Using *XhLEA*, a Group 1 Vegetative Late Embryogenesis Abundant Protein to Aid Water Deficit Tolerance in Plants and Microbes

by

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Abstract

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Late Embryogenesis Abundant (*LEA*) genes have been irrefutably linked to the osmotic stress response since their initial discovery in maturing cotton seeds. They have since been reported from a multitude of other organism where their occurrence is often associated with general responses to abiotic stress. Many studies have been conducted using *LEA* genes in over expression strategies to improve abiotic stress resistance. Of the known classes of *LEA*s, the group 1 *LEA*s have been widely reported, in plants, to only occur in seeds during late stages of development. Their expression coincides with the seeds acquisition of desiccation tolerance. In this thesis we present a group 1 *LEA* isolated from the desiccated vegetative tissues (leaves) of the resurrection plant *Xerophyta humilis*. Using *E.coli* and *Arabidopsis* we attempted to use *XhLEA* to improve salt and water deficit stress-responses, respectively. To this end we conducted soil-drought trials on two independent transgenic *Arabidopsis* lines expressing *XhLEA* under a drought inducible-promoter and monitored their responses as compared to untransformed WT (*Col-0*) controls. Solid substrate *E.coli* growth assays and liquid media growth curves under both stress and unstressed conditions were conducted. We found no obvious beneficial effect through the expression of *XhLEA* in either of the organisms.

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Contents

Declaration **2**

Abstract **3**

Contents **4**

List of Figures **6**

1 General Introduction **7**
1.1 Aims & Objectives 15

2 Over-expression of *XhLEA* in *E. coli* and its potential to confer osmotic stress resistance **16**
2.1 Introduction 16
2.2 Methods & Materials 18
2.2.1 *pProExHtC::XhLEA* Construction 18
2.2.2 RT-PCR to confirm presence of *XhLEA* transcripts 18
2.2.3 Protein purification 19
2.2.4 Plate-based *E. coli* stress test 19
2.2.5 Generation of growth curves 20
2.3 Results 21
2.3.1 Screen for directionality in *pProExHtC::XhLEA* 21
2.3.2 PCR on reverse transcribed RNA from induced *pProEx HtC::XhLEA* cultures 21
2.3.3 Recombinant *XhLEA* protein purification 22
2.3.4 Expression of *XhLEA* does not confer osmotic stress tolerance when grown on LBA supplemented with NaCl 23
2.3.5 Growth curves of *E. coli* expressing recombinant *XhLEA* . . 23
2.4 Discussion 25

3 Drought inducible expression of *XhLEA* in *Arabidopsis* and its effect on water deficit tolerance **27**
3.1 Introduction 27
3.2 Methods & Materials 29
3.2.1 *pMDC*$_{32}$*::XhLEA* construction 29
3.2.2 *Agrobacterium tumefaciens* (Gv3101) electroshock transformation 29

Stellenbosch University https://scholar.sun.ac.za

3.2.3 *pMDC*$_{32}$*::XhLEA/Col-0* floral dip 29
3.2.4 Confirmation of *pMDC*$_{32}$*::XhLEA/Col-0* transformation and expression 30
3.2.5 *Arabidopsis RD29A::XhLEA/Col-0* drought resistance trials . 30
3.2.5.1 Plant growth conditions 30
3.2.5.2 Drought tolerance trial 31
3.3 Results 32
3.3.1 Colony PCR on antibiotic resistant *Agrobacterium* colonies putatively containing *pMDC*$_{32}$*::XhLEA* 32
3.3.2 Confirmation of *XhLEA* insertion and expression in T-1 selection resistant *pMDC*$_{32}$*::XhLEA/Col-0* 32
3.3.3 *RD29A::XhLEA* drought tolerance trial 33
3.3.3.1 Confirmation of *RD29A::XhLEA* insertion 33
3.3.3.2 Drought responsive expression of *XhLEA* during the drought trial 34
3.3.3.3 Drought trial soil moisture content and relative leaf water content 34
3.4 Discussion 36

4 General conclusion **39**

List of References **41**

Stellenbosch University https://scholar.sun.ac.za

List of Figures

2.1 Screen for directionality in *pProExHtC::XhLEA* 21
2.2 PCR on reverse transcribed RNA from induced *pProEx HtC::XhLEA* cultures 22
2.3 Recombinant *XhLEA* protein purification 22
2.4 Expression of *XhLEA* does not confer osmotic stress tolerance when grown on LBA supplemented with NaCl 23
2.5 Growth curves of *E.coli* expressing recombinant *XhLEA* 24

3.1 Colony PCR on antibiotic resistant *Agrobacterium* Colonies putatively containing *pMDC*$_{32}$*::XhLEA* 32
3.2 Confirmation of *XhLEA* insertion and expression in T-1 selection resistant *pMDC*$_{32}$*::XhLEA/Col-0* 32
3.4 Confirmation of *RD29A::XhLEA* insertion 33
3.5 Drought responsive expression of *XhLEA* during the drought trial . . . 34
3.6 Drought trial soil moisture content and relative leaf water content . . . 35

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Chapter 1

General Introduction

Late Embryogenesis Abundant (*LEA*) Genes Have Been Found in a Large Variety of Organisms

The first genes in the late embryogenesis abundant (*LEA*) gene family were described in cotton seeds (Dure III *et al.*, 1981). They were identified and cloned from maturing seeds. Their expression coinciding with defined stages of development characterised by the seeds acquisition of desiccation tolerance. Following this discovery, *LEA*s have been found in all orthodox seeds studied to date. However, contrary to their nomenclature, numerous *LEA*s have been identified to be expressed in vegetative tissues (roots and leaves) under abiotic stress and abscisic acid (ABA) treatment (Bies-Etheve *et al.*, 2008; Hundertmark & Hincha, 2008). There have also been extensive reports of *LEA*s being identified outside of the plant kingdom including: (i) Bacteria, *Deinococcus radiodurans* (Battista *et al.*, 2001) and *Bacillus subtilis* (Stacy & Aalen, 1998) (ii) cyanobacteria (Close & Lammers, 1993) (iii) fungi, *Tuber borchii* (Truffle)(Ghignone *et al.*, 2006), the intracellular parasite *Encephalitozoon cuniculi* (Brosson *et al.*, 2006) and (iv) various higher order animals. All *LEA*s have a common feature that they are highly expressed under abiotic (cold, high salinity and drought) stress conditions, suggesting they fulfil functional roles in cellular protection under these conditions.

LEA genes occur in multi-gene families and are grouped according to defined amino acid motifs and repeats thereof. Genes in the *LEA* family have undergone many nomenclature and grouping changes throughout the years as our knowledge of the family grows

Bray (1993) was the first to group *LEA*s based on characteristic amino acid motifs. Unfortunately, subsequent grouping and naming of the *LEA* gene family by different academics has led to a chaotic situation with many groups being debated and refined (Wise, 2003; Hundertmark & Hincha, 2008). Three main *LEA* groups (Group 1, 2 and 3) are relatively free of controversy. However there are several smaller groups which are less consistent as different academics use different techniques to group them. Below is an outline of the characteristics of the three main *LEA* groups.

Group 1 *LEA*s (also known as Em, D_19 or LEA _ 5) have been characterised

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by the presence of the conserved amino acid sequence: GGQTRREQLGEEGYSQM-GRK (Battaglia *et al.*, 2008). In plants this sequence can appear as a tandem repeat (up to four times). However, in other organisms it may appear up to eight times (Battaglia *et al.*, 2008). These proteins have a high percentage of charged amino acids, specifically Glycine (Gly, G) which accounts for 18% of the total amino acid peptide. These charged amino acids allow for the proteins to have a largely unstructured form in an aqueous solution. Two other conserved motifs have been described in group 1 LEA proteins, one is located on the N terminal side just up-stream of the previously mentioned repeat, while the other is located at the C terminal end. These two motifs are not universally accepted as defining features of group 1 LEAs.

To date, plant group 1 *LEA*s have exclusively been observed in seeds during late embryogenesis. In *Arabidopsis* the two group 1 *LEA*s have been shown to mediate water loss from the developing seed but are not essential for germination (Manfre *et al.*, 2006). Group 1 LEA proteins are not exclusive to plants and have been described in *Bacillus subtilis* (Stacy *&* Aalen, 1998) and in methanogenic archaens. Sharon *et al.* (2009) described the presence of two Group 1 LEA proteins in the eggs of the crustacean *Artemia franciscana* which can remain dormant in a dehydrated state for up to 10 years. Recently four more group 1 LEA proteins were detected in *A. franciscana*.

Group 2 *LEA*s (also known as Dehydrins or D11) are the only group of LEAs that are, at the time of writing, unique to plants. This group of LEAs has three defining amino acid motifs: (i) the lysine (Lys, K) rich K- segment (EKKGIMD-KIKEKLPG) which can be found up to 11 times in the same polypeptide, (ii) the Y-segment ([V/T]D[E/Q]YGNP) found up to 35 times in a single polypeptide and (iii) the S-segment which features multiple serine (Ser, S) residues which may be phosphorylated (Battaglia *et al.*, 2008).

Group 3 *LEA*s (also known as D7, D29 and LEA_4) are characterized by the 11mer motif (TAQAAKEKAXE) which may be repeated numerous times. Group 3 has also been found to contain many charged and polar amino acids (Battaglia *et al.*, 2008). This group of *LEA*s is the most abundant in *Arabidopsis* representing 18 out of a total of 51 *LEA* encoding genes (Hundertmark *&* Hincha, 2008).

_LEA_s are highly expressed in all cellular structures upon osmotic stress and form part of the ABA stress response pathway

Genes (totalling 51) encoding for the numerous LEA proteins are found evenly distributed among *Arabidopsis*'s five chromosomes (Bies-Etheve *et al.*, 2008). Of these genes, many are arranged in tandem repeats (28%) while a further 24% of them present as gene pairs resulting from genome wide duplication (Bies-Etheve *et al.*, 2008). These tandem repeats and duplication events contribute to the disproportionately high number of *LEA* genes within the *Arabidopsis* genome. Genes (33 in total) from each of the various *LEA* groups have been shown to be expressed in seeds during late developmental stages. Their expression coinciding with the seeds acquisition of desiccation tolerance, However, during early seed development *LEA* transcripts are undetectable in the immature green siliques (Hundertmark *&* Hin-

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cha, 2008; Manfre *et al.*, 2006). Furthermore, different genes (22 in total) from each of the *LEA* groups have been shown to be expressed in vegetative tissues in both unstressed and abiotically (cold, high salinity and drought) stressed tissues. Only a small proportion of these genes (10) are expressed in both seeds and vegetative tissues (Bies-Etheve *et al.*, 2008; Hundertmark & Hincha, 2008). Expression profiles of the vegetative *LEA*s show 12 to be highly up-regulated during abiotic stress (more than 3 fold) (Hundertmark & Hincha, 2008). There is no one group of *LEA* genes that is heterogeneously expressed in both seeds and vegetative tissues. Likewise, abiotic stress does not cause one or all *LEA* groups to express heterogeneously but *LEA* genes from all groups are expressed to carry out their functions; either abiotic stress response or the development of the desiccation tolerant seed (Bies-Etheve *et al.*, 2008; Hundertmark & Hincha, 2008).

Changes in the expression of many genes during periods of stress are as a result of the ABA signalling pathway. When looking at the promoter sequences (2000 nt) upstream of the *LEA* genes it was found that 82% contained the well-defined ABA responsive element (*ABRE*) allowing these genes to be positively regulated by ABA. In the same study it was found that 69% of the *LEA* genes contained a low temperature responsive element (*LTRE*). These figures are disproportionally high considering that only 58% of *Arabidopsis* genes carry the *ABRE* and only 40% the *LTRE*, this is further evidence that the *LEA* gene family is indispensable to the plants abiotic stress response (Hundertmark & Hincha, 2008).

Due to the large number of LEA proteins that are expressed during either seed maturation or abiotic stress it would be interesting to know the sub-cellular localisation of these proteins. Various computer models have shown that the *Arabidopsis* LEA proteins are localised throughout the cell's various structures (Candat *et al.*, 2014). Recently the sub cellular localisation of the 51 *LEA* genes in *Arabidopsis* was determined by means of green fluorescent protein (GFP) fusion studies. In this study it was found that 36 LEAs were localised to the cytosol, with a large proportion of these being able to diffuse into the nucleus due to their small size. Mitochondrial and plastidial targeting accounted for 3 each, while 2 LEAs were targeted to both. The endoplasmic reticulum (ER) showed accumulation of 3 LEAs, 2 more LEAs resided in the vacuole and 2 were determined to be secreted (Candat *et al.*, 2014). From this study it is clear that the LEA proteins play a protective role throughout the cell's various sub-cellular components.

LEA proteins are highly hydrophilic due to their many charged amino acids, this has allowed them to be classified as Intrinsically Disordered Proteins (IDPs)

Typically, LEA proteins are small in size between 10 to 30 kDa, however, these small proteins secondary and tertiary structures are remarkably hard to model. McCubbin *et al.* (1985) first noted the peculiar characteristics of a LEA protein by using a variety of biochemical techniques. They noted that the LEA proteins did not display the characteristic properties of a globular protein and further experiments revealed a flexible conformation with little to no secondary structure. It was concluded that these characteristics are due in part to their large proportion of charged

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amino acids (Gly, Glu, and Gln). This amino acid bias in all LEA proteins leads to their common feature of being highly hydrophilic in nature (Wise & Tunnacliffe, 2004; Battaglia *et al.*, 2008).

One of the most intriguing characteristics of proteins which display such a high level of hydrophilicity is that they are largely unstructured in their natural, hydrated, state. For example the wheat *EM LEA* protein is predicted to be found with as much as 70% existing as a random coil in its hydrated state (McCubbin *et al.*, 1985). Indeed, computer modelling has predicted the intrinsic disorder of the majority of LEA proteins to be at least 50% disordered (Prilusky *et al.*, 2005). A further characteristic of intrinsically disordered LEA proteins is that they are heat stable.

LEA proteins display remarkable functional plasticity and redundancy due in part to their nature as IDPs

Over the last few years much work has been done to elucidate the *in vivo* function, as well as the mode of function, of LEA proteins. Their ability to gain a secondary structure under certain conditions: (i) dehydration, (ii) phosphorylation or (iii) physical interaction with membranes or proteins, is fundamental to their functions inside the cell. For a review of IDPs see (Sun *et al.*, 2013). There are currently two main models that have been observed when studying the function of the different LEA proteins. Firstly some LEA proteins behave as either unconventional chaperones, molecular shields and contribute to the formation of a glass state during stress conditions. Alternatively, other LEA proteins have been shown to bind certain metal cations upon phosphorylation to alleviating the cell from reactive oxygen species (ROS) under dehydrating conditions, as well as interacting with certain membranes bringing about conformational changes. These two models are discussed below in detail.

Unconventional chaperones, molecular shields and the formation of the glass state:

The first and most studied putative function of LEA proteins is their ability to prevent protein aggregation under osmotic stress conditions (desiccation, temperature and salt) (Kovacs *et al.*, 2008; Boucher *et al.*, 2010; Chakrabortee *et al.*, 2012; Goyal *et al.*, 2005). This protective function is similar to those of typical molecular chaperones, however, there exist some characteristic differences that has lead the LEA proteins to be classified as unconventional chaperones or molecular shield proteins (Sun *et al.*, 2013; Kovacs *et al.*, 2008). Chakrabortee *et al.* (2012) showed that, unlike conventional chaperones, a group 3 LEA protein from a nematode and a plant group 1 LEA protein could not be co-immunoprecipitated with their target proteins indicating a low affinity interaction. However, LEA proteins seem to display a low specificity but can bind to many targets enabling them to be highly versatile under stress conditions (Shimizu *et al.*, 2010).

Due to these characteristics it has been proposed that LEA proteins prevent protein aggregation by slowing the cohesion rates of denaturing proteins by surrounding them, physically preventing aggregation (Chakrabortee *et al.*, 2012). Boucher *et al.*

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(2010) demonstrated that a group 3 LEA protein was able to reverse protein aggregation when added after significant aggregation had occurred and postulated that this was due to the LEAs large hydration shell effectively dissolving protein aggregates. Additionally almost all characterised LEA proteins display a synergistic protective effect together with trehalose as well as sucrose which are known to accumulate in both seeds and cells undergoing osmotic stresses (Goyal *et al.*, 2005; Chakrabortee *et al.*, 2012).

Membrane stabilization and metal cation scavenging:
Membrane stabilization and metal cation binding is the second model that has been proposed, however, all experimental results seems to implicate only the group 2 LEA proteins (Dehydrins). Alsheikh *et al.* (2005) showed that 3 dehydrins from *Arabidopsis* displayed calcium binding activity when phosphorylated both *in vitro* and *in vivo*. Additionally they found that other cations (Zn and Fe (II)) can be bound by the dehydrins when present in high cellular concentrations. High concentrations of Zn and Fe (II) are known to occur *in vivo* during stress inducing conditions, thus, the scavenging of these cations reduces the formation of harmful ROS (Alsheikh *et al.*, 2005). Dehydrins are characterized by their signature amino acid motifs (K,Y and S segments), the lycine rich K segment has been found to bind acidic phospholipid membranes, resulting in a gain of secondary structure (Eriksson *et al.*, 2011; Koag *et al.*, 2003, 2009). This membrane interaction resulting in a gain of secondary structure has been proposed to raise the temperature of the phase change of these acidic phospholipid membranes, allowing these membranes to remain viable under stress conditions (drought, salt and heat) (Eriksson *et al.*, 2011).

Interestingly, a group 2 *LEA* has been shown to be involved in Fe transport in the phloem of Castor bean plants (Kruger *et al.*, 2002). Another Dehydrin from citrus was shown to bind Fe, Co, Ni, Cu and Zn. The metal binding domain of these proteins has been found to be related to the high His concentration within the dehydrins. Because the region that binds metal cations and the K segment that binds membranes are distinct from one another, it could be conceivable that these dehydrins scavenge metal cations and in turn bind to a membrane to isolate them. This in turn prevents the formation of ROS in the cell during osmotic or temperature stress (Hara *et al.*, 2005).

Much work still needs to be done before we can separate the LEA proteins by their mode of function. This task is further complicated due to the functional plasticity the LEA proteins display under stress and unstressed conditions. Forward and reverse genetic approaches which isolate only a handful of *LEA*s at any one time may not be able to tease apart the complex network displayed by the LEA protein family in response to stress.

Resurrection plants: Leaves behaving like seeds due in part to *LEA* expression
Resurrection plants are characterised by their ability to lose up to 95% of their leaf cellular water. These plants can become viable again within 24 h post watering. For a review see (Hartung *et al.*, 1998). Examples of resurrection plants can be

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found in small numbers throughout the taxonomic groups ranging from lower order plants to pteridophytes (mosses and ferns) to dicotyledons (Scott, 2000). Lower order resurrection species focus on rapid reparation of damaged tissues due to cellular water loss (Oliver *et al.*, 1998). Higher order resurrection plants, however, actively respond to water limitation and protect their cellular viability through a variety of mechanisms including: (i) cell wall folding or shrinkage (ii) sugar accumulation (sucrose and trehalose) (iii) degradation or shielding of chlorophyll (iv) up-regulation of anti-oxidants and ROS scavenging species and (v) up-regulation of various *LEA* genes (Oliver *et al.*, 1998).

Farrant (2000) showed that the cellular ultrastructure of resurrection plants upon dehydration varies in response from dramatic cell wall folding to the replacement of the large aqueous vacuoles with several smaller non-aqueous vacuoles. This cell wall folding and replacement of large aqueous vacuoles serve to provide sufficient cellular packing to prevent the plasmalemma from rupturing during dehydration (Scott, 2000; Farrant, 2000). Interestingly, this is similar to the way orthodox seeds survive cellular dehydration.

Resurrection plants have been shown to accumulate large amounts of sucrose upon dehydration (Scott, 2000; Illing *et al.*, 2005; Farrant & Moore, 2011). High concentrations of cellular sucrose has the ability to maintain hydrogen bonds in the desiccating cells, thus maintaining membrane and protein structure. Furthermore, the presence of large amounts of sucrose adds to the formation of a glass state which stabilises the cells internal structures (Illing *et al.*, 2005; Scott, 2000).

Oliver *et al.* (1998) proposed that desiccation tolerance in vegetative tissue evolved through the deregulation of seed-specific gene expression. To this end Illing *et al.* (2005) identified various *LEA* genes expressed in the desiccating vegetative tissues of the resurrection plant *Xerophyta humilis*. Many of these genes have orthologs in *Arabidopsis* which have a seed specific expression patterns. Many other resurrection plants have been shown to up-regulate *LEA* gene expression during desiccation (Scott, 2000; Illing *et al.*, 2005; Farrant & Moore, 2011). It has been proposed that this deregulation of seed-specific *LEA* expression in the vegetative tissues of resurrection plants allows these plants to maintain sufficient cellular water content while activating the above-mentioned protective mechanisms.

LEA proteins have been found to impart water deficit tolerance to various higher order animals

Many water-borne nematodes and insects are able to survive or propagate under extreme osmotic stress conditions. It is becoming clear that a similar mechanism is at play in both seeds and these desiccation tolerant organisms (Gal *et al.*, 2004; Kikawada *et al.*, 2006; Menze *et al.*, 2009). The presence of LEA proteins has been described in the nematodes *Artemia fransciscana* (Menze *et al.*, 2009; Sharon *et al.*, 2009), *Aphelenchus avenue* (Goyal *et al.*, 2005) and *Caenorhabditis elegans* (Gal *et al.*, 2004) as well as in the chironomid *Polypedilum vanderplanki* (Kikawada *et al.*, 2006), where their expression has been strongly correlated to these organisms ability to survive osmotic stress conditions.

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The importance of the LEA proteins to the survival of nematodes was first described in *Caenorhabditis elegans* when a group 3 *LEA* was silenced which lead to a loss of survival when the nematodes were placed under stress conditions (Gal *et al.*, 2004). Following this study, further group 3 LEA proteins have been detected in another nematode, *Aphelenchus avenue* (Goyal *et al.*, 2005). Interestingly, in this study it was noted through Western analysis that there were 2 groups of proteins hybridising to the LEA specific probe, one representing the full predicted protein and the other representing a smaller protein. Upon dehydration this ratio of full sized protein to smaller protein shifted until the smaller protein dominated. The authors further went on to show an enzymatic activity in dehydrated protein extracts which was able to process the full length LEA into smaller, yet still protective, fragments. This post-translational modification has also been shown for a group 3 *LEA* expressed in the desiccation tolerant larvae of *Polypedilum vanderplanki* (Kikawada *et al.*, 2006).

When an expressed sequence tag (EST) library constructed from desiccating larvae of *Polypedilum vanderplanki* was screened, 3 *LEA*-like cDNAs were identified (Kikawada *et al.*, 2006). It was shown that both their mRNA and protein levels were up-regulated during desiccation as well as hyper salinity (1 % NaCl) stress.

The brine shrimp *Artemia fransciscana* can survive extreme cellular water loss in the form of an encysted embryo (up to 95% total cellular water), termed anhydrobiosis. This survival is analogous to the acquisition of desiccation tolerance in orthodox seeds. 2 distinct groups (*LEA* 1 and *LEA* 3) of LEA proteins have been found in *A. franciscana.* These LEA proteins are present in the desiccation tolerant encysted embryos and not in the mature shrimp or the larvae. The group 3 *LEA* was found to localise to the mitochondrial matrix, where they showed its presence, together with trehalose, protected the organelle from water stress induced by freezing (Sharon *et al.*, 2009; Menze *et al.*, 2009).

These studies show a clear relationship between the mechanisms used by plants and certain anhydrobiotic organisms to combat desiccation stress. Although there are some functional differences, with the possibility of post translational modification of group 3 *LEA*s in animals, the sequence motifs and functional identity of the identified *LEA*s remains highly in accordance to *LEA*s isolated from plants.

Over-expression of *LEA* genes in various pro- and eukaryotic systems confers abiotic stress tolerance:

With the *LEA*s' role in plant stress responses becoming clearer there have been many attempts to improve the abiotic fitness of several plant species, both model (*Arabidopsis*), crop as well as a few non-plant systems. Various studies involving both over- and inducible expression of heterologous *LEA*s as well as over-expression of endogenous *LEA*s seem to confer increased abiotic stress tolerance.

In *Arabidopsis* the over-expression of a group 5 *LEA* (*JcLEA*) from *Jatropha curcas* increased tolerance to both salinity and drought (Liang *et al.*, 2013). Fur-

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thermore, freezing tolerance was increased through the over-expression of a wheat group 3 *LEA* gene (*WCS19*, (NDong *et al.*, 2002).

In rice (*Oryza sativa*) the over-expression of the barley gene *HVA1*, a group 3 *LEA*, conferred a higher tolerance to both salinity and drought stress. Furthermore, it was noted that the level of transgenic *LEA* expression correlated to the level of tolerance conferred by said *LEA* (Xu *et al.*, 1996). Additional studies using *HVA1* transgenic rice showed delayed loss in leaf water content compared to control plants, delaying wilting by 2 weeks (Babu *et al.*, 2004). The over-expression of *PMA80* (group 2 *LEA*) and *PMA1959* (group 1 *LEA*) from wheat in rice again improved both the salinity and drought tolerance in the transgenics as compared to the control (Cheng *et al.*, 2002). In field trials the over-expression of the native *OsLEA 3-1* in rice resulted in improved drought tolerance while not incurring any loss in yield quantity (Xiao *et al.*, 2007).

Over-expression of a *LEA* from *Salvia miltiorrhiza* (*SmLEA*) conferred tolerance, *in vitro*, to both NaCl stress and mannitol (osmotic stress) in *S. miltorrhiza*. Interestingly, IPTG induced expression of *SmLEA* improved both salt and osmotic stress in *E. coli* (Wu *et al.*, 2014). *Drosophila melanogaster* (fruit fly) cells have been shown to have increased tolerance to salt and desiccation through the over-expression of a group 1 *LEA* from *A. franciscana* (Marunde *et al.*, 2013).

These examples show that the over-expression of *LEA*s from various organisms is able to confer abiotic stress tolerance to various plants as well as animals.

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1.1 Aims & Objectives

A previously identified Group 1 *LEA* that was isolated from desiccated leaves in *Xerophyta humilis* represents, to the best of our knowledge, the first recorded group 1 *LEA* isolated from vegetative tissues. Group 1 *LEA*s are only expressed in the seeds of desiccation sensitive plant species during late stages of development where seeds are known to obtain their desiccation tolerance. In the following study we aimed to achieve the following:

1. Produce recombinant strains of *E. coli* (BL21codon plus ripple) with *XhLEA* cDNA in both sense and anti-sense orientations cloned downstream of an inducible (IPTG) promoter.

2. Use the product of 1 to test the ability of recombinant *E. coli* to withstand high osmotic stress conditions.

3. Produce T-3 generation transgenic *Arabidopsis* ($pMDC_{32}$*::XhLEA/Col-0*) with *XhLEA* cDNA cloned downstream of the *CaMV35S* constitutive promoter.

4. Produce T-3 generation transgenic *Arabidopsis* ($pMDC_{32}$*/RD29A::XhLEA/Col-0*) with *XhLEA* cDNA cloned downstream of the *RD29A* (stress inducible) promoter.

5. Use the products of 3 and 4 to test the transgenics ability to withstand vegetative water deficit conditions in relation to a WT (*Col-0*) control.

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Chapter 2

Over-expression of *XhLEA* in *E. coli* and its potential to confer osmotic stress resistance

2.1 Introduction

Since their discovery in cotton seeds, late embryogenesis abundant (*LEA*) proteins have been identified in a myriad of organisms ranging from bacteria to Nematodes to brine shrimp and plants (Stacy & Aalen, 1998; Dure III *et al.*, 1981; Kikawada *et al.*, 2006; Menze *et al.*, 2009; Sharon *et al.*, 2009). While they have been grouped according to conserved amino acid domains, their functional characteristics in their natural systems are often unclear. It is evident that *LEA*s increase upon exposure to multiple abiotic stress factors including: freezing, oxidative stress, desiccation and salt (Wise & Tunnacliffe, 2004; Xu *et al.*, 1996; Kikawada *et al.*, 2006).

Many reports define the use of heterologous systems to express *LEA*s in order to ascertain the mechanisms of any protective functions they may exert when recombinantly expressed. The heterologous systems of choice have been both the yeast and *E. coli* models (Liu & Zheng, 2005; Campos *et al.*, 2006; Dang *et al.*, 2014; Lan *et al.*, 2005). These approaches, however, have often yielded inconclusive results. A few notable examples with regards to plant *LEA*s follow.

Using *E. coli* as the heterologous expression platform, a group 3 *LEA* isolated from the immature seeds of the soybean was functionally characterised (Liu & Zheng, 2005). Cultures expressing recombinant protein were found to have improved survival to high salinity (500 mM NaCl and KCl) using a solid plate growth assay. However, that study further concluded that the same *LEA* was unable to promote survival under osmotic stress (1.1 M sorbitol). Contrary to this, a study on 3 different soy bean *LEA*s representing groups 1, 2 and 3 found that only the group 1 and 3 isoforms promoted improved *E. coli* survival under high salinity (800 mM NaCl and KCl) in liquid culture assay (Lan *et al.*, 2005). That study found no protective function for the group 2 LEA.

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Following the identification of 58 LEA sequences from *Arabidopsis* (Hundertmark & Hincha, 2008), functional characterisation of a subset of 15 of these LEAs has recently been reported (Dang *et al.*, 2014). That study heterologously expressed the subset of 15 *LEA*s in yeast (BY4742), particularly looking for functions under which recombinant protein provided abiotic stress protection (osmotic, cold, desiccation, salt). The study used gene sequences representing 6 *LEA* groups (including 1, 2 and 3). They found that only three isoforms from group 3 and three isoforms from group 2 were able to promote survival following desiccation stress (19h, 30°C). Conversely they found that the group 1 *LEA* (*AtEM-6*, homologue to *XhLEA*) was unable to positively influence yeast survival following desiccation. That study further noted that all *LEA*s under investigation did not provide any tolerance to yeast when grown in the presence of 1 M NaCl. Deleterious growth has been observed in *E. coli* when expressing a Group 2 and 4 *LEA* from *Arabidopsis* through IPTG induction in a liquid culture (Campos *et al.*, 2006). It is interesting to note that one of the group 2 *LEA*s shown to cause deleterious growth in *E. coli* (ERD10) was not shown to have any deleterious effect to the growth of yeast (Dang *et al.*, 2014).

Despite the apparent difficulties in determining LEA function through microbial heterologous expression systems, these systems still provide a rapid methodology to elucidate a potential function. In this chapter we describe a heterologous expression strategy (using *E. coli*) where the group 1 *XhLEA* identified from the desiccated leaves of the resurrection plant *Xerophyta humilis* was analysed for its ability to impart tolerance to NaCl-induced osmotic stress. Recombinant *XhLEA* expression was confirmed at both the RNA and protein levels. We used both a plate-based (solid substrate) stress assay and classical liquid cultures, specifically looking for enhanced growth as compared to untransformed control under conditions of high salinity up to 1 M NaCl.

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2.2 Methods & Materials

2.2.1 *pProExHtC::XhLEA* Construction

XhLEA coding strand (cds) was amplified in the following PCR 50 µl reaction: 1X GoTaq reaction buffer (Promega), 0.3 mM dNTP mix, 0.3 µM of each primer, *XhLEA* Fwd (5' - ATGGCTTCCCATCAAGAAAGGG - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG - 3') and 0.75 U GoTaq DNA polymerase. Thermal cycling conditions: 94°C – 2 min (one cycle), 72°C – 50 s, 58°C – 50 s, 72°C – 1 min s (30 cycles) and a final elongation of 72°C - 10 min. The PCR product was confirmed by agarose gel electrophoresis and then purified using the Wizard® SV Gel and PCR Clean-Up system (Promega).

pGEM T - easy was used in a T - A cloning reaction (4 h, RT) together with the purified A-tailed PCR product (3:1 insert:vector). This reaction was then used to transform ultra-competent *E. coli* (DH5α). Antibiotic (Ampicillin 100 µg/mL, amp^{100}) resistant colonies were used to inoculate liquid LB cultures (5 mL, amp^{100}). Overnight cultures were used to obtain plasmids using the Wizard® Plus SV Miniprep DNA purification system (Promega). Restriction digests (*Not*I) were performed on both the *pGEM T-easy::XhLEA* mini preparations and a purified *pProExHtC* empty vector in 1X orange buffer (Fermentas, Thermo Scientific, South Africa). These digests were run on an agarose gel and the fragments of interest were excised and purified using the Wizard® SV Gel and PCR Clean-Up system (Promega).

The *XhLEA* insert with *Not*I restriction overhangs was ligated (4 h, RT) into the *Not*I digested vector (*pProExHtC*, 3:1 insert:Vector) and transformed into *E. Coli* (BL21 Codon Plus). Antibiotic resistant (Spectinomycin 100 µg/mL, spec^{100}, Streptomycin 10 µg/mL, strep^{10}, Chloramphenicol 34 µg/mL, chl^{34}) colonies were screened for insert orientation in the following 50 µl PCR reaction containing 5 µl of a mini preparation from antibiotic resistant colony and: 1X GoTaq reaction buffer (Promega), 0.3 mM dNTP mix, 0.3 µM of each primer, M13 Rev (5' - ATGGCTTCCCATCAAGAAAGGG - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG - 3') for sense orientation (Primer pair 1) and M13 Rev (5' -ATGGCTTCCCATCAAGAAAGGG - 3') and *XhLEA* Fwd (5' - ATGCTTCCCATCAAGAAAGGG - 3') for anti-sense orientation (Primer pair 2) with 0.75 U GoTaq DNA polymerase. Thermal cycling conditions: 94°C – 2 min (one cycle), 72°C – 50 s, 58°C – 50 s, 72°C – 1 min (30 cycles) and a final elongation of 72°C - 10 min.

2.2.2 RT-PCR to confirm presence of *XhLEA* transcripts

Overnight cultures (20 mL, 37°C, 200 RPM, amp^{100}, strep^{10}, chl^{34}) of (i) *pProExHtC::XhLEA* sense (ii) *pProExHtC::XhLEA* anti-sense and (iii) *pProExHtC* empty vector were used to inoculate (1 mL) large culture flasks (200 mL). Total RNA was extracted 3 h post IPTG (1 mM) induction (37°C, 200 RPM, $OD_{(600)}$ 0.4 - 0.8). Samples (2 mL) were taken from 3 cultures representing (i) *pProExHtC::XhLEA*

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sense (ii) *pProExHtC::XhLEA* anti-sense and (iii) *pProExHtC* empty vector and pelleted (5 min, 10 000 x*g*, room temperature RT). Pellets were re-suspended in TE buffer (pH 8) supplemented with lysozyme (1 mg/mL) and incubated (5 min, RT). Following this incubation the standard RNAeasy plant kit (Quiagen, Thermo-scientific, South Africa) protocol was followed. Total RNA was quantified using a NanoDrop ND-100 and run on a gel to confirm its integrity.

Once confirmed, cDNA synthesis was performed by adding 0.5 µM random hexomer primer (Promega) to the appropriate amount of each RNA sample to a final concentration of 1 µg/µl and brought to a final volume with dH_2O (14 µl). These reactions were then incubated (70°C, 5 min) after which they were placed on ice (5 min). Following this incubation the following were added to a final reaction volume of 25 µl: 1X M-MLV reaction buffer (Promega), 0.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP) and 200 U M-MLV RT enzyme (Promega). The reaction tubes were then incubated (40°C, 10 min), then (55°C, 50 min). Aliquots (5 µl) were stored at -80°C.

Reverse Transcriptase-PCR was performed in the following 50 µl PCR reaction containing 2.5 µl reverse transcribed RNA: 1X GoTaq reaction buffer (Promega), 0.3 mM dNTP mix, 0.3 µM of each primer, *XhLEA* Fwd (5'- ATGGCTTCCCATCAAGAAAGGG - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG - 3') and 0.75 U GoTaq DNA polymerase. Thermal cycling conditions: 94°C – 2 min (one cycle), 72°C – 50 s, 58°C – 50 s, 72°C – 1 min (25 cycles) and a final elongation step of 72°C - 10 min.

2.2.3 Protein purification

Total protein was isolated from the above cultures following sampling for RNA extraction. Cells were pelleted 3 h post IPTG (1 mM) induction (5000 x*g*, 15 min, 4°C). The pellet (1 g) was resuspended in Lysis Equilibration Wash buffer (5 mL, LEW: 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Lysozyme was added (1 mg/mL) and left to stir on ice (30 min). Following this the suspensions were sonicated on ice (10 X 15 s at 15 s intervals). This crude lysate was centrifuged (10000 x*g*, 30 min, 4°C), the supernatant (soluble fraction) was separated from the pellet (insoluble fraction) and stored (-80°C). Purification of the recombinant His-tagged XhLEA was performed using Protino Ni-TED 1000 Packed columns (Macherey-Nagel, Duren, Germany) as per manufacturers' specifications. All fractions were collected and run on a 12 % SDS-PAGE gel.

2.2.4 Plate-based *E. coli* stress test

Liquid cultures of (i) *pProExHtC::XhLEA* sense (ii) *pProExHtC::XhLEA* anti-sense and (iii) *pProExHtC* empty vector (150 mL, 37°C, 200 RPM, amp^{100}, $strep^{10}$, chl^{34}) were grown until an $OD_{(600)}$ of 0.9 $\pm$0.01 was achieved. These cultures were induced with IPTG (1 mM) and incubated (conditions above) for a further hour. LBA plates were poured with a final NaCl concentration of (i) 0 M control (ii) 500 mM (iii) 1 M (iv) 1.5 M and (v) 2 M. Cultures were inoculated on the plates into their respective

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quadrants using an inoculation loop (Ø 5 mm). Plates were incubated overnight (37°C).

2.2.5 Generation of growth curves

Overnight cultures of (i) *pProExHtC::XhLEA* sense (ii) *pProExHtC::XhLEA* anti-sense and (iii) *pProExHtC* empty vector (10 mL, 37°C, 200 RPM, amp^{100}, $strep^{10}$, chl^{34}) were measured at $OD_{(600)}$ to determine optical density. From these overnight cultures a starter culture of 1 mL was obtained by diluting out the overnight cultures to obtain the same $OD_{(600)}$ for all 3 starter cultures cultures. Starter cultures (1 mL) were then inoculated into 200 mL LB in triplicate and incubated (9 h, 37°C, 200 RPM). Culture flasks were sampled and the $OD_{(600)}$ was measured in triplicate every hour for the first 5 h followed by every 2 h until completion. Protein expression was induced using IPTG at T=4 ($OD_{(600)}$ 0.6 - 0.8) to a final concentration of 1 mM. One hour post induction, (T=5), osmotic stress was added (NaCl) to a final concentration of 1 M.

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2.3 Results

2.3.1 Screen for directionality in *pProExHtC::XhLEA*

PCR was conducted on mini-preperations from *pProExHtc::XhLEA* transformed antibiotic resistant colonies to determine the orientation of *XhLEA*. Clones in the sense orientation were obtained using primer pair 1 (M13 Rev + *XhLEA* Rev, Figure 2.1). Clones in the anti-sense orientation were obtained using primer pair 2 (M13 Rev and *XhLEA* Fwd, Figure 2.1). Conversely amplicons were not obtained when using primer pair 2 on sense clones and primer pair 1 on anti-sense clones (Figure 2.1).

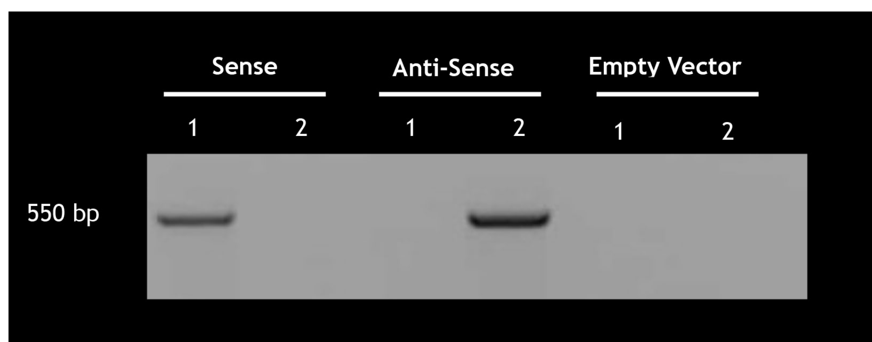

Figure 2.1: PCR on plasmid mini-preparations obtained from antibiotic marker resistant (amp^{100}) colonies following transformations of competent cells with the relevant *pROExHTc::XhLEA* constructs. The sense constructs were PCR screened with the primer pair 1 (M13 Rev + *XhLEA* Rev) and the anti-sense constructs with the primer pair 2 (M13 Rev + *XhLEA* Fwd)

2.3.2 PCR on reverse transcribed RNA from induced *pProEx HtC::XhLEA* cultures

PCR was performed on reverse transcribed RNA isolated from 3 h IPTG (1 mM) induced 200 mL liquid cultures. Using cds (*XhLEA* Fwd + Rev) specific primers amplification of a 500 bp band was achieved for both the sense and anti-sense variants (Figure 2.2). Conversely, no amplification was achieved when reverse transcribed RNA isolated from the empty vector was used as template DNA (Figure 2.2). This confirms the recombinant transcription of *XhLEA* in both the sense and anti-sense orientations.

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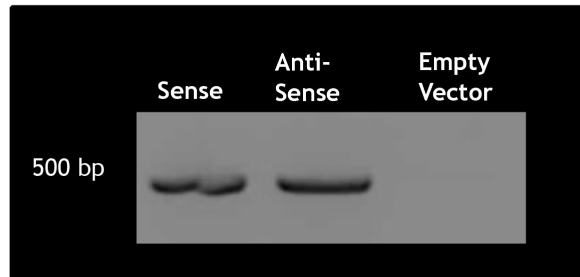

Figure 2.2: Reverse transcription PCR using the *XhLEA* cds specific primer pair (*XhLEA* Fwd + *XhLEA* Rev) on cDNA reverse transcribed from RNA isolates obtained after 3 h of IPTG-induced (1 mM) recombinant *XhLEA* expression in 200 mL liquid cultures. Both the sense and anti-sense variants of *pPROExHTc::XhLEA* were used.

2.3.3 Recombinant *XhLEA* protein purification

Total protein isolated from 200 mL liquid cultures 3 h post IPTG (1 mM) induced recombinant XhLEA expression was purified through a His-tag column (Protino Ni-TED 1000, Macherey-Nagel). Only the sense variant of *pProExHtC::XhLEA* was induced, isolated and purified while the empty vector was used as a negative control. Recombinant protein was detected to be approximately 25 kDa in size by the bands present in the first and second elution lane (E1 and 2, Figure 2.3). No band of this size was detected in the empty vector control. A protein of approximately 40 kDa can be seen in the elution lanes E1 and 2 in both the *pProExHtC::XhLEA* as well as the *pProExHtC* empty vector (Figure 2.3). This confirms the recombinant production of a 25 kDa protein in *pProExHtC::XhLEA* cultures which would correspond to the theoretical size of XhLEA.

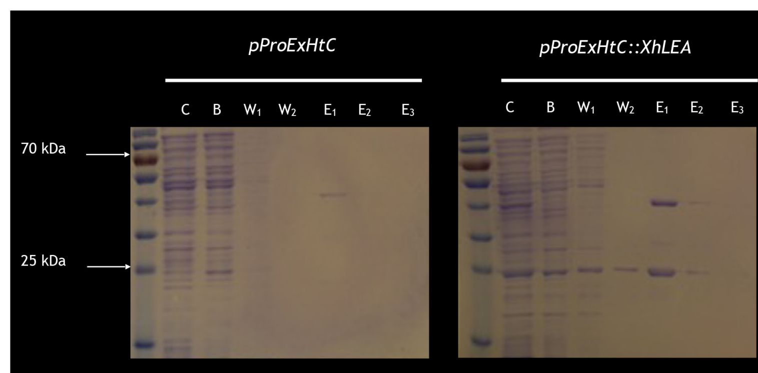

Figure 2.3: SDS-PAGE gels showing various protein fractions following His tag specific protein purification after 3 h of IPTG (1 mM) induced recombinant XhLEA expression in 200 mL liquid cultures. Cultures containing the sense variant were purified with the empty vector serving as the control. Recombinant XhLEA protein was detected at approximately 25 kDa in size. C: Crude extract, B: Binding, W1+2: Wash 1 and 2, E1+2+3: Elution 1, 2 and 3

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2.3.4 Expression of *XhLEA* does not confer osmotic stress tolerance when grown on LBA supplemented with NaCl

Growth of 3 *E. coli* strains containing (i) *pProExHtC::XhLEA* sense (ii) *pProEx-HtC::XhLEA* anti-sense and (iii) *pProExHtC* empty vector were inoculated from 1 h post IPTG (1 mM) induced liquid cultures. Growth of all the cultures were uninhibited on the control LBA plate (Figure 2.4 A). Growth on the 500 mM and 1 M NaCl LBA plate showed mild and severe reduction in growth respectively (Figure 2.4 B & C). The sense and anti-sense variants grew as well as the empty vector control at all of the tested NaCl concentrations. LBA plates containing higher NaCl concentrations (1.5 and 2 M) showed no observable growth (data not shown).

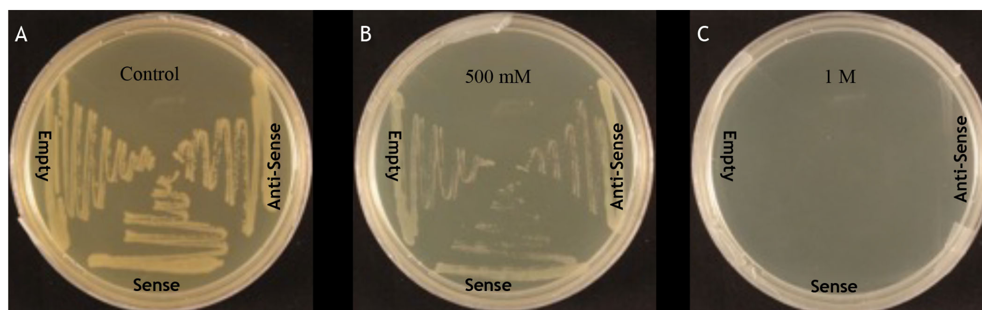

Figure 2.4: Plates showing the growth of *E. coli* inoculated from liquid cultures (150 mL) with an inoculation loop (Ø 5 mm) following 1 h of IPTG (1 mM) induced recombinant XhLEA expression. Cultures were inoculated onto LBA plates supplemented with either 500 mM NaCl (B) or 1 M NaCl (C). LBA (0 mM NaCl) was used as the control (A). Both the sense and anti-sense variants of *pProEx HtC::XhLEA* were used with an empty vector used as a control for plates B and C

2.3.5 Growth curves of *E. coli* expressing recombinant *XhLEA*

Growth curves monitoring the growth of *E. coli* (BL 21 Codon Plus) harbouring (i) *pProExHtC::XhLEA* sense (ii) *pProExHtC::XhLEA* anti-sense and (iii) *pProExHtC* empty vector were produced. Abiotic stress (NaCl) was added 1 h post IPTG (T=4, 1 mM) induction. The growth curves generated from the unstressed cultures all tracked together and reached a stationary phase after approximately 9 h (Figure 2.5, Solid line). Stressed cultures all grew together but they all achieved a lower $OD_{(600)}$ at their stationary phase (Figure 2.5, Dashed line). The graphs for both the stressed and unstressed control all tracked together until the addition of the abiotic stress (1 M NaCl) at T=5 where *E. coli* growth slowed significantly (Figure 2.5).

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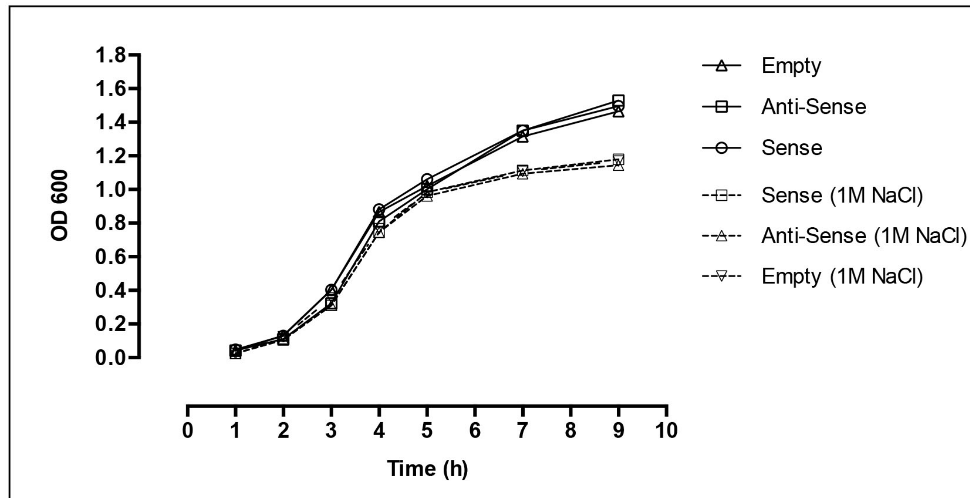

Figure 2.5: Graphs showing the growth of *E. coli* in liquid cultures (200 mL) by monitoring the optical density (600 nm). Recombinant XhLEA expression was induced with IPTG (1 mM) for both stressed and unstressed (solid line) experiments at T=4. Abiotic stress (NaCl) was added at T=5 to a final concentration of 1 M in the stressed experiment (Dashed).

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2.4 Discussion

The Group 1 *LEA*s are generally considered to be seed-specific in plants and their occurrence is often linked to the late maturation stages of seed development (when seeds acquire desiccation tolerance, Galau *et al.* (1986)). However, they have also been now described to occur in other Eukaryotes, archeae, and bacteria their expression coinciding with abiotic stress conditions (Stacy *&* Aalen, 1998; Battista *et al.*, 2001; Gal *et al.*, 2004).

This study focused on the ability of *XhLEA*, a group 1 *LEA* isolated from the desiccated vegetative tissues of *Xerophyta humilis*, to ameliorate the negative effects experienced by *E. coli* when growing on/in media high in NaCl (osmotic stress). The closest *Arabidopsis* homologues to *XhLEA* are the seed specific *AtEM-1* and *-6* (At3g51810 and At2g40170, respectively). Both genes are known group 1 *LEA*s and their expression patterns are strictly associated with late seed-maturation. Only *AtEM-6* has been functionally characterised (Manfre *et al.*, 2006). Using T-DNA insertion mutants, those studies were able to demonstrate a clear function in preventing water loss during seed maturation. It was proposed that *AtEM-6* fulfils the typical function of a *LEA* (*in-vivo*) where its large hydration shell allows it to physically bind water until the later stages of seed-maturation when this water is then lost.

It was of interest to identify *XhLEA* in desiccated leaf tissue. Amongst the most intriguing theories around the mechanisms which allow resurrection plants to survive complete cellular desiccation is the deregulation of seed specific genes into leaf tissue undergoing dehydrative processes (Illing *et al.*, 2005). One could draw many parallels between the processes of seed maturation and leaf desiccation of resurrection plants, given that both tissues remain viable after complete cellular desiccation. We thus wished to contextualise whether *XHLEA* could potentially exhibit a protective function in a heterologous system undergoing osmotic stress. We found that *E. coli* expressing recombinant *XhLEA* did not show any improvements in growth when grown either on 1 M NaCl in a solid plate growth assay or, in liquid cultures (figures 2.4 and 2.5).

Yeast recombinant expression systems have recently been used to test the ability of the *Arabidopsis* group 1 *LEA*, *AtEM-6*, (homologue of *XhLEA*) to ameliorate the effects of a variety of abiotic stresses (Dang *et al.*, 2014). That study reported that recombinant *AtEM-6* was unable to impart any improvement under conditions of freezing, desiccation, osmotic, oxidative or salt stresses. One could further question whether the *AtEM-6* protein was able to function in a yeast recombinant system as it does in its native role during seed maturation *in planta*. It is nevertheless, interesting that our heterologous approach using *E. coli* yielded very similar findings (under high salinity) when using *XhLEA*.

Other *LEA*s have been more successful in attenuating the negative effects on microbial growth associated with osmotic (NaCl or sorbitol/mannitol) and desiccation stress. Dang *et al.* (2014) showed that *LEA*s belonging to group 2 (*COR 47*, At1g20440; *ERD10*, At1g20450 and At2g21490) isolated from *Arabidopsis* protect

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yeast during desiccation stress. Additionally in that study is was found that a number of group 3 *LEA*s (At1g52690; At3g15670 and At4g13560) from *Arabidopsis* were able to protect yeast against desiccation. However, not one *LEA* out of 15 investigated in that study was able to protect yeast against high salt stress. There has only been one reported case of a group 1 *LEA* isolated from mature wheat seeds being able to act as a yeast osmoprotectant under 1 M NaCl and KCl as well as 1.5 M sorbitol stress when recombinantly expressed in yeast (Swire-Clark *&* Marcotte Jr, 1999). It would be interesting to perform *XhLEA* recombinant expression studies in yeast exposed to osmotic, desiccation and salt stress to determine if *XhLEA* behaves like the wheat group 1 *EM* gene or more like its homologue *AtEM-6*.

It would appear that using *LEA* genes in a microbial system is not necessarily consistent in revealing their functions (almost always associated with abiotic stress tolerance). Heterologous expression of 3 *LEA* genes isolated from soybean seeds in *E. coli* found that a group 3 and a group 1 *LEA* were able to improve growth in liquid cultures containing high KCl and NaCl (700 and 800 mM, respectively) while the third (a group 2 *LEA*) provided no protection under the same conditions (Lan *et al.*, 2005). Intriguingly, while *Arabidopsis ERD10* (group 2 *LEA*, At1g20450) was able to protect yeast against desiccation stress, its recombinant expression in *E.coli* caused deleterious growth (Campos *et al.*, 2006).

As previously mentioned there have been many parallels drawn between the advent of vegetative desiccation tolerance and seed desiccation tolerance. During the late stages of vegetative desiccation in *Xerophyta humilis*, 16 *LEA* genes are significantly up-regulated (Illing *et al.*, 2005). Many of these genes have seed specific homologues in *Arabidopsis*. In *Arabidopsis* these genes are expressed during the late maturation phase during the seeds acquisition of desiccation tolerance (Illing *et al.*, 2005). It is therefore conceivable that these *Xerophyta humilis* desiccation-induced vegetative *LEA* genes have evolved not in response to osmotic stress but as an analogous system such as the one found in developing seeds.

We suggest that removing *XhLEA* from this context and testing its function through salt induced osmotic stress in the isolation of a heterologous microbial systems may actually be insufficient to reveal this function. *XhLEA* may not be effective in the absence of other proteins/molecules which are involved in the desiccation tolerance machinery found in seeds and the vegetative tissues of many resurrection plants. Additionally, microbial heterologous expression systems may not be the best method to determine *LEA*s functions. In the following chapter we explored the ability of *XhLEA* to improve water deficit resistance when inducibly expressed in *Arabidopsis*.

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Chapter 3

Drought inducible expression of *XhLEA* in *Arabidopsis* and its effect on water deficit tolerance

3.1 Introduction

Late Embryogenesis Abundant (*LEA*) genes and their high expression levels in a variety of organisms and in organs undergoing desiccation (anhydrobiotic larvae, resurrection plants and seeds, (Farrant *&* Moore, 2011; Dure III *et al.*, 1981; Sharon *et al.*, 2009) or in tissues undergoing stress (salt, oxidative, temperature and desiccation, (Hundertmark *&* Hincha, 2008; Bies-Etheve *et al.*, 2008) has prompted researchers to use the LEA gene family to try and improve the stress-fitness of plants.

To this end wheat, rice, strawberry and tobacco have all been engineered to have either drought or freeze tolerance through the over-expression of a select group of *LEA* genes within their genetic backgrounds. Hara *et al.* (2003) used a cold induced citrus group 2 *LEA* (*CuCOR19*) to improve the electrolyte leakage of tobacco after freezing (-4°C, 3 h) which was constitutively expressed in the vegetative tissues indicating a membrane protective function. Houde *et al.* (2004) used a wheat group 2 *LEA* (*WCOR410*) gene to improve the freeze tolerance of strawberry plants. Interestingly in that study they tested cold acclimated transgenics as well us non cold acclimated transgenics against their respective WT controls and found that only the cold acclimated transgenics where able to improve freezing tolerance. Sivamani *et al.* (2000); Xu *et al.* (1996) both used the barely *HVA1* a group 3 *LEA* gene to promote the growth and recovery of maize and rice respectively when grown under water deficit conditions. To our knowledge no group 1 *LEA* has been used in vegetative tissues to genetically engineer any form of abiotic stress tolerance (freezing, salt or desiccation) *in planta*.

In *Arabidopsis, AtEM-6* (a homologue of *XhLEA*) was shown to mediate water loss in the seed during late maturation, indicating a role in water retention through its large hydration shell (Manfre *et al.*, 2006). The only other group 1 *LEA* gene in *Arabidopsis* is *AtEM-1*, and its expression, as with *AtEM-6*, is exclusive to the seeds

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(Manfre *et al.*, 2006).

In the following chapter we describe the production of *Arabidopsis* (*pMDC32::XhLEA/Col-0*) over expressing *XhLEA*, a group 1 *LEA* isolated from the desiccated vegetative tissues of *Xerophyta humilis*. We further present a drought trial of *Arabidopsis* (*RD29A::XhLEA/Col-0*) expressing *XhLEA* in its vegetative tissues under the control of a stress inducible promoter.

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3.2 Methods & Materials

3.2.1 *pMDC*$_{32}$*::XhLEA* construction

XhLEA::pCR8 was previously constructed through the insertion of *XhLEA* into the entry vector *pCR8* through a D-TOPO reaction. This entry vector (*pCR8::XhLEA*) was used in a LR recombinase reaction with *pMDC*$_{32}$ (Invitrogen, life technologies, South Africa) as per manufacturer's recommendations and transformed into ultra-competent *E.coli* Top10 . Correct *XhLEA::pMDC*$_{32}$ construction was confirmed for antibiotic (Kanamycin 50 µg/mL, kan^{50}) resistant colonies in a 50 µl PCR reaction containing 5 µl *XhLEA::pMDC*$_{32}$ plasmid mini-preparation and 1X GoTaq reaction buffer (Promega, Anatech, South Africa), 0.3 µM of each primer pMDC$_{32}$ Fwd (5'-AGAGGATCCCCGGGTACC - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCT-GTACTTGG -3'), 0.3 mM dNTP mix and 0.75 U GoTaq polymerase (Promega). Thermal cycling conditions: 95°C - 2 min (1 cycle) followed by 95°C - 50 sec, 58°C - 50 sec, 72°C - 1 min (30 cycles) with a final elongation of 72°C - 10 min.

3.2.2 *Agrobacterium tumefaciens* (Gv3101) electroshock transformation

Confirmed *pMDC*$_{32}$*::XhLEA* was used to transform *Agrobacterium tumefaciens* (GV3101) via electroshock. Electro-competent *Agrobacterium* aliquot (100 µl) was mixed with 1 µl (500 ng - 1 µg/ul) *pMDC*$_{32}$*::XhLEA* plasmid mini-preparation in a 2 mL cuvette. This was electroshocked (2.45 V, 2000 ohms, 25 µF) using a GenePulserX-cell (Bio Rad). Ice cold LB (1 mL) was added to the cuvette directly after the shock and then incubated (2 h, 28°C, with agitation). Aliquots (25 µl) of this culture was then plated onto antibiotic containing LBA (Gentamicin 25 µg/mL, gent^{25}, Rifampicin 50 µg/mL, rif^{50}, kan^{50}) and incubated (2 d, 28°C). Antibiotic resistant colonies were tested for correct insertion by colony PCR in the following 50 µl reaction containing colony spot (p10 pipette tip), 1X GoTaq reaction buffer (Promega), 0.3 µM of each primer pMDC$_{32}$ Fwd (5' - AGAGGATCCCCGGGTACC - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG - 3'), 0.3 mM dNTP mix and 0.75 U GoTaq polymerase (Promega). Thermal cycling conditions: 95°C - 4 min (1 cycle) followed by 95°C - 50 sec, 58°C - 50 sec, 72°C - 1 min (30 cycles) with a final elongation of 72°C - 10 min.

3.2.3 *pMDC*$_{32}$*::XhLEA/Col-0* floral dip

Confirmed colonies containing *XhLEA::pMDC*$_{32}$ were used to inoculate overnight starter cultures (kan^{50}, gent^{25}, rif^{50} ,5 mL, 28°C, 200 RPM). Starter cultures were used to inoculate 200 mL LB cultures (kan^{50}, gent^{25}, rif^{50}, 2 d, 28°C, 200 RPM). Cultures were pelleted (5500 x*g*, 10 min, RT) and re-suspended in a sucrose solution (5 % w/v) containing Silwet L-77 (0.05 % v/v) (Lehle Seeds, Texas, United States of America). *Arabidopsis* (*Col-0*) with developing floral buds were dipped twice (3 min) at 7 d intervals. Following each dipping event plants were dark incubated horizontally in an air tight container (RT, over night). Between dips the plants were returned to the controlled growth room. Following the second dip the plants were

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returned to the controlled growth room (16h:8h light period, 25°C:15°C) for seed maturation and collection.

3.2.4 Confirmation of *pMDC$_{32}$::XhLEA/Col-0* transformation and expression

Mature seeds collected from the *pMDC$_{32}$::XhLEA* dipped *Arabidopsis* (*Col-0*) plants were surface sterilised in a desiccation jar via gas sterilisation (100 mL bleach, 2 mL concentrated HCl (32%), 4 h). Sterilised seeds were sown on 1/2 MS supplemented with hygromycin (18 µg/mL). Inoculated plates were stratified (4 °C, overnight) before being placed in the controlled growth room (16h:8h light period) for germination and selection (7 - 10 d). Selection-resistant plants (T-1) were removed from tissue culture and hardened off in Jiffy peat pellets. Plants representing the T-1 generation were screened for *XhLEA* insertion and expression by genomic PCR and semi-quantitative (sq) RT-PCR respectively;

Genomic PCR: 50 µl PCR reaction containing 5 µl leaf gDNA extraction (Method adapted from Edwards et al., 1991) and 1X GoTaq reaction buffer (Promega), 0.3 µM of each primer pMDC$_{32}$ Fwd (5' - AGAGGATCCCCGGGTACC - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG -3'), 0.3 mM dNTP mix and 0.75 U GoTaq polymerase (Promega). Thermal cycling conditions: 95°C - 2 min (1 cycle) followed by 95°C - 50 sec, 58°C - 50 sec, 72°C - 1 min (30 cycles) with a final elongation of 72°C - 10 min.

sq RT-PCR: 50 µl PCR reaction containing 2.5 µl reverse transcribed RNA (extracted with RNeasy mini kit, Quigen, WhiteSci, as per manufactures instructions) and 1X GoTaq reaction buffer (Promega), 0.3 µM of each primer *XhLEA* Fwd (5'- ATGGCTTCCCATCAAGAAAGGG -3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG -3'), 0.3 mM dNTP mix and 0.75 U GoTaq polymerase (Promega). Actin 2 (*AtACT 2*) was used as in internal control *AtACT 2* Fwd (5'- ATGGCTGAGGCTGATGATAT -3') and *AtACT 2* Rev (5'- TTAGAAACATTTTCTGTGAACGAT -3'). Thermal cycling conditions: 95°C - 2 min (1 cycle) followed by 95°C - 50 sec, 58°C - 50 sec, 72°C - 1 min (25 cycles) with a final elongation of 72°C - 10 min

3.2.5 *Arabidopsis RD29A::XhLEA/Col-0* drought resistance trials

3.2.5.1 Plant growth conditions

Arabidopsis (*Col-0*) was transformed with *RD29A::XhLEA* (*XhLEA* driven by a drought inducible promoter) and seeded to T-3 generation. Drought trials were carried out on two independent lines of *RD29A::XhLEA* (*LEA 1-3* and *1-7*) and a WT *Col-0* control. Seeds representing the 3 genetic backgrounds were gas sterilised using chlorine gas in a desiccator jar (4 h). Sterile seeds were then spread on solid 1/2 MS plates containing BASTA (glyphosate 10 µg/mL), without BASTA for WT, and stratified (overnight, 4 °C). Selection resistant plantlets (15 per line) were selected

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one week later and planted out into saturated Jiffy discs. Plants were then grown in a controlled growth room until the desired size was reached (7-8 weeks).

3.2.5.2 Drought tolerance trial

The drought tolerance trial was conducted in the same growth room by withholding water and monitoring the plants and the soil moisture content of the Jiffy peat pellets as the trial progressed. Soil moisture was measured with a soil moisture probe (3 measurements per Jiffy pellet, GS3, Decagon devises, Washington, United States of America). Leaf samples were taken and snap frozen in liquid nitrogen at T=0 (beginning) T=4 (Middle) and T=8 (end) (days) for later molecular analysis. Relative leaf water content (RLWC) was determined by fresh leaf samples from each plant which were weighed (Fresh Weight, FW), soaked overnight in water, weighed again (Turgor Weight, TW), then dried overnight at 40 °C, then weighed again (Dry Weight, DW). RLWC was then calculated using the following formula:

$$RLWC = \frac{FW - DW}{TW - DW}$$

Genomic DNA PCR was performed on gDNA extracted from the tissue samples to confirm *XhLEA* insertion in the following 50 µl PCR reaction containing 5 µl gDNA extraction and 1X GoTaq reaction buffer (Promega), 0.3 µM of each primer $pMDC_{32}$ Fwd (5' - AGAGGATCCCCGGGTACC - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG - 3'), 0.3 mM dNTP mix and 0.75 U GoTaq polymerase (Promega). Thermal cycling conditions: 95 oC - 2 min (1 cycle) followed by 95 °C - 50 sec, 58 °C - 50 sec, 72 °C - 1 min (30 cycles) with a final elongation of 72 °C - 10 min.

Expression of *XhLEA* at each time point was determined via sq RT PCR in the following 50 µl PCR reaction containing 2.5 µl reverse transcribed RNA and 1X GoTaq reaction buffer (Promega), 0.3 µM of each primer *XhLEA* Fwd (5' - ATGGCTTCCCATCAAGAAAGGG - 3') and *XhLEA* Rev (5' - TTAAACATTGGTCCTGTACTTGG - 3'), 0.3 mM dNTP mix and 0.75 U GoTaq polymerase (Promega). Actin 2 (*AtACT 2*) was used as in internal control *AtACT 2* Fwd (5' - ATGGCTGAGGCTGATGATAT - 3') and *AtACT 2* Rev (5' - TTAGAAACATTTTCTGTGAACGAT - 3'). Thermal cycling conditions: 95 °C - 2 min (1 cycle) followed by 95 °C - 50 sec, 58 °C - 50 sec, 72 °C - 1 min (25 cycles) with a final elongation of 72 °C - 10 min.

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3.3 Results

3.3.1 Colony PCR on antibiotic resistant *Agrobacterium* colonies putatively containing *pMDC$_{32}$::XhLEA*

Following electro-shock transformation of *Agrobacterium* with *pMDC$_{32}$::XhLEA*, colony PCR was performed on the antibiotic resistant colonies. Figure 3.1 shows 5 colonies screened for the correct incorporation of *pMDC$_{32}$::XhLEA* using the *pMDC$_{32}$* fwd and *XhLEA* rev primer pair to amplify a 500 bp fragment. Colony #2 showed the correct amplification of a 500 bp band when compared to the positive control (Figure 3.1). Colonies #1, 3, 4 and 5 showed no amplification from the colonies and were therefore discarded. Colony #2 was used for all further work.

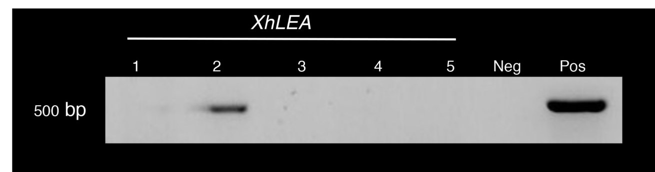

Figure 3.1: 1.2% agarose gel showing the amplification of a 500 bp amplicon with direction specific primers (*pMDC$_{32}$* Fwd and *XhLEA* Rev) after a *Agrobacterium* colony PCR was performed on antibiotic (gent^{25}, rif^{50}, kan^{50}) resistant colonies after *pMDC$_{32}$::XhLEA* transformation. Only colony 2 showed amplification (labelled 2) of the correct size when compared to the positive control

3.3.2 Confirmation of *XhLEA* insertion and expression in T-1 selection resistant *pMDC$_{32}$::XhLEA/Col-0*

Following the floral dip transformation, 2 selection-resistant *Arabidopsis* plantlets were recovered. These transgenics were screened for the correct *pMDC$_{32}$::XhLEA* insert via genomic PCR using the direction specific primer pair: *pMDC$_{32}$* Fwd and *XhLEA* Rev. Both plants showed amplification of the 500 bp fragment characteristic of *XhLEA* and this in turn corresponded to the positive control (Figure 3.2). contrary to this the WT (*Col-0*) control showed no amplification of any size fragment (Figure 3.2).

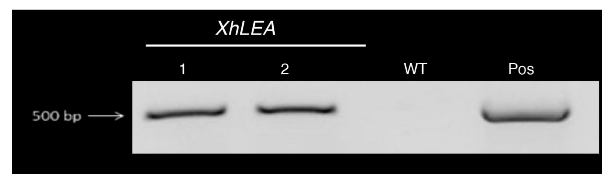

Figure 3.2: PCR on genomic DNA extractions from T-1 generation selection resistant (Hygromycin) *pMDC$_{32}$::XhLEA/Col-0*. Lines 1 and 2 showed amplification of a 500 bp amplicon with direction specific primers (*pMDC$_{32}$* Fwd and *XhLEA* Rev) which corresponded to the size of the positive control. Genomic DNA extracted from WT (*Col-0*) did not show any amplification

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Following insert confirmation, a PCR was performed on reverse transcribed RNA extracted from the 2 transgenics. The amplification of a 500 bp fragment was observed for both transgenics when using cds specific primers (*XhLEA* Fwd + Rev, Figure 3.3). The expression of the internal control gene Actin 2 (*AtACT 2*) was used as a positive control and showed amplification in both the transgenics (Figure 3.3).

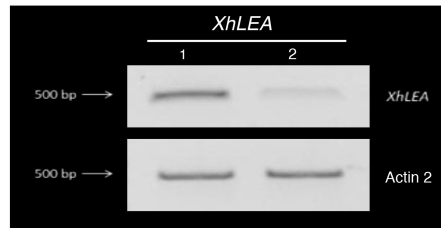

Figure 3.3: PCR on reverse transcribed RNA extractions from T-1 generation selection resistant (Hygromycin) *pMDC*$_{32}$*::XhLEA/Col-0*. Lines 1 and 2 showed amplification of a 500 bp amplicon with cds specific primers (*XhLEA* Fwd and Rev). Actin2 (*AtACT2*) was used as the internal positive control gene

3.3.3 *RD29A::XhLEA* drought tolerance trial

3.3.3.1 Confirmation of *RD29A::XhLEA* insertion

A PCR using cds specific primers (*XhLEA* Fwd + Rev) on genomic DNA extracted from *RD29A::XhLEA* lines *1-3* and *1-7* together with WT (*Col-0*) showed amplification of a 500 bp fragment for lines *1-3* and *1-7* as well as the positive control (Figure 3.4). Contrary to this, no amplification can be seen when using the WT genomic DNA as a template (Figure 3.4). The negative water control also shows no amplification of any sized band (Figure 3.4).

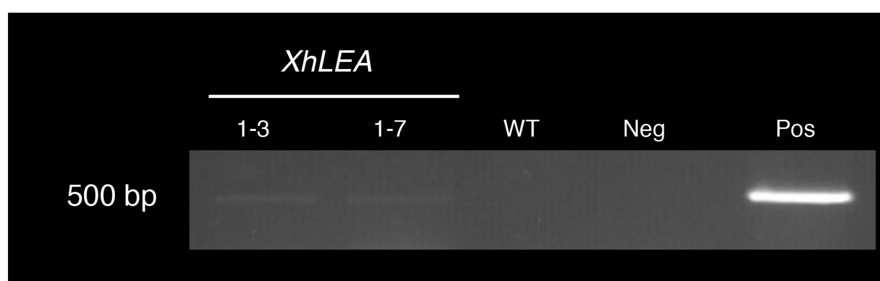

Figure 3.4: PCR on genomic DNA extractions from T-3 generation selection resistant (BASTA) *RD29A::XhLEA/Col-0*. Lines *1-3* and *1-7* showed amplification of a 500 bp amplicon with cds specific primers (*XhLEA* Fwd and *XhLEA* Rev) which corresponded to the size of the positive control. Genomic DNA extracted from WT *Col-0* did not show any amplification

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3.3.3.2 Drought responsive expression of *XhLEA* during the drought trial

PCR using cds specific primers (*XhLEA* Fwd + Rev) on reverse transcribed RNA extracted from *RD29A::XhLEA* lines *1-3* and *1-7* together with WT (*Col-0*) over time. Time points are representative of the start of the drought trial (T=0), Middle of the drought trial (T=4) and the end of the drought trial (T=8). Amplicons of 500 bp can be seen in the top panel for both lines *1-3* and *1-7* (Figure 3.5). The band intensities for both of these lines clearly increase from T=0 through to T=8. The band intensity is far greater in line *1-7* than in *1-3.* Contrary to this, no amplification can be seen of any size when WT reverse transcribed RNA extractions were used as a template (Figure 3.5). In the bottom panel Actin2 (*AtACT2*) was used as a internal positive control and one can see amplification for all 3 lines (*XhLEA 1-3* and *1-7* and WT) through all 3 time points (Figure 3.5).

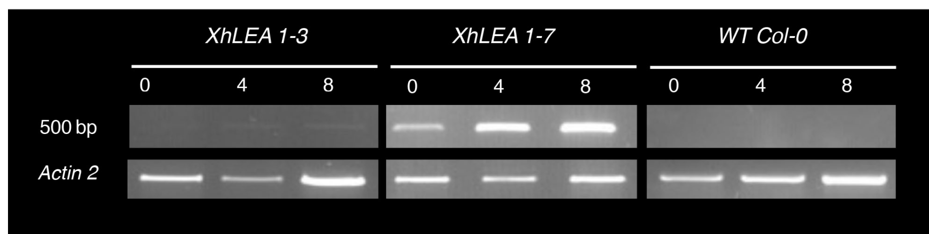

Figure 3.5: Semi-quantitative PCR on reverse transcribed mRNA extracted from lines *1-3* and *1-7* and the WT control at progressive time points (day 0, day 4 and day 8) representing start (no drought) middle (onset of drought) and end (wilting of leaves). Inducible expression can be seen for both lines *1-3* and *1-7*, however, line *1-3* shows significantly lower induction and over all expression when compared to line *1-7.* No expression was detected in the WT control. Actin 2 (*AtACT2*)was used as a constitutively expressed native control.

3.3.3.3 Drought trial soil moisture content and relative leaf water content

Figure 3.6 shows graph pairs (a & b), (c & d) and (e & f) that represent 3 independent drought tolerance trials conducted on T - 3 generation *RD29A::XhLEA/Col-0* plants in the same growth environment. Graphs a, c and e show the average water contained in the jiffy discs (plant growth substrate) over time during the drought trial while graphs b, d and f show the average water content of the leaves from the plants during the drought trials. One can see from the soil moisture readings that the rate of drying in all 3 drought trials differ slightly (Figure 3.6). In graph e one can see that the WT soil contained more water on average than the other 2 trays this difference was maintained through out that drought trial (Figure 3.6). While the time taken for the average soil moisture to reach 0 m^3/m^3 in a and c differ by 1 day indicating variable environmental conditions (Figure 3.6). Relative Leaf Water Content readings can also be seen to vary between the 3 drought trials with a much more pronounced loss of leaf water content in graphs b and f than in d. In the first 2 drought tolerance trials the soil moisture and the RLWC readings tracked together

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for both *XhLEA1-3* and *1-7* as well as the WT control. In the 3rd drought trial the initial soil moisture is higher in the WT control than the 2 transgenic lines this is in turn reflected in the RLWC graph which shows the WT plants retaining turgor for longer than the transgenic lines (Figure 3.6).

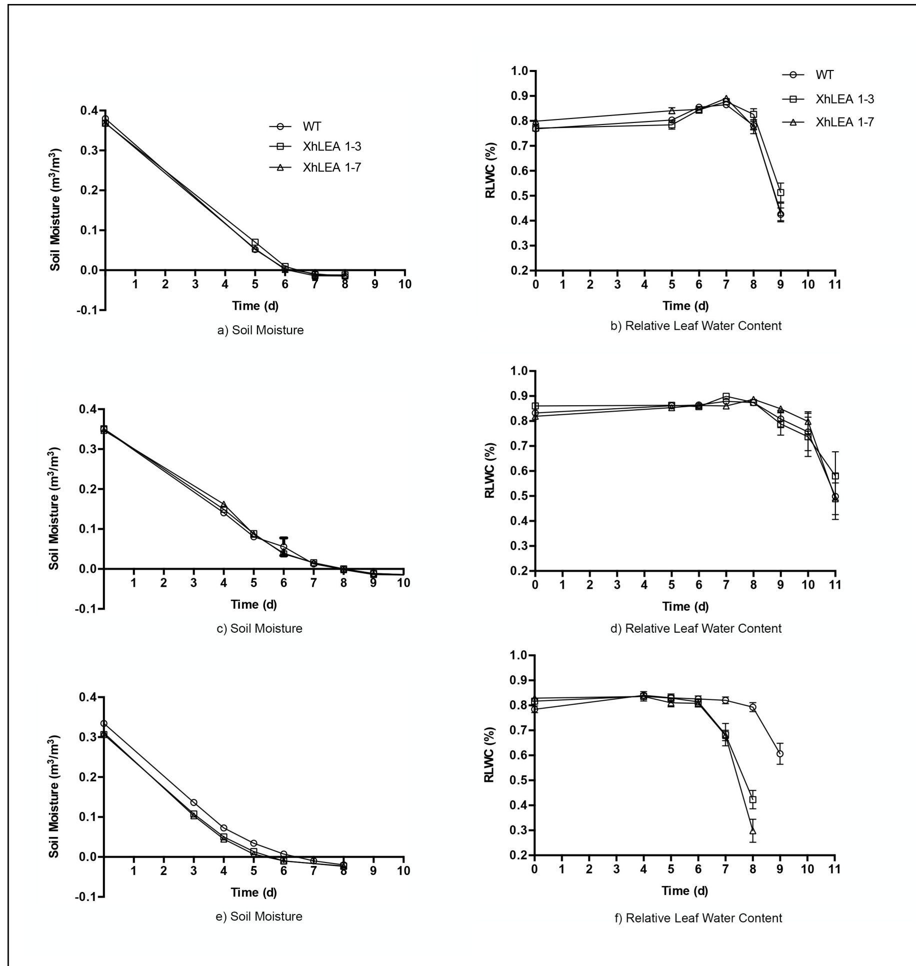

Figure 3.6: Graphs showing 3 independent drought experiments (a & b), (c & d) and (e & f). Graphs labelled a,c and e depict the average water loss of the soil within the drought experiments over time. Graphs b, d and f show the average relative leaf water content of the plants over time. n=12, error bars represent SE.

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3.4 Discussion

The *LEA* gene family were traditionally confined to seed specific expression but they have since been found in all plant tissues and a large number of bacteria, fungi and other higher order animals (Stacy & Aalen, 1998; Battista *et al.*, 2001; Gal *et al.*, 2004). Their expression in all organisms is almost exclusively linked to the onset of abiotic stress (Wise & Tunnacliffe, 2004). In desiccation-sensitive plants, the group 1 *LEA*s are the only group of *LEA* genes whose expression is exclusively seed specific (Hundertmark & Hincha, 2008).

This study focused on the inducible expression of *XhLEA*, a group 1 *LEA* isolated from the desiccated vegetative tissues of the resurrection plant *Xerophyta humilis*, to aid *Arabidopsis* vegetative tissues under drought conditions. *Arabidopsis* does not express any group 1 *LEA* genes in its vegetative tissues (Hundertmark & Hincha, 2008). The closest *Arabidopsis* homologues to *XhLEA* are the seed specific *AtEM-1* and *-6* (At3g51810 and At2g40170, respectively). Both genes are known group 1 *LEA*s and their expression patterns are strictly associated with late seed-maturation. Only *AtEM-6* has been functionally characterised (Manfre *et al.*, 2006). Using T-DNA insertion mutants, those studies were able to demonstrate a clear function in preventing water loss during seed maturation. It was proposed that *AtEM-6* fulfils the typical function of a *LEA* (*in-vivo*) where its large hydration shell allows it to physically bind water until the later stages of seed-maturation when this water is then lost.

Recently the over-expression of *AtEM-6* was shown to provide osmotic (salt stress, 150 - 300 mM NaCl) tolerance to cell cultures of rice, cotton and white pine (Tang & Page, 2013). In that study they found that in the rice cell line the over-expression of *AtEM-6* cause the increased expression of several Ca^{2+} dependent protein kinases which have been shown to improve the abiotic stress response (Sheen, 1996; Song & Matsuoka, 2009). It was thus of interest to use *XhLEA*, a group 1 *LEA*, expressed in the vegetative tissues of *Arabidopsis* to determine if its expression would aid its abiotic stress response specifically its response to water limiting conditions. Inducible vegetative expression of *XhLEA* was confirmed (figure 3.5), however, no consistent results were obtained in terms of the drought resistance-responses of the two independent transgenic lines (figure 3.6).

The evolution of desiccation tolerance in the vegetative tissues of a small group of plants known as resurrection plants has been postulated to be linked to the gene set expressed in orthodox developing seeds, late in the maturation phase when orthodox seeds loose water and gain desiccation tolerance. The loss of seed specific expression of a number of genes has been observed in the desiccated tissues of resurrection plants (Illing *et al.*, 2005; Mulako *et al.*, 2008). In those studies it was found that apart from several *LEA*s that are highly expressed in the desiccated vegetative tissues. also identified *XhPer1* (a 1 - cys - peroxiredoxin anti oxidant) and *Xhdsi-1^{VOC}* (a desiccation induced vicinal oxygenase chelate, (Illing *et al.*, 2005; Mulako *et al.*, 2008). Both of these genes share *Arabidopsis* homologues that are strictly expressed in seeds.

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Taking all this evidence together with our results we propose that the transgenic expression of *XhLEA* alone, a gene involved in the desiccation resistance pathway in the vegetative tissues of a resurrection plant, within a desiccation-sensitive system will not benefit the drought response. It is clear from the above studies that there are numerous factors that are not present in the vegetative tissues of *Arabidopsis* that are present in the desiccated vegetative tissues of resurrection plants. These factors are increasingly being linked to the seed-specific gene set involved late in orthodox seed maturation (Illing *et al.*, 2005; Mulako *et al.*, 2008). It may be possible that additional key seed specific factors that are not present in our *Arabidopsis* vegetative system are required for *XhLEA* to aid in water stress resistance.

As our knowledge of resurrection plant desiccation tolerance grows it would be interesting to see a full expression analysis of the desiccated tissues of *Xerophyta humilis* and compare that to the expression profiles in *Arabidopsis* seeds. This would give us a better understanding of the link between orthodox seed maturation and the mechanisms behind vegetative desiccation tolerance. Once a better overview of the genes involved has been elucidated it would be interesting to create transgenics to elucidate the simplest combination of genes that elicit a water deficit response in the vegetative tissues of *Arabidopsis* to aid water stress response. Additionally it would be interesting to test the current *XhLEA* transgenics for their ability to survive salt stress when *XhLEA* is expressed in the vegetative tissues to determine if *XhLEA* is able to protect vegetative tissues like *AtEM-6* can protects cell cultures either directly or by up regulating Ca^{2+} dependent protein kinases. It would be worthwhile to correspond with the authors who over-expressed *AtEM-6* in cell cultures to determine if they tested other abiotic stress conditions, specifically desiccation stress. This would give us insight into the possible stress protective functions of group 1 *LEA*s.

Wu *et al.* (2014) noted that the level of over expression of a stress induced *LEA* (LEA14) in *S. miltiorrhiza* was related to the salt and desiccation resistance afforded by that gene. With this in mind we have constructed *Arabidopsis* that over expresses *XhLEA* (figures 3.2 and 3.3) and the testing of these transgenics is ongoing. However, the *LEA* used was natively stress inducible therefore these findings would make sense and the over expression of *XhLEA* may still not provide water limiting stress protection for the reasons outlined above. Additionally, as can be seen from figure 3.5, the expression levels in the inducible system were very high for line *XhLEA 1-7* and transcripts were present even before there was any significant drought stress. This was due to the sub-optimal experimental conditions of this experiment.

The growth room in which these drought experiments were conducted suffered from severe fluctuations in humidity and temperature. These fluctuations had a twofold effect. Firstly they caused the stress inducible *RD29A* promoter to be active before there was any drought stress placed on the plants (Seki *et al.*, 2002), and secondly it influenced the rate at which the trays containing the test plants dried out (soil moisture graphs Figure 3.6). Additionally, despite the even distribution of plants within a growth tray, the time between the first and last wilting event (>65

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% RLWC) within each tray was unsatisfactory, often taking 3-4 days. Whether this was an artefact of the variable growth room conditions or an experimental design issue can be debated, however, it would be prudent to conduct these experiments in the highly controlled conditions of a growth cabinet which we have recently acquired. If the variability between first and last wilting events remains in the growth cabinet it may be worthwhile to remove the soil moisture variable all together by having a large number of larger pots with the 2 *XhLEA* lines and the WT control planted in the same pot. This setup would provide the same immediate environment for all the plants in the pot and each pot would act as an independent drought experiment.

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Chapter 4

General conclusion

The aim for this thesis was to use *XhLEA* a group 1 *LEA* isolated from the desiccated leaves of the resurrection plant *Xerophyta humilis* to aid in the osmotic (water deficit and NaCl) stress response in *Arabidopsis* and *E. coli* respectively when recombinantly expressed within these systems.

Homologues of *XhLEA* include the seed specific group 1 *LEA*s in *Arabidopsis, AtEM-1* and *-6*, *AtEM-6* was shown through T-DNA insertion mutants to be crucial in the mediation of water loss in the maturing seed (Manfre *et al.*, 2006). It was proposed that this function was due to *AtEM-6* ability to physically bind water through its large hydration shell. *AtEM-6* was further shown to provide protection to salt stressed plant cell cultures by up regulating the transcription of various Ca^{2+} dependent protein kinases which are known to aid in the abiotic stress response (Tang *&* Page, 2013). However, in the experiments described in this thesis we could not show definitively that *XhLEA* aids in the osmotic stress response in either *Arabidopsis* or *E. coli*.

Vegetative desiccation tolerance may have evolved through two, possibly not mutually exclusive, ways: (i) Due to an adaption of the abiotic stress response or (ii) due to the deregulation of the seed-specific gene set responsible for the developmental maturation of the orthodox seed (Illing *et al.*, 2005). Evidence for the second mechanism has been found by looking at the genes expressed in the desiccated vegetative tissues of *Xerophyta humilis*. Many of these expressed genes have homologues in *Arabidopsis* which have strict seed specific expression (Illing *et al.*, 2005; Mulako *et al.*, 2008). Therefore, if vegetative desiccation tolerance in resurrection plants is due to genes that are part of a developmental pathway which has lost its seed-specific expression profile, we propose that removing one gene involved in that pathway, *XhLEA*, is an inadequate method for improving osmotic stress tolerance. Genes involved in developmental pathways often are reliant on proteins or molecules produced by other members of that pathway to perform their function and in isolation are non-functioning. However, we have also highlighted problems within our research environment with respect to fluctuations in controlled growth conditions which led to clear variability and consistency in independent experiments.

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We showed that the expression of *XhLEA* is not detrimental to the normal growth of either *E. coli* or *Arabidopsis.* This may be a further indication that the protein was non-functional with out vital proteins or molecules produced by other genes in the pathway. *AtEM-6*s ability to protect cell cultures is further indication that the isolation of *XhLEA* in either bacterial cells or vegetative tissues does not allow it to interact with seed specific genes inhibiting its function, however, in cell cultures this gene interaction may be possible with in this single cell system possibly allowing *AtEM-6* access to other seed-specific gene sets to exhibit its protective function.

In conclusion, *XhLEA* was unable to provide the osmotic stress tolerance to *E. coli* and *Arabidopsis* due to it being investigated in an isolated system away from the regulated developmental pathway from which it was taken. We propose that the lack of protein products produced by other members of the desiccation tolerance pathway rendered *XhLEA* ineffective in protecting *E. coli* or *Arabidopsis* from osmotic stress (NaCl and drought respectively).

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