – were identified to have sequence similarities of 92% and 81% respectively. The multiple sequence alignment of XhLEA3-2, Xvis02_15976 and Xvis02_11331 is illustrated in Figure 2.8. Clearly, there is very little difference in sequence between the three LEA-homologs. Perhaps this is not unexpected as the three proteins belong to

two very closely related species. But, it is interesting to note the level of conservation between them. Their sequences are at appendices A1, A3 and A4.

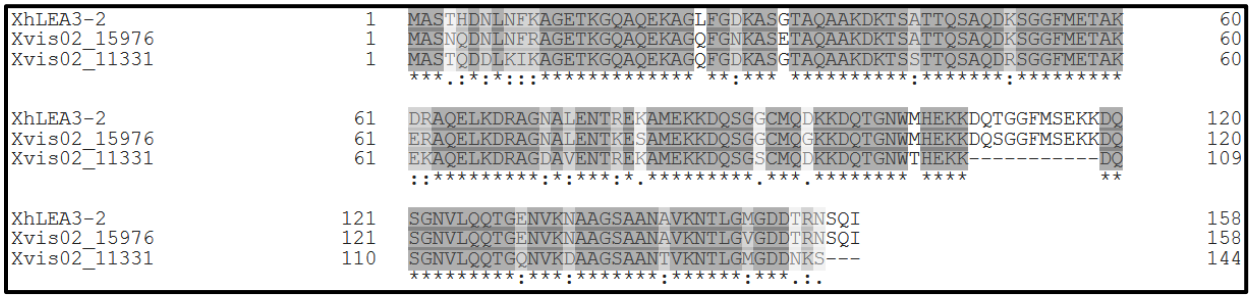

Figure 2.8 Multiple Sequence Alignment (MSA) of XhLEA3-2 and its two closest relatives in *X. viscosa*

Chapter 3 - The Production and Testing of XhLEA3-2 Antibodies

3.1 Introduction

3.1.1 Recombinant LEA Expression in *E. coli*

Recombinant protein expression in *E. coli* is a cheap and efficient way of obtaining a sufficient amount of protein for biochemical studies. *E. coli* reaches high culture volumes rapidly and the maintenance of an axenic culture is easily achieved with specific antibiotics in the growth medium coupled with antibiotic-resistance genes in the strain of interest. Furthermore, the rapid growth of *E. coli* means that optimizing protein expression can be done in a time-efficient manner and multiple expression cultures can be grown in parallel under different conditions in a single day. The suite of commercially-available vectors for protein expression in *E. coli* allows for: easy and flexible construction of a protein expression plasmid by standard DNA cloning techniques, the incorporation of antibiotic resistance into a specific bacterial strain, the option to add molecular tags to a protein of interest for downstream processing (e.g. purification), and the optimization of protein expression, *in vivo*, by using different vectors that perform better under certain circumstances (Rosano & Ceccarelli 2014). However, ectopic expression of LEAs in *E. coli* is by no means cut-and-dried. Low expression levels, no expression, and the presence of multiple or anomalously-sized bands on a Western Blot (Ried & Walker-Simmons 1993) are some of the problems that have been encountered when recombinant LEA expression has been attempted. For example, Liu et al. (2009) observed a discrepancy between the calculated *BhLEA2* size (13.5 kDa) and the size on a SDS gel (23 kDa). This is consistent with results from Velasco et al. (1998) who observed size discrepancies as well as multiple bands for the LEA-like protein pcCC2. Furthermore, other researchers (Fan, 2016; M. Artur, personal communication) have observed similar phenomena for *Xerophyta* LEA proteins.

Several reasons have been put forward in the literature to try explain the multiple bands/atypical migration of LEAs in SDS-PAGE gels. These include: weakened interactions between charged amino acids (abundantly found in LEAs) and SDS (Koag et al. 2009; Amara et al. 2014), and PTMs which are known to reduce the electrophoretic mobility of proteins in SDS-PAGE (Carruthers et al. 2015).

Despite these issues, many studies have shown successful recombinant LEA expression and purification (Ried & Walker-Simmons 1993; Svensson et al. 2000; Tolleter et al. 2007; Ginbot 2011; Waters 2015; Fan 2016; Gao & Lan 2016). Usually, a pool of LEAs identified in a certain species is narrowed down to those which are readily recombinantly expressed and these are then used for further study (Gao & Lan 2016). Recombinant LEA expression in *E. coli* was chosen for this study as an appropriate system to produce the required amount of protein for antibody production as well as for the other studies covered in Chapter 4.

3.1.2 XhLEA3-2 Polyclonal Antibody Production

Antibodies are iconic, 'Y-shaped' proteins produced by the immune system which target foreign molecules as well as microbial and/or viral toxins to trigger their inactivation and degradation (Alberts et al. 2002). The innate, high antigen-specificity of antibodies has led to their natural function being 'hijacked' by molecular biologists and consequently, antibodies are commonly used in research as accurate molecular probes. Polyclonal antibodies are named as such due to the fact that multiple B-cell clonal lines in an animal's immune system are each involved in producing their own unique type of antibodies which target the same antigen but at a different epitope. Monoclonal antibodies, on the other hand, are those produced using a single clonal line of B-cells. Consequently, monoclonal antibodies only recognize one specific epitope on the antigen. Therefore, monoclonal antibodies have a more distinct target-specificity, but they

are more expensive to produce. Polyclonal XhLEA3-2 specific antibodies were chosen for this study because they are cheap, easily purified and have the potential to detect XhLEA3-2 homologs provided that a common epitope is present in both targets. These antibodies were then used to detect XhLEA3-2 expression in *X. humilis* and *X. viscosa*, at different levels of hydration, by western blot.

3.2 Methods

3.2.1 Construction of the XhLEA3-2 Expression Plasmid

Previously, XhLEA3-2 was cloned into a modified pET-21a(+) expression vector (Novagen) and sequenced to confirm sequence-accuracy and in-frame cloning (Dennis 2015). This modified vector was mutagenized by Ginbot (2011) so that the stock N-terminal T7-tag was directly replaced with a 6X His-Tag. The XhLEA3-2:pET-21a (His) construct was transformed into the BL21 (DE3) pLysS *E.coli* (Novagen) expression strain (Dennis 2015). Transformed cells were stored at -80 ^{o}C in 50% glycerol.

3.2.2 Optimizing Recombinant XhLEA3-2 Expression in *E. coli*

The XhLEA3-2 expression strain was streaked onto sterile Luria Broth (LB) agar (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar) containing 100 µg/ml ampicillin (amp) and 25 µg/ml chloramphenicol (chlr). Overnight incubation followed at 37 ^{o}C. A single colony from the overnight growth was used to inoculate 5 ml sterile LB-chlr-amp liquid medium. This culture was grown overnight at 37 ^{o}C with shaking at 150 rpm. Next, 1 ml of the overnight culture was inoculated into 4X 50 ml sterile LB-chlr-amp media in 250 ml Erlenmeyer flasks and the 50 ml cultures were incubated until mid-log phase (~2 hrs, OD_{600} = 0.6). At this point, a final concentration of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each culture. The cultures were then incubated at 30 ^{o}C with shaking at 100 rpm. Each culture was

removed from incubation at different time points – 1 hr, 2 hrs, 4 hrs and 16 hrs (O/N) – and the entire 50 ml culture volume was immediately centrifuged at 10 000 g for 10 min at 4 ^{o}C. The supernatant was discarded and the pelleted cells were resuspended in 5 ml Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and lysed by sonication. A 10 µl volume of lysed cells from each culture was separated on a 12% SDS-PAGE gel and probed via western blot with a commercially available HRP-conjugated Anti-6X His-tag antibody (Abcam) at a concentration of 1:10 000 to detect recombinant XhLEA3-2 expression.

3.2.3 SDS-PAGE and Western Blot

Because of the small size of recombinant XhLEA3-2 (predicted 19.3 kDa with 6X His-tag), a 12% SDS-PAGE gel was determined to be the appropriate concentration for good resolution of XhLEA3-2. Unless otherwise stated, all SDS-PAGE gels were run at 90 V for 2 hrs with an initial protein-load of 10 µg in the required amount of 5X SDS Loading Buffer (0.25 M Tris-HCl pH 6.8, 0.1 g/ml SDS, 30% glycerol v/v, 5% β-Mercaptoethanol v/v, 0.2 g/L Bromophenol Blue (Sigma-Aldrich)) and boiled for 2 min before loading. A 5 µl volume of Colour Prestained Protein Standard (New England Biolabs) was used as the molecular weight marker unless otherwise stated. After electrophoresis, gels were either stained with Coomassie Blue Solution (2.5 g/L Coomassie Blue (Sigma-Aldrich), 50% v/v methanol, 10% v/v acetic acid) or used for western blotting. If used for staining, gels were incubated in Coomassie Blue Solution for 1 hr at room temperature with gentle shaking followed by an overnight destain step in Coomassie destain solution (45% v/v methanol and 10% v/v acetic acid). If used for western blot, gels were transferred to nitrocellulose membrane for 1 hr at 100 V in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol v/v). The appearance of the protein molecular weight marker on the membrane was considered indicative of successful protein transfer. After transfer,

the membrane was washed in TBS-Tween (137 mM NaCl, 2.7 mM KCl, 19 mM Tris Base, 0.1% Tween-20) for 5 min with gentle shaking. Empty protein binding sites on the membrane were then blocked in 10 ml blocking buffer (10% skim milk powder in TBS-Tween) for 1 hr at room temperature with gentle shaking. The primary antibody was then added to the blocking buffer at the manufacturer-specified or experimentally-determined concentration and allowed to incubate for 1 hr at room temperature with gentle shaking (or overnight at 4 ^{o}C, gentle shaking). If a secondary antibody was required, the primary antibody and excess blocking buffer were discarded and the membrane washed three times in TBS-Tween for 5 min each with gentle shaking. The secondary antibody was diluted in 10 ml blocking buffer to the manufacturer-specified or experimentally-determined (see 3.2.11) concentration, added to the membrane, and then allowed to incubate for 1 hr at room temperature with gentle shaking (or overnight at 4 ^{o}C, gentle shaking). After incubation, the secondary antibody and blocking buffer were discarded and the membrane was washed four times for 5 min each with TBS-Tween, gentle shaking followed by a 1 min rinse with dH_2O. The membrane was incubated with a chemiluminescent substrate from the WesternBright (Advansta) kit according to the manufacturer's instructions. The membrane was visualized under UV with a Molecular Imager ChemiDoc XRS+ system (Bio-Rad, Germany) according to the manufacturer's instructions.

3.2.4 Protein Quantification by Bradford Assay

The concentrations of all unknown protein samples were calculated by a standard 96-well plate Bradford Assay (Bradford 1976). Briefly, 10 µl of a protein solution with unknown concentration was added to a 96 well PolySorp® flat bottom plate (Sigma-Aldrich) in triplicate. Then, 10 µl of the buffer in which the protein samples of unknown concentration were in was added to the next row of wells in triplicate. Aliquots of 10 µl of Bovine Serum Albumin (BSA)

protein standards of the following concentrations, diluted in the unknown protein buffer, were added to empty wells in triplicate: 1.29 mg/ml; 1.00 mg/ml; 0.80 mg/ml; 0.65 mg/ml; 0.50 mg/ml; 0.32 mg/ml; 0.16 mg/ml; 0.08 mg/ml. Finally, 200 µl of 1X Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) diluted in dH_2O from a 5X stock solution was added to each protein-containing well and also to the three wells containing the buffer only. The absorbance of each well at 595 nm was measured by a Multiskan MCC Type 355 Microplate Reader (ThermoFisher Scientific). Ascent™ computer software (ThermoFisher Scientific) was used to generate a standard curve and calculate the unknown protein concentration according to the software instructions.

3.2.5 Recombinant XhLEA3-2 Expression in *E. coli*

A single colony of the XhLEA3-2:pET-21a BL21 (DE3) pLysS *E. coli* strain was used to inoculate 10 ml sterile LB-amp-chlr medium which was then incubated overnight at 37 ^{o}C with shaking at 150 rpm. This overnight culture was the initial inoculum for 1 L of fresh, sterile LB-chlr-amp medium contained in a 5 L Erlenmeyer flask. The culture was grown to mid-log phase (~3 hrs, OD_{600} = 0.6) at 37 ^{o}C with shaking at 200 rpm. At this point, IPTG was added to a final concentration of 1 mM and incubation continued for another 4 hrs at 30 ^{o}C with 100 rpm shaking. Cells were collected by centrifugation at 10 000 g for 10 min at 4 ^{o}C). The supernatant was discarded and the pellet stored at -20 ^{o}C overnight.

3.2.6 Recombinant XhLEA3-2 Purification

Protino Ni-TED Packed Columns (Macherey-Nagel) were used for recombinant XhLEA3-2 protein purification according to the manufacturer's instructions but with an additional boiling step included. Briefly, the pellet obtained from an *E. coli* expression culture was resuspended in 15 ml LEW buffer (Macherey-Nagel). Lysozyme (Sigma-Aldrich) was

added to the cell solution to a final concentration of 1 mg/ml and stirred on ice for 30 min. The cells were then sonicated to ensure complete cell lysis and the lysate centrifuged at 10 000 g for 30 min at 4 ^{o}C to remove cellular debris. The supernatant was separated from the pellet and boiled for 10 min. The boiled lysate was then centrifuged again at 10 000 g for 30 min at 4 ^{o}C. The supernatant was removed and the pellet discarded. One Protino Ni-TED Packed Column was equilibrated by passing 4 ml LEW buffer through the column by gravity flow. The supernatant from the boiled cell-lysate was passed through the column. The column was washed with 8 ml LEW buffer and the recombinant XhLEA3-2 eluted in 6 ml of elution buffer (Macherey-Nagel). Aliquots (1 ml) from all the above mentioned purification steps were collected and stored at -20 ^{o}C for analysis. The eluted fraction was dialyzed against 15 ml of dH_2O three times using an Amicon® Ultra-15 3K Centrifugal Filter Device (Merck Millipore) at 7000 g for 1 hr at 4 ^{o}C. The purified XhLEA3-2 as well as the 1 ml aliquots from the different purification steps were quantified using a standard Bradford assay as described earlier (3.2.4). A 10 µg sample of protein from all steps in the above process was run on a 12% SDS-PAGE gel and probed via western blot with a commercially available HRP-conjugated Anti-6X His tag antibody (Abcam) at a concentration of 1:10 000.

3.2.7 Testing for Recombinant XhLEA3-2 Phosphorylation

The Pro-Q® Diamond phosphoprotein stain (Invitrogen) is used as an efficient method for the preliminary identification of phosphorylated proteins in SDS-PAGE gels. The stain was used according to the manufacturer's instructions. Briefly, 10 µg of pure recombinant XhLEA3-2, 5 µl Colour Prestained Protein Standard (New England Biolabs) and 2 µl of PeppermintStick™ phosphoprotein molecular weight standards (Invitrogen) were run on a 12% SDS-PAGE gel for 2 hrs at 100 V. After electrophoresis, the gel was fixed in 100 ml 50% methanol, 10% acetic acid

for 30 mins twice. Then, the gel was washed three times in 100 ml dH_2O for 10 min each. After washing, the gel was stained with 60 ml Pro-Q® Diamond stain for 1 hr. The gel was then destained in 100 ml 20% acetonitrile, 50 mM sodium acetate, pH 4.0 for 30 min three times. The gel was then washed with 100 ml dH_2O twice for 5 min each. The gel was visualized under UV with a Molecular Imager ChemiDoc XRS+ system (Bio-Rad, Germany) according to the manufacturer's instructions.

3.2.8 Polyclonal XhLEA3-2 Antibody Production

Prior to the administration of antigen (in this case, recombinant XhLEA3-2), 10 ml of pre-immune blood was collected from the central ear artery of a female New Zealand White rabbit. Following this, 0.5 mg of purified XhLEA3-2 mixed with an equal volume of Incomplete Freund's Adjuvant (IFA) (Sigma-Aldrich) was injected subcutaneously into the back of each rabbit. The first booster injection (the same contents as above) was administered two weeks subsequent to the initial injection and two additional booster injections followed at one week intervals. Every two weeks after the initial injection, the rabbit was sedated and 5 ml blood was collected to monitor antibody development using a standard Enzyme-Linked Immunosorbent Assay (ELISA) as described later. After nine weeks from the initial injection, the rabbit was euthanised and exsanguinated to obtain the maximum antibody yield. All blood samples were spun down at 2000 g for 10 min at 4 ^oC. The serum (supernatant) was harvested and stored at -20 ^oC. The above work was done with assistance from the Animal Research Unit at the University of Cape Town.

3.2.9 Monitoring Antibody Production by ELISA

A 100 µl volume of a 3 ng/µl solution of recombinant XhLEA3-2 in PBS was added to 24 wells of a 96 well PolySorp® flat bottom plate (Sigma-Aldrich) and incubated, covered,

overnight at 4 ^{o}C. After incubation, the solution was removed and the wells were washed three times with 200 µl TBS-Tween. The wells were then blocked with 200 µl of 5% skim-milk powder in TBS-Tween (blocking buffer) for 2 hrs at room temperature followed by one wash with 200 µl TBS-Tween. A 100 µl volume of rabbit serum diluted to 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} in blocking buffer was added from each bleed and incubated, covered, for 2 hrs at room temperature. The wells were then emptied and again, washed three times with 200 µl TBS-Tween. Then, 100 µl of HRP-conjugated goat anti-rabbit secondary antibody (Agrisera) diluted according to the manufacturer's instructions (1:50 000) in blocking buffer was added to each well and incubated, covered, for 2 hrs at room temperature. The wells were emptied and washed three times with TBS-Tween. A 150 µl volume of 1-Step Slow TMB ELISA Substrate Solution (ThermoFisher Scientific) was added to each well and incubated for 30 mins until a colour change was observed. The reaction was stopped by the addition of 150 µl of 2 M H_2SO_4 to each well. The absorbance of each well was measured at 450 nm with a Multiskan MCC Type 355 Microplate Reader (ThermoFisher Scientific).

3.2.10 Purification of XhLEA3-2 Polyclonal Antibodies

Purification of the XhLEA3-2 polyclonal antibodies was performed according to the protocol published online by the Langdale Lab (https://langdalelab.com/). Recombinant His-tagged XhLEA3-2 was expressed in *E. coli* following the method described earlier. The cells were lysed and boiled as described above and the boiled lysate was passed through a Protino Ni-TED Packed Column (Macherey-Nagel). The column was then washed with 10 column volumes (10 ml) of equilibration buffer A (150 mM NaCl, 50 mM Tris-HCl, 100 ml dH2O, pH 7.4). Next, 2 ml of crude XhLEA3-2 rabbit antiserum was passed through the column. The flow-through was collected and re-passed through the column three times. The column was washed with 5 ml

of equilibration buffer A and then 5 ml of equilibration buffer B (2 M NaCl, 50 mM Tris-HCl, 100 ml dH2O, pH 7.5). A 1 ml volume of antibody elution buffer (4 M MgCl2, 4.3 ml dH2O) was added to the column and allowed to drain into the column so that it had replaced the previous solution. The column was then stoppered and left for 15 min at room temperature. The column was un-stoppered and 2 ml of antibody elution buffer was added and allowed to drain through. The three 1 ml antibody elution buffer fractions were collected and dialyzed against PBS. The fractions were used to probe crude tobacco plant extract and pure XhLEA3-2 by western blotting to test for specificity.

3.2.11 Optimizing Pure XhLEA3-2 Antibody Concentration by Dot Blot

One piece of nitrocellulose membrane was cut into 4 pieces each with 3 demarcated sections. Masses of 5 µg, 10 µg and 15 µg of pure recombinant XhLEA3-2 were spotted onto the three sections for each piece of membrane and allowed to dry. The membranes were blocked in 10 ml of 5% skim-milk powder in TBS-Tween (blocking buffer) for 1 hr at room temperature with gentle shaking. Primary antibody (purified rabbit serum) was added at different concentrations – 1:5000, 1:10 000, 1:30 000, 1:50 000 – and allowed to probe the membrane for 30 min at room temperature. The blocking buffer and primary antibody were then discarded and the membranes were washed three times for 5 min with 10 ml TBS-Tween. HRP-conjugated secondary antibody (Goat Anti-Rabbit) was diluted in 10 ml blocking buffer at the concentration specified by the manufacturer (1:50 000) and allowed to probe the membrane for 30 min at room temperature with gentle shaking. The membrane was washed three times for 5 mins with 10 ml TBS-Tween and then once with 10 ml dH_2O for 5 mins. The membranes were incubated with a chemiluminescent substrate from the WesternBright (Advansta) kit according to the

manufacturer's instructions and visualized under UV with a Molecular Imager ChemiDoc XRS+ system (Bio-Rad, Germany) according to the manufacturer's instructions.

3.2.12 XhLEA3-2 Expression in *X. humilis* during Dehydration

Previously, *X. humilis* plants, acclimated in plant growth chambers (Conviron) to conditions of 60% relative humidity, light intensity of 150-200 µmol/m^{-2}/s^{-1}, and a photoperiod of 16 hour light and 8 hour dark, were subject to dehydration (Waters 2015). Leaf samples at different relative water contents (RWCs) were sampled and total protein extracted. This process is similar to that described in detail for *X. viscosa* below in 3.2.13, 3.2.14 and 3.2.15. Samples (40 µg) of *X. humilis* protein extracted by (Waters 2015) at 100% RWC, 50% RWC and 10% RWC were run on a 12.5% SDS-PAGE gel, blotted onto nitrocellulose membrane and then probed using the XhLEA3-2 specific antibody produced in 3.2.8 – 3.2.10 to detect for XhLEA3-2 expression.

3.2.13 *X. viscosa* Plant Material

X. viscosa plants were collected from Buffelskloof Nature Reserve near Lydenberg, Mpumalanga Province, South Africa. They were immediately transported to the University of Cape Town, Plant Stress Lab where they were kept under greenhouse conditions as described by (Sherwin & Farrant 1996). Two weeks prior to dehydration, plants were acclimatized in plant growth chambers (Conviron) with the following environmental parameters: 16 hrs light (100 µmol.m^{-2}.s^{-1}; 25 ^{o}C) and 8 hrs dark (15 ^{o}C) with 50% relative humidity. These conditions were maintained throughout the dehydration and rehydration described below.

3.2.14 *X. viscosa* Dry-Down, Rehydration and Sampling

Four mature *X. viscosa* plants were watered thoroughly to soil saturation at dusk and then four leaves from each plant were removed at dawn the following day. The sampled leaves were

immediately cut into three small sections with the sections from 'leaf one' being weighed individually to determine fresh weight (FW) and the sections from the other leaves immersed in liquid nitrogen and stored at -80 ^{o}C for subsequent experimentation. The sections from leaf one were then dried in a 70 ^{o}C oven for 2 days after which they were placed in a desiccator for 10 min and then weighed immediately to determine dry weight (DW). The absolute water content (AWC) was calculated according to the formula below for each leaf section and then averaged to get the mean AWC for the four *X. viscosa* plants.

$$AWC = \frac{(FW - DW)}{DW}$$

This initial AWC is the AWC at full turgor (AWCft) and is used to calculate the percentage relative water content (% RWC) according to following formula where AWCn represents the AWC at time point 'n':

$$\% RWC = \left(\frac{AWCn}{AWCft}\right) \times 100$$

The fully watered *X. viscosa* plants were allowed to dry to an air-dry state over a period of 15 days. Four leaves per plant were excised at 10 AM on days 3, 5, 7, 9, 11, 13 and 15. Again, one leaf was sectioned in three and the sections weighed immediately to determine the FW for each day. Then, the sections were dried for 2 days in a 70 ^{o}C oven and placed in a desiccator for 10 min after which they were weighed immediately to determine DW. The AWC was then calculated followed by the % RWC for each day. On day 16, plants were rehydrated to soil saturation and leaf samples (four per plant) were excised after 9 hrs, 24 hrs and 72 hrs post-rehydration. All leaf samples that were not used to determine % RWC were immediately immersed in liquid nitrogen and stored at -80 ^{o}C. Note that the protocol described in detail here was also utilized in experimentation described for *X. humilis* (above). This is a relatively

standard procedure in determining RWC of the same tissues in which molecular studies are subsequently performed.

3.2.15 *X. viscosa* Protein Extraction and Analysis

Frozen, stored leaf material at various pre-determined RWC's, was thoroughly ground with a small plastic pestle that was able to fit into a 1.5 ml microcentrifuge tube. Then, 750 µl of Trizol Reagent (Invitrogen) was added to the homogenized leaf material and vortexed for 5 min. The mixture was incubated at room temperature for 5 min before 200 µl of chloroform was added and mixed by inversion. Again, the mixture was incubated at room temperature for 5 min and then centrifuged at 12 000 g for 15 min at 4 ^{o}C. The aqueous phase was removed and stored at -80 ^{o}C for future use in extraction of total RNA (if desired). The organic phase was re-suspended in 300 µl ethanol and then incubated at room temperature for 5 min. This mixture was then centrifuged at 2500 g for 10 min at room temperature. The supernatant (soluble protein) was removed, added to 1.5 ml of isopropanol and incubated for 10 min at room temperature. Then, the mixture was centrifuged at 10 000 g for 10 min at 4 ^{o}C. The supernatant was removed and the protein pellet was washed three times with 2 ml 0.1 M ammonium acetate (prepared in methanol), once with 2 ml cold acetone and then air dried for 10 min. The dried protein was resuspended in 100 µl Laemmli Buffer (0.625 M Tris-HCl pH 6.8, 0.2 g/L SDS, 10% glycerol v/v, 5% β-mercaptoethanol v/v). The protein extracts were quantified by Bradford Assay and then 40 µg of each extract was run on a 12% SDS-PAGE gel and probed, with the previously produced XhLEA3-2 antibody (3.2.8 – 3.2.10), by western blot.

3.3 Results and Discussion

3.3.1 Optimizing Recombinant XhLEA3-2 Expression in *E. coli*

Optimization of recombinant protein expression in *E. coli* is important so as to obtain the maximum yield of protein per round of expression thus saving time and materials. Figure 3.1 shows a time-series depicting recombinant XhLEA3-2 expression in *E. coli* after 1 hr, 2 hrs, 4 hrs and 16 hrs (O/N) after induction with IPTG.

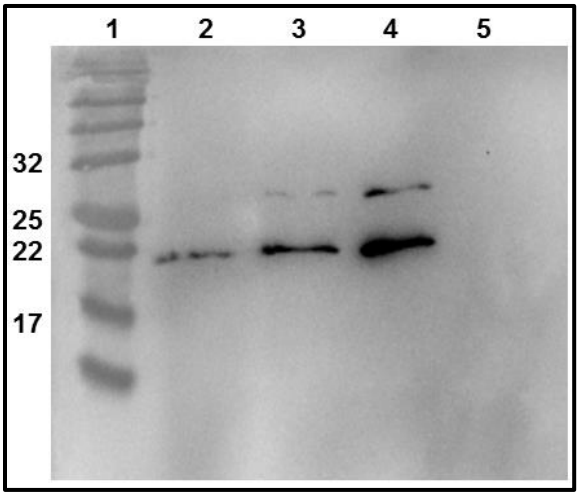

Figure 3.1 A time series of recombinant XhLEA3-2 expression in *E. coli* culture after induction with IPTG. Lane 1, Colour Prestained Protein Standard (kDa). Lanes 2-5, 1 hr, 2 hrs, 4 hrs and 16 hrs (O/N) post-induction respectively.

After expression, two bands are observed: an intense band at the predicted molecular size of recombinant XhLEA3-2 (19.3 kDa – including His-tag) and a faint band at a higher molecular weight (~27 kDa). The presence of two bands as opposed to a single band is discussed in 3.3.2 and 3.3.3. The highest expression levels (most intense bands) were obtained 4 hrs after induction with IPTG. However, it cannot be ruled out that even higher expression could have been achieved if induction was carried out for a longer period of time (e.g. 5 hrs, 6 hrs). However, it is evident that leaving the culture for periods of up to 16 hrs (O/N) resulted in the complete degradation of recombinant XhLEA3-2, as shown by no apparent bands in lane 5 (Figure 3.1).

Therefore, in order to restrict the duration of the process of culture-growing, induction and cell harvesting to one day, 4 hrs was decided as the maximum time that could practically be allowed for induction.

3.3.2 Recombinant XhLEA3-2 Purification

An aliquot at each step in the extraction and purification procedure – cell lysate, boiled cell lysate, column flow-through, column wash, protein elution and protein concentration – was quantified and equal quantities run on an SDS-PAGE gel as seen in Figure 3.2.

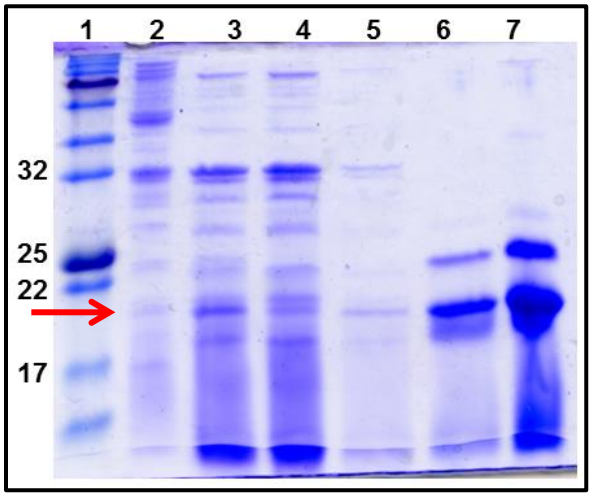

Figure 3.2 Coomassie-stained 12% SDS-PAGE gel showing all the steps involved in the expression and purification of recombinant XhLEA3-2. Lane 1, Colour Prestained Protein Standard (kDa). Lane 2, crude cell lysate. Lane 3, boiled crude cell lysate. Lane 4, column flow-through. Lane 5, column wash. Lane 6, XhLEA3-2 elution. Lane 7, concentrated pure XhLEA3-2. The expected size of XhLEA3-2 (19.3 kDa) is indicated by the red arrow.

A band at the expected size of recombinant XhLEA3-2 (19.3 kDa) stands out strongly in the elution and concentration steps and faintly in the boil, flow-through and wash steps. The absence of a clear band at 19.3 kDa in the cell lysate step is most probably due to the presence of many other cellular proteins that, as a whole, exist in a much higher concentrations relative to recombinant XhLEA3-2. Boiling was included as an additional step to aid in purification by exploiting the heat-stable nature of LEA proteins (Russouw et al. 1995; Tunnacliffe & Wise

2007). Boiling resulted in the aggregation and precipitation of the non-heat-stable *E. coli* proteome while XhLEA3-2 remained soluble. The insoluble proteins were then removed by centrifugation. Because of this, the band at the size of recombinant XhLEA3-2 (19.3 kDa) stands out more strongly in lane 2 as compared to lane 1 due to the relative concentrations of other proteins having been decreased. A faint band at the expected size of recombinant XhLEA3-2 in the wash step suggests that some of the recombinant protein is lost during this step possibly due to the saturation of free Nickel-binding sites on the column. In the elution and concentration steps, an intense band at 19.3 kDa was observed as well as a fainter band at about 27 kDa as observed previously (Figure 3.1). The very faint bands in these lanes are likely contaminating proteins but are at acceptably low levels. Dialysis of the eluted proteins removed unwanted salts; in particular the imidazole found at high concentrations in the elution buffer. Imidazole strongly absorbs UV light and so therefore it must be removed from protein samples before they can be used in spectrophotometric assays.

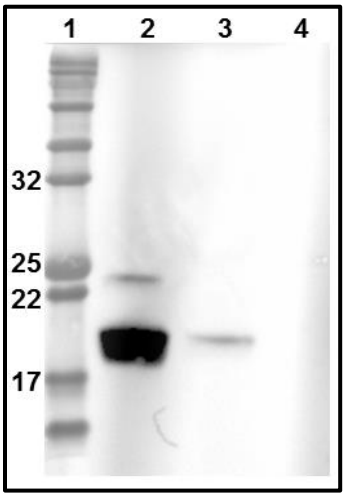

Figure 3.3 Probing for His-tagged recombinant XhLEA3-2 via Western Blot. Lane 1, Colour Prestained Protein Standard (kDa). Lane 2, pure XhLEA3-2. Lane 3, crude cell lysate of XhLEA3-2, *E. coli* expression culture. Lane 4, crude cell lysate of *E. coli* expression culture containing empty expression vector.

To confirm that the two bands observed after purification of recombinant XhLEA3-2 on a Coomassie-stained SDS-PAGE gel were, in fact, the recombinantly expressed His-tagged XhLEA3-2 (as opposed to non-specific binding), the purified recombinant XhLEA3-2 was probed using a commercially available HRP-conjugated Anti-6X His-tag antibody (Figure 3.3). Again, the intense band at the expected size and faint band at a higher molecular weight were observed. This confirms that both proteins represented by each band are His-tagged. Furthermore, no bands were observed when *E. coli* culture containing the empty pET-21a (His) expression vector was induced with IPTG and the lysed cell extract probed with the antibody. This demonstrates that there are no non-specific *E.coli* proteins that the Anti-6X His-tag antibody is binding to. Thus, both bands are recombinant XhLEA3-2 or part of recombinant XhLEA3-2. It is possible that the larger molecular weight band is a separate protein somehow produced from the XhLEA3-2:pET-21a(His) construct (perhaps as a result of the *E. coli* polymerase erratically missing the stop codon in the expression vector) or, it is the same molecular weight as the more intense band but for some other reason is migrating consistently at a slower rate.

3.3.3 Testing for Recombinant XhLEA3-2 Phosphorylation

Because LEAs are known to have a decreased electrophoretic mobility and PTMs have been proposed as a reason for this (see 3.1.1), it was decided to test to see if recombinant XhLEA3-2 was partially phosphorylated to try to determine a reason for the slower migration of the faint band in 3.3.2. Pro-Q® Diamond phosphoprotein staining is a cheap and efficient method for the preliminary identification of phosphorylated proteins in SDS-PAGE gels. The results from this phosphoprotein-specific stain are illustrated in Figure 3.4.

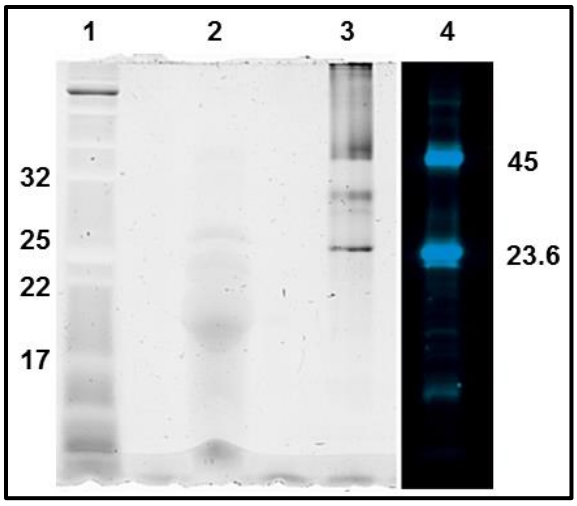

Figure 3.4 Pro-Q® Diamond stained 12% SDS-PAGE gel and PeppermintStick™ phosphoprotein standards. Lane 1, Colour Prestained Protein Standard (kDa). Lane 2, pure XhLEA3-2. Lane 3, visualized PeppermintStick™ phosphoprotein standards (kDa). Lane 4, theoretical PeppermintStick™ phosphoprotein standards (kDa).

A dark band represents phosphorylation of a serine, threonine or tyrosine residue(s). The poor visualization of this gel is due to the fact that a UV Molecular Imager was used as opposed to visualization under light of 500-600 nm (the manufacturer's recommendation). The reason for the use of a UV Molecular Imager was because there was no other appropriate imager available in the department. Furthermore, smearing of the PeppermintStick™ phosphoprotein molecular weight standards appears to have occurred making it unclear where the phosphorylated bands in the marker are. Thus, an image of what PeppermintStick™ should look like has been appended to Figure 3.4 to assist in identification of the bands. What is clear is that there are no dark bands in the recombinant XhLEA3-2 lane and thus it is concluded that phosphorylation is not what is causing the appearance of a faint higher molecular weight band. This is somewhat at odds with the prediction in 2.3.5 that indicates that XhLEA3-2 does have phosphorylation sites, although, this does not necessarily mean that those site are constitutively phosphorylated. To further try and more accurately identify the recombinant XhLEA3-2 bands as well as their phosphorylation

state, the two bands could be isolated from an SDS-PAGE gel and sent for protein sequencing and analysis by Mass Spectrometry (MS).

3.3.4 Monitoring XhLEA3-2 Antibody Production

A colorimetric ELISA was used to determine the increase in immune response of one New Zealand White Rabbit to XhLEA3-2 after injection with recombinant XhLEA3-2 in the presence of an adjuvant (Figure 3.5). Because the level of colour change measured by light absorbance at 450 nm is directly proportional to the level of XhLEA3-2 antibodies bound to the recombinant XhLEA3-2 which coats the wells, it is evident that the highest antibody yields were in the third bleed.

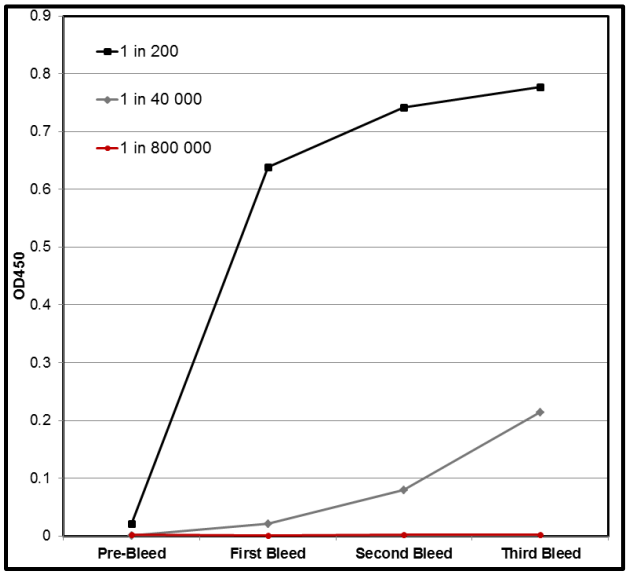

Figure 3.5 ELISA absorbance readings at OD_{450} for each bleed at different serum dilutions: 1 in 200 (black squares); 1 in 40 000 (grey diamonds); 1 in 800 000 (red circles)

Figure 3.5 shows an increase in XhLEA3-2 antibody production over the course of the 49 day protocol. The rabbit serum was diluted to 1:200; 1:40 000 and 1:800 000 to ensure that the change in OD_{450} fell within the detectable range for the assay. Clearly, a serum dilution of 1: 800 000 is too great a dilution for antibody detection in this assay as is evident by the flat red line

(i.e. not enough of a color change for detection). Because it contained the most XhLEA3-2 antibodies, the serum from the third bleed was used for antibody purification. It is possible that higher antibody yields could have been achieved had blood samples been taken over a longer period of time. However, the curve does appear to flatten out and thus the higher yield would likely not be worth the extra time, material and cost.

3.3.5 XhLEA3-2 Antibody Purification

Rabbits are exposed to many foreign molecules during their lives and thus naturally produce antibodies to these molecules which remain in the bloodstream. Of these foreign molecules, some are likely to be generic plant proteins (e.g. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) – a critical enzyme involved in carbon fixation in photosynthetic organisms which is known to come up strongly when using antibodies produced in rabbits (personal correspondence, BRU lab)) and therefore any antibodies to these proteins would cause unwanted background when probing crude plant extract with serum produced by rabbits. Similarly, any bacterial proteins that the rabbit has been exposed to will result in antibody production to these and thus unwanted background when probing bacterial crude extract. Therefore, purification of the serum is required to isolate only those antibodies which are specific to XhLEA3-2.

The XhLEA3-2 antiserum was purified using a protocol adapted from the Langdale Lab in the Department of Plant Sciences at the University of Oxford. Instead of using more expensive columns designed for antibody purification, this protocol makes use of relatively cheap Nickel-Affinity columns – the same as those originally used for recombinant XhLEA3-2 purification (3.2.6). This protocol exploits the His-tagged antigen's (in this case, recombinant XhLEA3-2)

affinity for nickel residues in the column and the antibodies' specificity for that antigen. The antibody and antigen can then be eluted off the column sequentially.

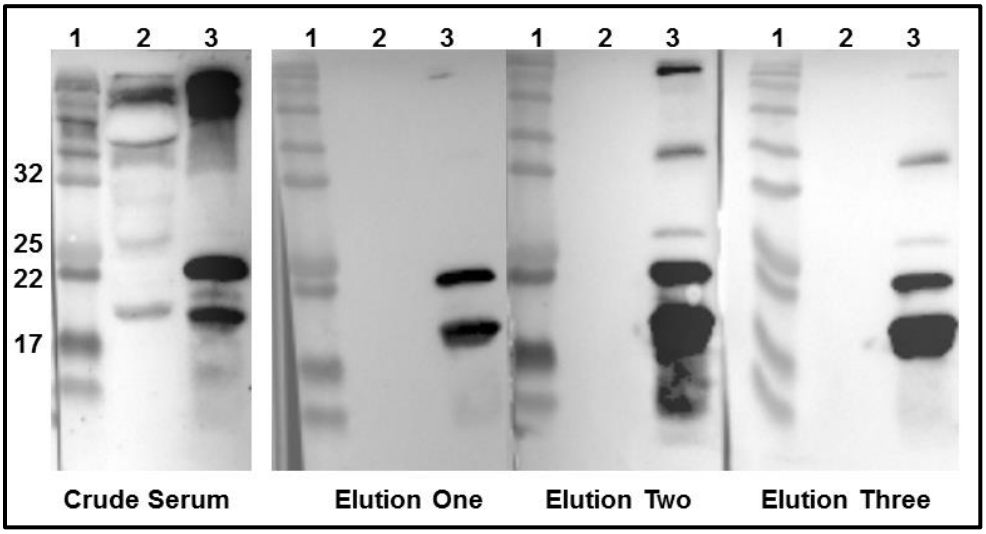

Figure 3.6 Four Western Blots each probed with rabbit serum containing XhLEA3-2 antibodies at different stages of purification (crude serum and elutions 1-3). Lane 1, Colour Prestained Protein Standard (kDa). Lane 2, crude tobacco protein extract. Lane 3, pure XhLEA3-2.

Figure 3.6 shows the effectiveness of this purification system. Crude serum, as expected, evidently picked up non-specific proteins in crude tobacco (*N. benthamiana*) extract. Furthermore, it strongly picked up two high molecular weight bands in the pure recombinant XhLEA3-2. These are likely *E. coli* protein contaminants that were unable to be removed during the recombinant XhLEA3-2 purification process. After purification, the three elutions of pure XhLEA3-2 antibodies did not pick up any non-specific proteins in crude tobacco extract. Furthermore, the intensity of the pure XhLEA3-2 bands in elutions two and three are at least as intense (if not more) than these bands for the crude serum thus eliminating the worry that the concentration of antibodies is too low to pick up non-specific bands in crude tobacco extract for the elutions. Also, the intensity of the non-specific bands in the pure XhLEA3-2 lane for the elutions was significantly decreased which demonstrates that most of these non-specific

antibodies, likely generated due to bacterial contaminants in the injected recombinant XhLEA3-2, did pass through the column during purification. Based on the above image, 'elution three' was assessed to be the most pure and thus was chosen for the determination of optimal concentration for western blotting (3.3.6).

3.3.6 Optimizing Pure XhLEA3-2 Antibody Concentration for Western Blots

A dot blot was used to determine the optimal concentration of pure XhLEA3-2 antibody to use for visible, high resolution detection in western blots (Figure 3.7). Furthermore, determination of the optimal antibody concentration prevents wastage of the purified antibody, and non-specific binding of the antibody to the membrane due to the concentration being too high.

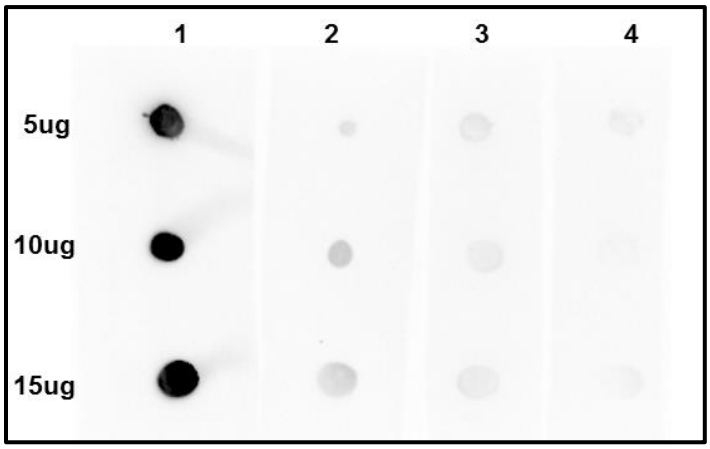

Figure 3.7 A Dot Blot of XhLEA3-2 of different masses probed with pure XhLEA3-2 Antibody at different concentrations. Lane 1, 1:5000 dilution. Lane 2, 1:10 000 dilution. Lane 3, 1:30 000 dilution. Lane 4, 1:50 000 dilution.

Pure recombinant XhLEA3-2 was spotted onto four nitrocellulose membranes at different masses. Each membrane was probed with a different dilution of pure XhLEA3-2 antibody: 1:5000, 1:10 000, 1:30 000 and 1:50 000. Concentrations lower than a 1:5000 dilution (i.e. 1:10 000, 1:30 000 and 1:50 000) resulted in inadequately intense spots after probing with an HRP-conjugated secondary antibody and detection with a chemiluminescent substrate. Thus, 1:5000

was considered to be an appropriate antibody dilution when doing western blots, as it bound XhLEA3-2 effectively and resulted in a clearly visible dark spot under UV.

3.3.7 XhLEA3-2 Expression in *X. humilis*

The XhLEA3-2 specific antibody, produced, purified and quantified, was used to show that XhLEA3-2 was expressed in *X. humilis* leaf tissue upon dehydration to 10% RWC (Figure 3.8). The band detected by the XhLEA3-2 specific antibody sits at approximately the expected size of XhLEA3-2 (16.8 kDa). Expression at this RWC and not at higher RWCs is not unexpected and is linked to qPCR data produced by Waters (2015) which showed that XhLEA3-2 transcripts are maximally expressed below 30% RWC in *X. humilis* leaf tissue. Interestingly, no double band is observed here as opposed to when the XhLEA3-2 was recombinantly expressed in *E. coli*. In summary, the XhLEA3-2 specific antibody picked up a single protein band at the expected size of XhLEA3-2 at a RWC typical of LEA expression.

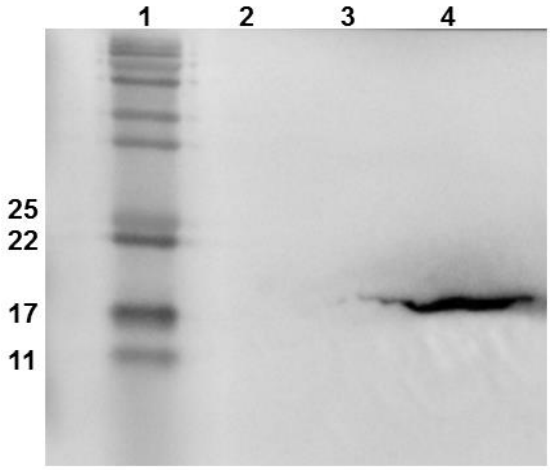

Figure 3.8 Western Blot of *X. humilis* protein extract at different RWCs during dehydration, probed with XhLEA3-2 specific antibody. Lane 1, Colour Prestained Protein Standard (kDa). Lane 2, full turgor. Lane 3, 50% RWC. Lane 4, 10% RWC.

3.3.8 Identification of Two Putative XhLEA3-2 Homologs in *X. viscosa*

It was decided to test the XhLEA3-2 antibody on *X. viscosa*; a close relative of *X. humilis*. Total protein from *X. viscosa* at different stages of drying was extracted, quantified and separated by SDS-PAGE. Following this, the protein extracts were probed with the XhLEA3-2 antibody by western blotting to detect the presence of XhLEA3-2 homologs, two of which were identified in *X. viscosa* (see 2.3.7 above).

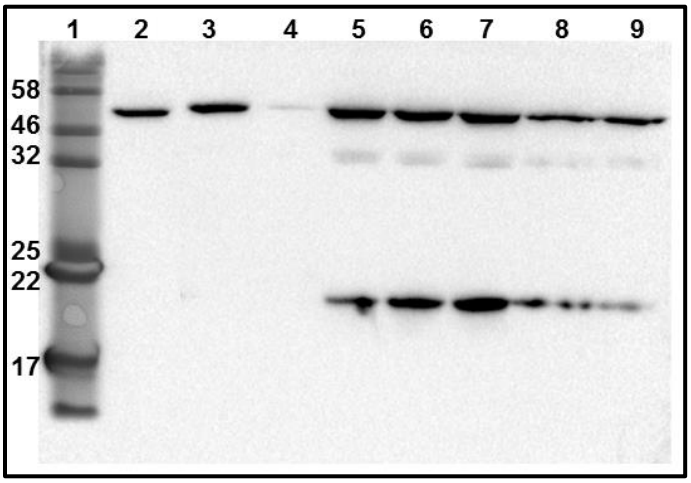

Figure 3.9 Western blot of *X. viscosa* protein extract at different RWCs during a dehydration/rehydration cycle probed with XhLEA3-2 specific antibody. N.B the molecular weight ladder has been overlaid due to the antibody binding to it and obscuring the image. Lane 1, Colour Prestained Protein Standard (kDa). Lane 2, full turgor. Lane 3, 74% RWC. Lane 4, 58% RWC. Lane 5, 33% RWC. Lane 6, air dry. Lanes 7-9, 9 hrs, 24 hrs and 72 hrs after rehydration respectively.

Figure 3.9 shows three protein bands that were detected by the XhLEA3-2 antibody. It is important to note that the molecular weight ladder has been overlaid due to the antibody binding to it and obscuring the image and the original image can be found in Appendix B1. At approximately 50 kDa, a band of fairly consistent intensity was observed throughout the dehydration and rehydration cycle. It is unlikely to be a LEA due to its large mass – LEAs are typically less than about 30 kDa (Hong-Bo et al. 2005). It could be the result of non-specific binding although it is also possible that it is the result of dimer-formation of the two smaller

bands (32 kDa and 20 kDa). Most probably, the faint band in lane 4 is due to an error in protein quantification and thus less protein was loaded than intended. However, repetition of this experiment and quantification of the sample would be required to confirm this. Unfortunately, due to low yields of plant total protein and time constraints, repetition of the dry down and rehydration plus subsequent protein extraction and probing could not be completed.

Bands at approximately 32 kDa and 20 kDa appeared in leaf tissues as the plant dehydrated to 33% RWC. The band at 20 kDa is similar in size to that observed when probing *X. humilis* leaf tissue for XhLEA3-2 (16.8 kDa). These two bands gained intensity as the plant dehydrated further. The most intense bands were observed at 9 hrs after rehydration and they decreased in intensity so that by 72 hrs post rehydration the band was very faint. This pattern where expression increases at low water contents is typical of *X. humilis* LEAs (Illing et al 2005; Waters 2015) and LEAs in general (Ingram & Bartels 1996; Bray 1997). Furthermore, this expression pattern suggests that these putative LEAs are functional or at least present during the very dry stages and early stages of rehydration in the dehydration/rehydration cycle, but not at the higher RWCs.

The XhLEA3-2 homologs from 2.3.7, Xvis02_15976 and Xvis02_11331, have predicted sizes of 16.8 kDa and 15.3 kDa respectively (see sequences in Appendices A3 and A4). These predicted sizes are smaller than the smallest band in Figure 3.9. The two homologs would overlap at the resolution in Figure 3.9 and so only one band would be observable. Additionally, it cannot be ruled out that the lowest molecular weight band could be one (or both) of the XhLEA3-2 homologs predicted in 2.3.7 and that the band is observed at a higher molecular weight because of the tendency of LEAs to move more slowly in SDS-PAGE .

In summary, Figure 3.9 shows that the XhLEA3-2 antibody can pick up two putative LEA proteins in *X. viscosa* and the link between *X. humilis* and *X. viscosa* is worth further study.

Chapter 4 - XhLEA3-2 Functional Tests

4.1 Introduction

4.1.1 LEA Functions and Functional Tests

The insight that LEAs play some role in abiotic stress tolerance has been extensively reported (see also Chapter 1) but the precise molecular function(s) of the majority of LEAs remain unclear. LEAs have been predicted to have roles in protein protection (chaperones), membrane protection, ion sequestration, antioxidant activity, hydration buffering and molecular crowding (reviewed in Tunnacliffe & Wise 2007; Amara et al. 2014). These predictions are based on LEA sequence analysis and their intrinsically disordered nature, but carry relatively little evidence. The most well studied putative LEA function is molecular chaperone activity (Goyal et al. 2005; Reyes et al. 2005; Reyes et al. 2008; Furuki & Sakurai 2016). These studies show that certain LEAs can prevent *in vitro* globular protein denaturation and aggregation to some degree. Evidently, some LEAs play a molecular chaperone role under stress conditions.

4.1.2 Abiotic Stress Tests using Transgenic Model Microorganisms

Because of their upregulation during abiotic stress (Collett et al. 2004; Gao & Lan 2016; Costa et al. 2017), LEAs have been predicted to play some role in stress tolerance. An easy way to test this is using a transgenic model with microorganisms like *E.coli* or yeast. Transforming microorganisms with LEA gene(s) has been shown to improve tolerance to stresses such as dehydration, salt and heat (Honjoh et al. 1999; Swire-Clark & Marcotte 1999; Liu et al. 2010; Liu et al. 2013; Gao & Lan 2016; Boothby et al. 2017 *inter alia*). Although this model does not give any clues towards the precise molecular function(s) of a particular LEA *in planta*, it does give information about whether or not LEAs improve abiotic stress tolerance *in vivo*.

4.1.3 LEA Enzyme Protection Assays

Heat shock proteins (HSPs) are known to be upregulated during abiotic stress and function as molecular chaperones (Lindquist 1986). Because of a similar pattern of LEA upregulation during stress, LEAs were predicted to have molecular chaperone activity similar to HSPs. Indeed, several studies have shown this to be the case (Goyal et al. 2005; Reyes et al. 2005; Reyes et al. 2008; Furuki & Sakurai 2016). Using lactate dehydrogenase (LDH) and citrate synthase (CS), enzymes that are known to aggregate and lose activity under certain stress conditions, researchers could show that the addition of LEAs prevented the loss of enzyme activity to a significant degree. Notably, this effect is often increased by the addition of trehalose; a sugar known to accumulate during plant dehydration (Goyal et al. 2005).

The LDH activity assay used in this thesis is based on the catalysis of pyruvate to lactate and the simultaneous conversion of NADH to NAD^+ (Figure 4.1). It is the formation of NAD^+, measured spectrophotometrically, that is used to determine the rate of the reaction and thus infer the activity of LDH. By stress-treating the LDH with and without the presence of LEAs and then measuring its activity, one can determine the protective ability of the LEA protein on LDH activity which gives evidence towards its putative chaperone function.

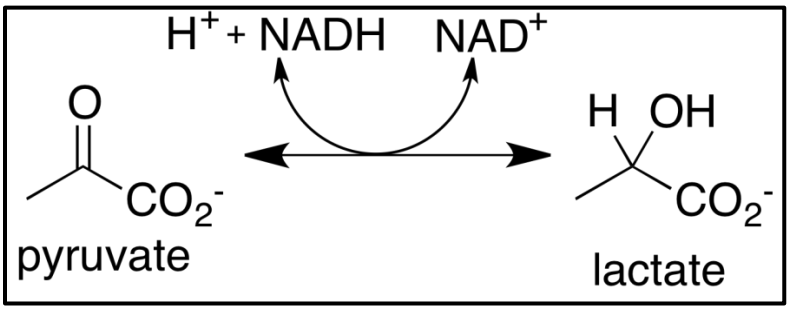

Figure 4.1 Catalysis of pyruvate to lactate by lactate dehydrogenase and the resultant formation of NAD^+ from NADH

4.1.4 Abiotic Stress Tests using Transgenic Model Plants

Many transgenic studies have shown an improved abiotic stress-tolerance ability in plants that ectopically express LEAs (Xu et al. 1996; Wang et al. 2006; RoyChoudhury et al. 2007; Liu et al. 2014; Wang et al. 2014; Zhang et al. 2016 *inter alia*). These studies give credence to the hypothesis that LEAs play some role in abiotic stress tolerance and could be potential candidate genes for genetically-engineered drought-resistant plants. The traditional approach towards the development of stable transgenic tobacco lines can take between 2 – 4 months (Dugdale et al. 2014). One potential way around this lengthy process is to transiently transform tobacco plants using *Agrobacterium*-mediated transformation. This method has been successfully used for *in planta* protein localization in the past (Kokkirala et al. 2010). The Fast *Agrobacterium*-mediated Seedling Transformation (FAST) method was developed by Li et al. (2009). This method for studying *in vivo* protein function reduces the time required for plant growth and eliminates the need to produce stable transgenic lines. It is based on the co-cultivation of tobacco seedlings in an *Agrobacterium* cell suspension, transformation and subsequent protein expression (reviewed in Gelvin 2003). Briefly, *Agrobacterium* carrying the expression vector for the gene of interest infects the plant tissue. Then, exploiting the natural ability of *Agrobacterium* to transfer genetic information into plant cells, the expression vector is transferred to the plant cells during infection. The excess *Agrobacterium* is then killed by appropriate antibiotics and the plant, now carrying the expression vector for the gene of interest is allowed to recover. Then, the *in vivo* expressed proteins can be localized by microscopy using fluorescent tags or probing with specific antibodies. Furthermore, the transformed seedlings can be submitted to a number of abiotic stresses and then studied to assess any physiological advantage provided by the gene of interest.

In this chapter, the ability of XhLEA3-2 to protect *E. coli* from osmotic and desiccation stress was tested. Furthermore, its ability to act as a molecular chaperone was assessed. Finally, the XhLEA3-2 DNA coding sequence was cloned into an *Agrobacterium* expression vector and it was expressed, transiently, in tobacco seedlings.

4.2 Materials and Methods

4.2.1 *E. coli* Osmotic Stress Assay

Cultures of XhLEA3-2:pET-21a (His) in BL21 DE3 pLysS *E. coli* cells (see 3.2.1) and the same strain carrying empty pET-21a (His) (control) were grown overnight from glycerol stocks in LB-chlr-amp media at 37 ^{o}C with shaking at 150 rpm. A 50 µl volume from each overnight culture was added to one 5 ml LB-chlr-amp and allowed to grow until OD_{600} = 0.6 (~2 hrs) at 37 ^{o}C with shaking at 150 rpm. The cultures were then induced with 1 mM IPTG and continued to be incubated at 37 ^{o}C with shaking at 150 rpm for 4 hrs. A volume from each culture that resulted in a starting OD_{600} of 0.05 was added to three sterile 10 ml LB-chlr-amp with 1 mM IPTG. These three LB-chlr-amp media were treated with either 300 mM NaCl, 300 mM mannitol, 20% polyethylene glycol (PEG) or no stress treatment. The treated cultures were allowed to grow until stationary phase with an OD_{600} reading taken every hour. The growth curves for each culture were compared to see if XhLEA3-2 expression had any effect on *E.coli* growth under stress conditions.

4.2.2 *E. coli* Dehydration Stress Assay

This protocol was adapted from Boothby et al. (2017). Two 3 ml LB-chlr-amp media were inoculated with 5 µl of a glycerol stock of XhLEA3-2:pET-21a (His) in BL21 DE3 pLysS *E. coli* cells (see 3.2.1) and the same strain carrying empty pET-21a (His) (control) and both cultures were incubated overnight at 37 ^{o}C with shaking at 150 rpm. The OD_{600} of each culture

was checked to see if they were the same (not more than OD_{600} = 0.05 between them). A 1 ml aliquot from each overnight culture was used as the inoculum for three fresh 5 ml LB-chlr-amp media (replicates) per overnight culture. These cultures were allowed to grow at 37 ^{o}C with 150 rpm shaking until OD_{600} = 0.6 (~2 hrs) and were then induced with 1 mM IPTG for 4 hrs under the same growth conditions. After the 4 hr induction, the OD_{600} of each culture was recorded. Approximately 10^8 cells were transferred to sterile microcentrifuge tubes according to the formula below:

$$OD_{600}\ of\ 1.0 = 8 \times 10^8 cells/ml$$

These cells were centrifuged for 20 min at 4000 g at room temperature. The supernatant was discarded and the harvested cells were then dehydrated for 16 hrs in a SpeedVac™ Plus SC210A (Savant™) at room temperature. The cells were resuspended to their original volume in dH_2O and the entire volume was spread out onto LB-chlr-amp agar plates and incubated at 37 ^{o}C overnight. The colonies were counted and the counts for each of the three replicates were averaged.

4.2.3 Lactate Dehydrogenase Activity Assay

This assay is an adaptation of that described by Reyes et al. (2008). In short, LDH from rabbit muscle (Sigma-Aldrich) diluted to 100 nM in 25 mM Tris-HCl pH 7.5 was dried down in the presence and absence of 100 nM XhLEA3-2 in 25 mM Tris-HCl pH 7.5 with 100 nM BSA in 25 mM Tris-HCl pH 7.5 as a control. Next, LDH activity was measured spectrophotometrically to assess whether or not XhLEA3-2 had a protective effect on LDH activity loss due to dehydration. The experiment was done in triplicate. The experimental setup is shown in Table 4.1.

Table 4.1 Reaction setup for LDH dehydration in the presence and absence of putative stabilizing agents

	LDH Only	LDH;XhLEA3-2	LDH;BSA
LDH	100 nM	100 nM	100 nM
XhLEA3-2	-	100 nM	-
BSA	-	-	100 nM
25 mM Tris-HCl pH 7.5	Up to 75 µl	-	-
Total	75 µl	75 µl	75 µl

A 75 µl volume of 100 nM LDH, 75 µl of 100 nM LDH;100 nM XhLEA3-2 (produced and purified as in Chapter 3) and 75 µl of 100 nM LDH;100 nM BSA in microfuge tubes were all dehydrated in a centrifugal evaporator (Savant^{TM} SpeedVac^{TM} Plus SC210A) at room temperature for 0 min, 30 min, 60 min, 90 min and 120 min. After dehydration for 60 min, full dehydration had occurred (i.e. only a dry pellet was observable by eye) and all tubes were rehydrated back to their original volume with 25 mM Tris-HCl pH 7.5. Tubes labelled 90 min and 120 min were then dehydrated and rehydrated again according to the same process above. A 10X LDH Reaction Buffer was made (22.0 mg Sodium Pyruvate and 10.6 mg NADH in 25 mM Tris-HCl pH 7.5). A 1 ml volume of 1X LDH Reaction Buffer (diluted in 25 mM Tris-HCl pH 7.5) was dispensed into a 2 ml plastic cuvette and the initial absorbance was recorded at 360 nm. Each dehydrated and rehydrated sample was assayed for LDH activity by adding 52.6 µl of the sample into the 1 ml of 1X LDH Reaction Buffer. Readings were taken every 10 sec for 1 min. The OD_{360} readings were normalized to the initial 1X LDH Reaction Buffer reading. LDH activity was defined as the rate in OD_{360} change over 1 min multiplied by negative one.

4.2.4 Plasmid Miniprep of pEAQ-HT and XhLEA3-2:pBlueScript

5 ml LB-kanamycin (kan) was inoculated with 5 µl of a glycerol stock of pEAQ-HT in DH5α *E. coli* cells, kindly provided by the Biopharming Research Unit (BRU) at the University of Cape Town (UCT). A 5 µl volume of XhLEA3-2 in pBueScript, was inoculated into 5 ml LB-

amp. Both cultures were left to incubate overnight at 37 ^{o}C with shaking at 150 rpm. Pure pEAQ-HT DNA and XhLEA3-2:pBlueScript was extracted from the overnight culture cells using the Zyppy™ Plasmid Miniprep Kit (Zymoclean) according to the manufacturer's instructions.

4.2.5 Primer Design for Cloning XhLEA3-2 in pEAQ-HT

Primers (Forward: 5'- TTAATATTCCCGGGATGGCCTCCACCCACG -3'; Reverse: 5'-GCGGCGCTCGAGCTTATATCTGGGAATTTCTTG -3') specific for the XhLEA3-2 coding sequence were designed using DNAman software (Lynnon) to PCR-amplify XhLEA3-2 while simultaneously adding a XmaI site directly upstream of the ATG (start codon) and a XhoI site directly after the stop codon. The primers were synthesized at the oligonucleotide facility in the Molecular Biology Department at UCT.

4.2.6 PCR Amplification of XhLEA3-2 with XmaI and XhoI Ends

The PCR parameters are contained in Table 4.2 and the reaction mix was made up using the HiFi Hotstart Readymix kit (KAPA Biosystems) according to manufacturer's instructions. A 10 µl aliquot of the finished PCR amplification was run on a 1% agarose gel at 90 V for 30 min. The remaining product was cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturer's instructions.

Table 4.2 PCR parameters for the amplification of XhLEA3-2

	Temperature	Time	Cycles
Initial Denaturation	95 ^{o}C	3 min	X1
Denaturation	98 ^{o}C	20 sec	X35
Annealing	54 ^{o}C	30 sec	
Extension	72 ^{o}C	30 sec/kbp	
Final Extension	72 ^{o}C	5 min	X1

4.2.7 Digestion of pEAQ-HT and PCR Amplified XhLEA3-2

Both the amplified XhLEA3-2 coding sequence and the pEAQ-HT vector were double digested with XmaI and XhoI restriction enzymes (FastDigest, Thermo Scientific) in separate reactions set up according to Table 4.3.

Table 4.3 Experimental set-up for the digestion of PCR-Amplified XhLEA3-2 and pEAQ-HT with XmaI and XhoI

	pEAQ-HT	PCR-Amplified XhLEA3-2
Reaction Mix	3 µl XmaI; 3 µl XhoI; 6 µl FastDigest Buffer; 3 µg pEAQ-HT DNA; Make up to 60 µl with dH2O	3 µl XmaI; 3 µl XhoI; 6 µl FastDigest Buffer; 3 µg XhLEA3-2 DNA; Make up to 60 µl with dH2O
Incubation	37 ^{o}C for 30 mins	

The cut fragments were run on a 1% agarose gel at 90 V for 30 min and then gel-extracted with the Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research) according to manufacturer's instructions.

4.2.8 Ligation of XhLEA3-2:pEAQ-HT and Transformation into DH5α *E. coli* cells

The gel-extracted, cut pET-21a (His) and XhLEA3-2 fragments were ligated together with T4 DNA Ligase (Thermo Scientific) at 22 ^{o}C for 1 hr in a reaction mix as set out in Table 4.4.

Table 4.4 Reaction mix for the ligation of pEAQ-HT and PCR-Amplified XhLEA3-2

	pET-21a (His) and XhLEA3-2 Ligation Reaction
Reaction Mix	50 ng cut pEAQ-HT DNA; 10 ng cut XhLEA3-2 DNA; 2 µl Ligase Buffer; 2 µl T4 DNA Ligase; Make up to 20 µl with dH2O
Incubation	22 ^{o}C for 1 hour

The entire ligation mix was added to 100 µl of chemically competent *E. coli* cells and left on ice for 30 mins. Then, the mixture was heat-shocked for 90 s at 42 ^{o}C followed by incubation

on ice for 2 min. The mixture was incubated at 37 ^{o}C with shaking at 150 rpm for 1 hr before being spread out on LB-kan agar plates. The plates were incubated overnight at 37 ^{o}C.

4.2.9 Colony PCR of Transformed Cells

Transformation was confirmed by colony-PCR (HiFi Hotstart Readymix kit, KAPA Biosystems) with the parameters outlined in Table 4.2. Primers designed for the pEAQ-HT vector were kindly donated by the BRU. These primers bind approximately 100 bp on either side of the multiple cloning site of the pEAQ-HT vector. Successfully transformed colonies were grown in LB-kan media and the cells were then stored at -80 ^{o}C in 50% glycerol.

4.2.10 Transformation of XhLEA3-2:pEAQ-HT into *A. tumefaciens*

XhLEA3-2:pEAQ-HT DNA was extracted from DH5α cells as outlined above. A 100 µl volume of electro-competent *A. tumefaciens* cells of strain LBA4404 were thawed on ice for approximately 2 min. A 400 ng amount of XhLEA3-2:pEAQ-HT was added to the electro-competent cells and incubated on ice for 5 min. This mixture was then pipetted into a pre-chilled, electroporation cuvette. Electroporation was carried out at 1.8 kV, 25 µF and 200 Ω. The cuvette was placed back on ice and 900 µl of sterile LB was added. The cells were added to a fresh microcentrifuge tube and incubated at 27 ^{o}C for 2 hrs with no shaking. A 500 µl volume of the transformed cells were plated out onto LB-rifampicin (rif) –kan plates and incubated at 27 ^{o}C for 2 days. Successful transformation was confirmed by colony PCR with pEAQ-HT primers as described above.

4.2.11 Growing *N. benthamiana* (Tobacco) Seedlings

A 100 µl volume of *N. benthamiana* seeds were surface sterilized by placement in a microcentrifuge tube followed by the addition of 1 ml 10% bleach and 0.1% Tween-20, at room temperature, for 10 min. The bleach solution was removed and 1 ml 70% ethanol was added for

1 min at room temperature. The ethanol was removed and the seeds were rinsed five times by vortexing for 5 min with 1 ml sterile dH_2O. The now sterile seeds were spread out evenly on a Murashige-Skoog (MS) medium containing 1% sucrose at pH 6.0 plate (2.15 g/L MS Salts (Sigma Aldrich), 10 g/L sucrose, 2.2 g/L Phytagel (Sigma Aldrich). The plate was wrapped in Parafilm™, and the seeds germinated in a cycle of 16 hr light/22°C followed by 8 hr dark/18°C for 6 days.

4.2.12 Co-Cultivation of Tobacco Seedlings with LBA4404:XhLEA3-2:pEAQ-HT

LBA4404 *A. tumefaciens* cells containing XhLEA3-2:pEAQ-HT were grown from 1 ml of glycerol stock in 20 ml of LB-rif-kan medium overnight at 27 ^{o}C with shaking at 250 rpm. The overnight cultured bacterial cells were harvested by centrifugation at 6000 g for 10 min at room temperature. The supernatant was discarded and the harvested cells were resuspended in 20 ml washing solution (2.03 g/L $MgCl_2.6H_2O$, 0.02 g/L acetosyringone). The suspension was centrifuged again at 6000 g for 10 min at room temperature and the supernatant discarded. The pellet was resuspended in a volume of co-cultivation medium (2.15 g/L MS Salts (Sigma Aldrich), 10 g/L sucrose, 0.02 g/L acetosyringone, 0.05% Silwet 806) so that the OD_{600} = 0.5. In a clean Petri dish, 30–40 6-day-old tobacco seedlings were soaked in 20 ml of the co-cultivation cell suspension. The seedlings and *Agrobacterium* cell suspension were incubated at 22 ^{o}C in the dark for 60 hrs. Excess co-cultivation suspension was blotted off with sterile filter paper and surfaced sterilized in 1% bleach, 500 µg/ml carbenicillin for 15 min followed by three brief rinses with sterile dH_2O. The seedlings were aseptically placed (with autoclaved tweezers) on a MS medium containing 1% sucrose and 500 µg/ml carbenicillin (pH 6.0) and left to grow in a cycle of 16 hr light/22 ^{o}C followed by 8 hr dark/18 ^{o}C.

4.2.13 Protein Extraction from Tobacco Seedlings

Twenty transformed seedlings were placed in a microcentrifuge tube and homogenized by grinding with a micropestle in the presence of 40 µl extraction buffer (1.5 g/L Dithiothreitol (DTT), 0.7 g/L Ethylenediaminetetraacetic acid (EDTA), 0.01% Triton X-100, 1 Complete Protease Inhibitor (Roche) per 10 ml, 17.4 mg/L Phenylmethylsulfonyl fluoride (PMSF) in 10 ml Tris-HCl, 150 mM NaCl, pH 7.4). The ground material was centrifuged for 10 min at 10 000 g at room temperature and the pellet was discarded. The supernatant was run on a 12.5% SDS-PAGE gel and probed by western blotting (as described in 3.2.3) using the antibody produced in 3.2.8 – 3.2.10 at a concentration of 1:1000.

4.3 Results and Discussion

4.3.1 Measuring the Effect of XhLEA3-2 on *E.coli* Growth Rate during Osmotic Stress

In general, osmotic stress treatments of *E. coli* reduced the growth rate (Figure 4.2). The greatest reduction in growth rate occurred when the growth medium contained 20% PEG. Treatments of 300 mM mannitol and 300 mM NaCl seem to have reduced the growth rate approximately equally. Furthermore, in all treatments, no difference in the growth rate of *E.coli* expressing, and not-expressing XhLEA3-2 was observed. This suggests that the expression of XhLEA3-2 in *E. coli* has no effect on its growth rate under stress conditions.

This is in contrast to other studies which have shown that *E.coli* strains expressing LEA proteins have had improved growth and/or survival when osmotically stressed. For example, Gao & Lan (2016) showed that ten of thirteen LEAs identified in *Pinus tabuliformis* increased *E.coli* survival by 2-7 fold when exposed to high NaCl concentrations. Of these ten LEAs, two were LEA_4-like LEAs. Gao & Lan (2016) saw more distinct differences in *E.coli* survival between

the control and LEA-expressing strains at higher NaCl concentrations (>500 mM). Perhaps, XhLEA3-2 has no effect on *E.coli* osmotic stress tolerance but it could also be that the stress was not enough for an XhLEA3-2-mediated tolerance effect to be observed. Repeating this experiment with higher osmotic concentrations could yield further insight.

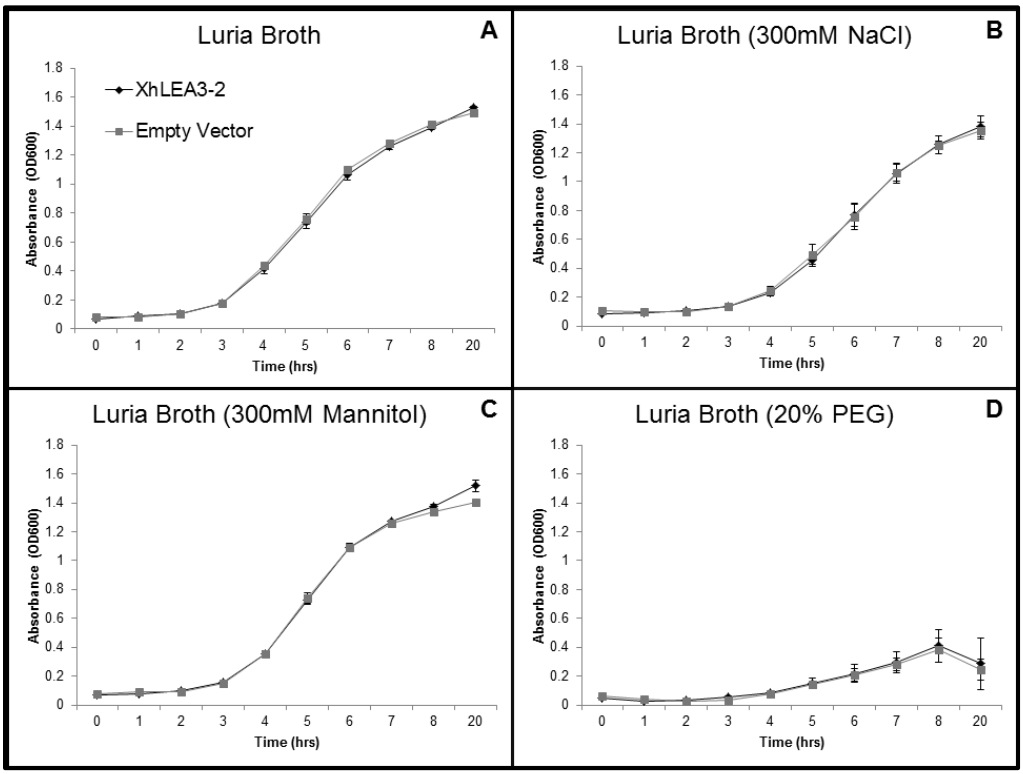

Figure 4.2 The growth rate of *E. coli* expressing XhLEA3-2 (black diamonds) or nothing (grey squares) under different osmotic stress conditions. A, no stress. B, 300 mM NaCl. C, 300 mM mannitol. D, 20% polyethylene glycol.

4.3.2 Improved Survival of *E.coli* Expressing XhLEA3-2 after Dehydration

The *E.coli* strain that expressed XhLEA3-2 showed significantly increased survival (approximately 8-fold) after desiccation (Figure 4.3). This is consistent with results from Boothby et al. (2017) who demonstrated that Tardigrade-specific Intrinsically Disordered Proteins (TDPs) improved *E.coli* survival of dehydration by up to 1000-fold. Although this evidence does little to explain the precise role of XhLEA3-2, it does suggest that it plays some

role in desiccation stress tolerance. The putative, specific role of XhLEA3-2 is further investigated in section 4.3.3.

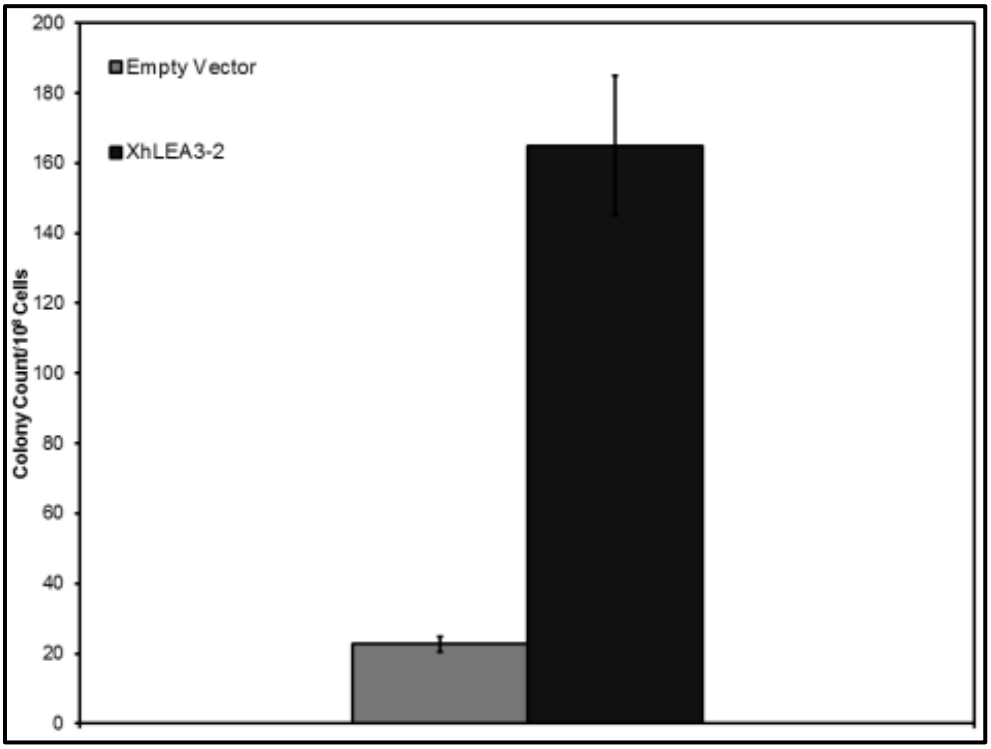

Figure 4.3 Colony count of *E. coli* expressing XhLEA3-2 (black) or nothing (grey) after dehydration

4.3.3 XhLEA3-2 Protects Lactate Dehydrogenase Activity after Dehydration

The best studied putative LEA function is protein protection. This 'molecular chaperone' activity has been demonstrated in several studies (Goyal et al. 2005; Reyes et al. 2005; Reyes et al. 2008; Furuki & Sakurai 2016). These studies used LDH as a model enzyme – a well-established system to test potential molecular chaperone candidates. Goyal et al. (2003) and Reyes et al. (2005, 2008) demonstrated that the presence of LEAs during dehydration of LDH (which causes aggregation) reduced the loss of LDH activity significantly.

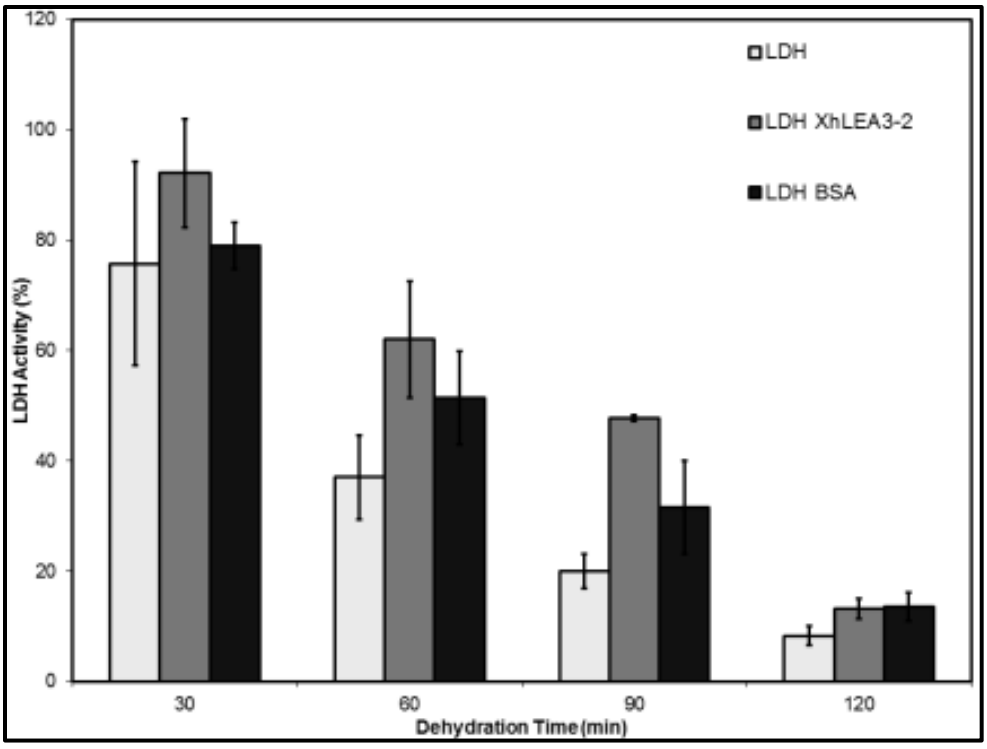

Figure 4.4 LDH activity measured after different time periods of dehydration in the presence of XhLEA3-2 (grey), in the presence of BSA (black) or alone (white).

The effect of XhLEA3-2 on the preservation of LDH activity after dehydration is shown in Figure 4.4. LDH in the presence of XhLEA3-2 had less dehydration-related loss of activity over time as compared to LDH alone or in the presence of BSA. The presence of BSA did reduce LDH loss of activity to some degree but this was not as noticeable as the presence of XhLEA3-2. This result is consistent with Goyal et al. (2003) and Reyes et al. (2005, 2008) suggesting that XhLEA3-2 has some molecular chaperone activity and this could be its function in protecting a desiccating plant cell. However, a positive control in this experiment, such as a known HSP, would make it easier to discern whether the molecular chaperone effect of XhLEA3-2 is of a similar magnitude to well-characterized molecular chaperones. Furthermore, it cannot be ruled out that the protective effect of XhLEA3-2 is as a result of molecular crowding which, by filling up empty space, prevents the hydrophobic regions of LDH from physically coming into contact

and thus aggregating. If this was the case, it might be expected that BSA would have an equal protective effect on LDH activity after dehydration because the molar amounts of XhLEA3-2 and BSA used in the assay were the same. However, the intrinsic disorder of XhLEA3-2 could be playing a role here, filling up more space and thus having a more dramatic molecular crowding effect.

4.3.4 PCR Amplification of XhLEA3-2

XhLEA3-2 was successfully amplified out of pBluescript and the XmaI and XhoI restriction sites added as seen in Figure 4.5. Lane 2 contained a negative control – no template DNA was added to this PCR reaction thus confirming that the band in Lane 3 was not due to primer dimers.

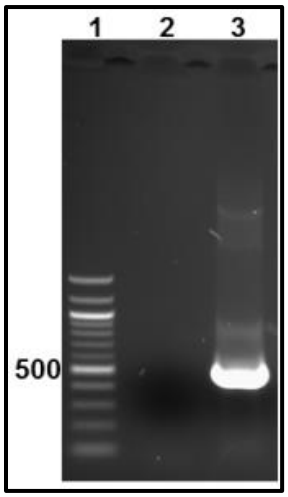

Figure 4.5 PCR amplification of XhLEA3-2 with the addition of XmaI and XhoI restriction sites. Lane 1, Quick-Load® 100 bp DNA Ladder (bp). Lane 2, no template DNA control. Lane 3, XhLEA3-2 PCR product.

4.3.5 Digestion, Ligation and Transformation of pEAQ-HT:XhLEA3-2

pEAQ-HT and XhLEA3-2 were digested with XmaI and XhoI restriction enzymes and then run on an agarose gel (Figure 4.6). The digested bands at the correct sizes (pEAQ-HT ~ 10 kb and XhLEA3-2 ~ 0.5 kb) were extracted and purified for ligation. After ligation, the mixture

was transformed into competent DH5α *E.coli* cells and screened by antibiotic selection and colony PCR.

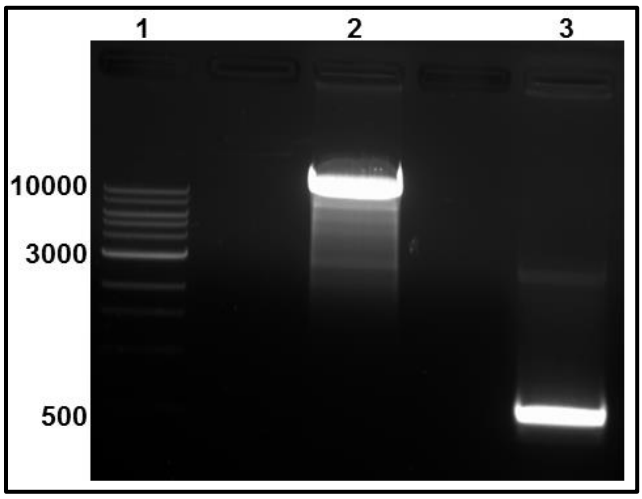

Figure 4.6 Restriction enzyme digestion of pEAQ-HT and XhLEA3-2 with XmaI and XhoI. Lane 1, Quick-Load® 1 kb DNA Ladder (bp). Lane 2, digested pEAQ-HT. Lane 3, digested XhLEA3-2.

4.3.6 Colony PCR of *E. coli* DH5α Cells Transformed with XhLEA3-2:pEAQ-HT

Transformed colonies were screened by colony PCR (Figure 4.7) with pEAQ-HT specific primers. These primers bind about 100 bp on either side of the pEAQ-HT multiple cloning site (MCS) and thus successfully transformed colonies yielded a band on an agarose gel that was approximately 200 bp larger than the expected size. Therefore, a 700 bp band indicated successful transformation and the presence of XhLEA3-2:pEAQ-HT. A 200 bp band indicated a failed transformation or the presence of the pEAQ-HT vector alone. The negative control for this PCR experiment is in lane two and contains only the empty pEAQ-HT DNA. Lane 3 is the PCR product of a successfully transformed DH5α colony.

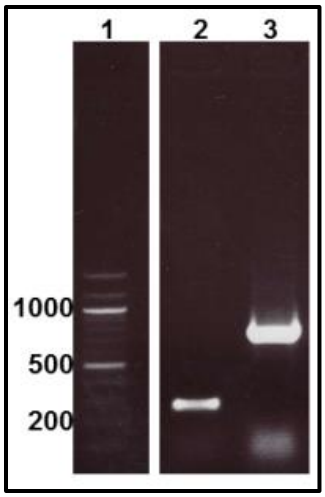

Figure 4.7 Colony PCR of DH5α cells containing XhLEA3-2:pEAQ-HT. Lane 1, Quick-Load® 100 bp DNA Ladder (bp). Lane 2, empty pEAQ-HT template only. Lane 3, XhLEA3-2 amplified out of XhLEA3-2:pEAQ-HT.

4.3.7 Colony PCR of LBA4404 Cells Transformed with XhLEA3-2:pEAQ-HT

Next, XhLEA3-2:pEAQ-HT needed to be transformed into electrocompetent *A. tumefaciens* LBA4404 cells. DH5α cells containing XhLEA3-2:pEAQ-HT were grown up and the expression construct was extracted and purified.

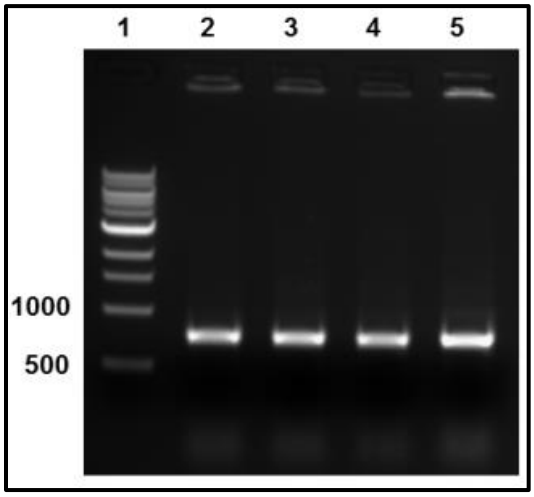

Figure 4.8 Colony PCR of XhLEA3-2:pEAQ-HT in LBA4404 cells. Lane 1, Quick-Load® 100 bp DNA Ladder (bp). Lanes 2-5, LBA4404 colonies containing XhLEA3-2:pEAQ-HT.

This pure XhLEA3-2:pEAQ-HT was transformed into LBA4404 cells by electroporation and successfully transformed colonies were screened by colony PCR (Figure 4.8) again, using pEAQ-HT specific primers. As above, successfully transformed colonies yielded a band of about 700 bp which is approximately 200 bp more than the expected size of XhLEA3-2.

4.3.8 Expression of XhLEA3-2 in Tobacco Seedlings

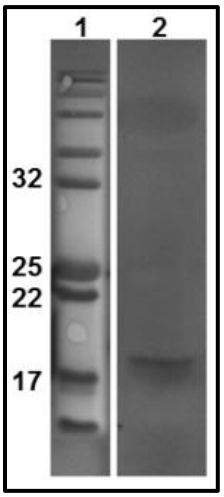

Figure 4.9 Expression of XhLEA3-2 in tobacco seedlings. N.B the molecular weight ladder has been overlaid due to the antibody binding to it and obscuring the image. Lane 1, Colour Prestained Protein Standard (kDa). Lane 2, crude tobacco protein extract expressing XhLEA3-2.

XhLEA3-2 was successfully detected by western blot after 60 hrs of co-cultivation of XhLEA3-2:pEAQ-HT in LBA4404 and 6 day old tobacco seedlings (Figure 4.9 – N.B. ladder overlaid and original image is at Appendix B2). Thus, the tobacco seedlings were expressing XhLEA3-2 after 60 hrs. This is conclusive as crude tobacco extract probed with the purified XhLEA3-2 antibody produced in Chapter 3 at an equal concentration showed no non-specific bands. Furthermore, the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter is specific for plant protein expression (Sainsbury et al. 2009) and thus the XhLEA3-2 could not have been expressed in the *Agrobacterium* cells that carried the expression construct. To further support the identity of the protein band detected in Figure 4.9, tobacco seedlings transformed with empty

expression vector could be probed using the same antibody. In this case, no band would confirm that the expression is a result of the XhLEA3-2:pEAQ-HT construct.

The band at about 17 kDa is about the expected size of XhLEA3-2 (16.8 kDa). Based on the intensity of the band, the expression level is low. This would need to be taken into account if using these transiently transformed tobacco seedlings for localization or stress tests. Furthermore, repeated rounds of co-cultivation and expression did not yield consistent levels of XhLEA3-2 expression – in several cases there was no expression at all (data not shown). Co-cultivation with *A. tumefaciens* was ultimately too much of a stress on the seedlings for them to survive even when plated on solid carbenicillin-containing MS-media which in theory would kill any *A. tumefaciens* but not affect tobacco growth. Thus, due to the low expression levels and the lack of seedling survival this method, although quick, is inappropriate to test for the beneficial effects of XhLEA3-2 on seedling survival under stress conditions. However, Li et al. (2009) and Weaver (2015) have shown that this method can be successful for quick localization of proteins in model plant systems and perhaps this could be attempted for XhLEA3-2 in the future.

Chapter 5 - Conclusion

In this thesis, an XhLEA3-2 specific polyclonal antibody was produced, purified and successfully used for the identification of XhLEA3-2 in dry *X. humilis* and *X. viscosa* leaf tissue as well as in transiently transformed tobacco seedlings. Apart from these applications, this antibody will be a useful tool for future work regarding XhLEA3-2 in the future.

LEAs have already shown themselves to be good potential candidates for transgenic studies looking to improve abiotic stress tolerance in model plants species (Wang et al. 2006; RoyChoudhury et al. 2007; Liu et al. 2009; Liu et al. 2014). The transient expression of XhLEA3-2 in tobacco seedlings was a first attempt to try and test the ability of XhLEA3-2 to protect a model plant from stress. To this end, to the major challenge was tobacco seedling survival after transformation and consistency of XhLEA3-2 ectopic expression. Although the FAST method (Li et al. 2009) may be more efficient on paper, a more sound result is likely to be provided by the construction of a true transgenic tobacco line as done by Liu et al. (2009) with transgenic tobacco stably expressing a LEA_4 from the resurrection plant *Boea hygrometrica* showing increased levels of dehydration stress tolerance.

The detection of XhLEA3-2 expression at a low RWC in *X. humilis* gives more evidence that LEAs are expressed in the dry *X. humilis* leaf tissue (and not in hydrated leaf tissue) and consequently are predicted to play a role in dehydration stress tolerance. This is a follow-on to XhLEA3-2 qPCR data presented by (Waters 2015) that shows the upregulation of XhLEA3-2 gene expression at low RWCs in *X. humilis*. A more detailed *X. humilis* dry down curve and subsequent detection of XhLEA3-2 expression would be useful to more accurately narrow down the precise stage at which XhLEA3-2 is expressed during dehydration.

Putative LEA expression was detected by the XhLEA3-2 specific antibody in *X. viscosa*. Seemingly the antibody detected multiple proteins with the characteristic expression of XhLEA3-2 at low RWCs. The fact that the XhLEA3-2 antibody picked up any proteins in *X. viscosa* leaf extract is indicative of the high sequence homology of LEA_4s (Dure 1993; Battaglia et al. 2008), particularly in closely related species but also illustrates how conserved the expression pattern of LEAs is across species.

An XhLEA3-2 expressing *E. coli* strain was shown to have improved survival after desiccation although XhLEA3-2 did nothing to aid in osmotic stress tolerance. This result helps elucidate the stress-tolerant nature and abilities of XhLEA3-2 and that its properties can act across some species. Dehydration and osmotic stress are linked and therefore it is unusual that XhLEA3-2 does not protect *E. coli* from both types of stresses. Further work could bring clarity to this end.

LDH activity was shown to be more conserved in the presence of XhLEA3-2 leading to the conclusion that XhLEA3-2 has some molecular chaperone or molecular crowding activity. The mechanism remains unclear but perhaps XhLEA3-2 binds to LDH with low specificity and high affinity, due to its intrinsic disorder (Pazos et al. 2013), and protects the LDH from denaturation and aggregation. An alternative hypothesis is that XhLEA3-2 acts as a 'crowding agent' thus filling up space and preventing denatured LDH from coming into contact with other denatured proteins and therefore preventing aggregation to some degree. The role of LEA proteins in abiotic stress tolerance continues to be of interest to scientists hoping to mimic desiccation tolerance in sensitive species. Understanding, the precise functional mechanisms of these proteins will be valuable to this end.

The bioinformatic work in Chapter 2 predicted many LEA-characteristics of XhLEA3-2. Furthermore, the *in silico* localization suggests that XhLEA3-2 potentially plays some role at the cell membrane. Using the XhLEA3-2 antibody produced in Chapter 3, this localization could be confirmed via immunolocalisation studies and could help in the determination of the precise role of XhLEA3-2.

As the global population grows and the climate continues to change, the issue of food security becomes more and more pressing. The study and production of drought tolerant crops is of utmost importance if we are to continue to provide sufficient food for both humans and livestock. The function(s) of the small and somewhat mysterious LEAs could hold key insights in this regard.

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Appendices

Appendix A: XhLEA3-2 and XhLEA3-2 *Xerophyta viscosa* homologs sequence information

A1: Amino acid sequence of XhLEA3-2

MASTHDNLNFKAGETKGQAQEKAGLFGDKASGTAQAAKDKTSATTQSAQDKSGGFMETAK
DRAQELKDRAGNALENTREKAMEKKDQSGGCMQDKKDQTGNWMHEKKDQTGGFMSEKKDQ
SGNVLQQTGENVKNAAGSAANAVKNTLGMGDDTRNSQI

A2: Amino acid sequence of recombinant XhLEA3-2

MHHHHHHRGSEFELRRQAYMASTHDNLNFKAGETKGQAQEKAGLFGDKASGTAQAAKDKT
SATTQSAQDKSGGFMETAKDRAQELKDRAGNALENTREKAMEKKDQSGGCMQDKKDQTGN
WMHEKKDQTGGFMSEKKDQSGNVLQQTGENVKNAAGSAANAVKNTLGMGDDTRNSQI

A3: Amino acid sequence of Xvis02_15976

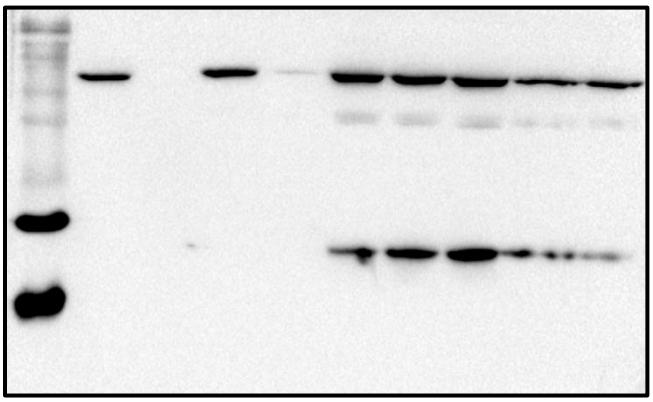
MASNQDNLNFRAGETKGQAQEKAGQFGNKASETAQAAKDKTSATTQSAQDKSGGFMETAK
ERAQELKDRAGNALENTKESAMEKKDQSGGCMQGKKDQTGNWMHEKKDQSGGFMSEKKDQ
SGNVLQQTGENVKNAAGSAANAVKNTLGVGDDTRNSQI

A4: Amino acid sequence of Xvis02_11331

MASTQDDLKIKAGETKGQAQEKAGQFGDKASGTAQAAKDKTSSTTQSAQDRSGGFMETAKE
KAQELKDRAGDAVENTREKAMEKKDQSGSCMQDKKDQTGNWTHEKKDQSGNVLQQTGQNVK
DAAGSAANTVKNTLGMGDDNKS

Appendix B: Original imagery

B1: Figure 3.8



B2: Figure 4.9