2017

Expression, Purification, and Characterization of an Intrinsically Disordered Late Embryogenesis Abundant (LEA) Protein from Artemia franciscana Utilizing Escherichia coli and Nicotiana tabacum

Md Fazlul Karim
Eastern Illinois University

The Graduate School
EASTERN ILLINOIS UNIVERSITY

Thesis Maintenance and Reproduction Certificate

FOR: Graduate Candidates Completing Theses in Partial Fulfillment of the Degree
Graduate Faculty Advisors Directing the Theses

RE: Preservation, Reproduction, and Distribution of Thesis Research

---

Preserving, reproducing, and distributing thesis research is an important part of Booth Library's responsibility to provide access to scholarship. In order to further this goal, Booth Library makes all graduate theses completed as part of a degree program at Eastern Illinois University available for personal study, research, and other not-for-profit educational purposes. Under 17 U.S.C. § 108, the library may reproduce and distribute a copy without infringing on copyright; however, professional courtesy dictates that permission be requested from the author before doing so.

Your signatures affirm the following:

- The graduate candidate is the author of this thesis.
- The graduate candidate retains the copyright and intellectual property rights associated with the original research, creative activity, and intellectual or artistic content of the thesis.
- The graduate candidate certifies her/his compliance with federal copyright law (Title 17 of the U. S. Code) and her/his right to authorize reproduction and distribution of all copyrighted materials included in this thesis.
- The graduate candidate in consultation with the faculty advisor grants Booth Library the non-exclusive, perpetual right to make copies of the thesis freely and publicly available without restriction, by means of any current or successive technology, including by not limited to photocopying, microfilm, digitization, or internet.
- The graduate candidate acknowledges that by depositing her/his thesis with Booth Library, her/his work is available for viewing by the public and may be borrowed through the library's circulation and interlibrary loan departments, or accessed electronically.
- The graduate candidate waives the confidentiality provisions of the Family Educational Rights and Privacy Act (FERPA) (20 U. S. C. § 1232g; 34 CFR Part 99) with respect to the contents of the thesis and with respect to information concerning authorship of the thesis, including name and status as a student at Eastern Illinois University.

I have conferred with my graduate faculty advisor. My signature below indicates that I have read and agree with the above statements, and hereby give my permission to allow Booth Library to reproduce and distribute my thesis. My adviser's signature indicates concurrence to reproduce and distribute the thesis.

Graduate Candidate Signature	Faculty Adviser Signature
Printed Name	Printed Name
Biological Sciences	7/21/17
Graduate Degree Program	Date

Please submit in duplicate.

Expression, Purification, and Characterization of an intrinsically disordered Late Embryogenesis Abundant (LEA) protein from Artemia franciscana utilizing Escherichia coli and Nicotiana tabacum

(TITLE)

BY

MD FAZLUL KARIM

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science in Biological Sciences

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

2017

YEAR

I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

THESIS COMMITTEE CHAIR	7/20/17 DATE	DEPARTMENT/SCHOOL CHAIR OR CHAIR'S DESIGNEE	7/20/17 DATE
THESIS COMMITTEE MEMBER	7/20/17 DATE	THESIS COMMITTEE MEMBER	DATE
THESIS COMMITTEE MEMBER	7/20/17 DATE	THESIS COMMITTEE MEMBER	DATE

Expression, Purification, and Characterization of an intrinsically disordered Late Embryogenesis Abundant (LEA) protein from *Artemia franciscana* utilizing *Escherichia coli* and *Nicotiana tabacum*

A Dissertation

submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

The Department of Biological Sciences

at

Eastern Illinois University

By

Md Fazlul Karim

Charleston, Illinois

July 2017

ABSTRACT

Anhydrobiosis is an astounding strategy that allows certain species (both animals and plants) to survive severe environmental conditions such as desiccation, extreme cold, or heat in the habitat. Despite the occurrence of several different molecular strategies, expression of highly hydrophilic polypeptides termed LEA proteins has been most conclusive identified as a requirement for the survival of plants and animals during periods of water stress such as freezing and drying. Several classification schemes for LEA proteins have been proposed and the brine shrimp, *Artemia franciscana,* is the only known animal that naturally expresses LEA proteins from three different classification groups (groups 1, 3, and 6). LEA proteins occur in different subcellular compartments including the cytosol and mitochondria. To understand the biochemical properties of LEA proteins, it is important to characterize their structure. LEA proteins are intrinsically disordered in aqueous solution and the exact structure and function of these proteins in the dry and/or hydrated states is still poorly defined and understood. We found, that a purified group 1 LEA protein from *A. franciscana* (*Afr*LEA 1.1) helped to retain enzyme activity after desiccation of *lactate dehydrogenase (*LDH) for land 7 days in the presence or absence of BSA or trehalose or other purified LEA protein. Increased concentration of purified *Afr*LEA 1.1, increased the percentage of LDH activity retained after desiccation. To further characterize *Afr*LEA 1.1, we cloned, expressed, and purified the protein in *E. coli*. We purified untagged *Afr*LEA 1.1 protein by affinity chromatography *via* Intein Mediated Purification with an Affinity Chitin-binding Tag system; a novel protein purification system which utilizes the inducible self-cleavage activity of protein splicing elements

(termed inteins) to separate the target protein from the affinity tag. Furthermore, *AfrLEA1.1* was expressed in *Nicotiana tabacum* to investigate if the protein increases drought tolerance of this model plant. Tobacco plants with confirmed transgenic *AfrLEA1.1* were subjected to water stress in the presence of polyethylene glycol (PEG; 10,000 MW) at increasing percentages to investigate the impact of osmotic stress on plant survival. PEG-stressed transgenic LEA plants showed significantly faster growth of roots compared to non-transgenic GUS control plants under the same conditions both if measured as an increase in fresh weight ($P=0.033$, $P<0.05$) or dry weight ($P=0.028$, $P<0.05$). This result clearly indicates a better capability to cope with water stress in presence of *AfrLEA1.1* and points to a function of this protein not only during desiccation but also under less severe osmotic stress conditions. Transgenic LEA plants also showed a significantly increased level of total growth compared to controls, measured as an increase in total fresh weight ($P=0.0461$, $P<0.05$) and total dry weight ($P=0.0342$, $P<0.05$) under standard growth conditions. Along with the better growth of roots under osmotic stress condition and better overall somatic growth under control condition, they also showed a significantly higher amount of chlorophyll content after freezing condition compares to room temperature.

Dedicated to my Parents

ACKNOWLEDGEMENTS

I am sincerely thankful to all individuals who helped me in completing this project while working on my degree. It would not have been possible without their constant support and encouragement. It is my pleasure to acknowledge them for their motivation, guidance and numerous suggestions.

I would like to express the highest gratitude to my research adviser Dr. Gary A. Bulla and committee members Dr. Michael A Menze, Dr. Yordan S Yordanov and Dr. Gopal R Periyannan for their immense guidance and support.

I express my deep sense of appreciations and gratefulness to Dr. Yordan S Yordanov, for his keen interest and above all, his overwhelming attitude in helping his students. He has been largely responsible for my completing of this work. His timely assistance, meticulous scrutiny, scholarly advice and scientific approach have helped me to accomplish this work in an interesting and expanding field.

Finally, I would like to extend my appreciation to the entire EIU Biological Science faculty for challenging me to grow in the classroom and providing a wonderful experience at EIU. The assistance, cooperation, and experience of my fellow lab members were essential for completion of this project. Of course, family and friends who were there to give me the extra push necessary to succeed. Last, but not least, I am thankful to the Department of Biological Sciences and Eastern Illinois University for this opportunity to be trained and to be an alumnus.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

OVERVIEW

INTRODUCTION

LEA proteins are known as "late embryogenesis abundant" because they are most abundant at the late embryogenesis state than at any other developmental state of plant seeds (Galau et al. 1986). LEA proteins were first discovered in mature wheat (*Triticum aestivum*) and cotton (*Gossypium hirsutum*) over 35 years ago (CUMING & LANE 1979; Dure et al. 1981), subsequently they were found in many other plant seeds (Olvera-Carrillo et al. 2011; Tunnacliffe & Wise 2007; Shih et al. 2008; Hoekstra et al. 2017; Shewry & Casey 1999) as well as in vegetative organs (J. Ingram & Bartels 1996). Some of these LEA proteins might protect other proteins by preventing aggregation because of osmotic stresses or desiccation which is mostly occurred due to low temperatures as well as high temperatures (Thomashow 1999). Although these proteins are abundant in seeds and pollens of plants, they are also found in a variety of organisms like bacteria (*Deinococcus radiodurans*) (Leon Dure 2001; Stacy & Aalen 1998; Battista et al. 2001), cyanobacteria (Close & Lammers 1993), slime mold (Eichinger et al. 2005), fungi (Mtwisha et al. 1998; Katinka et al. 2001; Abba' et al. 2006), nematodes (Gal et al. 2004; Tyson et al. 2007; Solomon et al. 2000; Haegeman et al. 2009; Browne et al. 2004; Browne et al. 2002), brine shrimp (*Artemia*) (Hand et al. 2007; Chen et al. 2009; Menze et al. 2009; Marunde et al. 2013; Warner et al. 2010; Warner et al. 2012; Sharon et al. 2009), bdelloid rotifers (Pouchkina-Stantcheva et al. 2007; Tunnacliffe et al. 2005), *Bacillus subtilis* (Stacy & Aalen 1998), and a chironomid insect larvae *(Polypedilum vanderplanki)* (Kikawada et al. 2006a). They

are most prevalent in those organisms to protect against different environmental stress conditions like cold, drought and high salinity (Battaglia et al. 2008).

Late Embryogenesis Abundant own their name due to the late accumulation in the maturation process of plant seeds (Galau et al. 1986). However, besides seed maturation, in many plants accumulation of LEA proteins can take place in response to abscisic acid (ABA) and water stress (Bartels 2005; Cuming 1999). From the studies of Tunnacliffe & Wise (2007) and Hand et al. (2011), it has been shown that LEA proteins in other organisms than plants show function in desiccation tolerance along with other chemical compounds, especially no-reducing sugars such as trehalose (Hand et al. 2011; Tunnacliffe & Wise 2007). The proposed function of LEA proteins regarding desiccation tolerance also supported directly by the study of Gal et al. (2004); Battista et al. (2001). In their study, they found that nematode and bacteria conferred reduced LEA protein expression eventually resulting decrease tolerance in water stress.

However, the exact molecular functions of LEA proteins are still unclear and LEA proteins have been suggested to act as protein and membrane protectants, cell membrane stabilizers, hydration buffers, antioxidants, organic glass formers and ion chelators (Tunnacliffe & Wise 2007). LEA proteins are suggested to perform specific functions like stabilization of sugar glasses (encouraged by sugar like trehalose) (Wolkers et al. 2001; Shimizu et al. 2010; Hoekstra 2005), This glassy state is very important for certain animals to protect themselves from severe cold or drought. However, this glass seems to shield proteins from damage (Zhang et al. 1998). LEA proteins also participated in the formation of structural networks (Wise & Tunnacliffe 2004) in accordance with protein stabilization via protein-

protein interaction (molecular shield) (Johnson et al. 2010; Chakrabortee et al. 2012) and membrane stabilization (Tolleter et al. 2010; Tunnacliffe & Wise 2007).

Most LEA proteins are highly hydrophilic and lack or are underrepresented in the number of some specific amino acids (Cys and Trp residues) while others are more abundant compared to other globular proteins and are overrepresented (Gly, Ala, Glu, Lys, Arg and Thr residues). The composition of amino acid residues in most LEA proteins led them to be considered as members of the large hydrophilin group (DURE III 1993; Dure 1994; Garay-Arroyo et al. 2000). Further research utilizing bioinformatics tools to understand LEA structure in solution indicated random coil in solution for most LEA proteins. However, some specific LEAs do form defined secondary structures, but these are considered exceptions from the majority of LEAs that remain mostly random coil in solution. Most LEA protein, therefore, belongs to the larger family of intrinsically disordered proteins (IDPs). (Mouillon et al. 2006; Tompa 2005; Shih et al. 2004; Goyal et al. 2003; Ismail et al. 1999; Close 1996; McCubbin, Kay & Lane 1985; Dure et al. 1989; DURE III 1993; Kovacs et al. 2008; Dure 1993).

LEA proteins are abundant in the plant kingdom, though recent studies confirmed their presence in animals too (Hand et al. 2011). Most of the proteins and their corresponding mRNAs are found in a high concentration in tissues at the late embryonic stage of seed development. Nevertheless, some transcripts are also found in different vegetative tissues treated with environmental stressors such as cold stress, osmotic stress, dehydration, and desiccation (Thomashow 1998; Baker et al. 1988; Hughes & Galau 1989; Dure et al. 1989;

Robertson & Chandler 1994; J. Ingram & Bartels 1996; Oliveira et al. 2007; Bies-Ethève et al. 2008; Hundertmark & Hincha 2008).

The classification of LEA proteins is based on a computational analysis which compares similarities based on the amino acid sequences of proteins. This type of analysis has some limitations and may underscore the physiochemical properties of the amino acid composition, and might overlook short but conserved amino acid motifs, which may be important for specific functions (Wise 2002; Wise & Tunnacliffe 2004). The first classification of LEA proteins was introduced by Dure et al. (1989) and categories LEA proteins into six families based on their amino acid sequences and compositions (Galau & Hughes 1987; Baker et al. 1988; Dure et al. 1989; DURE III 1993; Colmenero-Flores et al. 1997; Cuming 1999).

Considering previous classifications, Battaglia et al. (2008) grouped LEA proteins into seven (7) different groups or families and named them group 1-7 correspondingly, based on their hydrophobic characteristics and consensus amino acid sequences **(Table A and B)**. According to this grouping scheme a given LEA protein falls within group 1 (D-19, PFAM LEA_5), group 2 (D-11, PFAM Dehydrin) and group 3 (3A & 3B) (D-7, PFAM LEA_4 & D-29, PFAM LEA_4), group 4 (4A & 4B) (PFAM LEA_1 & D-113, PFAM LEA_1), group 5 (5A & 5B $ 5C) (D-34, PFAM SMP & D-73, PFAM LEA_3 $ D-95, PFAM LEA_2) (DURE III 1993; Cuming 1999), group 6 (PFAM LEA_6) (Colmenero-Flores et al. 1997) or group 7 (PFAM ABA_WDS) (Silhavy et al. 1995; Rossi et al. 1996). (ABA; Abscisic Acid).

Table A: Classification and nomenclature of LEA proteins with the corresponding PFAM number; adapted from Battaglia et al. (2008).

Group	Dure	PFAM	PFAM No.	Example
1	D-19	LEA_5	PF00477	Em1, Em6
2	D-11	Dehydrin	PF00257	Dehydrin, RAB
3A	D-7	LEA_4	PF02987	ECP63, PAP240, PM27
3B	D-29	LEA_4	PF02987	D-29
4A	–	LEA_1	PF03760	LE25_LYCES
4B	D-113	LEA_1	PF03760	PAP260, PAP051
5A	D-34	SMP	PF04927	PAP140
5B	D-73	LEA_3	PF03242	AtD121, Sag21, lea5
5C	D-95	LEA_2	PF03168	LEA14
6	–	LEA_6	PF10714	LEA18
7	–	ABA_WDS	PF02496	ASR

Table B: Consensus amino acid sequences of the different motifs characteristic of each LEA protein group; adapted from Battaglia et al. (2008)

GROUP	MOTIF	CONSENSUS SEQUENCE
LEA 1 (D-19)	1	T V V P G G T G G K S L E A Q E H/N L A E
PF00477	2	T R K E Q L/M G T/E E G Y Q/K E M G R K G G L/E
	3	D K/E S G G E R A A E/R E G I E/D I D E S K F/Y
LEA 2 (D-11)	K	E/K/R K K G I/L/M/F M/L/K D/E K I K E/D K L/I P G
PF00257	S	L/H H R S G S/H W/S S S S S D/E D/E D/E
	Y	R/Q T/V D E Y G N P V/I H/Q
LEA 3 (D-7)	1	G G/S V/F L Q/S Q T/A G E Q/K V
PF02987	2	A A/K/T D/E A V K H/N T L/F G M
	3[a]	T A Q A/S A K D/E K T S/Q E/Q
	5[a]	A/T T/A E/Q A A/T K Q/E K A S/A E
	4	S Y K/R A G E T K G/A R/K/Q K T/A
LEA 3 (D-29)	1[a]	T A/A E K A G E Y K D Y
PF02987	4[a]	T V/A/M E/Q K A/T K/R E A/T K D T/A
	2[a]	A Y E/D K A G S A K D M/A
	3[a]	A A Q K A/S/T K D/E Y A G D/D/S
	5	E S W T/A E/G W A K E/D K I
LEA 4 (D-113)	1	A/V Q/E E K A/V E K/R A/M T A/T R/H D/T P/K x E/Q K E M/I/L A H/T E/Q K/R K E/K A/E K/R
PF03760	2	M Q/E S/A/T A K E K/T A/V S/A/K N/D M/V A/G A/S S/A A K A/E/S G/K M/V E/D K/I T K A K/T
	3	E/Q A E/K M D/E K/L H/Q Q/A/E A/E K/R A/E H H/N A/R A/E E K/A/R Q/E
	4	P/G/H T/M G T/A H Q M/T S A L/M P G H G T G Q P T/A G H V V/T E/D G
LEA 6 (LEA-18)	1	L E D Y K M/R Q/K/R G/A Y G T/A Q/E G H Q/L Q/E/P P/V K/S P/D G/V R/P G/H
PF10714	2	G/S S/G/A T D/E A P T L/I S/P G G/A/T A V/L
	3	T D A/T I N R H G/Q V P
	4	G/Q L P T/V E/D/S T/D S/P P Y V/T
LEA 7 (ASR)	1	A A G A Y/F A L H E K/E H K A K K D P E H A H R H K I
PF02496	2	E I/V A A A/V A A A V/A G A/S G G F/Y A/V F/Y H E H H E/Q K K E/D A/D K/H
	3	D/K Y K/R K E E K H H K H M/L E/Q H/Q L/I G E L/M G A/T V/M
	4	H/W H H H H L/F F/R H H H K/Q D/Q
	5	E/K E E/D E/Q E A/H H/E G K K H H L/F F

The color in letters indicates the type of amino acid. Non-polar: violet = aliphatic (A, V, G, M, L, I, P); grey = aromatic (Y, W, F); Polar: green = uncharged (S, Q, N, T); blue = positively charged (R, K, H); red = negatively charged (D, E).

[a] 11-mers as described by Dure (2001).

Numerous algorithmic analysis of LEA proteins predicting that most of the LEA proteins should adopt defined secondary structures like α-helix and β-sheet in the solution (Chou & Fasman 1978; Rost & Liu 2003; Rost & Sander 1993). The study of Dure et al. (1989) has predicted that most of the LEA proteins primarily assumed with a α-helical structure but in aqueous solution several LEA proteins mostly discovered unstructured and failure to crystallize (McCubbin, Kay & Lane 1985). Those studies and findings of LEA proteins attributed these proteins as an intrinsically disordered and has a high degree of hydration (Hincha & Thalhammer 2012; Hand et al. 2010; Tunnacliffe & Wise 2007; Wise & Tunnacliffe 2004). At the same time, the studies of Dure (1993) and Imai et al. (1996) have predicted that the 20-mer motif of group 1, the K-segment of group 2, the 11-mer repeat of group 3 and additional hydrophilic domain in several LEA proteins form amphiphilic α-helical structures in a solution.

Due to the importance of LEA proteins in desiccation tolerance, many experiments were performed to determine the structures of LEA proteins under different conditions especially in the hydrated versus desiccated state. Secondary structures of different LEA proteins were deduced by using techniques like circular dichroism (CD), nuclear magnetic resonance (NMR), or Fourier transform IR (FTIR) spectroscopy in presence of different solvents and solutes like sodium dodecyl sulfate (SDS) in water, trifluoroethanol (TFE), glycerol, etc (Gilles et al. 2007; Russouw et al. 1997; Russouw et al. 1995; Eom et al. 1996; Gokce et al. 2005; Soulages et al. 2002; Boudet et al. 2006). These studies demonstrated that some LEA proteins which lacked well-defined secondary structures in pure water formed α-helical structures in presence of SDS (Ismail et al. 1999).

A group of LEA protein expressed in *Typha latifolia* pollen was found to remain completely disordered in the hydrated state but gained structure upon desiccation (Wolkers et al. 2001). Another LEA protein from *Aphelenchus avenue* (*Aav*LEA1) (Browne et al. 2002) was also found to be disordered in the hydrated state using CD and FTIR spectroscopy and the study suggested the existence of alpha-helices at low temperature (Goyal et al. 2003). On the other hand, the mitochondrial LEA protein from pea seed (PsLEAm) is mostly a disordered protein but showed α-helices in the presence of SDS and TFE (Tolleter et al. 2007; Grelet 2005). The study of Shih et al. (2004) also showed that the soybean GmPM16 protein has a high degree of disordered in solution but again adopted α-helical structures upon addition of SDS or TFE or at low temperatures (Shih et al. 2004). Almost all the LEA proteins are hydrophilic, but group 5 is unique and is more hydrophobic and heat unstable compared to the other groups. This is the only group of LEA proteins for which three-dimensional structure data exist (Singh et al. 2005).

There is a common dogma that functional protein must have a defined three-dimensional structure, but in the 80s, several studies showed that proteins do not necessarily lack function due to the absence of the defined secondary structure motifs. Proteins lacking defined secondary and tertiary structure motifs are termed "natively unfolded proteins" or "intrinsically disordered protein" (Dunker et al. 2001; Schweers et al. 1994; Uversky et al. 2000). Numerous studies demonstrated that in *Arabidopsis* and *Drosophila* genome, around 29-41% of functional proteins are partially and 8-17% are fully disordered respectively (Dunker et al. 2001). It is not always necessary for functional proteins to be folded. After comparing ordered and disordered proteins Dunker and colleagues found

that proteins with disordered regions have some common sequence features that are different from structured proteins. Those disordered proteins display some special features like low sequence complexity, biases in amino-acid composition, noncompact, extended sequence, and high flexibility. They are rich in hydrophilic amino acids (ex: Glu, Lys, Gly, Gln, Ser, Pro, and Ala) and depleted in specific amino acids (ex: Ile, Leu, Val, Trp, Phe, Try, Cys and Asn) residues (Uversky 2002; Dunker et al. 2001).

There are significant differences between folded proteins and unfolded LEA proteins. Folded proteins generally contain a hydrophobic core and are surrounded by polar and hydrophilic side chains which interact with water or other molecules. Most LEA proteins do not have a hydrophobic core and the entire polypeptide chain and eventually participate in water-protein interactions. That is another reason, they fail to obtain/or obtain any particular structure, as a result, most of the LEA proteins found as an unfolded state in a solution.The study of Goyal et al. and McCubbin et al. stated that due to having that water loving properties the molecular weight of wheat LEA1 and nematode *Aav*LEA1 proteins has significantly higher compared to the same size folded proteins. This is another indication of high water association. The *Aav*LEA1 protein, for example, displayed a 20-fold increased association with water compared to a similar globular protein (Goyal et al. 2003; McCubbin, Kay, Lane, et al. 1985). Intrinsically disordered LEA proteins might interact with other molecules instead of water and can assume different conformations due to this interaction. They may interact with other proteins, nucleic acids, or sugar molecules. A common feature of many LEA proteins is that conformational changes are observed during water removal (Goyal et al. 2003). This is because that LEA protein when losing

interaction with water is forced to undergo inter- or intramolecular interaction, which may eventually lead to a defined conformation in the dry state (Prestrelski et al. 1993). The biochemical feature of LEA proteins had been changed under dehydration. They have a tendency to interact with oligosaccharide glasses, eventually, increases of hydrogen bonding and turned into the glassy matrix (Shih et al. 2004; Wolkers et al. 2001). The study of Goyal et al. and Wolkers et al. showed that although some LEA proteins that are in the same group they may not form the same secondary structures during desiccation and display different folding mechanisms (Wolkers et al. 2001; Goyal et al. 2003). Overall, it can be said that LEA proteins are mostly intrinsically disordered proteins and may have different folding mechanisms during desiccation even if they belong to the same group and have an identical length.

It has been suggested that expression of LEA proteins is one of the key factors involved in conferring desiccation tolerance. LEA proteins generally disappeared in seeds during the time of germination and are degraded into their amino acids, ultimately used for seed maturation. The accumulation of LEA proteins during dehydration was confirmed by using orthodox seeds treated with exogenous ABA (Abscisic Acid) or PEG (Polyethylene glycol) or mannitol (ROSENBERG & RINNE 1989; Bartels et al. 1988; Blackman et al. 1991; Hsing et al. 1990; Hsing & Wu 1992). These studies confirmed that LEA proteins and desiccation are closely associated. The leakage of electrolytes from cells after desiccation was drastically reduced in plants with increased levels of LEA protein compared to non-transgenic controls. Blackman et al. (1995) could show the relevance of LEA proteins in desiccation tolerance using germinating seedlings of soybean. Contrary to the original

belief that LEA proteins play only a role in seed desiccation tolerance, this and other studies demonstrated that LEA proteins also accumulate in vegetative tissues of plants under water stress (Hong et al. 1992). Different studies showed that LEA proteins mostly appeared at the early stage of seed maturation and during the time of dehydration. LEA proteins in plants and animals disappeared rapidly after rehydration and their presence depends on stresses like chilling, drought, freezing and salt stress (Ried & Walker-Simmons 1993). The study of Moons et al. (1995) compared proteins profiles by giving an ABA treatment to both salts tolerant and salt sensitive rice. This study found the significantly higher percentage of LEA (a group of LEA II and LEA III) in roots of salt-tolerant rice varieties.

Several studies had been showed that LEA proteins can act as a cryoprotectant to protect enzymes activity such as lactate dehydrogenase (LDH) (Tamiya et al. 1985; Carpenter & Crowe 1988). LEA proteins from different sources were used for the cryoprotective assays. Compared to other cryoprotectants (ex: sucrose, BSA, and other proteins), LEA proteins were more effective in protecting LDH activity (HONJOH et al. 2000; Goyal et al. 2005). Other studies also showed that besides protecting LDH activity, LEA proteins also took part in protecting other enzymes like fumarase, LEAM, and rhodanese respectively (Goyal et al. 2005; Grelet 2005). The study of Pouchkina-Stantcheva et al. (2007) showed that LEA protein (*Ar*LEA1 and *Ar*LEA2) from bdelloid rotifers helped to prevent the aggregation of desiccation-sensitive enzymes.

Besides cryoprotection properties, LEA proteins also have radical scavenging or dehydration protection activity. Some LEA proteins have a high affinity for metal ions and

ultimately helped in retention of metal ions (Svensson et al. 2000; Kruger et al. 2002; Herzer et al. 2003). These are the most metal ions; Ca^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Mn^{2+}, interacted with LEA proteins (Hetherington & Brownlee 2004). These metal ions also have very important role in signal transduction pathways. LEA protein has a high proportion of His residues which is probably helped the plants to survive through abiotic stresses (Knight 1996; MINORSKY & SPANSWICK 1989).

OBJECTIVES OF THE DISSERTATION

The overall objective of this dissertation is to improve our current understanding for the role of LEA proteins in desiccation tolerance through molecular characterization, protein expression data, and functional studies of group 1 LEA proteins from embryos of *A. franciscana*. In chapter 1, I have cloned and purified untagged *Afr*LEA1.1 by using the IMPACT Kit. Protein expression was confirmed by SDS-PAGE and Western blotting. Finally, I have used that the purified protein for LDH assay before and after desiccation.

In chapter 2, I summarize the results of transgenically transferring the *afrlea1.1* into *Nicotiana tabacum*. Transfer of the LEA1.1 gene into the tobacco genome was confirmed by PCR, double restriction digestion, and semi-quantitative RT-PCR. After confirming different lines of LEA1.1 plants, we exposed the transgenic tobacco plants to water stress using polyethylene glycol (PEG; 10,000 MW). I measured the growth by recording increases in plant material per unit of time for leaf, root, and stem under fresh and dried condition. I also measured the chlorophyll content of both transgenic LEA plants and control plants both at 25°C (room temperature) and 4°C.

CHAPTER 1

Expression, Purification and, Characterization of group 1 LEA protein from the embryo of *Artemia franciscana* by using *Escherichia coli.*

1.1 INTRODUCTION

Water is vital for the survival of any kind of life (both plants and animals). Extreme loss of water is detrimental for life (Yancey et al. 1982). Anhydrobiotic organisms are considered as the most extreme example of water-loss tolerance and can survive almost complete desiccation. This phenomenon is known as anhydrobiosis (Crowe & Clegg 1973; Crowe & Madin 1974; Cornette & Kikawada 2011; Keilin 1959; Watanabe et al. 2005). Anhydrobiosis is a unique metabolic state that enables organisms to remain viable even after losing 97% of their body water (Gusev et al. 2014). Anhydrobiotic organisms possess the ability to survive desiccation in nature to water contents around 0.02 – 0.05 g H_2O g^{-1} dry mass and enter into a state that approaches suspended animation (Crowe & Clegg 1973; Crowe & Madin 1974; Crowe & Madin 1975; Hinton & Needham 1968).

Animals that exhibit anhydrobiosis are small and relatively simple invertebrates including insects, nematodes, rotifers, tardigrades, and the crustacean *Artemia franciscana* (Wharton 2015). The brine shrimp *Artemia franciscana* is a primitive arthropod that lives in saline water (Kim et al. 2015) and undergoes either oviparous or ovoviviparous development (MacRae 2003). *Artemia franciscana,* has served as an important model for animal desiccation tolerance, and multiple LEA proteins that belong to group 1 (PF00477), 3

(PF02987), and 6 (PF04927) **(Table 1.1)** are found in developmental stages that survive severe desiccation (Wu et al. 2011; Sharon et al. 2009; Hand et al. 2007).

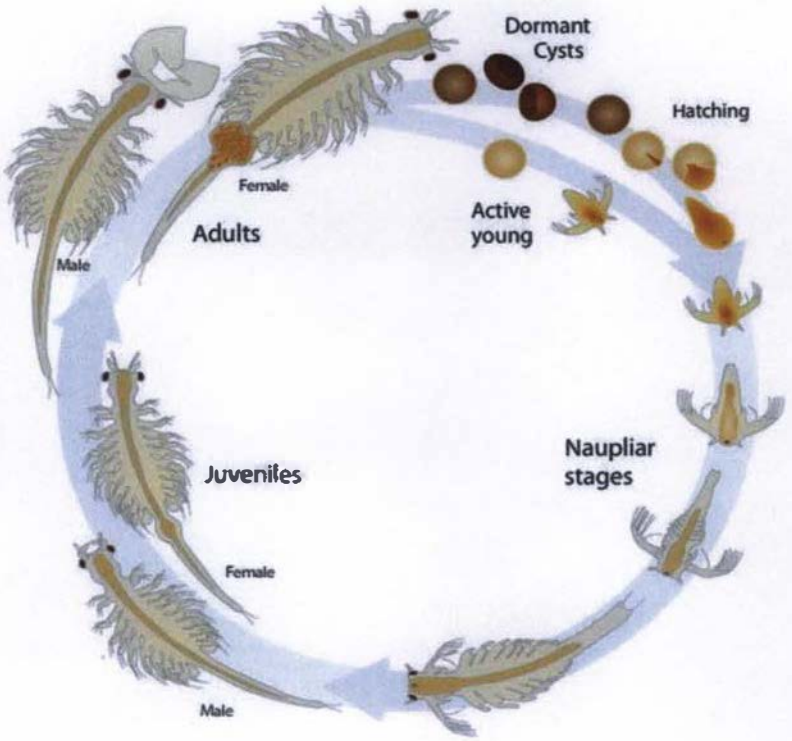


Figure 1.1: Life cycle of brine shrimp (*Artemia franciscana*).

Source: http://learn.genetics.utah.edu/content/gsl/artemia/

At the time of unfavourable environmental conditions (such as high salt concentrations, high pH, temperature fluctuations, or anoxic conditions), brine shrimp develop ovoviviparous by yielding free-swimming larvae (nauplii), however, under adverse conditions oviparous developing embryos arrest at gastrulation and are released from females as cysts before entering diapause (MacRae 2016) (**Fig 1.1**). Embryos arrested in the diapause state can stay dormant for a long time, until favorable conditions occur. This form of developmental arrest is a genetically programmed and can occur at the embryonic,

larval, pupal, or adult stage, depending on the species (Danilevskii 1965; Tauber et al. 1986). Entering diapause promotes survival of some organisms during exposure to temperature fluctuation, desiccation, and hypoxia (Robbins et al. 2010). The cyst of the brine shrimp is able to tolerate complete desiccation, long-term anoxia, and low temperatures without an appreciable loss in viability (Clegg et al. 2000; Liu et al. 2009). Previous experiments have verified that these cysts remain in a severely reduced metabolic state until more favorable conditions have returned (Clegg 2002; Lavens & Sorgeloos 1987; Sorgeloos et al. 2001).

The exact molecular mechanisms by which *Artemia* cysts tolerate environmental insults remain unknown, but recent research has reported the occurrence of several LEA proteins in cysts from *Artemia franciscana* and suggests that the accumulation of these proteins might provide tolerance to environmental extremes (Goyal et al. 2003; Hand et al. 2007; Kikawada et al. 2006b; Menze et al. 2009; Sharon et al. 2009). In addition to LEA proteins small heat shock proteins (sHSP), and artemin is also expressed before the onset of water stress (Kim et al. 2015). LEA proteins are believed to be critical for desiccation tolerance since an organism's expression levels of LEA protein and mRNA are closely related to its capacity for water loss (Menze et al. 2009; Browne et al. 2004). Indeed, knockout of Group1 LEA proteins reduce survival of *Artemia franciscana* embryos after desiccation and freezing (Toxopeus et al. 2014). In addition to LEA proteins, trehalose which is a non-reducing disaccharide contributes to the extreme desiccation tolerance in this animal (Hand et al. 2011; Crowe et al. 1997; MacRae 2016).

The overall classification of LEA proteins got dimension in ages. Lea proteins were first classified into six groups or families based on amino acid compositions (Dure et al. 1989), but later on, reclassified into seven distinct groups based on specific domains and motifs **(Table A & B)** (Amara et al. 2014; Battaglia et al. 2008). However, most LEA proteins in plants belong to group 1, 2 and 6. So far the research has been done over *Artemia franciscana,* it was found that most of the LEA proteins expressed by this animal are belong to group 1, 3 and 6 (Hand & Menze 2015).

LEA proteins in *Artemia franciscana* belong to group 1 (*Afr*LEA1.1 and *Afr*LEA1.3) (Marunde et al. 2013), group 3 (*Afr*LEA1, *Afr*LEA2 and *Afr*LEA3m) (Hand et al. 2007; Boswell et al. 2014; Menze et al. 2009) and group 6 (*Afr*LEA6, also known as SMP) (Wu et al. 2011; Hand & Menze 2015). Group 1 LEA proteins are mostly acidic and hydrophilic due to their high proportion of acidic amino acids (Sharon et al. 2009). Group 3 LEA proteins have a repeating motif of 11 amino acids as a differentiating feature (Dure 1993). Among three different group 3 LEA proteins found in *A. franciscana*, *Afr*LEA1 and *Afr*LEA2 are cytosolic and hydrophobic in nature. Another group 3 LEA protein, *Afr*LEA3m, is enriched in α-helices and has a mitochondrial pre-sequence. The mRNA of LEA proteins is more abundant in diapause-destined embryos than in swimming larvae and adult (Boswell et al. 2014; Menze et al. 2009).

Table 1.1: LEA proteins found in the brine shrimp *Artemia franciscana*

Protein	Group	Location	Number of amino acids	References
*Afr*LEA1.1	1	Cytoplasm	182	(Sharon et al. 2009)
*Afr*LEA1.3	1	Mitochondria	197	(Warner et al. 2010)
*Afr*LEA1	3	Cytoplasm	357	(Hand et al. 2007)
*Afr*LEA2	3	Cytoplasm	364	(Hand et al. 2007)
*Afr*LEA3m	3	Mitochondria	307	(Menze et al. 2009)
*Afr*LEA6	6	Cytoplasm	257	(Wu et al. 2011)

An LEA6 protein from *Artemia franciscana* (*Afr*LEA6) that has recently been identified, exhibits strong sequence homologies to SMP in plants (Hand & Menze 2015; Wu et al. 2011). *Afr*LEA6 is less hydrophilic than groups 1 and 3 LEA proteins, which is a characteristic of SMPs like MtPM25 (Boucher et al. 2010). It is assumed that *Afr*LEA6 has an important role in improving long-term desiccation tolerance in animal cells as suggested for SMP's in plants (Chatelain et al. 2012).

The presence of multiple LEA proteins in a single organism suggests different subcellular targets of LEA proteins to protect vital cellular components from damage exerted by desiccation. The group 3 LEA protein, *Afr*LEA3m from *A. franciscana* is the first protein from an animal species reported to be targeted to the mitochondria (Menze et al. 2009). This group 3 LEA protein is composed of 307 amino acids and contains a 29-amino acid

pre-sequence at the N-terminus. Group 1 LEA proteins are found in the cytosol and mitochondria of *Artemia* cyst (Warner et al. 2010).

Structural and biochemical analysis of LEA proteins from *A. franciscana* has been done based on their amino acid composition. Most LEA proteins in Artemus have a hydrophilic nature like LEA proteins found in other organisms (Amara et al. 2014). Hand et al. found that desiccation of *Afr*LEA2, a member of group 3 proteins from *A. franciscana*, caused an increase in α-helix content from 4% in solution to 46% in the dried state. Similarly, *Afr*LEA3m which was predominantly disordered in solution adopted a more α-helical structure after drying. However, *Afr*LEA3m possessed a greater percentage of β-sheet in the dry state compared to *Afr*LEA2, which could explain the lower α-helix content in *Afr*LEA3m (Hand & Menze 2015; Boswell et al. 2014).

The exact molecular functions of LEA proteins are still unclear and LEA proteins have been suggested to act as protein and membrane protectants, cell membrane stabilizers, hydration buffers, antioxidants, organic glass formers and ion chelators (Tunnacliffe & Wise 2007). LEA proteins have the potential to protect target proteins from inactivation and aggregation during water stress. A role in protein stabilization is supported by the fact that some LEA proteins preserve enzyme activity *in vitro* during water stress (Reyes et al. 2005). Many proteins, including the enzymes citrate synthase and lactate dehydrogenase, form insoluble aggregates when dried or frozen, but aggregation is reduced in the presence of LEA proteins from groups 1, 2, and 3 (Amara et al. 2014).

The protection conferred by *Afr*LEA1.3 is interesting because it worked during moderate water stress, a condition in which cellular water content is above 20% and LEA proteins usually do not form α-helical structure. This observation is an example that folding is not always a prerequisite for LEA protein activity (MacRae 2016; Marunde et al. 2013). Hand et al. observed that cells loaded with trehalose and expressing *Afr*LEA2 or *Afr*LEA3m showed 98% membrane integrity compared with 0% intact membranes for control cells without LEA proteins or trehalose. Even without intracellular trehalose, *Afr*LEA3m conferred 94% protection based on membrane integrity (Liu et al. 2009; Hand & Menze 2015). The LEA proteins of *Artemia* have the potential to protect proteins from drying-induced aggregation by forming glasses with trehalose, an abundant cyst sugar (Sharon et al. 2009; Warner et al. 2010; Hand et al. 2011; Toxopeus et al. 2014).

1.2 MATERIALS AND METHODS

1.2.1 MATERIALS

The original nucleic acid sequences of *Afrlea1.1* was cloned from *A. franciscana* which was previously published (ABR67402) (Sharon et al. 2009). All the chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Water for different experiments was purified with a Milli-Q Reagent System (Millipore, Billerica, MA) to an electrical resistance of 18 mΩ. All the rotors used for the ultracentrifugation were by Beckman Coulter™ and Fisher Scientific (accuSpin Micro 17).

1.2.2 METHODS

Cloning of *Afrlea1.1* to specific Vector, pTXBI

The LEA1.1 gene was amplified with gene-specific primers (**Table 1.2**) following the protocol from Thermo Scientific® using Phusion High-Fidelity DNA Polymerase.

Table 1.2: Primers sequences for the amplification of *Afrlea1.1* to be cloned into the intein tag system.

Primers	**Sequences**
Forward	5`ggcggc**catatg**GAGAGCGAACAGGGTAAATTGAGTCGC 3`
Reverse	5`tata**actagt**GCATCTCCCGTGATGCACTTCTGCCGGGCAAGCCCCC 3`

*Bold letters are denoting restriction enzyme cutting sites.

The online program OligoAnalyzer Tool (www.idtdna.com/calc/ analyzer) was used to design all primers. Moreover, the online program, NEB cutter (http://nc2.neb.com/ NEBcutter2/) and Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) developed by Max Heiman at Yale University, were used to examine whether the target genes have any cutting site for the used restriction enzymes. The common criteria to design the primers were as follow:

- The length of the primers was between 17-28 bases.
- Guanine and cytosine (GC) content were at least 50 % of total bases.
- Melting temperature of the primers was kept between 60-80°C.
- The annealing temperature of the primers was around 72°C.

- 3-6 extra bases were added at the 5' end, outside of the restriction enzymes cutting sites, to facilitate restriction enzyme activity.

All reactions that were set up for PCR are shown in **Table 1.3**. PCR products were run on 1.2% agarose gel for 1 h at 120 volts. TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA) was used to make and run the agarose gels. A 2-log plus DNA ladder with mass ranges from 0.1-10 kb was used as a standard to identify the correct PCR products (www.neb.com/products/n3200-2-log-dna-ladder-01-100-kb). Then the DNA was purified by gel extraction following manufacturer instructions of the NucleoSpin Gel and PCR clean up kit (Macherey-Nagel, Bethlehem, PA). Purified samples were quantified using the Epoch Microplate Spectrophotometer (BioTeK® Instruments, Winooski, VT). Purified samples were stored at - 20°C.

Table 1.3: Reagents used to amplify DNA through polymerase chain reaction.

Ingredients	Volume
Reaction Buffer (5X)	10 µL
Deoxy- nucleotide triphosphate (dNTP)	1 µL
Primers mixture	1 µL
Template DNA	1 µL
DNA polymerase	0.5 µL
dH_2O	36.5 µL
Total volume	50 µL

After PCR clean up, the LEA 1.1 gene and Vector (pTXB1) was used for double restriction digestion using NdeI (catatg) and SpeI (actatg) at 37 °C for 1h using the composition listed in **Table 1.4**.

Table 1.4: The reagents used to set up reactions for restriction enzyme digestion.

Ingredients	Vector digestion	Insert digestion
Cutsmart buffer (10X)	10 µL	5 µL
DNA	2.5 µg	1 µg
Restriction enzyme 1	2 µL	1 µL
Restriction enzyme 2	2 µL	1 µL
Deionized H_2O	varied	varied
Total volume	100 µL	50 µL

Digested DNA usually possesses a 5'-phosphate group that is required for ligation. In order to prevent self-ligation, the 5'-phosphate groups at the vector have to be removed prior to ligation. The dephosphorylation was accomplished by adding 0.5 µL calf intestinal alkaline phosphatase (CIP) to the digestion reaction for the vector. The reactions for vector dephosphorylation were run for 1 h at 37°C. Following dephosphorylation, the digested vectors were run on 1.2 % agarose gel and purified by using NucleoSpin PCR and gel cleanup kit (Macherey-Nagel, Bethlehem, PA). On the other hand, digested inserts were not run on agarose gel rather purified by using the same clean-up kit. Restriction enzymes used in these experiments were purchased from New England BioLabs, Ipswich, MA.

The enzyme T4 DNA Ligase (New England BioLabs, Ipswich, MA) was used for the ligation reaction of digested vector and the inserts. The reaction was kept at room

temperature for 1 h followed by16 °C for 16 h. The reaction composition is noted in **Table 1.5**. Ligated plasmids were either frozen at - 20°C or immediately used to transform chemically competent *E. coli* cells.

Table 1.5: The reagents used to set up ligation reactions.

Ingredients	Volume
Vector	3-4 µL (>120 ng)
Insert	1-2 µL (>50 ng)
T4 DNA Ligase Buffer	1 µL
T4 DNA Ligase	1 µL
dH_20	Varied
Total volume	10 µL

Transformation in *E. coli* cells, Clone selection, and plasmids DNA purification

One vial of One Shot® BL21 cells (DE3) chemically competent *E. coli* cells (Thermo-Fisher Scientific, Carlsbad, CA) was used for each transformation. Briefly, one vial of the competent cell was removed from - 80°C and placed on ice to thaw frozen bacteria. Then 5 µL of isolated plasmid was added to the bacteria and mixed by gentle tapping. The mixture of bacteria and plasmids were incubated on ice for 30 minutes. After incubation on ice, heat shock was given at 42°C for exactly 30 seconds in a pre-heated water bath. Following heat shock, the bacteria were kept on ice for 2 minutes and 250 µL of SOC media (Thermo-Fisher Scientific, Carlsbad, CA) was added. The bacteria were cultured for 1 h at 37°C on a shaker rotating at 225 rpm. Finally, bacteria were spread on ampicillin (100 µg/mL) containing LB (Luria-Bertani) plates and grown in the incubator overnight at

37°C. then the plasmid was isolated and confirmed the insert with PCR and double restriction digestion. After having confirmation of insert, the Isolated plasmid was used for transformation in BL21 chemically competent *E.coli* cells for protein expression.

Following the day of transformation, 3-6 colonies were selected and grown in 5 mL of LB media containing 100 µg/mL ampicillin. The bacteria were cultured overnight (12-16 hours) at 37°C on a shaker rotating at 225 rpm. The next day, 500 µL of overnight grown bacteria were mixed with 250 µL 3X glycerol solution (65% glycerol, 0.1 M $MgSO_4$, 0.2 M Tris·Cl, pH 8) in cryopreservation vials and preserved at - 80°C for long term storage. The remaining 4.5 mL of bacteria were used for plasmid isolation using the NucleoSpin Plasmid isolation kit using the corresponding protocol (Macherey-Nagel, Bethlehem, PA).

Purified plasmids were quantified with the Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, VT) following the manufacturer's guideline. The insertion of LEA genes in the plasmids was verified by two methods. One way was by running PCR products on 1.2% agarose gel where isolated plasmids from cloned bacteria were used as template DNA. Another way to verify the success of cloning was digestion of the isolated plasmids by restriction enzymes and comparing plasmids size with the empty vector on 1.2% agarose gels.

Protein (LEA1.1) expression, confirmation, and on-column purification:

After confirmation of insert, a single colony was selected for overnight culture in 5 ml LB medium containing ampicillin. The culture was grown overnight at 37°C. The following day 100 µl of the bacterial culture was added to 4.9 ml of fresh ampicillin containing LB

and grown until the OD_{600} reached 0.4-0.5 absorbance units (mid-log phase). Generally, it took 2-3.30 hrs to reach mid-log phase. Upon reaching mid-log phase protein expression was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 0.5 mM. The culture was allowed to grow until OD_{600} reached 0.8-1.0, which generally occurred after 2-3 hrs. Then the cells were harvested by centrifugation at 6000 rpm for 5 mins. The pellet was saved and the supernatant discarded.

The pellet was collected from 5 ml culture, lysed in 500ul column buffer (lysis buffer) containing 0.4% Triton X-100 and 1mM PMSF. Cells were sonicated on ice for ~10 seconds for 3-6 times spaced by about 10 sec interval to disrupt cell membranes and liberate proteins. Tubes were kept on ice while sonicating to avoid increases in temperature and foam formation. About 1ml of lysis buffer (column buffer) containing PMSF and Triton X-100 was collected for Bradford Assay. Then the lysates were centrifuged for 1 hour at 14,000 rpm at 4°C to separate dissolve proteins from other cellular components. After centrifugation, the supernatant was transferred into a fresh micro tube.

The Bradford assay was used to determine total protein concentration in the samples. The standard curve was generated by taking absorbance readings of Bradford reagent (Bio-Rad Laboratories, Hercules, CA) containing 0, 125, 250, 500, 750, 1000, and 1500 μg/mL bovine serum albumin (BSA) **(Table 1.6).** The optical density (OD) of the samples were taken at 595 nm using a spectrophotometer (Evolution 300 UV-Vis, Thermo-Fisher Scientific, Carlsbad, CA)

For Bradford assay, a total volume was made 20 µl was taken, where protein sample and column buffer (same one used as lysis buffer) was used in a 1:1 dilution. Then 1ml of Coomassie blue was added to every tube of standard and samples for protein dilution. Then incubated for 15 minutes in a dark place and then absorbance was taken at OD_{595}.

Table 1.6: Optical density of standard BSA samples in Bradford reagent at 595nm.

Sample	Protein concentration (µg/mL)	OD at 595nm
A	1500	0.97
B	1000	0.68
C	750	0.54
D	500	0.36
E	250	0.18
F	125	0.08

After the Bradford Assay, the concentration of protein sample was calculated and then the protein samples were prepared for SDS-PAGE. About 40-50 µg of total protein was loaded into each well of the gel used for SDS-PAGE.

Preparing SDS-PAGE

In order to run protein samples, nine welled 0.75 mm thick sodium dodecyl sulfate-polyacrylamide gels were used. To prepare two 10 % resolving gels, 4.1 mL dH_2O, 3.3 mL acrylamide/bis (37.5:1) solution, 2.5 mL gel buffer (1.5 M Tris-HCl, pH 8.8) and 0.1 mL of 10% SDS were mixed together and degassed for 15 minutes. Then, 50 µL of 10% fresh ammonium persulfate and 5 µL TEMED solutions were mixed properly with a degassed solution and cast. After 45 minutes, 5% stacking gel was prepared by mixing 5.7 mL dH_2O,

1.7 mL acrylamide/bis (37.5:1) solution, 2.5 mL gel buffer (0.5 M Tris-HCl, pH 6.8) and 0.1 mL of 10% SDS. The mixture of the stacking gel also degassed for 15 minutes. Similar to resolving gel, 50 µL of 10% fresh ammonium persulfate and 5 µL TEMED solutions were mixed properly with the degassed solution and cast on top of stacking gel. After polymerization, gels were used either immediately or kept in a refrigerator at 4°C for up to 5 days.

Running SDS-PAGE

All the protein samples were made up to 60 µl by using 30 µl sample and 30 µl Lameli buffer solution. Two polymerized gels were placed together in the gel running box (Bio-Rad Laboratories, Hercules, California) and half of the box was filled with 1X running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS, pH 8.3). Before loading, all samples and standards were heated at 95°C for 10 minutes. Then, 20 µL protein samples, 10 µL Kaleidoscope prestained protein standards (Bio-Rad Laboratories, Hercules, CA) were loaded into different wells. The gels were run at 120 volts until the lowest band (10 kDa) of the Kaleidoscope separated from other bands which took on average 1 h.

Then the gel was carefully removed from the cassettes and placed into staining solution for 1-2 hours followed by destaining (7.5% glacial acetic acid + 5% methanol in dH_2O overnight. The destaining solution was changed 3-4 times. The following day images were taken.

Western Blotting (Immunoblotting)

After electrophoresis, gels were washed in transfer buffer (25 mM Tris, 190 mM glycine, 0.1% SDS, 20% methanol, pH 8.3) for 15 minutes on a shaker. The small amount of SDS in the transfer buffer may give the proteins enough charge to move unidirectionally towards the anode. I used nitrocellulose membrane to transfer proteins from the gel for Western blotting. A sandwich was made by combining a fiber pad with soaking paper, SDS gel, membrane, soaking paper, and a final fiber pad (bottom to top), to transfer proteins. The sandwich was placed into the transfer cassette and the tank was filled with transfer buffer and run for 1 h at 60 V. While transferring proteins, the tank was kept on ice to avoid high temperature due to the electric current.

To see whether the transfer was successful, membranes were stained with Ponceau Red (0.2% w/v Ponceau S, 5% glacial acetic acid) for 5 minutes. Then the membranes were washed with water for three times and bands of proteins on the membrane became visible.

Blocking buffer was made by dissolving 5% nonfat dry milk powder in TBS-T (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) solution. The membranes were incubated in the blocking buffer for 1 h at room temperature. Blocking the membrane in milk solution prevents unspecific binding of primary and secondary antibodies.

Anti-Rabbit primary antibodies (Rockland Immunochemicals, Limerick, PA) was used for detecting intein tagged LEA proteins. The primary antibody was diluted in the blocking buffer at 1:1000 and membranes were incubated overnight on a shaker at 4°C. The

following morning, the membranes were washed with TBS-T three times (each 5 minutes) prior to incubation with secondary antibody.

CBD (Chitin-Binding Domain) antibody and anti-mouse IgG1 were used as secondary antibodies (Cell Signaling Technologies, Danvers, MA). The secondary antibodies were also diluted in TBS-T solution at 1:1000. After incubation in secondary antibody for 1 h at room temperature, membranes were washed with TBS-T for three times (each 5 minutes).

The membranes were incubated with Lumiglow (Cell Signaling Technologies, Danvers, MA) for 1-2 minutes at room temperature. Lumiglow is a substrate for horseradish peroxidase (HRP) and was diluted with deionizing water at 1:10. Finally, the membranes were exposed to X-ray film in a dark room for 30-120 seconds and films were developed.

On-column purification

For on-column purification, 500 ml of LB medium, containing 100 µg/ml ampicillin, with a freshly grown colony was inoculated and IPTG induced for the protein expression. The lysate was collected as previously mentioned protocol. Clarified lysate (supernatant) was run onto the chitin column which was followed by washing with 20-bed volumes of washing buffer (500 mM NaCl, 20mM Tris-HCl) to thoroughly remove the unbound proteins. Then the column was washed with 3 column volumes of cleavage buffer (150 mM NaCl, 20 mM Tris-HCl, 50 mM DTT) for purification. Then the flow was stopped and the column was kept at 4°C for 48 hrs for on-column cleavage of LEA1.1 protein. After that, the target protein was eluted with column buffer (150mM NaCl, 20mM Tris-HCl, 0.4% Triton X-100, 20mM PMSF), PMSF was added to the buffer right before the use.

Then the elution was collected in microfuge tubes and a sample was run in SDS-PAGE for confirmation and rest was stored at -80 °C for future use upon dialysis with storage buffer (0.05M phosphate buffer, pH 7.4+ 0.1M NaCl). Then the column was regenerated by washing with 3-bed volumes of stripping solution (0.3 M NaOH). Firstly allowed the resin to soak for 30 minutes and wash the resin with additional 7-bed volumes of stripping solution. Then wash with 20-bed volumes of water, followed by 5-bed volumes of column buffer.

Activity Assay of Lactate Dehydrogenase (LDH)

The LDH used for the assay was obtained from Sigma-Aldrich (St Louis, MO; product code L2500). Prior to use, LDH was exchanged into LEA storage buffer (0.05M phosphate buffer, pH 7.4+ 0.1M NaCl) using Amicon® Ultra Centrifugal Filters (Ultracel®-10K; Millipore, Billerica, MA). Then 10 µl droplets of 50 µg/ml LDH, with or without protectants, were dried in 1.5 ml microcentrifuge tubes at room temperature for one week in a dry box containing Drierite. All the samples were rehydrated with 20 µl of LEA storage buffer (diluted two-fold) for 1 h on ice. Control assays of LDH activity were performed prior to desiccation by adding 10 µl of LDH sample (50 µg/ml) to a final reaction volume of 1.0 ml, which contained 0.2 M Tris-HCl buffer (pH 7.3), 220 µM NADH and 6.6 mM sodium pyruvate. LDH activity after desiccation was measured as described for controls, except that 10 µl of LDH sample were added to account for the two-fold dilution of the enzyme during rehydration. Change in A340 was recorded for 1.5 min, and LDH activity was reported as a percentage of the rate measured for non-dried controls. Each sample was compared to control values that contained the same mixture of protectants in order to

account for an observed increase in LDH activity in the presence of higher concentrations of protectant protein. LDH activity was also measured by comparing the activity in presence of *Afr*LEA6 (SMP), bovine serum albumin (BSA) and trehalose (Tre). Reported values are the average of two separate drying trials each with three nested replicates (n = 6, results were shown as a Mean ± SD).

Statistical Analysis:

For statistical analysis, One-way ANOVA with Holm-Sidak posthoc test and Microsoft excel 2016 was used. Statistical significance value was taken, $p<0.05$

1.3 RESULTS

Confirmation of *Afrlea1.1* in target Vector after transformation

Afrlea1.1 was cloned into the destination vector and was amplified by PCR and purified through PCR clean up gel electrophoresis in a concentration of 326 ng/µl. Then purified LEA 1.1 gene and target vector, pTXB1 was set for double restriction digestion and purified from gel and set for ligation reaction and then successfully transformed into TOP 10 *E.coli* competent cells and isolated colonies were confirmed for insert by PCR through gel electrophoresis and eventually purified plasmids with the desired insert was transformed into BL21 bacterial competent cells for protein expression. The transformation in BL21 was confirmed by following same PCR protocol (**Fig 1.2**).

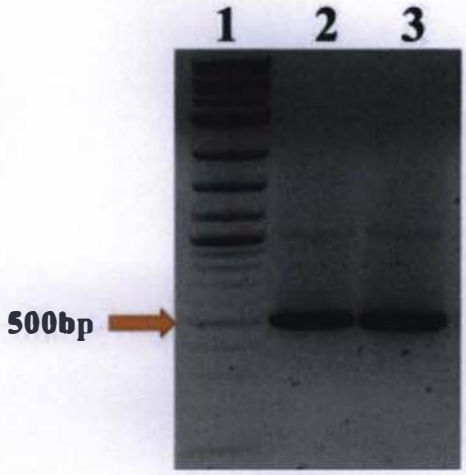


Figure 1.2: Confirmation of the *Afrlea1.1* (495 bp) in the pTXBl Vector after transformation into TOP 10 *E.coli* competent cells. **Lane 1**: 2-log Plus DNA ladder, **Lane 2-3:** PCR products of LEA 1.1 from two isolated plasmids. All two plasmids successfully yielded LEA1.1 DNA when used as a template.

Protein expression and on-column purification by affinity chromatography

After successful transformation, the BL21 transformed cells were induced by IPTG for protein expression and the confirmation of expression was done by SDS-PAGE (**Fig 1.3**) and Western blot (**Fig 1.4**) and protein concentration was quantitated by using the Bradford assay. *Afr*LEA1.1 (18.45 kDa) was tagged with intein along with chitin binding region (28 kDa) and final molecular weight was 46.45 kDa. In SDS-PAGE and Western blot the band for the confirmation was found at 46.45 kDa range.

During primer design, last six bases (**GGC GGA**), were removed from the 3′ end of the gene sequence cause this two codons code for Glycine and the rate of cleavage according to IMPACT kit is very low (10%) for Glycine (https://www.neb.com/products/e6901-

impact-kit). The next one is AAG (Lys), the cleavage rate is higher (75-90%). So reverse primer designed by considering AAG as the last codon.

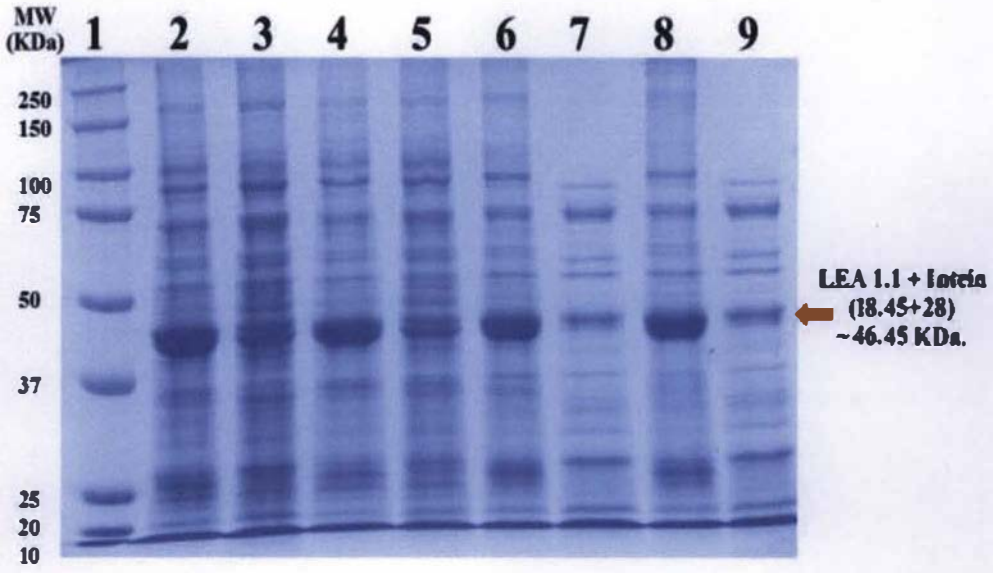


Figure 1.3: SDS-PAGE to detect the IPTG-induced expression of the LEA1.1 protein in BL2I *E. coli* competent cells. **Lane 1**: Kaleidoscope™ Prestained SDS-PAGE Standards, **Lane 2,4,6,8**: IPTG-induced BL21 cells lysate, **Lane 3,5,7,9**: Un-induced BL21 cells lysate. All the induced cells lysate showing a positive result for LEA1.1 protein extraction along with intein tag at a range of 46.45 kDa.

Figure 1.4: Western blot analysis to confirm the expression of LEA1.1 protein along with intein tag by using Anti CBD antibody. **Lane 1 & 3:** IPTG-induced BL2I cells lysate, **Lane 2:** Un-induced BL21 cells lysate.

Purified protein was concentrated by using Amicon Ultra Centrifugal Filters (Ultracel-10K; Millipore) from 10ml to 350µl. The final concentrated concentration of LEA1.1 protein was 28 mg/ml (**Fig 1.5, 1.6 & 1.7**).

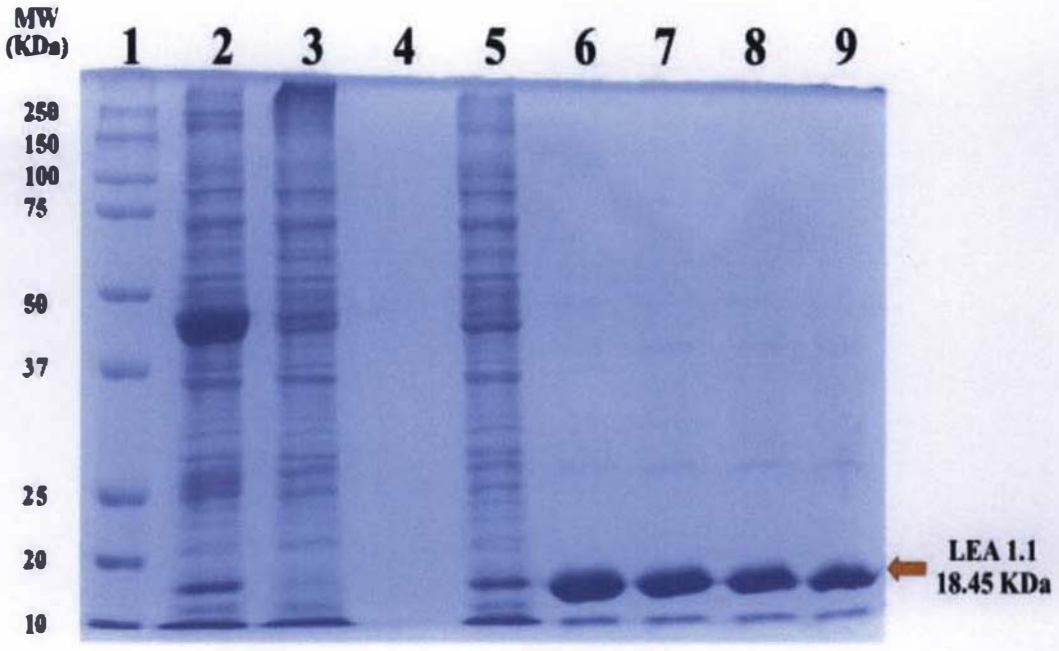


Figure 1.5: SDS-PAGE after on-column purification of LEA1.1(18.45 kDa) protein without intein tag. **Lane 1**: Kaleidoscope™ Prestained SDS-PAGE Standards, **Lane 2**: clarified IPTG-induced lysate, **Lane 3**: Un-induced Lysate, **Lane 4**: First Flow-through (FT-F) from chitin column, **Lane 5**: Last Flow-through (FT-L) from chitin column, **Lane 6-9**: Elution of LEA1.1 after stopping column flow and inducing a cleavage reaction at 4°C for 16 hours.

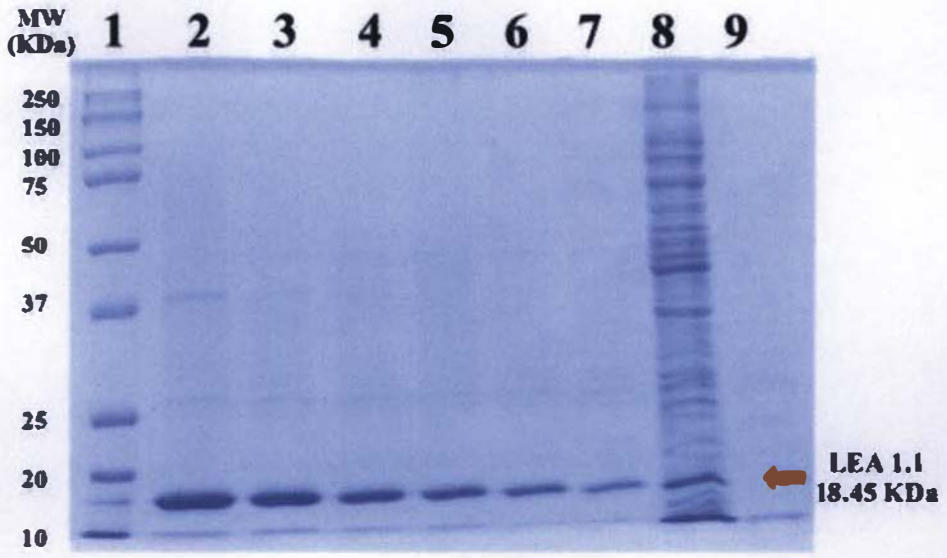


Figure 1.6: SDS-PAGE after on-column purification of LEA1.1(18.45 kDa) protein without intein tag. **Lane 1**: Kaleidoscope™ Prestained SDS-PAGE Standards, **Lane 2-7**: Elution of LEA1.1 after stopping column flow and inducing a cleavage reaction at 4°C for 16 hours, **Lane 8**: Wash first, **Lane 9**: wash last.

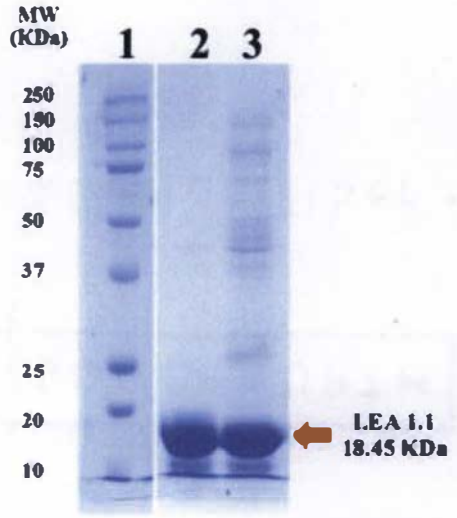


Figure 1.7: SDS-PAGE after on-column purified and concentrated of LEA1.1(18.45 kDa) protein without intein tag before and after heat treatment. **Lane 1**: Kaleidoscope™ Prestained SDS-PAGE Standards, **Lane 2**: LEA1.1 protein after heat treatment at 95°C for 15 minutes, **Lane 3**: LEA1.1 protein before heat treatment.

Protection of LDH enzyme Activity upon desiccation along with LEA1.1 proteins and other protectants

Drying studies of target enzyme and LEA 1.1 protein along with other protectants performed to see the protection rate of the LEA1.1 protein against dehydration-induced damage. After desiccation for a week, LDH enzymes when rehydrated it shows significant protection rate compared to initial activity for LEA1.1 along with Trehalose (77%±3%) and LEA6 (82%±8%). Both combinations showed this rate of activity for 100mM of Trehalose and 400 µg/ml of LEA6 respectively.

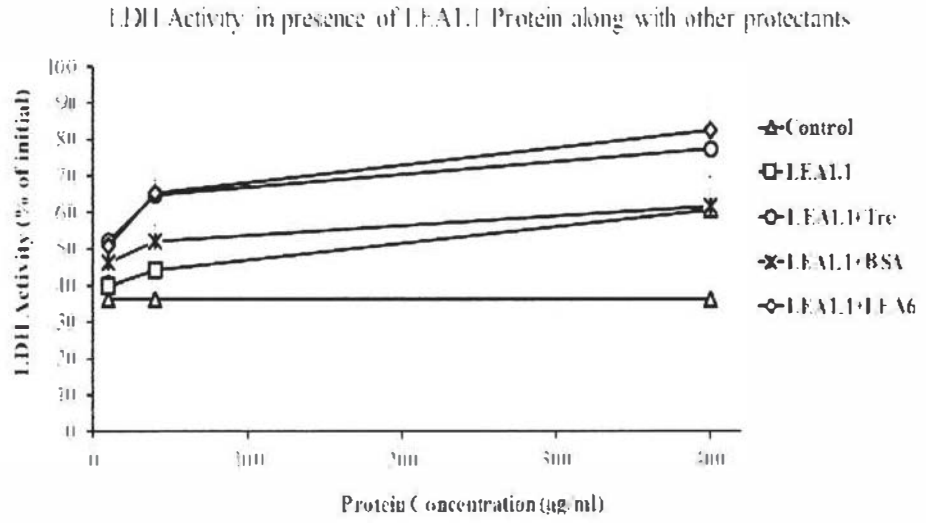


Figure 1.8: LDH activity after desiccation for one week with and without protectants. Late Embryogenesis Abundant (LEA) protein concentrations were 10, 40, and 400 µg/mL. The protective capability of purified untagged LEA1.1 protein was measured with or without the addition of protectants (Bovine Serum Albumin(BSA), Trehalose (Tre), and LEA 6) before and after desiccation (Mean ±SE, n=6). A significant level of protection was for LEA1.1 along with 100mM Trehalose and 400 µg/mL of LEA6. Statistical analysis was done by one-way ANOVA with Holm-Sidak post hoc test ($p<0.05$). For the clarification, symbols to indicate significance was removed from the graph.

1.4: DISCUSSION

Anhydrobiosis represents a unique example of the adaptation of an organism to water loss, where an organism can exist in an ametabolic state until water returns (Gusev et al. 2014). The cyst of the brine shrimps *Artemia franciscana* enter diapause, a state of developmental arrest and greatly enhanced stress tolerance (MacRae 2016; Hand et al. 2007; Clegg et al. 2000). Survival of animals and plants during the time of extreme water loss is key for the expression of LEA proteins. Upon the first discovery of LEA proteins in cotton seeds at maturation till now these were found to be present in several anhydrobiotic animals and plants especially in desiccation tolerant stages. From the study of Hand et al. (2011), we came to know its availability in several animal phyla like Arthropoda, Rotifera, and Nematoda (Hand et al. 2011).

Probably the main feature distinguishing anhydrobiotic organisms including *Artemia* is that they produce many types of highly hydrophilic proteins in preparation for severe dehydration (Tunnacliffe et al. 2010). LEA proteins are hydrophilic and non-globular proteins and recent findings show that they play various roles in dehydrating cells, including homeostasis of proteins and nucleic acids, stabilizing cell membranes, redox balance, and the formation and stability of a glassy state (Tunnacliffe & Wise 2007). Despite several studies on *Artemia* LEA proteins, their functions, roles, and localizations in the anhydrobiotic cyst remain unknown (Kim et al. 2015). The goal of this study was to purify one particular LEA protein, LEA1.1 cloned from *Artemia* and introduced into *E.coli*.

Due to their hydrophilic, unstructured nature, LEA proteins themselves are not vulnerable to aggregation on desiccation, freezing, or boiling (Tunnacliffe et al. 2010). When the enzyme phosphofructokinase was dried in the presence of *Afr*LEA2 plus 100 mM trehalose, 98 % of control (non-dried) activity was preserved, and 103 % of control activity remained intact in the presence of *Afr*LEA3m plus 100 mM trehalose (Boswell et al. 2014). A group 1 protein from the brine shrimp, *Afr*LEA1.3 preserved mitochondrial function and improved viability of transgenic *Drosophila melanogaster* Kc167 cells during freeze-thawing, drying, and hyper osmotic stress.

To my knowledge, our method using intein for protein purification has not been reported before for LEA1.1. The system was used for the protein production; IMPACT™ is mostly used nowadays. Where intein was used as a continuous fusion partner (Chong et al. 1997). The unwanted auto-splicing occurs when the fusion protein is expressed in the host cells (Volkmann et al. 2009). In some cases, the rate of in vivo auto-cleavage of the fusion protein is more than 90%, which ultimately leads to low purification of the target proteins (Cui et al. 2006). To avoid self-cleavage and the low cleavage rate of the target protein (LEA1.1) from intein tag in the column, the last six bases (GGCGGA) of the intein tag sequence were removed from the 3` end. This was done because these codons code for glycine and the rate of cleavage according to IMPACT kit is very low (10%) for Glycine **(Section 1.2)**. The amino acid encoded by the adjacent AAG encode lysine, with which the cleavage rate is much higher (75-90%). Therefore, a reverse primer designed to include AAG as the last codon of the gene sequence. After purification, a higher cleavage rate of the target protein was confirmed (Figure 1.5 and 1.6).

BL21 cells were transformed with *Afilea1.1*. This BL21-LEA line was used for protein expression and the expression was confirmed by comparing with the uninduced BL21-LEA cells. From figure 1.3, it was shown that induced cells produced a band with a predicted molecular weight of LEA1.1 protein along with intein tag at around 46.45 kDa. The expression was also confirmed by western blot (Figure 1.4), using the anti-CBD antibody as a primary antibody. The concentration of LEA1.1 protein was measured by Bradford assay where it was found around 0.8mg/mL.

Having confirmation of protein expression by SDS-PAGE (Figure 1.3), Western blot (Figure 1.4) and the Bradford assay, the procedure was scaled up for on-column purification. The results showed, for the first time, purification of the LEA1.1 protein without any tag using the IMPACT kit (Figure 1.5, 1.6). The purified LEA1.1 protein was found to be very heat stable (Figure 1.7) with the minimal loss after 15 minutes at 95°C.

It was predicted that purified LEA1.1 protein might have a various supporting role during desiccation. Experiments evaluating the capacity of the *Afi*·LEA1.1 protein to protect desiccation-sensitive, target enzymes from damage during drying showed that this ability depends on the target protein chosen. For LDH, *Afi*·LEA1.1 was able to afford better protection than that provided by BSA (Bovine Serum Albumin), which is in apparent contrast with reports for other LEA proteins in the literature. However, it should be noted that *Afi*·LEA1.1 did afford a high degree of protection to LDH similar to that seen with other LEA proteins (Goyal et al. 2005); the difference is that BSA-stabilized LDH in my study far more than previously reported but resembling the findings of Boswell et al. (2014). Reyes et al. (2005) reported that in the presence of BSA, LDH exhibited 75%

residual activity after being dried to 2% water content, but activity dropped below 40% at a water content <2%. Another aspect that has differed substantially among studies is the concentration of LDH in the test mixture. In the present study, LDH was dried at an initial concentration of 50 μg/ml because preliminary observations showed that at lower concentrations the enzyme lost activity in a time-dependent fashion if simply stored on ice for 1 h during rehydration. In comparison, multiple groups have reported the use of dried or frozen LDH at concentrations lower than 10 μg/ml (Goyal et al. 2005; Reyes et al. 2005). The use of such low concentrations of LDH could result in unreliable results due to non-specific adsorption of LDH to vial surfaces. The study also showed that two LEA proteins along with each other help to retain more LDH activity than individual protein or in presence of any other protectants (Figure 1.8). From Figure 1.8, it has been seen that LEA1.1 along with LEA6 showed more protective capability for LDH than any other protectants, resembling the findings of Boswell et al. 2014, though these authors used LEA protein combinations distinct from those used in our study. (Boswell et al. 2014).

1.5: CONCLUSION

In conclusion, it can be said that *Afr*LEA1.1 protein is a heat stable protein and able to protect desiccation-sensitive enzymes from the deleterious effects of desiccation and subsequent rehydration. These findings serve to not only further define the molecular characteristics and possible functions of *Afr*LEA1.1, but also add to the pool of evidence that supports a role for LEA proteins in desiccation tolerance.

CHAPTER 2

Heterologous expression and functional characterization of LEA1.1 from *Artemia franciscana* in *Nicotiana tabacum*

2.1 INTRODUCTION

Late Embryogenesis Abundant Proteins are mostly found as a group of hydrophilic proteins. In plants, most of the LEA proteins accumulate at high concentrations in embryonic tissues during the last stages of seed development when desiccation occurs (J Ingram & Bartels 1996). As most seeds acquire the ability to withstand severe dehydration at this stage, LEA proteins have been associated with desiccation tolerance (Dure et al. 1981). Plants can be affected by different abiotic stresses such as drought, freezing and high salinity in the long run of their life cycle, which has a negative impact on their survival, which also impacts the agriculture industry. It has been found by statistical analyses that almost half of the total crop production is lost in every year due to abiotic stress such as drought (Boyer 1982; Vinocur & Altman 2005). Being exposed to the abiotic stresses, most of the plants exposed to reduced levels of water ultimately become accustomed to the extreme environment by having modified desiccation tolerant structures. To withstand desiccation, plants not only have modified pollen, seeds or spores but also have modified vegetative organs, like leaves and roots (Bray 1993; Blum 2013; J Ingram & Bartels 1996).

Abiotic stresses bring remarkable changes both externally and internally. Drought and high salinity usually resulted in increased level of ABA, which basically stimulates the

expression of stress-related genes (Raghavendra et al. 2010; Cramer et al. 2011). Abiotic stresses also produce extensive changes in gene regulation (gene activation/suppression) through signal transduction pathways ultimately controlling protein production profiles (Ahuja et al. 2010).

There are extensive physiological changes that take place upon desiccation. Consequently, synthesis of osmotically active molecules compatible with metabolism is required for plant survival. These osmotically active proteins result in effective adjustments in the intracellular compartment of cells solutes with low molecular weight. Several studies showed that among many osmoprotectants, trehalose (a nonreducing disaccharide) is mostly used in nature, while other disaccharides like sucrose, raffinose, stachyose, and cyclitols are accumulating in a large amount during seed maturation. These non-reducing sugars mostly act as a replacement of water molecules and also contribute to the formation of bioglasses as well as act as a vitrification agent to prevent the cell damage (Ahuja et al. 2010). It has been shown by several studies in transgenic *Arabidopsis thaliana* plants that LEA proteins presumed to play a very important protective role by enhancing tolerance to drought, freezing and salt stress (Liang et al. 2013; Amara et al. 2013).

Most of the LEA proteins from different groups accumulate during the embryonic development at the desiccated state and usually localize in different cytoplasmic organs such as the cytoplasm, nucleus, chloroplast, vacuole, nucleus, mitochondrion, endoplasmic reticulum, peroxisome and plasma membrane (Hoekstra et al. 2001). Both LEA proteins and their mRNAs accumulate in a high concentration in embryonic tissues at the last stage of development during desiccation (Hand et al. 2011; J Ingram & Bartels 1996).

LEA proteins have a wide variety of expression profiles and functions by correlating with stress resistance and many studies showed that during salt and osmotic stress, plants conferred protection by the involvement of LEA proteins (Hand et al. 2011; Shih et al. 2008; Tunnacliffe et al. 2010). Different scientists introduced heterologous LEA genes into microorganisms and plants to alter stress tolerance. Over-expression of LEA proteins has been documented through transgenic expression in different species like tobacco, Arabidopsis, rice, wheat, maize, lettuce or cabbage. Most of these organisms showed improved abiotic stress resistant phenotypes (Guo et al. 2013; Goday et al. 1994; Hanin et al. 2011; Delahaie et al. 2013; Wasilewska et al. 2008).

The introduction of a single LEA gene (LEA 1.1) in a model system might provide some systematic tool to understand the degree of stress tolerance. Tobacco (*Nicotiana tabacum*) is a model plant was transgenically expressed a single LEA 1.1 gene from a heterogeneous organism *Artemia franciscana* and observed significant improvement over control plants under various osmotic stresses which is resembling the result of Iturriaga et al. (1992). In the course of time, many different plants have been transgenically exposed with LEA genes from different organisms like transgenic rice (*Oryza sativa*) with HAV1 gene (LEAIII) which was successfully transferred and conferred resistance to drought or salinity resistance (Xu et al. 1996). Likewise, wheat (*Avena sativa* overexpressing the HAV1 gene showed improved desiccation tolerance, biomass productivity, and water efficiency under high salt, osmotic, or drought conditions via protection of the plasma membrane (Babu et al. 2004; Maqbool et al. 2002; Sivamani et al. 2000).

The study of NDong et al. (2002) showed that the accumulation of wheat (WCS19) LEAIII protein (a cold-regulated chloroplast LEA protein) in transgenic Arabidopsis has shown improved ion leakage, increased resistance to photoinhibition in leaves under freezing stress. This suggests that WCS19 proteins have the capacity of enhancing freezing tolerance (NDong et al. 2002). Another experiment conducted by Cheng et al. (2002) also showed that a LEA gene from wheat PMA1595 (LEAI) or PMA80 (LEAII), transgenically transferred into rice (*Oryza sativa*) was also associated with increased salt and drought stress tolerance (Cheng et al. 2002). Transgenic tobacco plant accumulated with hot pepper hydrophobic LEA V showed enhanced tolerance to dehydration and salt stress (Kim et al. 2005). Moreover, accumulation of LEA V protein in Arabidopsis showed early germination as well as better growth under salt and osmotic stress (Borrell et al. 2002; Hara et al. 2003).

Different LEA proteins from different sources were transgenically accumulated in different model plants (ex: Tobacco) under various stress conditions and the analyses of those transgenic plants demonstrate that LEA proteins have a presumptive protective role in dehydrating cells (Bartels 2005). However, the precise mechanistic molecular function of LEA proteins is still unclear but it has been suggested that LEA proteins have been working as stabilizers, hydration buffers, membrane protectants, antioxidants, ion chelators and bioglass formers (Honjoh et al. 1995).

2.2: MATERIALS AND METHODS

2.2.1: MATERIALS

All the chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Water for different experiments was purified with a Milli-Q Reagent System (Millipore, Billerica, MA) to an electrical resistance of 18 mΩ. All the rotors used for the ultracentrifugation were by Beckman Coulter™ and Fisher Scientific (accuSpin Micro 17).

2.2.2: METHODS

Cloning and transformation of LEA 1.1 gene in *Agrobacterium tumefaciens*

The original nucleic acid sequences for *Afrlea1.1* was cloned from *A.franciscana* (ABR67402) (Sharon et al. 2009) and the resulting cDNA was amplified with primers **(Table 2.1)** from the pTXB1 plasmid where the *Afrlea1.1* was subcloned.

Table 2.1: Primers for Gateway Cloning of *Afrlea1.1* gene.

Primers	Sequences
Forward	5`GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACC**ATGGAGAGCGAACAGGGT** 3`
Reverse	5`GGGGACCACTTTGTACAAGAAAGCTGGGTC**AGGCGGGAA GACGGCCCG** 3`

***Bold letters** are denoting portions of the gene sequence.

The primers were designed based on the instructions by Gateway® Technology (Invitrogen™ by Life Technologies) (See Appendix Gateway® Technology, Designing *att*B PCR Primers). The PCR reaction was followed by manufacturer instructions from Thermo Scientific (details in appendix Thermo Scientific Dream Taq green PCR Master Mix (2X)) **(Table 2.2)** as well as confirmed by using another protocol from *Pfx50*™ DNA Polymerase **(Table 2.3)** by Invitrogen Technology (see Appendix *Pfx50*™ DNA Polymerase). Then the PCR products were run on a 1.2% agarose gel at 120V for 1 hour. The thermal cycling conditions set to run the PCR using Dream Taq green PCR Master Mix (2X) were as follows: (Initial temperature: (94°C for the 5 mins) x 1X*, (Denaturation: 94°C for the 30s, Annealing: 55 °C for the 30s, Final extension: 72 °C for the 30s)x 30X* and 4 °C for ∝. Alternatively, the thermal cycling conditions using *Pfx50*™ DNA Polymerase protocol was as follows:; (Initial temperature: (94°C for the 2 mins) x 1X*, (Denaturation: 94°C for the 15s, Annealing: 65 °C for the 20s, Extension: 68 °C for the 45s Final extension: 68 °C for the 5 mins)x 30X* and 4 °C for ∝. **[X*denoting the number of times (Example: 30X* = 30 times)].**

Table 2.2: Composition of PCR reaction, run with Thermo Scientific Dream Taq Green PCR Master Mix for LEA1.1 gene.

Ingredients	Volume
dH_20	19 µL
2X Green Master Mix	25 µL
Primers (2 µM)	5 µL
DNA (10X/100X/1000X)*	1 µL(>50 ng)
Total volume	50 µL

*10X/100X/1000X denoted the dilution of original DNA samples which was 61 ng/ µL.

Table 2.3: Composition of PCR reaction, run with *Pfx50*™ DNA Polymerase for LEA1.1 gene.

Ingredients	Volume
10X PCR Mix	5 µL
10 mM dNTP Mix	1.5 µL
Primers Mix (2 µM)	1.5 µL
Template DNA (100X) *	2 µL (>50 ng)
Pfx50™ DNA Polymerase	1 µL
dH_20	39 µL
Total volume	50 µL

100X* denoted the dilution of original DNA samples which was 61 ng/ µL.

Eventually, the *att*B-flanked cDNA of *Afrlea1.1* was purified from the gel by PCR clean-up followed by instructions from NucleoSpin® Gel and PCR Clean-up by MACHERY-NAGEL (attached at Appendix PCR Cleanup and Gel Extraction). The concentration of product with *att*B-flanked cDNA of LEA1.1 was measured by using NanoDrop™ Lite Spectrophotometer from Thermo Scientific. The PCR Cloning system with Gateway® Technology includes a choice of donor vector, pDONR™/Zeo, which has M13 forward and reverse priming sites. The BP Recombination Reaction was performed by following the instructions of Gateway® Technology (**Figure 2.1**).

Gateway cloning system

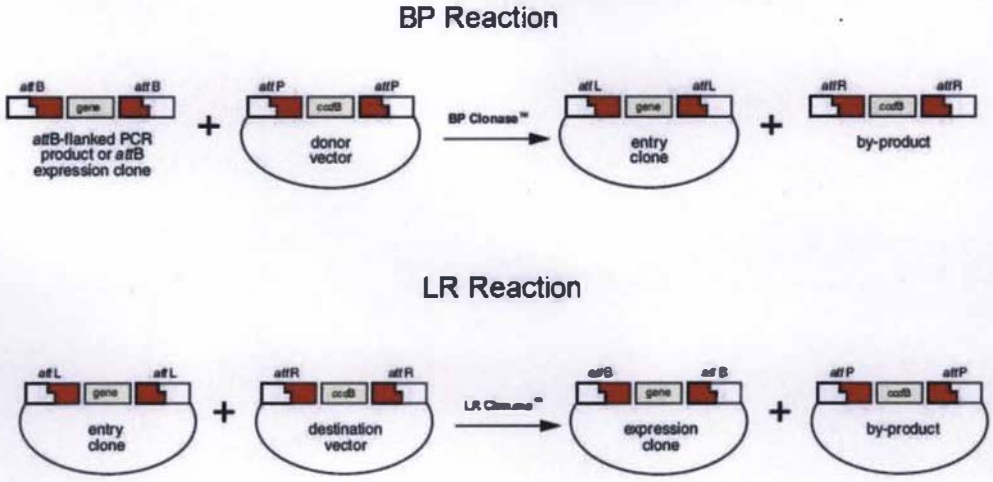


Figure 2.1: Gateway Cloning System by BP Reaction and LR Reaction into Destination Vector (adapted from Gateway® Technology, Invitrogen by Life technologies)

The BP Recombination Reaction was then transformed into One Shot® TOP10 Electrocompetent *E.coli* cells by electroporation by following the manufacturer instructions (see the Appendix Performing the BP Recombination Reaction). Then the BP reaction was plated onto prewarmed LB-agar plates containing 50µg/ml Zeocin. Two different volumes were spread on two plates to ensure well-spaced colonies. The plates were kept overnight at 28°C for more than 18 hours to produce sizable colonies. Isolated colonies were grown in liquid LB for overnight at 28°C with gentle shaking followed by plasmid purification using the manufacturer instructions of Thermo Scientific GeneJET Plasmid Miniprep Kit. Those plasmids contained the supercoiled *attL*-containing entry clone. The presence of the desired LEA1.1 gene was confirmed by PCR reaction by following same PCR protocol of Thermo Scientific Dream Taq Green PCR Master Mix.

After having entry clone containing the desired gene, it was set for LR Recombination Reaction in a supercoiled *att*R-containing destination vector, pPZP222.

The LR Recombination Reaction was performed by following the same instructions of Gateway® Technology. Then the resulting LR Recombination Reaction was transformed into One Shot® TOP10 Electrocompetent *E.coli* cells by electroporation using the manufacturer instructions of Gateway® Technology. As a positive control, pENTR™-gus plasmid was used, which was allowed to generate an expression clone containing the gene encoding β-glucuronidase (*gus*) (Kertbundit et al. 1991). Then the LR reaction was poured on pre warmed LB-agar plates having a zeocin antibiotic (50μg/ml) on it for the overnight growth at 28°C of transformed bacteria with expression clones. This time also Two different volume was used in two plates to ensure well-spaced colonies. After having the colonies in selected plates, isolated colonies were grown in liquid LB for overnight at 28°C with gentle shaking which was followed by plasmid purification by following the manufacturer instructions of Thermo Scientific GeneJET Plasmid Miniprep Kit. Then the presence of desired LEA1.1 gene was confirmed by PCR reaction by following same PCR protocol. Being confirmed of the insert in purified plasmids were sent for sequencing at the center of DNA Core Sequencing Facility, 1201 W. Gregory Drive, 334 ERML, Urbana, IL 61801. The insertion of desired LEA1.1 gene into the vector after LB and LR reactions were also confirmed by double restriction digestions using XhoI and SacI.

The plasmids with the desired insert confirmed with PCR, double restriction digestions and verified with DNA sequencing were selected for the transformation in *Agrobacterium tumefaciens* by following Manufacturer Instructions of Bio-Rad Gene

Pulser electroporation unit (Bio-Rad Laboratories, USA). For the *Agrobacterium tumefaciens* (AGL1) (Lazo et al. 1991) transformation three selective antibiotics (Rifampin, Carbenicillin, and Streptomycin) were used in a 50 mL LB agar plates. Plates were kept overnight at 28°C for growth. Then the following day after having colonies on the selective plates, isolated colonies were grown in 5 mL of LB media with selective antibiotics at 250 rpm, 28°C for 48 hours. After having growth on LB media, plasmids were purified by following the same plasmid purification protocol. Then the confirmation of insert was also confirmed by PCR and double restriction digestion by following same protocol used for LB and LR reactions of Gateway® Technology.

Transformation into tobacco (*Nicotiana tabaccum*):

The AGLI bacterium with the desired gene was grown in LB medium in three different ways. AGL1, which was transformed with p35S-LEA1.1 plasmid was cultured on 5ml of LB with three antibiotics (Rifampin, Carbenicillin, and Streptomycin). Antibiotics were used at 200mg/L, 100mg/L and 100mg/L for Cefotaxime, Streptomycin, and Carbenicillin respectively. As a positive control, AGL1 was used which was transformed with p35S-GUS and cultured on 5 ml of LB with same set up of antibiotics. As a negative control, AGLI alone was used. For the negative control, no streptomycin was used. All the growth cultures were incubated at 28°C with 228 rpm for 48 hours.

Preparation of MS media was done by following the content of **Table 2.4**. To make one liter of MS media firstly 4.44g of MS media with B5 Vitamins was added with 30g of sucrose along with BAP and NAA. The volume was made up to 1 liter and pH was adjusted

pH 5.8. Then 8g of agar was added and autoclaved for 20 mins then the media cooled to 55-60°C and selective antibiotics (gentamicin, cefotaxime) added, mixed and poured on Petri plates. The process was done inside an under laminar flow hood

For the transformation, tobacco plant (*Nicotiana tabaccum*), SR1 variety (Streptomycin resistance) was used. Tobacco leaves were cut into small pieces at around 0.6-0.8 cm by avoiding the midrib and dipped into 25 ml MS medium (**(Table 2.4)** separately. The change of MS medium was done three times. All washed leaves were transferred into Petri plates (15x150mm) containing MS medium without antibiotics and were kept in the dark growth chamber to check any contamination for 48 hours at 25°C. Leaves were placed upside down and at approximately 8-10 disks per regeneration plate. The transformation process was done following the protocol from Plant Transformation Core Facility, the University of Missouri (https://plantsciences.missouri.edu/muptcf/protocols/tobacco.html).

Next, sliced leaves were transferred in Petri plates made with MS agar medium by using streptomycin (50µg/ml) and grown in a growth chamber under continuous light at 25°C. It was kept for 30 days until callus formation. Each callus was transferred into the magenta box (Magenta™ vessel, Sigma-Aldrich) with MS agar medium with antibiotics gentamicin (100 mg/L) and cefotaxime (500 mg/L) as a first transfer. After having the growth of the plants in presence of gentamicin and cefotaxime antibiotics, second and third propagation was done in presence of cefotaxime (500 mg/L) and cefotaxime (200 mg/L) respectively and without gentamicin.

Table 2.4: Media recipe for Tobacco (*Nicotiana tabacum*) (Murashige and Skoog (MS) medium) per liter:

Ingredients	Amount (per liter)
MS media with B5 Vitamins	4.44 g
Sucrose	30 g
BAP (6-Benzylaminopurine) (10mM)	888 µl
NAA (Naphthalene acetic acid) (10mM)	108 µl
Agar	8 g
Gentamicin (100mg/L)	100 mg
Cefotaxime (500mg/L)	500 mg

The transformation of *gus* in tobacco plants was confirmed by using GUS testing solution. Typically GUS activity in solution is determined with the fluorogenic substrate 4-methyl-umbelliferyl β-D-glucuronide (MUG)

MUG (non-fluorescent) + GUS ⟹ glucuronic acid + 4MU (fluorescent)

The reaction product 4-methylumbelliferone (4MU) is maximally fluorescent at high pH, where the hydroxyl group is ionized. The addition of a basic solution of sodium carbonate simultaneously stops the assay, so for the continuation, the reaction product was kept on 70% ethanol. A small part of leaves was dipped into the solution and centrifuged at maximum speed for 1 min. Then it was kept overnight in the solution and the following day the solution was exchanged with 70% ethanol for several times and the changing was

followed by once in a day for a couple of days until leaves turned into visible blue and complete disappearance of green color observed.

Profile of Transgenic Expression

After the third propagation, being confirmed with the growth of the transgenic plants, fresh leaves were collected from the plants for DNA extraction. The DNA extraction was done by following manufacturer instructions from Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit. All the extracted DNA from transgenic LEA plant, transgenic GUS plants, and non-transgenic plants (SR1) were tested for the presence of virulence gene as well as for gentamicin resistance gene by using respective primers (**Table 2.5**). The same set of primers also applied for AGL1 plasmids too.

Table 2.5: Primers for Gentamicin Resistance gene.

Primers	Sequences
GmF	5`CAA CGA TGT TAC GCA GCA GG 3`
GmR	5`CAA CAA CCG CTT CTT GGT CG 3`

In addition to confirming the presence of the gene in the DNA, RNA extraction was also performed by following the manufacturer instructions from Thermo Scientific GeneJET Plant RNA Purification, Mini Kit. RNA was also extracted from all transgenic lines and control plants. The cDNA library was then created following manufacturer instructions of Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. Then RT-PCR was done

to confirm expression of both LEA and Ubiquitin gene expression from cDNA by using specific primers **(Table 2.6)** in transgenic *Nicotiana tabacum.*

Table 2.6: Primers for LEA and Ubiquitin specific gene.

Primers	Sequences
Exprlea1.1	**Forward** 5`GGT CAT GAA GGG TAC GTG GA 3` **Reverse** 5`GAG CTG CTC GGC TCT CTT TT 3`
NtUbi	**Forward** 5` AGC TGA GGG GAG GAA TG 3` **Reverse** 5`GCA ACC TAG AAA CCA CC 3`

Growth conditions and Stress Treatment

Tobacco plants, either transgenic with LEA1.1 or GUS, were grown in a growth chamber under continuous light at 25°C. All the data presented here are from plants grown in the growth chamber unless stated otherwise. The plants were grown in a closed magenta box to prevent evaporation. To improve the drought stress Polyethylene Glycols PEG (MW 10,000) was used with the nutrient solution (MS medium without antibiotics). PEG 10,000 was too big to be taken up by the intact roots (LAWLOR 1970) and provide the imposition of the uniform and controllable drought stress. The experiments were started with 4-weeks old tobacco plants. The experiment was designed in two ways. For one set of treatment, all the fresh transgenic tobacco plants were treated with 10% of PEG initially. The same set of plants were untreated for comparison. Another set of experiments was designed by growing the plants without PEG treatment then, after 15 days, drought stress was imposed

on the treated groups by adding PEG 10,000 to a final concentration of 5% and again after 15 days drought stress further increased in the treated groups by adding PEG 10,000 to a final concentration of 10%. It was seen that subjection to PEG up to 10%, caused reversible wilting of the plants. Plants growth were followed by measuring the height of the stem. The fresh weight of different parts of the plants (root, leaf, and stem) was recorded. To have the dry weight, firstly different parts of plants were dried at 37°C for 5 days and then dried at 80°C for 48 hours in an oven (Despatch LBB Series Owner's Manual, Protocol Plus™).

Total Chloroform measurement

Fresh leaves were taken from both transgenic LEA plants and GUS plants for chlorophyll measurement. Filter paper (9.0 cm, Whatman International Ltd. Maidstone, England) was used in 100mm x15mm Petri Plates and soaked with MS media. Freshly cut leaves were kept in Petri plates by putting upside down. Two exact set of Petri Plates were made. One set kept on 25°C by covering with aluminum foil and another set kept on 4°C by covering with aluminum foil too. After 48 hours both sets transferred into -80°C until chlorophyll extraction. ~100mg of fresh weight of leaf tissue was measured and homogenized with 500 µl of 80% (v/v) acetone in a 1.5ml microfuge tube. For homogenization, a blue plastic homogenizer and quartz sand were used. Then it was centrifuged at maximum speed for 5 mins. It was repeated for three times and then all the supernatant were collected very carefully in a separate 1.5 ml microfuge tube. It was adjusted up to 1.5 ml with 80% acetone and centrifuged again for 5 mins with maximum speed after that 1.0 ml of supernatant was taken very carefully in a spec-20 cuvette and in another cuvette 1.0 ml of 80 % acetone

was taken to set the blank at 660 nm and the absorbance of clear pigments extract was taken, if the absorbance was greater than 0.4, diluted with 80% acetone to keep the absorbance in between 0.3-0.4.

The absorbance of the chlorophyll extract was taken at 645nm and 663nm (Amon 1949). The process of calculation of chlorophyll extract by Arnon (1949) was also shown by the study of (Richardson et al. 2002). Arnon (1949) equations of calculations were as follows.

In a mg of Chl a/ml solution in cuvette $= 0.0127\ (A663) - 0.00269\ (A645)$

In a mg of Chl b/ml solution in cuvette $= 0.0029\ (A663) - 0.00468\ (A645)$

In a mg of total Chl/ml solution in cuvette $= 0.0202\ (A663) + 0.00802\ (A645)$.

Statistical Analysis:

For statistical analysis, One-way ANOVA with Holm-Sidak posthoc test and Microsoft excel 2016 was used. Statistical significance value was taken, $p<0.05$

2.3: RESULTS

Confirmation of the presence of *Afrlea1.1* in pTXB1 plasmid by Gateway Primers

LEA 1.1 gene cloned from *A. franciscana* was subcloned in pTXB1 plasmid and before the Gateway Cloning it was confirmed by using the gateway primers (**Table 2.1**) and general PCR was done by following two different PCR protocol for the confirmation of the gene. It was previously published that the size of LEA1.1 gene is around 495 bps and the product found after 1.2 % agarose gel run confirming exactly the same size (Figure 3.2). This is the gel image of PCR by Dream Taq Green Master Mix and the gel image of PCR by *Pfx50*™ DNA Polymerase is attached in Appendix (**APPENDIX A**). Then the *att*B-PCR product was extracted by PCR Clean-up process and the plasmid DNA concentration after PCR clean-up was about 13.1 ng/µl.

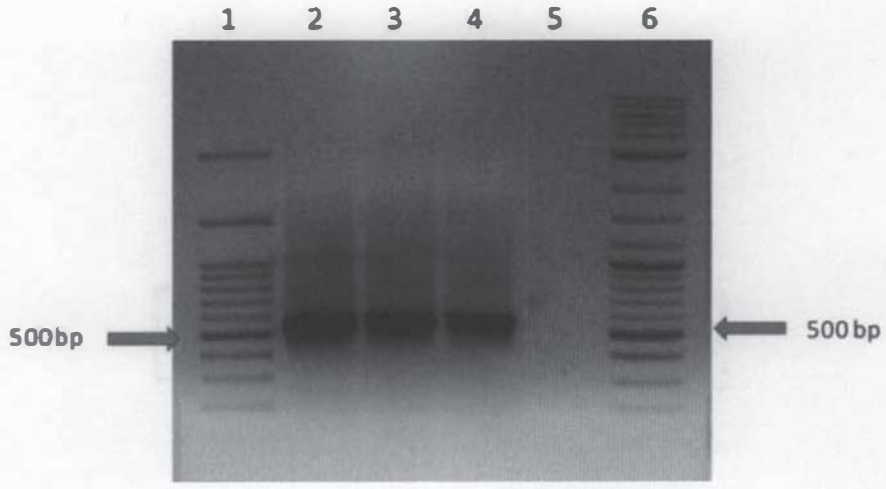


Figure 2.2: PCR products of LEA1.1(540 bps) from the pTXB1 plasmid by Gateway Primers by using Dream Taq Green Master Mix protocol from Thermo Scientific. **Lane 1**: 100bp plus DNA ladder, **Lane 2-4**: amplification of LEA1.1 from pTXB1 plasmid after 10,

100 and 1000 times dilution of original plasmid concentration, **Lane 5**: empty plasmid as a negative control, **Lane 6**: 1 Kb plus DNA ladder. All three plasmids contain LEA 1.1 DNA

Gateway Cloning of LEA1.1 gene into Destination Vector, pPZP222-S.

Destination Vector

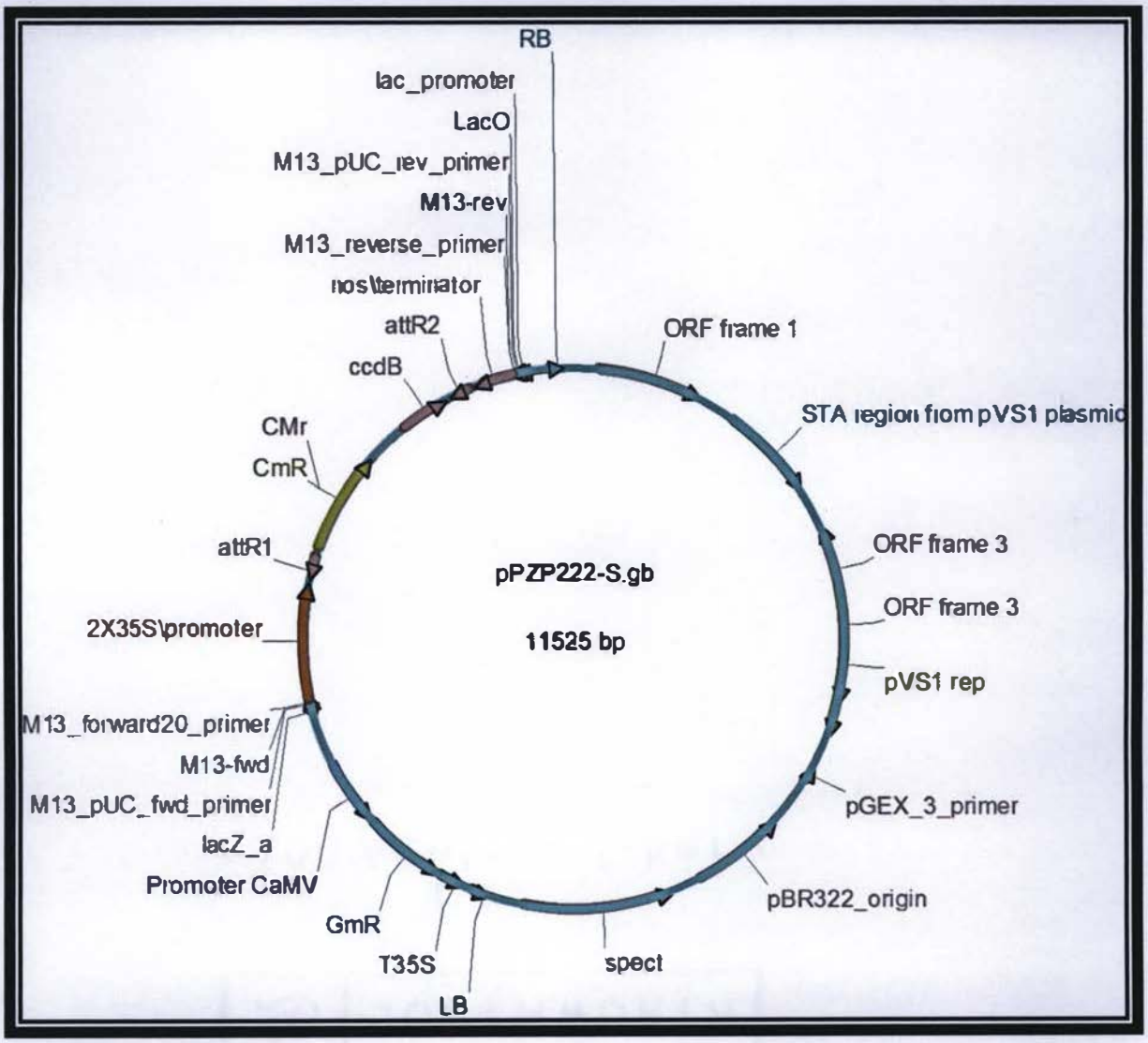


Figure 2.3: Map of Destination Vector pPZP222-S.

The *att*B-PCR product was used for BP Recombination Reaction and successfully transformed into Entry Vector (pDONR™/Zeo). Then BP Reaction was plated into pre-warmed MS-agar Petri Plates with zeocin antibiotics and distinct colonies were found after 24 hours incubation at 28°C. Extracted plasmid from individual colonies was set for PCR Reaction and confirmed the presence of insert with the *att*L site by Agarose Gel Electrophoresis.

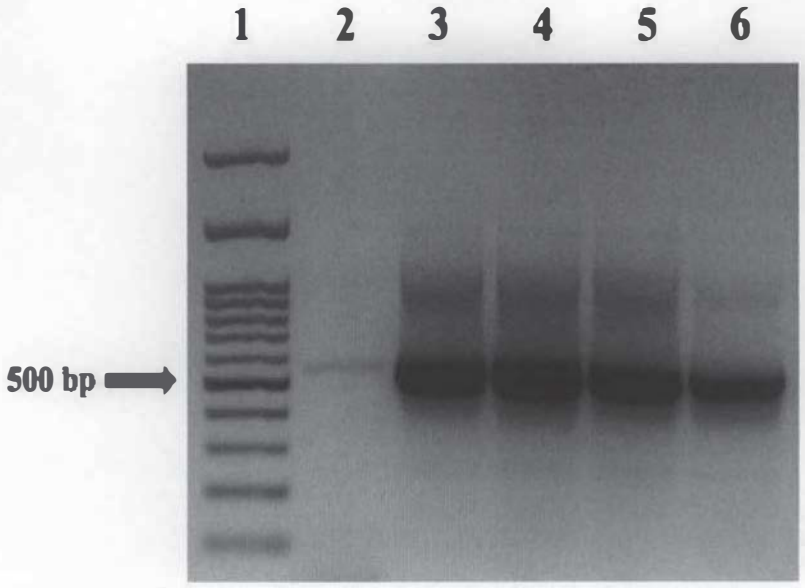


Figure 2.4: Confirmation of PCR products of LEA1.1(540 bps) after BP Recombination Reaction of Gateway Cloning by using Dream Taq Green Master Mix protocol from Thermo Scientific. **Lane 1**: 100bp plus DNA ladder, **Lane 2**: Water as a negative control, **Lane 3-6**: amplification of LEA1.1 from BP Recombination Reaction (4 distinct plasmids from four distinct colonies). All four plasmids contain LEA1.1 DNA insert with the *att*L site.

The *att*L-PCR product was used for the LR Recombination Reaction and successfully transformed into Destination Vector (pPZP222-S). Then LR Reaction was plated into pre-warmed MS-agar Petri Plates with streptomycin antibiotics and distinct colonies were found after 24 hours incubation at 28°C. Extracted plasmid from individual colonies was amplified by PCR and run on an agarose gel to confirm the presence of the insert.

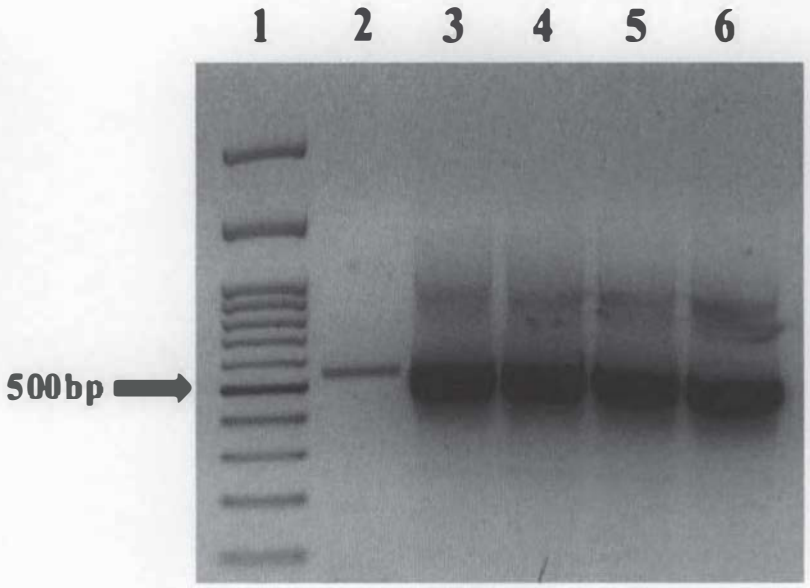


Figure 2.5: Confirmation of PCR products of LEA1.1(540 bps) after LR Recombination Reaction of Gateway Cloning by using Dream Taq Green Master Mix protocol from Thermo Scientific. **Lane 1**: 100bp plus DNA ladder, **Lane 2**: Water as a negative control, **Lane 3-6**: amplification of LEA1.1 from LR Recombination Reaction (4 distinct plasmids from four distinct colonies). All four plasmids contain LEA1.1 DNA.

After purification of plasmids from the LR Recombination Reaction, plasmids were restriction digested with EcoRI and HindIII set. The reaction took place at 37°C for 30 mins

and followed by 80°C for 5 mins. All the purified plasmids were expected to show bands at around 1100 bps, which was observed **(Figure 2.6)**.

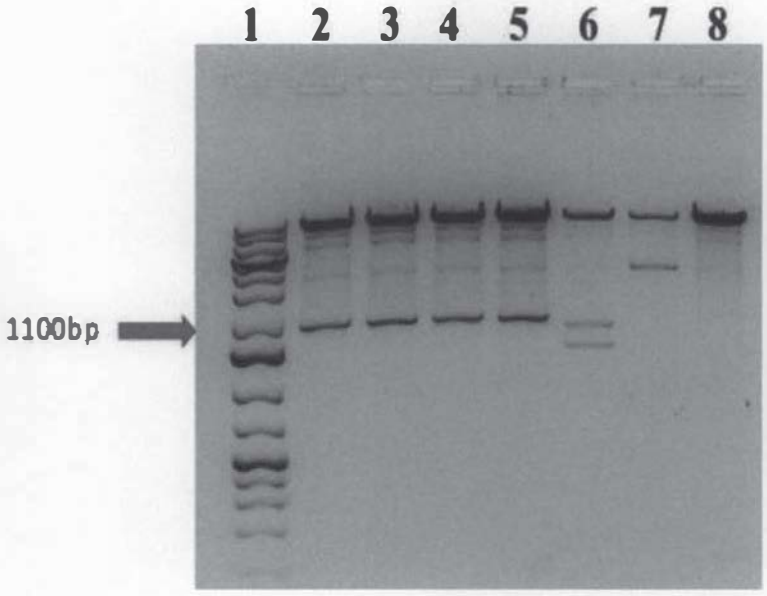


Figure 2.6: Double Restriction Digestion by EcoRI and HindIII of four purified plasmids after transformation of LR Recombination Reaction into the destination vector. **Lane 1**: IKb plus DNA ladder. **Lane 2-5:** Isolated plasmids after transformation of LR Recombination Reaction. **Lane 6:** p2Z2-S Vector as a negative control. **Lane 7:** p2Z2-GUS Vector as a negative control **Lane 8:** pPBZ222 Vector as a negative control. All four plasmids contain LEA1.1 DNA.

Transformation into *Agrobacterium tumefaciens* and the confirmation of insertion of *Afrlea1.1*

Confirmation of insert by PCR;

Transformation of purified plasmids after LR Recombination Reaction into the AGL1 Vector was confirmed by PCR reaction with the exact reaction set of Table 8 except the 35 reaction cycles instead of 30 reaction cycles. Out of four purified plasmids, only culture #2 did fail to show a positive result.

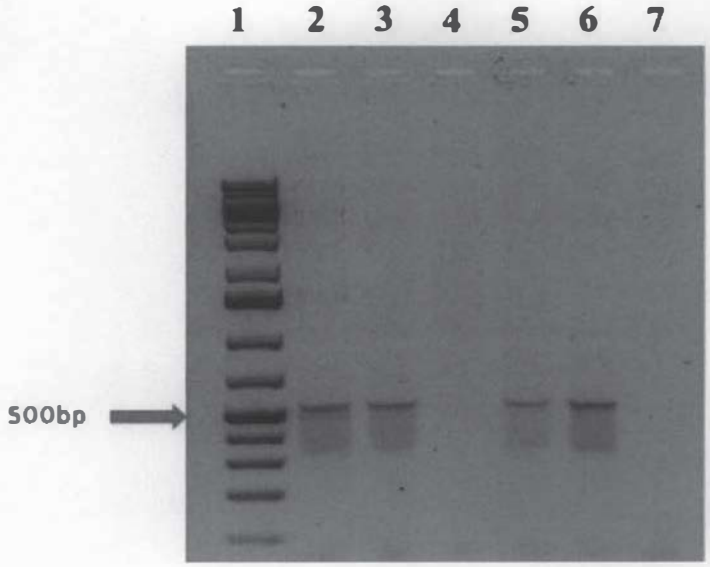


Figure 2.7: Confirmation of PCR products of LEA1.1(540 bps) after LR Recombination Reaction of Gateway Cloning by using Dream Taq Green Master Mix protocol from Thermo Scientific. **Lane 1**: 1Kb Plus DNA ladder, **Lane 2-5**: four different purified AGL1 plasmids, **Lane 6**: purified plasmid after transformation of LR Recombination Reaction, **Lane 7**: p2Z2-S Vector as a negative control. All four plasmids contain LEA1.1 DNA as indicated by agarose gel electrophoresis.

Confirmation of insert by Double Restriction Digestion (EcoRI and HindIII);

After purification of plasmids from AGL1 were set for double restriction digestion with EcoRI and HindIII set The reaction took place at 37°C for 30 mins and followed by 80°C for 5 mins. All the purified plasmids were showing bands at around 1100 bps **(Figure 2.8)**. The confirmation was also checked by the set of restriction endonucleases XhoI and SacI **(APPENDIX B)**.

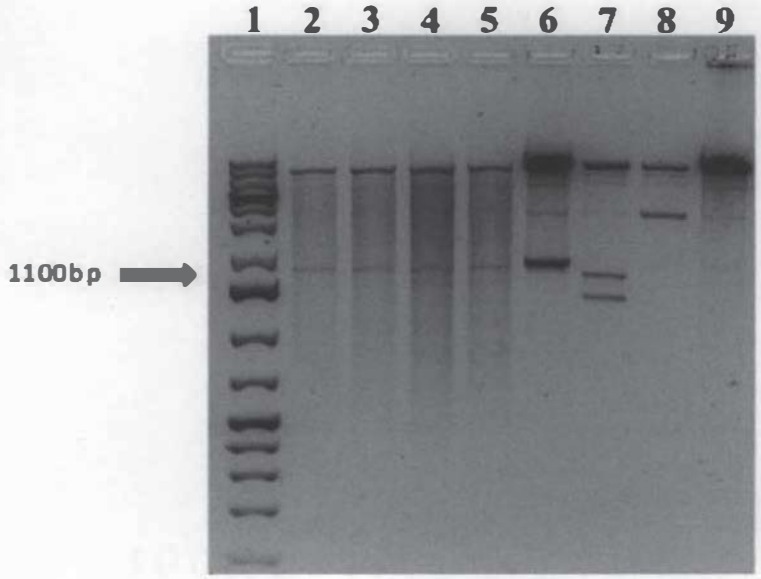


Figure 2.8: Double Restriction Digestion by EcoRI and HindIII of four purified plasmids from AGL1 after transformation. **Lane 1**: IKb plus DNA ladder. **Lane 2-5:** Isolated plasmids after transformation in AGL1. **Lane 6**: purified plasmid after transformation of LR Recombination Reaction, **Lane 7:** p2Z2-S Vector as a negative control. **Lane 8:** p2Z2-GUS Vector as a negative control **Lane 9:** pPBZ222 Vector as a negative control. All four plasmids contain LEA1.1 DNA.

Transformation of LEA1.1 Gene in Tobacco (*Nicotiana tabacum*):

After the confirmation of the insertion of LEA1.1 gene in the AGL1 bacterium, it was set for the transformation in the tobacco plant. For the transformation, SR1 (Streptomycin Resistance 1) variety of *Nicotiana tabacum* was used. By following the transformation protocol.

Table 2.7: Total number of regeneration, rooting, and shooting of transgenic tobacco plants transformed with *Afrleal.1* and *gus*.

Sample	Explants	Regeneration	Roots & Shoots	PCR*
LEA 1.1	35	24 (69%)	9 (26%)	**5/7**
GUS	30	19 (63%)	11 (37%)	**3/3**

***In PCR, LEA1.1 was found 5 positives out of 7 and GUS was found 3 positives out of 3.**

A total of 35 transgenic LEA1.1 and 30 transgenic GUS explants were transferred into Petri plates with MS-agar for the regeneration. For LEA 1.1 24, 69% formed a callus and 63% were GUS positive **(Table 2.7).** The regenerated plants were then transferred to the small magenta box with MS-agar medium containing gentamicin and cefotaxime antibiotics to check the root and shoot formation. After 45 days it was found 9 (26%) of the LEA1.1 plants and 37% of the 11 GUS plants rooted and produced shoots. After that, the transgenic LEA 1.1 and GUS plants were propagated for three times to check the bacterial contamination. Gradually, the number of antibiotics were reduced and the final antibiotic used was only cefotaxime at 200 mg/L.

Confirmation of the insert through genomic DNA extraction:

The genomic DNA was extracted from the fresh leaves of transgenic LEA and GUS plants to check the presence of insert and any check for bacterial contamination by following the manufacturer instructions of GeneJet Plant Genomic DNA Purification Mini Kit of Invitrogen life technologies. The concentration of DNA was found in between (25-100)ng/μL. The presence of gus gene was done using gus leaf testing solution. The purity of DNA was checked by checking the presence of the virulence-resistance gene and gentamicin resistance gene.

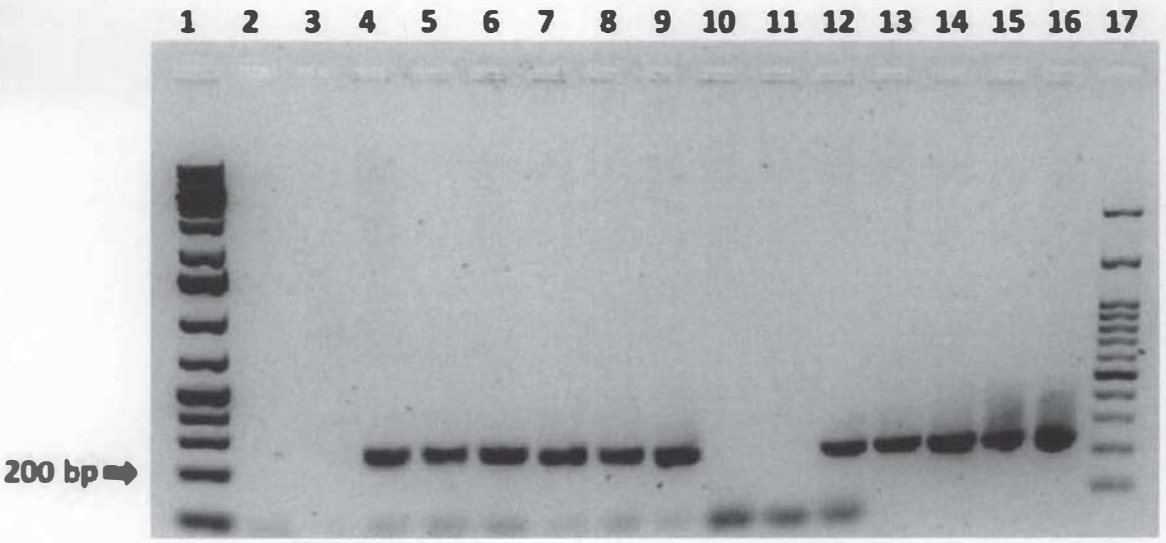


Figure 2.9: Confirmation of PCR products of gentamicin resistance gene in the genomic DNA of transgenic plants. **Lane 1**: 1Kb Plus DNA ladder, **Lane 2**: water blank as a negative control. **Lane 3**: SR1 plants DNA as a negative control. **Lane 4-10**: Seven genomic DNA from transgenic LEA plants. **Lane 11-13:** Three genomic DNA from transgenic GUS plants. **Lane 14-16**: Purified plasmids from AGL1, AgL2, and AgL4 respectively as a positive control. **Lane 17:** 100bp Plus DNA ladder.

All the transgenic lines including LEA 1.1 and GUS along with control SR1 were tested for the virulence gene to check the bacterial contamination. Virulence gene is only present in bacterial cells so the transgenic should be negative for this gene. Figure 2.9 shows no virulence gene PCR band using DNA from transgenics LEA1.1, GUS, and SR1 indicating the absence of bacterial contamination.

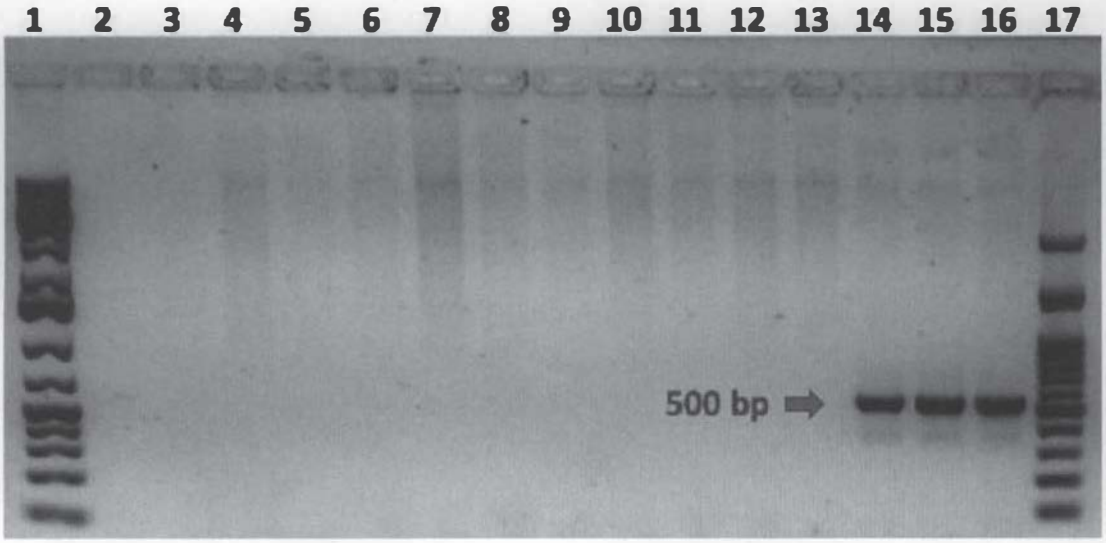


Figure 2.10: Confirmation of PCR products of Virulence resistance gene in the genomic DNA of transgenic plants. **Lane 1**: 1Kb Plus DNA ladder, **Lane 2**: water blank as a negative control. **Lane 3**: SR1 plants DNA as a negative control. **Lane 4-10**: Seven genomic DNA from transgenic LEA plants. **Lane 11-13:** Three genomic DNA from transgenic GUS plants. **Lane 14-16**: Purified plasmids from AGL1, AgL2, and AgL4 respectively as a positive control. **Lane 17:** 100bp Plus DNA ladder.

Finally, all the extracted DNA from both LEA1.1, GUS, and SR1 were tested for the presence of the LEA1.1 gene with LEA1.1 specific primers (**Table 2.6**). Out of 7 LEA1.1 lines, 6 show bands and number 1 showed very light band. In contrast, GUS and SR1 genes were not detected. As a positive control, all the AGL1 extracted plasmids showed predicted bands which are the indication of the presence of the LEA1.1 specific gene in the extracted DNA from transgenic LEA 1.1 plants.

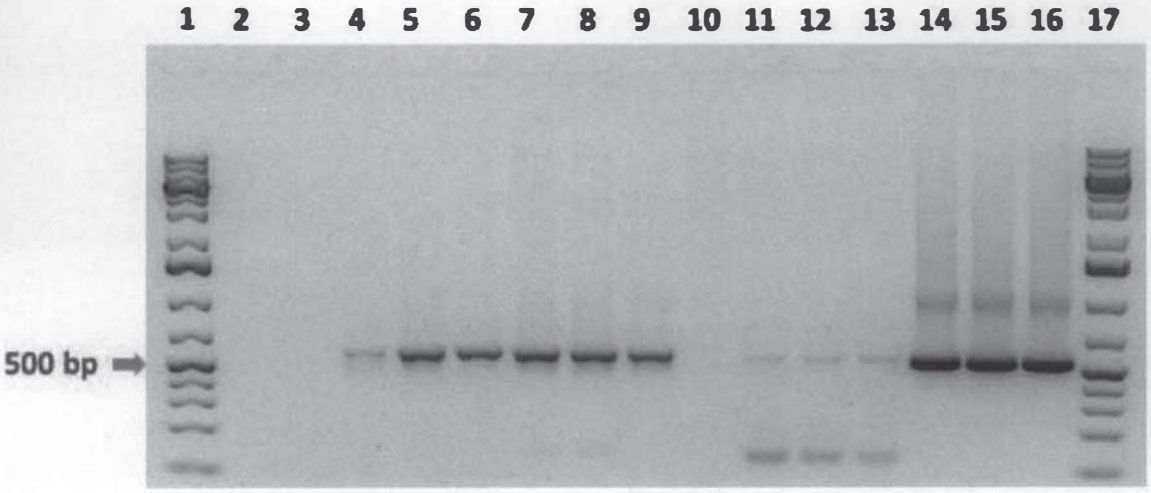


Figure 2.11: Confirmation of PCR products of *Afrleal.1* and *gus* gene in the genomic DNA of transgenic plants. **Lane 1**: 1Kb Plus DNA ladder, **Lane 2**: water blank as a negative control. **Lane 3**: SR1 plants DNA as a negative control. **Lane 4-10**: Seven genomic DNA from transgenic LEA plants. **Lane 11-13:** Three genomic DNA from transgenic GUS plants. **Lane 14-16**: Purified plasmids from AGL1, AgL2, and AgL4 respectively as a positive control. **Lane 17:** 100bp Plus DNA ladder.

RNA extraction and Over-expression (OX) profiling by RT-PCR:

RNA was extracted from all transgenic cell lines, cDNA generated and tested using RT-PCR followed by gel electrophoresis. Out of 7 cell lines, 5 were found to be positive for LEA1.a expression **(Figure 2.12, top).** Ubiquitin gene expression was used as a control **(Figure 2.12, bottom)**. Respective primers are listed in **Table 2.6**.

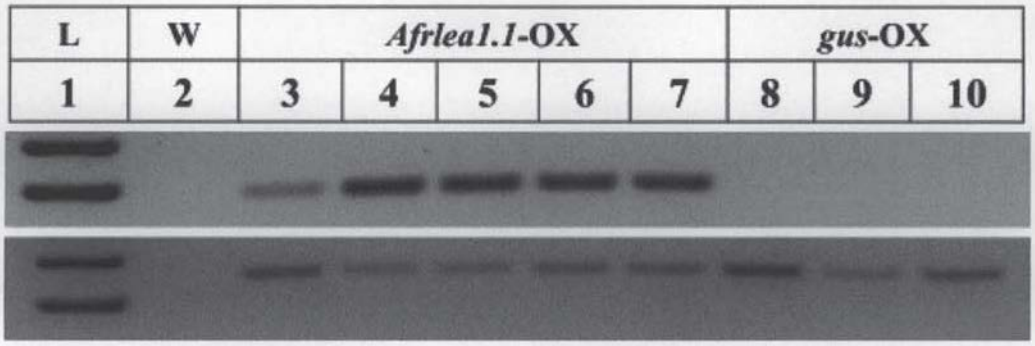


Figure 2.12: Overexpression of LEA1.1 and ubiquitin in all transgenic cell lines. **Lane 1**: 1 Kb Plus DNA ladder, **Lane 2**: Water as a negative control. **Lane 3-7**: Transgenic LEA1.1 cell lines from 3 to 7 respectively. **Lane 8-10**: Transgenic GUS cell lines from 1 to 3 respectively.

Transformation of *gus* in *Nicotiana tabacum:*

All the gus transformed tobacco was treated with a gus solution and out of 6 transformed plants, 4 showed positive in the gus solution (**Fig 2.13**).

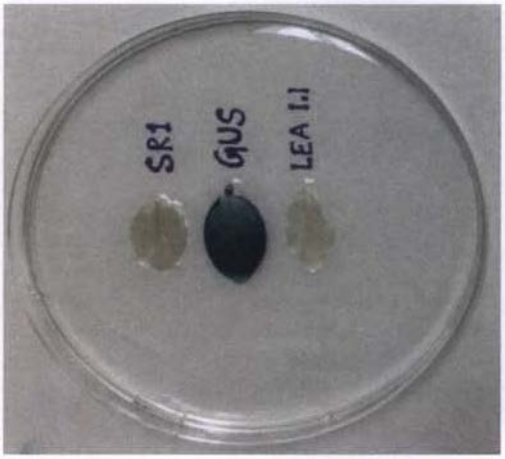


Figure 2.13: Confirmation of transformation of *gus* into tobacco. The left leaf is SR1 variety as a negative control and the middle one is transgenic gus leaf and the right one is a transgenic LEA1.1 leaf as a negative control.

Growth Performance of Transgenic Plants:

The growth performance was investigated for both LEA and GUS transgenic tobacco plants under PEG 10,000-induced drought stress. There were not any significant differences observed between stressed and non-stressed plants for both control and transgenic (**Fig 2.14**). Both LEA and GUS plants were grown almost at the same rate under normal condition measured after 15 days after that both GUS and LEA plants were stressed by 5 % and 10 % PEG 10,000 for 15 days consecutively and there was not a significant difference in height. In comparison to the 5% and 10% PEG 10,000 treatment, there was more growth noted under 5 % PEG treatment, but not at a significant rate (**Fig 2.14**).

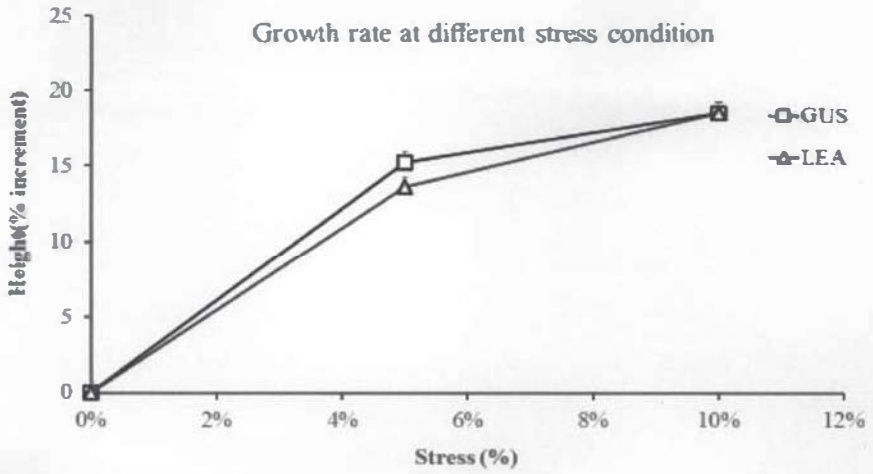


Figure 2.14: Growth rates (%) of transgenic LEA and GUS tobacco plants under stressed by 5% and 10% PEG 10,000. Firstly all LEA and GUS transgenic plants were grown at 0% stressed condition for 15 days then stressed with 5% PEG for 15 days and then with 10% PEG for 15 days more. The growth rate represents as a % increment of height under 5% and 10% PEG treatment. The result was an average of 20 LEA plants and 12 GUS plants and also showing as an average±SE.

The growth performance was also determined by comparing fresh weight and dry weight of different parts of transgenic LEA and GUS plants. The differences were significantly pronounced for transgenic LEA plants compared to the GUS plants in a comparison of total weight both in fresh weight ($p=0.0461$, $p<0.05$) and dry weight ($p=0.0342$, $p<0.05$) (**Figure 2.15 and 2.17**). The significance observed the difference in weight between stressed LEA and GUS plants was especially pronounced for the roots. There is significant growth of roots for transgenic LEA plants ($p=0.033$, $p<0.05$) compared to GUS plants ($p=0.028$, $p<0.05$) both in fresh weight and dry weight under stressed condition (**Figure 2.16, 2.18 and 2.19**).

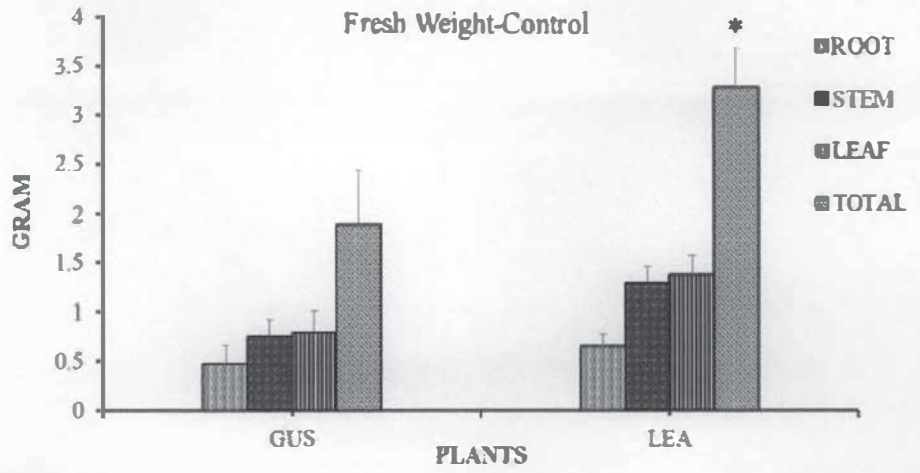


Figure 2.15: Fresh weight of transgenic LEA and GUS tobacco plants grown in a growth chamber under low continuous light at 25°C. These plants were without drought stress. Values are averages±SE of 12 plants for GUS and 24 for LEA plants.

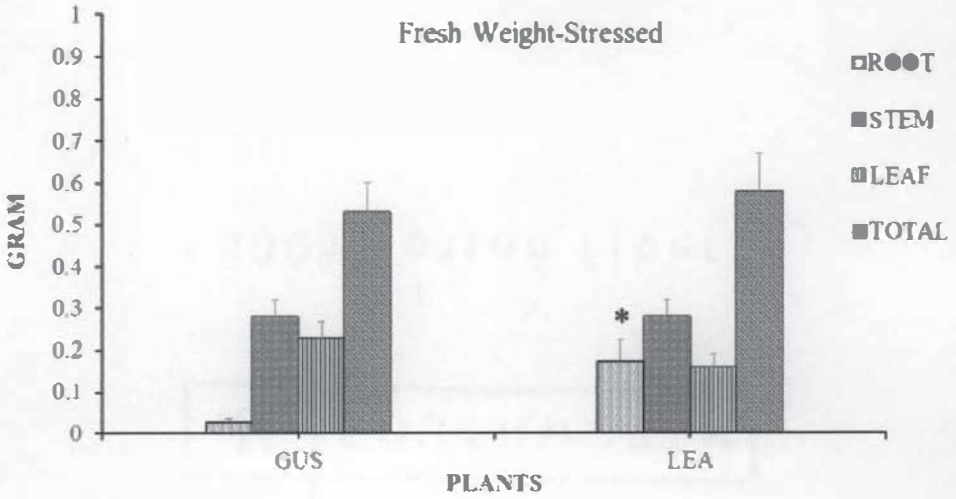


Figure 2.16: Fresh weight of transgenic LEA and GUS tobacco plants grown in a growth chamber under low continuous light at 25°C. These plants were in drought stress. Values are averages±SE of 12 plants for GUS and 24 for LEA plants.

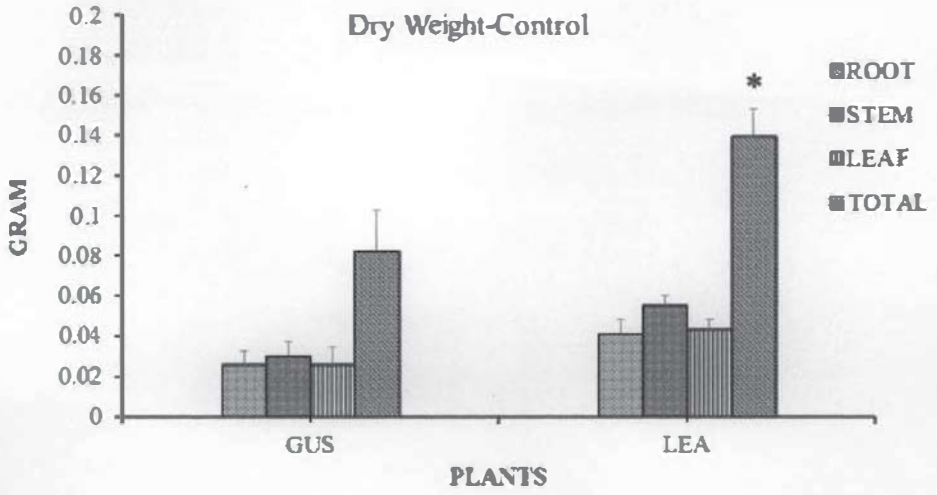


Figure 2.17: Dry weight of transgenic LEA and GUS tobacco plants grown in a growth chamber under low continuous light at 25°C. These plants were without drought stress. Values are averages±SE of 12 plants for GUS and 24 for LEA plants.

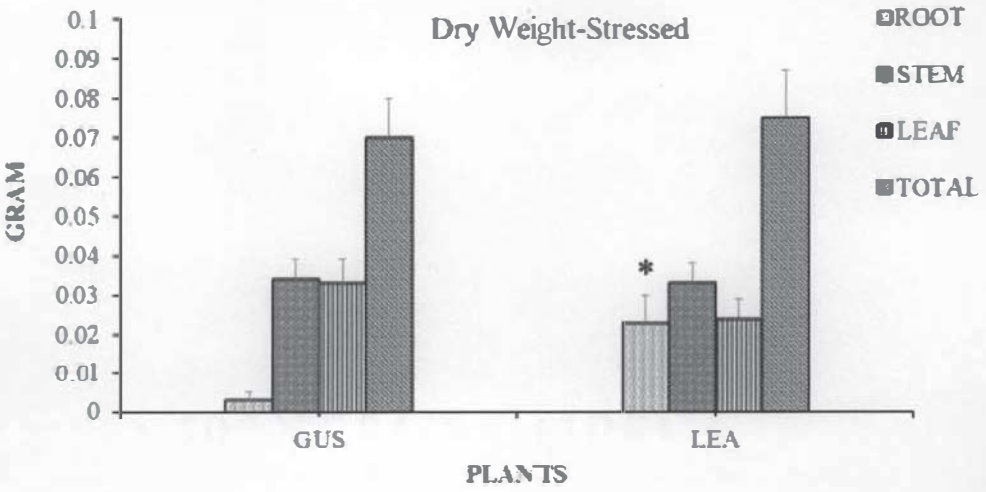


Figure 2.18: Dry weight of transgenic LEA and GUS tobacco plants grown in a growth chamber under low continuous light at 25°C. These plants in drought stress. Values are averages±SE of 12 plants for GUS and 24 for LEA plants.

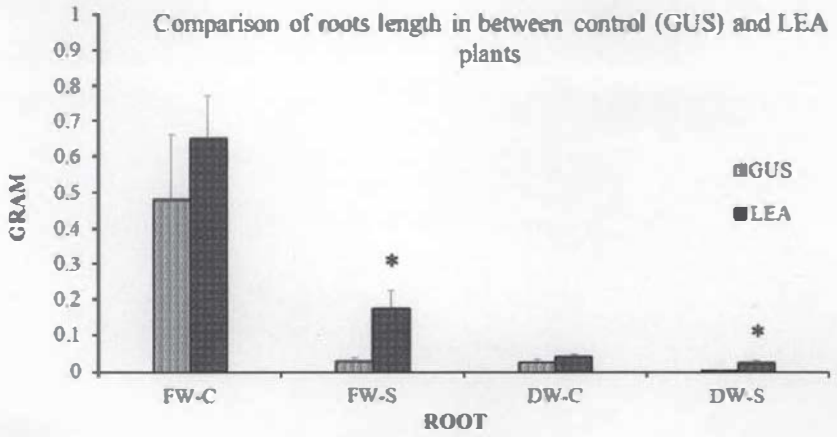


Figure 2.19: Comparison of roots weight for both transgenic LEA and GUS tobacco plants grown in a growth chamber under low continuous light at 25°C. FW-C (Fresh Weight-Control) FW-S (Fresh Weight-Stressed) DW-C (Dry Weight-Control) DW-S (Dry Weight-Stressed). Values are averages±SE of 12 plants for GUS and 24 for LEA plants.

Chlorophyll measurement in Transgenic Plants

To investigate the low-temperature tolerance of the transgenic plants, the tobacco plants leaves were treated at 4°C and 25°C for 24h and were transferred to normal conditions and then kept on -80°C for storage till the measurement of chlorophyll content. As shown in **Figure 2.20** that the transgenic LEA plants have significantly more chlorophyll content than the control GUS plants. Chlorophyll content can be used to estimate the degree of the leaf senescence. Under normal conditions, there is no significant difference in chlorophyll level ($p=0.09$, $p<0.05$), but the chlorophyll content was significantly reduced in the control plants ($p=0.0003$, $p<0.05$) compared with the transgenic LEA plants.

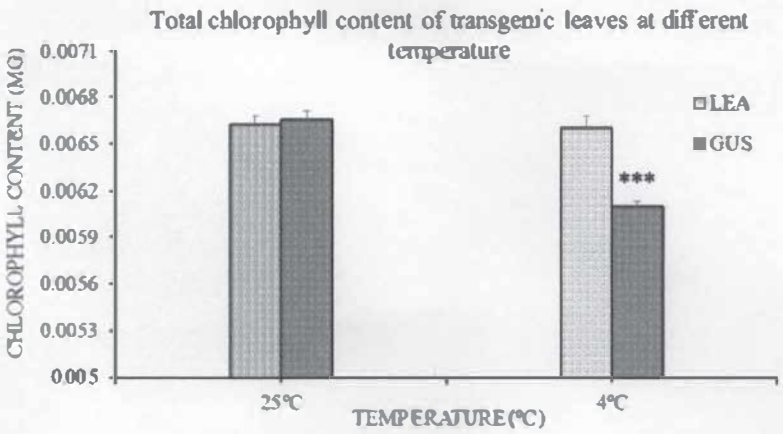


Figure 2.20: Measurement of total chlorophyll content in transgenic leaves at two different temperature (4°C and 25°C) for both transgenic LEA and GUS plants. Values are averages±SE for 3 GUS and 6 LEA plants.

2.4 DISCUSSION

LEA proteins are highly hydrophilic are speculated to retain water molecules and protect other proteins from desiccation (Tunnacliffe & Wise 2007). *Afrlea1.1* was successfully transgenically introduced into a model system to measure the degree of stress tolerance. As a model system, Tobacco (*Nicotiana tabacum* SR1 variety) was selected as it was previously used to express three different groups of LEA genes from *C.plantagineum* (Iturriaga et al. 1992). The SR1 variety was used because it has a gene coding for the secreted *Serratia marcescens* endonuclease, which was fused with the mannopine synthase promoter of Agrobacterium tumefaciens Ti plasmid and transferred to *Nicotiana tabacum* SR1 plants. The promoter is leaf- and root-specific. The resulting transgenic plants demonstrated elevated nuclease activity (Trifonova et al. 2002) which is known to non-

specifically degrade RNA and DNA. It has a very active antiviral activity which was previously shown both in animals and in plants exogenously. Transgenic tobacco plants (*Nicotiana tabacum* L cv. SR1) expressing *S. marcescens* chimeric, mutant, and intracellular mutant nuclease gene variants were used against many plants specific viruses and shown a higher level of resistance compared to the control non-transgenic tobacco plants (Trifonova et al. 2015).

As a destination vector, pPZP222-S, an Agrobacterium binary vector was used. This vector is versatile, relatively small, stable and fully sequenced. Bacterial marker genes in the vectors confer resistance to spectinomycin (pPZP200 series), allowing their use in Agrobacterium strains with different drug resistance markers. Plant marker genes in the binary vectors confer resistance to kanamycin or to gentamycin (GmR) (used here) and are adjacent to the left border (LB) of the transferred region. The multiple cloning sites (MCS) are located between the left border and the right border (Hajdukiewicz et al. 1994). We were able to successfully transform the transgenic plants, with extracted DNA from all transgenic plants showing the presence of the introduced by gentamycin resistance gene (Figure 2.9).

The GUS reporter system (GUS: β-glucuronidase) is a reporter gene system, principally useful in plant molecular biology. It functions by using the cauliflower mosaic virus (CaMV) 35S promoter to direct the expression of beta-glucuronidase in transformed plants. Expression of GUS can be measured accurately using fluorometric assays (Figure 3.13) of very small amounts of transformed plant tissue (Jefferson et al. 1987).

To impose drought stress on the transgenic plants, PEG 10,000 was added to the nutrient solution. PEG 10,000 is too large to be taken up by the roots of plants and therefore provided a uniform and controllable drought stress to the experimental plants (LAWLOR 1970). The transgenic plants and the control plants were exposed to different concentration of PEG but no significant difference was found based on length (Fig 2.14). We assumed that enhanced root development might serve to enhance adaptation and survival of transgenic LEA plants over controls in drought conditions. After the drought experiment, the most significant difference was found under the stressed condition, especially for the root formation. The transgenic LEA plants have higher root formation compared to control plants under a stressed condition (figure 2.16 and 2.18). In contrast to the root enhancement, significant differences were not observed in other parts of transgenic LEA and control plants (**Figures 2.15 and 2.17**). However, under fresh and dry weight there is a significant difference were found in terms of total weight; fresh weight ($p=0.0461$, $p<0.05$) and dry weight ($p=0.0342$, $p<0.05$). These results showing increased plant mass, which suggests that the LEA proteins could provide enhanced survival rates under drought conditions, as previously suggested by Babu et al. (2004) and Maqbool et al. (2002). Though there was no significant difference found for stems and leaves formation between transgenic LEA and control GUS plants. Different studies showed that LEA protein mostly found in seeds and also in vegetative organs; mostly in roots. This results which conferred significant growth for roots in transgenic LEA plants compared to the control GUS plants, resembling the findings.

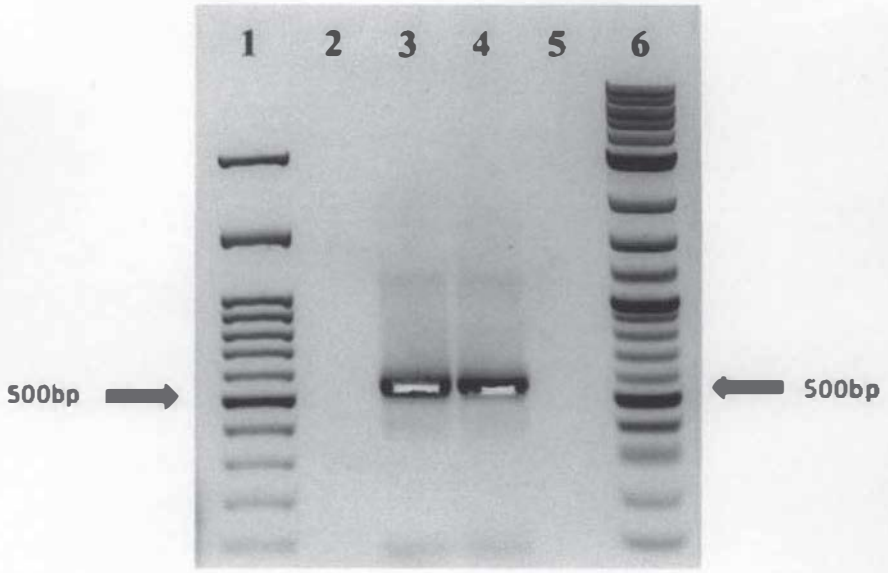
Several reports have demonstrated that LEA proteins enhanced transgenic plant low-temperature tolerance (Yu et al. 2005). To investigate that low-temperature tolerance, green leaves of both transgenic LEA plants and control plants were incubated 4°C and 25°C respectively for 48 hours in presence of MS growth media without any sunlight. The subsequent chlorophyll content measurements showed a significant difference between LEA and control plants (Figure 2.20), suggesting that LEA proteins contribute to the low-temperature tolerance of transgenic LEA plants, supported the findings of Liu et al. (2016).

3.5 CONCLUSION

A recent rapid increase in studies from diverse fields have shown LEA proteins to be a versatile family of proteins. They exhibit myriads of functions: chaperone, antifreeze, radical-scavenger, ion-binding functions, some of which likely contribute to their reported roles in response to stress conditions (cold, drought, heavy-metal stress as well as biotic stress). Despite a lack of understanding concerning molecular mechanisms of LEA protein action, it can be said of a deep body of research that LEA proteins play a critical role in the survival of several types of plants and animals under extreme conditions. An understanding of these mechanisms has the potential provide a major contribution to agriculture and biotechnology.

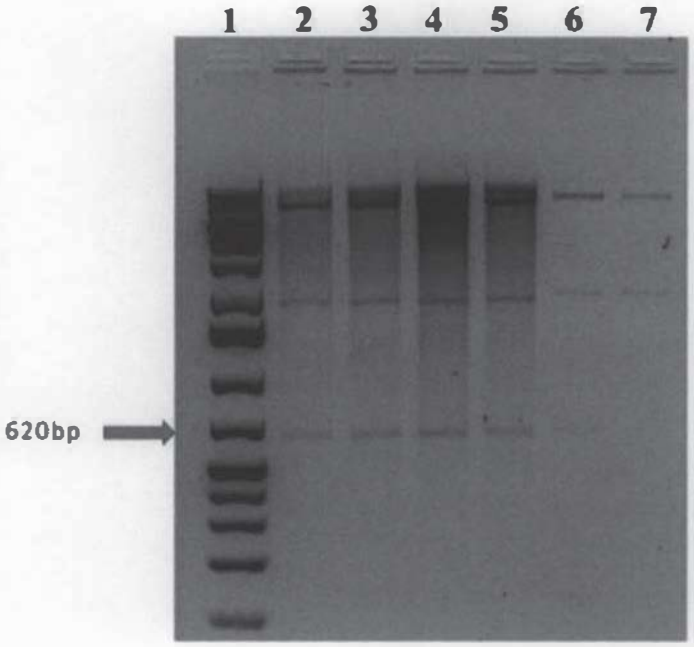
APPENDICES

APPENDIX A



PCR products of LEA1.1(540 bps) from the pTXB1 plasmid by Gateway Primers by using *Pfx50*™ DNA Polymerase protocol from Thermo Scientific. **Lane 1**: 100bp DNA ladder, **Lane 2 &5**: empty plasmid as a negative control, **Lane 3 & 4**: amplification of LEA1.1 from pTXB1plasmid, **Lane 6**: 1Kb DNA ladder. All three plasmids contain LEA1.1 DNA as indicated by agarose gel electrophoresis.

APPENDIX B



Double Restriction Digestion by XhoI and SacI of four purified plasmids from AgL1 after transformation. **Lane 1**: IKb DNA ladder. **Lane 2-5:** Isolated plasmids after transformation in AgL1. **Lane 6**: purified plasmid after transformation of LR Recombination Reaction (100 times dilution), **Lane 7:** p2Z2-S Vector as a negative control. All four plasmids contain LEA1.1 DNA as indicated by agarose gel electrophoresis.

REFERENCES

Abba', S., Ghignone, S. & Bonfante, P., 2006. A dehydration-inducible gene in the truffle Tuber borchii identifies a novel group of dehydrins. *BMC genomics*, 7(1), p.39.

Ahuja, I. et al., 2010. Plant molecular stress responses face climate change. *Trends in plant science*, 15(12), pp.664–674.

Amara, I. et al., 2013. Enhanced water stress tolerance of transgenic maize plants over-expressing LEA Rab28 gene. *Journal of Plant Physiology*, 170(9), pp.864–873.

Amara, I. et al., 2014. Insights into Late Embryogenesis Abundant (LEA) Proteins in Plants: From Structure to the Functions. *American Journal of Plant Sciences*, 5(5), pp.3440–3455.

Arnon, D.I., 1949. Copper Enzymes in Isolated Chloroplasts. Polyphenoloxidase in Beta Vulgaris. *Plant physiology*, 24(1), pp.1–15.

Babu, R.C. et al., 2004. HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. *Plant Science*, 166(4), pp.855–862.

Baker, J., Van Dennsteele, C. & Dure, L. 3rd, 1988. Sequence and characterization of 6 Lea proteins and their genes from cotton. *Plant molecular biology*, 11(3), pp.277–291.

Bartels, D., 2005. Desiccation Tolerance Studied in the Resurrection Plant raterostigma ,

plantagineum. *Integr. Comp. Biol.*, 45, pp.696–700.

Bartels, D., Singh, M. & Salamini, F., 1988. Onset of desiccation tolerance during development of the barley embryo. *Planta*, 175(4), pp.485–492.

Battaglia, M. et al., 2008. The Enigmatic LEA Proteins and Other Hydrophilins. *Plant Physiology*, 148(1), pp.6–24.

Battista, J.R., Park, M.J. & McLemore, a E., 2001. Inactivation of two homologues of proteins presumed to be involved in the desiccation tolerance of plants sensitizes Deinococcus radiodurans RI to desiccation. *Cryobiology*, 43(2), pp.133–139.

Bies-Ethève, N. et al., 2008. Inventory, evolution, and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in Arabidopsis thaliana. *Plant Molecular Biology*, 67(1–2), pp.107–124.

Blackman, S.A., Obendorf, R.L. & Leopold, A.C., 1995. Desiccation tolerance in developing soybean seeds: The role of stress proteins. *Physiologia Plantarum*, 93(4), pp.630–638.

Blackman, S. a et al., 1991. Maturation proteins associated with desiccation tolerance in soybean. *Plant Physiology*, 96(3), pp.868–74.

Blum, A., 2013. The interdrought conference in perspective. *Journal of Experimental Botany*, 64(18), pp.5773–5774.

Borrell, A. et al., 2002. Arabidopsis thaliana Atrab28: A nuclear-targeted protein related to

germination and toxic cation tolerance. *Plant Molecular Biology*, 50(2), pp.249–259.

Boswell, L.C., Menze, M.A. & Hand, S.C., 2014. Group 3 late embryogenesis abundant proteins from embryos of *Artemia franciscana*: structural properties and protective abilities during desiccation. *Physiological and biochemical zoology : PBZ*, 87(5), pp.640–651.

Boucher, V. et al., 2010. MtPM25 is an atypical hydrophobic late embryogenesis-abundant protein that dissociates cold and desiccation-aggregated proteins. *Plant, cell & environment*, 33(3), pp.418–30.

Boudet, J. et al., 2006. Comparative Analysis of the Heat Stable Proteome of Radicles of Medicago truncatula Seeds during Germination Identifies Late Embryogenesis Abundant Proteins Associated with Desiccation Tolerance. *Plant Physiology*, 140(4), pp.1418–1436.

Boyer, J.S., 1982. Plant Productivity and Environment. *Science*, 218(4571), p.443 LP-448.

Bray, E. a., 1993. Molecular Responses to Water Deficit. *Plant Physiology*, 103, pp.1035–1040.

Browne, J.A. et al., 2004. Dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode Aphelenchus avenae. *Eukaryotic Cell*, 3(4), pp.966–975.

Browne, J., Tunnacliffe, A. & Burnell, A., 2002. Anhydrobiosis: Plant desiccation gene found in a nematode. *Nature*, 416(6876), p.38.

Carpenter, J.F. & Crowe, J.H., 1988. The mechanism of cryoprotection of proteins by solutes. *Cryobiology*, 25(3), pp.244–255.

Chakrabortee, S. et al., 2012. Intrinsically disordered proteins as molecular shields. *Mol. BioSyst.*, 8(1), pp.210–219.

Chatelain, E. et al., 2012. Temporal profiling of the heat-stable proteome during late maturation of *Medicago truncatula* seeds identifies a restricted subset of late embryogenesis abundant proteins associated with longevity. *Plant, Cell, and Environment*, 35(8), pp.1440–1455.

Chen, W.-H. et al., 2009. A gene catalogue for the post-diapause development of an anhydrobiotic arthropod Artemia franciscana. *BMC genomics*, 10, p.52.

Cheng, Z. et al., 2002. Wheat LEA genes, PMA80, and PMA1959 enhance dehydration tolerance of transgenic rice (Oryza sativa L.). *Molecular Breeding*, 10(1–2), pp.71–82.

Chong, S. et al., 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene*, 192(2), pp.271–281.

Chou, P.Y. & Fasman, G.D., 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Advances in enzymology and related areas of molecular biology*, 47, pp.45–148.

Clegg, J.S., 2002. Cryptobiosis - A peculiar state of biological organization. *Tsitologiya*, 44(10), pp.1011–1014.

Clegg, J.S., Jackson, S.A. & Popov, V.I., 2000. Long-term anoxia in encysted embryos of the crustacean, Artemia franciscana: viability, ultrastructure, and stress proteins. *Cell and Tissue Research*, 301(3), pp.433–446.

Close, T.J., 1996. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum*, 97(4), pp.795–803.

Close, T.J. & Lammers, P.J., 1993. An Osmotic Stress Protein of Cyanobacteria Is immunologically Related to Plant Dehydrins '. *Plant Physiology*, 101, pp.773–779.

Colmenero-Flores, J.M. et al., 1997. Characterization of Phaseolus vulgaris cDNA clones responsive to water deficit: identification of a novel late embryogenesis abundant-like protein. *Plant molecular biology*, 35(4), pp.393–405.

Cornette, R. & Kikawada, T., 2011. The induction of anhydrobiosis in the sleeping chironomid: Current status of our knowledge. *IUBMB Life*, 63(6), pp.419–429.

Cramer, G.R. et al., 2011. Effects of abiotic stress on plants: a systems biology perspective. *BMC Plant Biology*, 11(1), p.163.

Crowe, J.H. et al., 1997. Stabilization of Dry Membranes by Mixtures of Hydroxyethyl Starch and Glucose: The Role of Vitrification. *Cryobiology*, 35(1), pp.20–30.

Crowe, J.H. & Clegg, J.S., 1973. *Anhydrobiosis*, Stroudsburg, Pa.: Dowden, Hutchinson &

Ross.

Crowe, J.H. & Madin, K.A., 1974. Anhydrobiosis in tardigrades and nematodes. *Transactions of the American Microscopical Society*, pp.513–524.

Crowe, J.H. & Madin, K.A.C., 1975. Anhydrobiosis in nematodes: evaporative water loss and survival. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 193(3), pp.323–333.

Cui, C. et al., 2006. Elimination of in vivo cleavage between target protein and intein in the intein-mediated protein purification systems. *Protein expression and purification*, 50(1), pp.74–81.

Cuming, A.C., 1999. LEA Proteins. In P. R. Shewry & R. Casey, eds. *Seed Proteins*. Dordrecht: Springer Netherlands, pp. 753–780.

CUMING, A.C. & LANE, B.G., 1979. Protein Synthesis in Imbibing Wheat Embryos. *European Journal of Biochemistry*, 99(2), pp.217–224.

Danilevskii, A.S., 1965. Photoperiodism and seasonal development of insects. *Photoperiodism and seasonal development of insects*.

Delahaie, J. et al., 2013. LEA polypeptide profiling of recalcitrant and orthodox legume seeds reveals ABI3-regulated LEA protein abundance linked to desiccation tolerance. *Journal of Experimental Botany*, 64(14), pp.4559–4573.

Dunker, A.K. et al., 2001. Intrinsically disordered protein. *Journal of Molecular Graphics*

and Modelling, 19(1), pp.26–59.

Dure, L., 1993. A repeating 11-mer amino acid motif and plant desiccation. *The Plant journal : for cell and molecular biology*, 3(3), pp.363–369.

Dure, L., 1994. Structure/Function Studies of Lea Proteins. In G. Coruzzi & P. Puigdomènech, eds. *Plant Molecular Biology: Molecular Genetic Analysis of Plant Development and Metabolism*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 245–255.

Dure, L. 3rd et al., 1989. Common amino acid sequence domains among the LEA proteins of higher plants. *Plant molecular biology*, 12(5), pp.475–486.

Dure, L., Greenway, S.C. & Galau, G.A., 1981. Developmental biochemistry of cotton seed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis. *Biochemistry*, 20(14), pp.4162–8.

DURE III, L., 1993. Structural motifs in Lea proteins. *Plant responses to cellular dehydration during environmental stress*, pp.91–103.

Eichinger, L. et al., 2005. The genome of the social amoeba Dictyostelium discoideum. *Nature*, 435(7038), pp.43–57.

Eom, J. et al., 1996. The embryo-specific EMB-1 protein of Daucus carota is flexible and unstructured in solution. *Plant Science*, 115(1), pp.17–24.

Gal, T.Z., Glazer, I. & Koltai, H., 2004. An LEA group 3 family member is involved in survival of C. elegans during exposure to stress. *FEBS Letters*, 577(1–2), pp.21–26.

Galau, G.A. & Hughes, D.W., 1987. Coordinate accumulation of homeologous transcripts of seven cotton Lea gene families during embryogenesis and germination. *Developmental biology*, 123(1), pp.213–221.

Galau, G.A., Hughes, D.W. & Dure, L. 3rd, 1986. Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. *Plant molecular biology*, 7(3), pp.155–170.

Garay-Arroyo, A. et al., 2000. Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *The Journal of biological chemistry*, 275(8), pp.5668–74.

Gilles, G.J. et al., 2007. A predicted N-terminal helical domain of a Group 1 LEA protein is required for protection of enzyme activity from drying. *Plant Physiology and Biochemistry*, 45(6–7), pp.389–399.

Goday, A. et al., 1994. The maize abscisic acid-responsive protein Rabl 7 is located in the nucleus and interacts with nuclear localization signals. *The Plant cell*, 6(3), pp.351–360.

Gokce, I. et al., 2005. Single peptide bonds exhibit poly (Pro)II ("random coil") circular dichroism spectra. *Journal of American Chemical Society*, (127), pp.9700–9701.

Goyal, K. et al., 2003. Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein. *Journal of Biological Chemistry*, 278(15), pp.12977–12984.

Goyal, K., Walton, L.J. & Tunnacliffe, A., 2005. LEA proteins prevent protein aggregation due to water stress. *The Biochemical journal*, 388(Pt 1), pp.151–7.

Grelet, J., 2005. Identification in Pea Seed Mitochondria of a Late-Embryogenesis Abundant Protein Able to Protect Enzymes from Drying. *Plant Physiology*, 137(1), pp.157–167.

Guo, F. et al., 2013. Induced Expression of AtLEC1 and AtLEC2 Differentially Promotes Somatic Embryogenesis in Transgenic Tobacco Plants. *PLoS ONE*, 8(8).

Gusev, O. et al., 2014. Comparative genome sequencing reveals the genomic signature of extreme desiccation tolerance in the anhydrobiotic midge. *Nature Communications*, 5, p.4784.

Haegeman, A. et al., 2009. Expressed sequence tags of the peanut pod nematode Ditylenchus africanus: The first transcriptome analysis of an Anguinid nematode. *Molecular and Biochemical Parasitology*, 167(1), pp.32–40.

Hajdukiewicz, P., Svab, Z. & Maliga, P., 1994. The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant molecular biology*, 25(6), pp.989–994.

Hand, S.C. et al., 2011. LEA Proteins During Water Stress: Not Just for Plants Anymore. *Annual Review of Physiology*, 73(1), pp.115–134.

Hand, S.C. et al., 2010. LEA Proteins During Water Stress: Not Just for Plants Anymore. *Annual review of physiology*, (October), pp.1–20.

Hand, S.C. et al., 2007. Life without water: Expression of plant LEA genes by an anhydrobiotic arthropod. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 307(1), pp.62–66.

Hand, S.C. & Menze, M.A., 2015. Molecular approaches for improving desiccation tolerance: insights from the brine shrimp *Artemia franciscana*. *Planta*, 242(2), pp.379–388.

Hanin, M. et al., 2011. Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms. *Plant Signaling & Behavior*, 6(10), pp.1503–1509.

Hara, M. et al., 2003. Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco. *Planta*, 217(2), pp.290–298.

Herzer, S. et al., 2003. Purification of native dehydrin from Glycine Max cv., Pisum sativum, and Rosmarinum officinalis by affinity chromatography. *Protein Expression and Purification*, 28(2), pp.232–240.

Hetherington, A.M. & Brownlee, C., 2004. THE GENERATION OF Ca 2+ SIGNALS IN PLANTS. *Annual Review of Plant Biology*, 55(1), pp.401–427.

Hincha, D.K. & Thalhammer, A., 2012. LEA proteins: IDPs with versatile functions in cellular dehydration tolerance: Figure 1. *Biochemical Society Transactions*, 40(5), pp.1000–1003.

Hinton, H.E. & Needham, A.E., 1968. Reversible Suspension of Metabolism and the Origin of Life [and Contribution to the Discussion]. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 171(1022), pp.43–57.

Hoekstra, F.A., 2005. The differential in Desiccated Anhydrobiotic Plant Systems â€™ Longevities. *Integr.Comp.Biol*, 45, pp.725–733.

Hoekstra, F.A., Golovina, E.A. & Buitink, J., 2017. Mechanisms of plant desiccation tolerance. *Trends in Plant Science*, 6(9), pp.431–438.

Hoekstra, F.A., Golovina, E.A. & Buitink, J., 2001. Mechanisms of plant desiccation tolerance. *Trends in Plant Science*, 6(9), pp.431–438.

Hong, B., Barg, R. & Ho, T.H., 1992. Developmental and organ-specific expression of an ABA- and stress-induced protein in barley. *Plant molecular biology*, 18(4), pp.663–674.

Honjoh, K. et al., 1995. Isolation and characterization of hardening-induced proteins in *Chlorella vulgaris C-27*: identification of late embryogenesis abundant proteins. *Plant & cell physiology*, 36(8), pp.1421–1430.

HONJOH, K. et al., 2000. Cryoprotective Activities of Group 3 Late Embryogenesis

Abundant Proteins from Chlorella vulgaris C-27. *Bioscience, Biotechnology, and Biochemistry*, 64(8), pp.1656–1663.

Hsing, Y.-I.C. & Wu, S.J., 1992. Cloning and characterization of cDNA clones encoding soybean seed maturation polypeptides. *Bot Bull Acad Sin*, 33, pp.191–199.

Hsing, Y.C. et al., 1990. Expression of Maturation-Specific Genes in Soybean Seeds. *Crop Science*, 30, pp.1343–1350.

Hughes, D.W. & Galau, G.A., 1989. Temporally modular gene expression during cotyledon development. *Genes & Development*, 3(3), pp.358–369.

Hundertmark, M. & Hincha, D.K., 2008. LEA (late embryogenesis abundant) proteins and their encoding genes in Arabidopsis thaliana. *BMC genomics*, 9, p.118.

Imai, R. et al., 1996. A lea-class gene of tomato confers salt and freezing tolerance when expressed in Saccharomyces cerevisiae. *Gene*, 170(2), pp.243–248.

Ingram, J. & Bartels, D., 1996. the Molecular Basis of Dehydration Tolerance in Plants. *Annual review of plant physiology and plant molecular biology*, 47, pp.377–403.

Ingram, J. & Bartels, D., 1996. THE MOLECULAR BASIS OF DEHYDRATION TOLERANCE IN PLANTS. *Annual review of plant physiology and plant molecular biology*, 47, pp.377–403.

Ismail, A.M., Hall, A.E. & Close, T.J., 1999. Purification and partial characterization of a dehydrin involved in chilling tolerance during the seedling emergence of cowpea.

Plant Physiology, 120(1), pp.237–244.

Iturriaga, G. et al., 1992. Expression of desiccation-related proteins from the resurrection plant *Craterostigma plantagineum* in transgenic tobacco. *Plant molecular biology*, 20(3), pp.555–8.

Jefferson, R.A., Kavanagh, T.A. & Bevan, M.W., 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal*, 6(13), pp.3901–3907.

Johnson, J.N., Ahrendt, E. & Braun, J.E. a., 2010. CSPα: the neuroprotective J protein this paper is one of a selection of papers published in this special issue entitled "Canadian Society of Biochemistry, Molecular & Cellular Biology 52nd Annual Meeting — Protein Folding: Principles and Diseases" and has. *Biochemistry and Cell Biology*, 88(2), pp.157–165.

Katinka, M.D. et al., 2001. Genome sequence and gene compaction of the eukaryote parasite Encephalitozoon cuniculi. *Nature*, 414(6862), pp.450–453.

Keilin, D., 1959. The Problem of Anabiosis or Latent Life: History and Current Concept. *Proceedings of the Royal Society of London. Series B - Biological Sciences*, 150(939), p.149 LP-191.

Kertbundit, S. et al., 1991. In vivo random B-glucuronidase gene fusions in *Arabidopsis thaliana* (plant gene expression/insertional mutagenesis/promoter cloning/transgenic plants/phloem-specific gene expression). *Botany*, 88(June), pp.5212–5216.

Kikawada, T. et al., 2006a. Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid. *Biochemical and Biophysical Research Communications*, 348(1), pp.56-61.

Kikawada, T. et al., 2006b. Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid. *Biochemical and Biophysical Research Communications*, 348(1), pp.56-61.

Kim, B.Y. et al., 2015. DISTINCTIVE LOCALIZATION OF GROUP 3 LATE EMBRYOGENESIS ABUNDANT SYNTHESIZING CELLS DURING BRINE SHRIMP DEVELOPMENT. *Archives of Insect Biochemistry and Physiology*, 89(3), pp.169–180.

Kim, H.-S. et al., 2005. Molecular and functional characterization of CaLEA6, the gene for a hydrophobic LEA protein from Capsicum annuum. *Gene*, 344, pp.115–123.

Knight, H., 1996. Cold Calcium Signaling in Arabidopsis Involves Two Cellular Pools and a Change in Calcium Signature after Acclimation. *the Plant Cell Online*, 8(3), pp.489–503.

Kovacs, D. et al., 2008. Chaperone Activity of ERD10 and ERD14, Two Disordered Stress-Related Plant Proteins. *Plant Physiology*, 147(1), pp.381–390.

Kruger, C. et al., 2002. A metal-binding member of the late embryogenesis abundant protein family transports iron in the phloem of Ricinus communis L. *Journal of Biological Chemistry*, 277(28), pp.25062–25069.

Lavens, P. & Sorgeloos, P., 1987. The cryptobiotic state of *Artemia* cysts, its diapause deactivation and hatching: a review. *Artemia Research and its Applications*, 3, pp.27–63.

LAWLOR, D.W., 1970. Absorption of Polyethylene Glycols By Plants and Their Effects on Plant Growth. *New Phytologist*, 69(2), pp.501–513.

Lazo, G.R., Stein, P.A. & Ludwig, R.A., 1991. A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. *Bio/technology (Nature Publishing Company)*, 9(10), pp.963–967.

Leon Dure, 2001. Occurrence of a Repeating 11-Mer Amino Acid Sequence Motif in Diverse Organisms. *Protein & Peptide Letters*, 8(2), pp.115–122.

Liang, J. et al., 2013. JcLEA, a novel LEA-like protein from Jatropha curcas, confers a high level of tolerance to dehydration and salinity in Arabidopsis thaliana. *PLoS ONE*, 8(12), pp.1–13.

Liu, Y. et al., 2016. Group 3 LEA Protein, ZmLEA3, Is Involved in Protection from Low Temperature Stress. *Frontiers in Plant Science*, 7, p.1011.

Liu, Y.L. et al., 2009. Formation of diapause cyst shell in brine shrimp, Artemia parthenogenetica, and its resistance role in environmental stresses. *Journal of Biological Chemistry*, 284(25), pp.16931–16938.

MacRae, T.H., 2003. Molecular chaperones, stress resistance and development in Artemia

franciscana. *Seminars in cell & developmental biology*, 14(5), pp.251–258.

MacRae, T.H., 2016. Stress tolerance during diapause and quiescence of the brine shrimp, *Artemia*. *Cell Stress and Chaperones*, 21(1), pp.9–18.

Maqbool, S.B. et al., 2002. Competence of oat (*Avena sativa L.*) shoot apical meristems for integrative transformation, inherited expression, and osmotic tolerance of transgenic lines containing hva1. *Theoretical and Applied Genetics*, 105(2–3), pp.201–208.

Marunde, M.R. et al., 2013. Improved tolerance to salt and water stress in Drosophila melanogaster cells conferred by late embryogenesis abundant protein. *Journal of insect physiology*, 59(4), pp.377–386.

McCubbin, W.D., Kay, C.M., Lane, B.G., et al., 1985. Hydrodynamic and optical properties of the wheat germ Em protein. *Canadian Journal of Biochemistry and Cell Biology*, 63(8), pp.803–811.

McCubbin, W.D., Kay, C.M. & Lane, B.G., 1985. Hydrodynamic and optical properties of the wheat germ Em protein. *Canadian Journal of Biochemistry and Cell Biology*, 63(8), pp.803–811.

Menze, M.A. et al., 2009. Occurrence of mitochondria-targeted late embryogenesis abundant (LEA) gene in animals increases organelle resistance to water stress. *Journal of Biological Chemistry*, 284(16), pp.10714–10719.

MINORSKY, P. V. & SPANSWICK, R.M., 1989. Electrophysiological evidence for a role for calcium in temperature sensing by roots of cucumber seedlings. *Plant, Cell & Environment*, 12(2), pp.137–143.

Moons, A. et al., 1995. Molecular and physiological responses to abscisic acid and salts in roots of salt-sensitive and salt-tolerant Indica rice varieties. *Plant Physiology*, 107(1), pp.177–86.

Mouillon, J., Gustafsson, P. & Harryson, P., 2006. Structural investigation of disordered stress proteins. Comparison of full-length dehydrins with isolated peptides of their conserved segments. *Plant Physiology*, 141(2), pp.638–50.

Mtwisha, L. et al., 1998. Hsp 12 is an LEA-like protein in Saccharomyces cerevisiae. *Plant Molecular Biology*, 37(3), pp.513–521.

NDong, C. et al., 2002. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. *Plant Physiology*, 129(3), pp.1368–81.

Oliveira, E. et al., 2007. LC-MSMS identification of Arabidopsis thaliana heat-stable seed proteins: enriching for LEA-type proteins by acid treatment. *Journal of mass spectrometry : JMS*, 42(11), pp.1485–1495.

Olvera-Carrillo, Y., Luis Reyes, J. & Covarrubias, A. a., 2011. Late embryogenesis abundant proteins. *Plant Signaling & Behavior*, 6(4), pp.586–589.

Pouchkina-Stantcheva, N.N. et al., 2007. Functional divergence of former alleles in an ancient asexual invertebrate. *Science (New York, N.Y.)*, 318(5848), pp.268–71.

Prestrelski, S.J. et al., 1993. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophysical Journal*, 65(2), pp.661–671.

Raghavendra, A.S. et al., 2010. ABA perception and signaling. *Trends in Plant Science*, 15(7), pp.395-401.

Reyes, J.L. et al., 2005. Hydrophilins from distant organisms can protect enzymatic activities from water limitation effects in vitro. *Plant, Cell, and Environment*, 28(6), pp.709–718.

Richardson, A.D., Duigan, S.P. & Berlyn, G.P., 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytologist*, 153(1), pp.185–194.

Ried, J.L. & Walker-Simmons, M.K., 1993. Group 3 Late Embryogenesis Abundant Proteins in Desiccation-Tolerant Seedlings of Wheat (Triticum aestivum L.). *Plant Physiology*, 102(1), pp.125–131.

Robbins, H.M. et al., 2010. Diapause termination and development of encysted Artemia embryos: roles for nitric oxide and hydrogen peroxide. *Journal of Experimental Biology*, 213(9), pp.1464–1470.

Robertson, M. & Chandler, P.M., 1994. A dehydrin cognate protein from pea (Pisum sativum L.) with an atypical pattern of expression. *Plant molecular biology*, 26(3),

pp.805–816.

ROSENBERG, L.A. & RINNE, R.W., 1989. Protein synthesis during rehydration, germination and seedling growth of naturally and precociously matured soybean seeds (Glycine max). *Annals of Botany*, 64(1), pp.77–86.

Rossi, M. et al., 1996. Asr genes belong to a gene family comprising at least three closely linked loci on chromosome 4 in tomato. *Molecular & general genetics : MGG*, 252(4), pp.489–492.

Rost, B. & Liu, J., 2003. The PredictProtein server. *Nucleic Acids Research*, 31(13), pp.3300–3304.

Rost, B. & Sander, C., 1993. Prediction of Protein Secondary Structure at Better than 70% Accuracy. *Journal of Molecular Biology*, 232(2), pp.584–599.

Russouw, P.S. et al., 1995. Isolation and characterization of a heat-soluble protein from pea (Pisum sativum) embryos. *Seed Science Research*, 5(3), pp.137–144.

Russouw, P.S. et al., 1997. The most prevalent protein in a heat-treated extract of pea (Pisum sativum) embryos is a LEA group I protein; its conformation is not affected by exposure to high temperature. *Seed Science Research*, 7(2), pp.117–124.

Schweers, O. et al., 1994. Structural studies of tau protein and Alzheimer paired helical filaments show no evidence for beta-structure. *The Journal of biological chemistry*, 269(39), pp.24290–7.

Sharon, M. a et al., 2009. Characterization of a group 1 late embryogenesis abundant protein in encysted embryos of the brine shrimp *Artemia franciscana*. *Biochemistry and cell biology = Biochimie et biologie cellulaire*, 87(2), pp.415–30.

Shewry, P.R. & Casey, R., 1999. Seed proteins. In *Seed proteins*. Springer, pp. 1–10.

Shih, M. et al., 2004. Gene cloning and characterization of a soybean (Glycine max L.) LEA protein, GmPM16. *Plant Molecular Biology*, 56(5), pp.689–703.

Shih, M.-D., Hoekstra, F.A. & Hsing, Y.-I.C., 2008. Late Embryogenesis Abundant Proteins. In J.-C. K. and M. D. B. T.-A. in B. Research, ed. Academic Press, pp. 211–255.

Shimizu, T. et al., 2010. Desiccation-induced structuralization and glass formation of group 3 late embryogenesis abundant protein model peptides. *Biochemistry*, 49(6), pp.1093–1104.

Silhavy, D. et al., 1995. Isolation and characterization of a water-stress-inducible cDNA clone from Solanum chacoense. *Plant molecular biology*, 27(3), pp.587–595.

Singh, S. et al., 2005. Solution structure of a late embryogenesis abundant protein (LEA14) from Arabidopsis thaliana, a cellular stress-related protein. *Protein science : a publication of the Protein Society*, 14(10), pp.2601–2609.

Sivamani, E. et al., 2000. Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley

HVA1 gene. *Plant Science*, 155(1), pp.1–9.

Solomon, A. et al., 2000. Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. *Parasitology*, 121 (Pt 4, pp.409–416.

Sorgeloos, P., Dhert, P. & Candreva, P., 2001. Use of the brine shrimp, Artemia spp., in marine fish larviculture. *Aquaculture*, 200(1–2), pp.147–159.

Soulages, J.L. et al., 2002. Temperature-induced extended helix/random coil transitions in a group 1 late embryogenesis abundant protein from soybean. *Plant Physiology*, 128(3), pp.822–32.

Stacy, R.A.P. & Aalen, R.B., 1998. Identification of sequence homology between the internal hydrophilic repeated motifs of Group 1 late-embryogenesis-abundant proteins in plants and hydrophilic repeats of the general stress protein GsiB of Bacillus subtilis. *Planta*, 206(3), pp.476–478.

Svensson, J., Palva, E.T. & Welin, B., 2000. Purification of recombinant Arabidopsis thaliana dehydrins by metal ion affinity chromatography. *Protein expression and purification*, 20(2), pp.169–178.

Tamiya, T. et al., 1985. Freeze denaturation of enzymes and its prevention with additives. *Cryobiology*, 22(5), pp.446–456.

Tauber, M.J., Tauber, C.A. & Masaki, S., 1986. *Seasonal adaptations of insects*, Oxford University Press on Demand.

Thomashow, M.F., 1999. PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology*, 50(1), pp.571–599.

Thomashow, M.F., 1998. Role of cold-responsive genes in plant freezing tolerance. *Plant physiology*, 118(1), pp.1–8.

Tolleter, D. et al., 2007. Structure and Function of a Mitochondrial Late Embryogenesis Abundant Protein Are Revealed by Desiccation. *the Plant Cell Online*, 19(5), pp.1580–1589.

Tolleter, D., Hincha, D.K. & Macherel, D., 2010. A mitochondrial late embryogenesis abundant protein stabilizes model membranes in the dry state. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1798(10), pp.1926–1933.

Tompa, P., 2005. The interplay between structure and function in intrinsically unstructured proteins. *FEBS Letters*, 579(15), pp.3346–3354.

Toxopeus, J., Warner, A.H. & MacRae, T.H., 2014. Group 1 LEA proteins contribute to the desiccation and freeze tolerance of *Artemia franciscana* embryos during diapause. *Cell Stress and Chaperones*, 19(6), pp.939–948.

Trifonova, E.A. et al., 2015. [Transgenic Expression of Serratia marcescens Native and Mutant Nucleases Modulates Tobacco Mosaic Virus Resistance in *Nicotiana tabacum* L]. *Genetika*, 51(7), pp.835–840.

Trifonova, E.A. et al., 2002. [Transgenic tobacco (*Nicotiana tabacum* SR1) plants expressing the gene coding for Serratia marcescens nuclease]. *Genetika*, 38(2), pp.274–277.

Tunnacliffe, A. et al., 2010. Dormancy and Resistance in Harsh Environments. , 21, pp.91–108.

Tunnacliffe, A., Lapinski, J. & McGee, B., 2005. A Putative LEA Protein, but no Trehalose, is Present in Anhydrobiotic Bdelloid Rotifers. *Hydrobiologia*, 546(1), pp.315–321.

Tunnacliffe, A. & Wise, M.J., 2007. The continuing conundrum of the LEA proteins. *Naturwissenschaften*, 94(10), pp.791–812.

Tyson, T. et al., 2007. Gene induction by desiccation stress in the entomopathogenic nematode Steinernema carpocapsae reveals parallels with drought tolerance mechanisms in plants. *International Journal for Parasitology*, 37(7), pp.763–776.

Uversky, V.N., 2002. What does it mean to be natively unfolded? - Uversky - 2003 - European Journal of Biochemistry - Wiley Online Library. *European Journal of Biochemistry*, 12(May 2001), pp.2–12.

Uversky, V.N., Gillespie, J.R. & Fink, A.L., 2000. Why are "natively unfolded" proteins unstructured under physiologic conditions? *Proteins*, 41(3), pp.415–427.

Vinocur, B. & Altman, A., 2005. Recent advances in engineering plant tolerance to abiotic

stress: achievements and limitations. *Current Opinion in Biotechnology*, 16(2), pp.123–132.

Volkmann, G., Sun, W. & Liu, X., 2009. Controllable protein cleavages through intein fragment complementation. , 18(February), pp.5–7.

Warner, A.H. et al., 2012. Complexity of the heat-soluble LEA proteome in Artemia species. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 7(3), pp.260–267.

Warner, A.H. et al., 2010. Evidence for multiple group 1 late embryogenesis abundant proteins in encysted embryos of *Artemia* and their organelles. *Journal of biochemistry*, 148(5), pp.581–92.

Wasilewska, A. et al., 2008. An update on abscisic acid signaling in plants and more ···. *Molecular Plant*, 1(2), pp.198–217.

Watanabe, M. et al., 2005. Induction of anhydrobiosis in fat body tissue from an insect. *Journal of insect physiology*, 51(6), pp.727–731.

Wharton, D.A., 2015. Anhydrobiosis. *Current Biology*, 25(23), pp.R1114–R1116.

Wise, M.J., 2002. The POPPs: clustering and searching using peptide probability profiles. *Bioinformatics (Oxford, England)*, 18 Suppl 1, pp.S38-45.

Wise, M.J. & Tunnacliffe, A., 2004. POPP the question: what do LEA proteins do? *Trends in Plant Science*, 9(1), pp.13–17.

Wolkers, W.F. et al., 2001. Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses in vitro. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, 1544(1–2), pp.196–206.

Wu, G. et al., 2011. Diverse LEA (late embryogenesis abundant) and LEA-like genes and their responses to hypersaline stress in post-diapause embryonic development of Artemia franciscana. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 160(1), pp.32–39.

Xu, D. et al., 1996. Expression of a Late Embryogenesis Abundant Protein Gene, HVA1, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice. *Plant Physiology*, 110(1), pp.249–257.

Yancey, P.H. et al., 1982. Living with water stress: evolution of osmolyte systems. *Science (New York, N.Y.)*, 217(4566), pp.1214–1222.

Yu, J.-N. et al., 2005. Two New Group 3 LEA Genes of Wheat and Their Functional Analysis in Yeast. *Journal of Integrative Plant Biology*, 47(11), pp.1372–1381.

Zhang, P. et al., 1998. Solid-state 13C NMR investigations of the glycosidic linkage in alpha-alpha' trehalose. *Solid state nuclear magnetic resonance*, 12(4), pp.221–225.