Functional characterization of a LEA protein and a defensin variant for manipulation of stress responses in plants

Thesis submitted to the University of Hyderabad

for the award of

Doctor of Philosophy

By

Akanksha Sharma

(Enrolment No. 07LPPH02)

Department of Plant Sciences

School of Life Sciences

University of Hyderabad

Hyderabad-500046

India

May 2016

University of Hyderabad
(A Central University established in 1974 by act of parliament)
HYDERABAD – 500 046, INDIA

"DECLARATION"

I, **Akanksha Sharma** hereby declare that this thesis entitled **"**Functional characterization of a LEA protein and a defensin variant for manipulation of stress responses in plants**"** submitted by me under the supervision of **Prof. P. B. Kirti**, is an original and independent research work. I also declare that it has not been submitted previously in part or in full for any degree or diploma of any other University or Institution.

Date:

Akanksha Sharma
(Research Scholar)

University of Hyderabad
(A Central University established in 1974 by act of parliament)
HYDERABAD – 500 046, INDIA

"CERTIFICATE"

This is to certify that this thesis entitled "Functional characterization of a LEA protein and a defensin variant for manipulation of stress responses in plants" is based on the results of the work done by **Ms. Akanksha Sharma**, a research scholar for Ph.D programme in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, under my supervision. The work presented in this thesis is original and plagiarism free. No part of this thesis has been submitted for any degree or diploma of any other University or Institution.

Prof. P.B. Kirti
Supervisor

HEAD
Dept. of Plant Sciences

DEAN
School of Life Sciences

Dedicated to my parents and my beloved brother

Amit Sharma

Acknowledgements

At the outset, I would like to acknowledge the contributions made by different people in their special way to make the completion of this thesis possible.

I would like to express my profound and heartfelt thanks to my research supervisor, Prof. P. B. Kirti for giving me a chance to work in the lab and for abundant facilities provided throughout my work and for his constant support, expert guidance, patience and calm personality which provided a comfortable working atmosphere.

I am thankful to Prof. Ch. Venkat Ramana, Head, Department of Plant Sciences, former Heads Prof. A. R. Reddy and Appa Rao Podile for allowing me to use the infrastructural facilities in the department.

I thank Prof. P. Reddanna, Dean, School of Life Sciences, and former Deans Prof. R. P. Sharma, Prof. A. S. Raghavendra, Prof. Aparna Dutta Gupta and Prof. M. Ramanadham for the necessary facilities provided to the school for smooth running of the research work.

I am grateful to Prof. G. Padmaja and Dr. J. S. S. Prakash, my doctoral committee members for their valuable suggestions and kind support.

I take this as an opportunity to thank all the faculty members and research scholars of the School of Life Sciences, for providing the necessary help in several aspects throughout my research work.

I am very much thankful to the past and present labmates Dr. Rajesh, Dr. Vijayan, Dr Srinivasan, Dr. Divya, Dr. Ashraf, Dr. Pushyami, Dr. Dilip, Dr. Pawan, Dr. Ahan, Dr. Jinu, Dr. Uday, Dr. Naveen, Jyotsana, Triveni, Deepankar, Israr, Trishla, Sakshi, Prasanna, Achala, Moin and other lab members for their support and timely help.

I extend my heartfelt appreciation to the Lab attendants Kishan, Abuzer, Satish, Manohar and Naresh for their help in lab and in greenhouse.

I acknowledge the help of Ms. Nalini, Center for Nanotechnology for Confocal microscopic studies.

I thank all my friends at School of life sciences for their help and support. My special thanks to Sumit & Venkat.

I want to thank all my non University friends Ankita, Avantika, Nancy, Reeti and Manosi. They were behind me in all the ups and downs and supported me till now and forever. Thank you all for being there for me.

I would also like to acknowledge all the non-teaching staff members of Department of Plant Sciences and School of Life Sciences for their in official works during my research period.

I acknowledge UGC, CSIR, DBT, DST, DBT-CREBB, DST-FIST, UGC-SAP, and ICAR for research funding in form of various project to our laboratory and the Department.

I specially thank DBT for providing fellowship and funding my research.

I want to thank my parent in laws, sister in law and brother in law for their love and support.

A special gratitude and heartfelt thanks to my beloved parents for their unfailing support and unconditional love. I thank my lovely son, Pranshu, for his sweet smile and understanding. I want to express my deepest love and thanks to my loving husband, Manjul, for his support, patience, constant encouragement and taking care of our son.

Finally, I am thankful to God for His immeasurable blessing upon my life.

Akanksha Sharma

Abbreviations

Mg	Microgram
Ml	Microliter
µM	Micromolar
ABA	Abscisic acid
Bp	Base pairs
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
Cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
D	Day
DEPC	Diethyl pyrocarbonate
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
DPI	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
G	Gram
GFP	Green fluorescent protein
GSP	Gene specific primers
H	Hours
H_2DCFDA	2', 7'- dichlorodihydroflourescien diacetate
Kb	Kilobases
KDa	Kilodalton
LB	Luria Bertani
M	Molar
MES	2-(N-Morpholino)-ethane sulfonic acid
Min	Minutes
MJ	Methyl jasmonate
Ml	Milliliter
MS	Murashige and Skoog
MSH	Half Murashige and Skoog
NAA	Naphthalene acetic acid
Ng	Nanogram

OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pI	Isoelectric point
PVPP	Polyvinyl polypyrrolidone
RNA	Ribo nuceic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
TE	Tris.EDTA
Tris	Tris (hydroxymethyl) aminomethane
U	Units
UPM	Universal primer mixture
UTR	Untranslated regions
WT	Wild type

Table of contents

Chapter 1

General Introduction

Most plants are sessile organisms that are unable to evade unfavorable changes in their environment. Thus, they have to deal with varied and complex types of interactions involving various environmental factors. These factors can be abiotic that include drought, salinity, high and low temperatures, chemical toxicity and oxidative stresses or biotic in the form of pests and diseases. Abiotic stresses adversely affect growth and productivity in plants and initiate a number of morphological, physiochemical and molecular changes in them. These abiotic stress factors have a huge impact on world agriculture, and it has been proposed that they decrease average yields by greater than 50% for most major crop plants (Wang et al. 2003). Additionally, plants must defend themselves from invasion by a vast range of pests and pathogens, including viruses, fungi, bacteria, nematodes, and herbivorous insects (Hammond-Kosack, Kim 2000). Pathogen attack results in 10-16% loss of the global harvest (Chakraborty and Newton 2011) and this figure for pest damage alone is about 14-25% of the total agricultural yield (DeVilliers and Hoisington 2013). Each stress elicits a complex cellular and molecular response system utilized by the plant in order to reduce damage and ensure survival, but often at the expense of its own growth and yield (Herms, Daniel A. 1992). In the course of evolution, plants have evolved specific mechanisms, allowing them to cope up with and adapt to these stressful abiotic and biotic events. The biology of a cell or cells in tissue is very complicated. Multiple pathways of cellular signaling that have complex interactions or crosstalk are activated with any given stimulus from the environment. These interactions probably evolved as mechanisms that enable the live systems to respond to any form of stress with the activation of relevant biological processes.

Defense response genes in plants are transcriptionally activated by pathogens or by different forms of environmental stresses. The induction of defense genes expression in response to certain pathogens is further dependent on humidity and temperature, suggesting the existence of a complicated signaling network, which allows the plant to recognize and protect itself against pathogens and environmental stress. Extensive studies in the past have shown that reactive oxygen species (ROS) and calcium function as secondary messengers in the early response to abiotic and biotic stress. For instance, there is an increase in cytosolic Ca^{+2} levels in plant cells in response to various abrasive environmental conditions of water stress, osmotic stress, cold and wounding including pathogen onslaught. This increase in Ca^{+2} concentration in intracellular spaces, leads to the activation of several simultaneous pathways, activated by several calcium-interacting proteins such as Ca^{+2} dependent protein kinases (CDPK's), calmodulin and calcineurin like

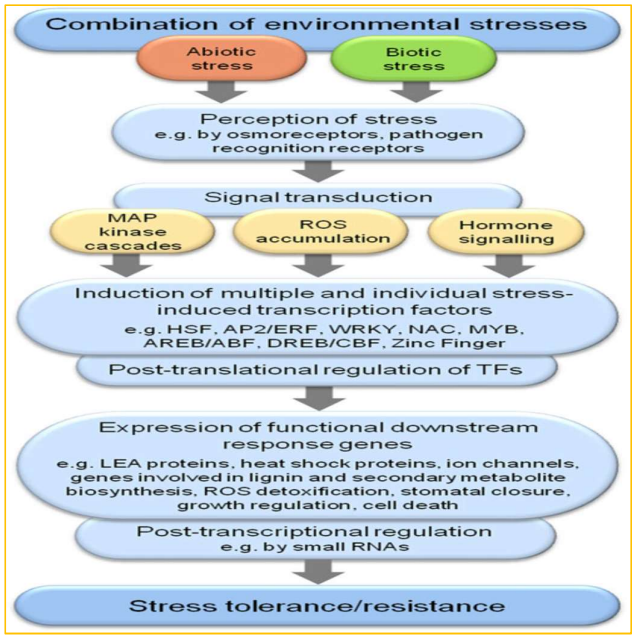
proteins (CBL's) all of which have structural "EF hand" calcium binding motif (Errakhi et al. 2008; Dey et al. 2009; Arimura and Maffei 2010; Takahashi et al. 2011). Also, it is well known fact that plants respond to avirulent microbial invaders as well as to the abiotic stress factors with an oxidative burst (Allan and Fluhr 1997; Lamb and Dixon 1997; Torres and Dangl 2005; Galletti et al. 2008). In this response, O_2^- is generated by NADPH-dependent oxidases and apoplastic oxidases, which is quickly converted to H_2O_2 by superoxide dismutase (SOD). This peroxide entity diffuses into the extracellular medium via cell wall and enters into the cell (Hammond-Kosack and Jones 1996).

Phytohormones also play important pivotal roles in abiotic and biotic stress signaling. ABA is the most studied stress-responsive hormone, which is extensively involved in responses to abiotic stresses such as osmotic stress, water stress and low temperature (Wasilewska et al. 2008; Peleg and Blumwald 2011) whereas phytohormones JA, SA and ET play central roles in biotic stress signaling upon pathogen infection. Most commonly, the signaling networks involved in biotic defense are mediated by phytohormones and are dependent on the nature of the pathogen and its mode of pathogenicity. SA plays an important role in the installation of systemic acquired resistance (SAR) and in the initiation of defense responses against hemibiotrophic and biotrophic pathogens (Grant and Lamb 2006; Vlot et al. 2009). By contrast, ET and JA are frequently related to defense against herbivorous insects and necrotrophic pathogens (Bari and Jones 2009). Experimental evidence also established that JA and ET signaling pathways act together whereas SA and JA are mutually antagonistic. Studies on the mutants *coi1* (jasmonate insensitive) and *ein2* (ethylene insensitive) reported that JA response marker gene *PDF1.2* induced by *Alternaria brassicicola* requires both JA and ET signaling pathways (Penninckx et al. 1998; Thomma et al. 2001). On the other hand, mutations in the JA signaling (*coi1*) results in increased basal and, inducible expression of the SA marker gene *PR1*, and mutations that disturb SA signaling (*npr1*) lead to coordinated increase in the induced or basal levels of the JA marker gene *PDF1.2* (Kazan and Manners 2008).

Many transcription factors, for instance, AtMYC2, RD26, BOS1 and the members of WRKY transcription family also act as mediators in plant response to abiotic and biotic stress in multiple hormones signaling pathways (Abe et al. 2003; Mengiste et al. 2003; Wu et al. 2009). Expression profile of *VvWRKY11*, *AtWRKY39,* and *AtWRKY53* genes indicate that their protein products are co-regulators in responses of the plant against pathogens, water and heat stresses (Rushton et al. 2010). Additionally,

WRKY transcription factors (OsWRKY24 and OsWRKY45*)* antagonize the function of ABA by suppression of ABA-inducible promoters, suggesting multifaceted potentiality of these molecules (Qiu and Yu 2009). The signaling factors or components in plant responses to different abiotic and biotic stress clearly and most frequently overlap. Usually, the activated signaling cascades operate via synergistic and antagonistic actions.

The onset of any generic signal transduction pathway in plants initiates with signal perception, followed by the generation of second messengers that includes Ca^{+2}, ROS and inositol phosphates. These second messengers further regulate the intracellular calcium level. This disruption in cytosolic Ca^{2+} is perceived by calcium binding proteins, also known as Ca^{2+} sensors. These sensory proteins then interact with their respective interacting partners and initiate a phosphorylation cascade. These signals finally result in the expression of specific sub-sets of defense genes that helps in the assembly of overall defense reaction (Fujita et al. 2006) which ultimately aid the plant to survive and surpass the undesirable stress environment (**Fig. 1.1**). Thus, the plant responds to stresses as a distinct entity as cells and synergistically as a whole organism.



Source: Atkinson and Urwin 2012; Journal of Experimental Botany.

Figure1.1. Major events in the signal transduction pathway activated in response to biotic and abiotic stresses. The extracellular stress signal is first perceived by the membrane receptors which then activate large and complex

signaling cascade intracellularly including the generation of secondary signal molecules. The signal cascade results into the expression of multiple stress responsive genes, the products of which can provide the stress tolerance directly or indirectly.

When a plant is subjected to any stress, abiotic or biotic, a number of genes are turned on, resulting in elevated levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of defense against these stresses. Understanding the changes in physiological, biochemical, and molecular machinery that occur in response to stress and exploiting this knowledge to develop stress tolerant crops is crucial towards breeding superior and exceeding crops under stress. Additionally, wild relatives of many plant species particularly crops like wheat, maize, potato, tomato, cotton, tobacco and sugar cane (Hawkes 1977; Rao et al. 2003) are extremely important as they carry genes for high levels of resistance to several important biotic and abiotic stresses. Discovery and incorporation of resistant genes from these wild species to less resistant or susceptible species hold a great potential for genetic enhancement and sustaining crop improvement and productivity.

Presently, little success has been achieved in developing advanced plants with enhanced stress tolerance under field conditions by exploiting traditional breeding strategies or techniques (Flowers and Yeo 1995). Traditional methods are germplasm dependent and consumes a lot of time and resources (Roy et al. 2011), and are often limited by divergent adverse conditions (Ribaut et al. 1996, 1997; Frova et al. 1999). Hence, the development of genetically modified or engineered plants by the introduction and/or overexpression of desired beneficial genes would be a faster and feasible method for the breeding of ‘‘improved’’ plants than through a conventional breeding method. Additionally, it would be the only choice available when genes of interest belong to distant relatives, cross barrier species, or originate from non-plant sources.

Current engineering strategies bank on the transfer of one or several genes that encode either end products of signaling pathways or biochemical pathways under the control of a suitable promoter. The products of these genes provide shield against environmental stress either directly or indirectly (Bohnert and Sheveleva 1998; Holmberg and Bülow 1998; Smirnoff 1998). Overexpression of biosynthetic enzymes for osmoprotectants, scavengers of reactive oxygen species and stress-induced proteins e.g. late embryogenesis abundant (LEA) proteins are among the approaches reported to develop plants, tolerant to environmental factors that impose water-deficit stress, such as drought, salinity and temperature extremes (Reguera et al.

2012; Agarwal et al. 2013). Table 1.1 represents a recent example of transgenic plants engineered for enhanced resistance to different abiotic stresses exploiting different approaches.

Table 1.1. Examples of transgenic plants engineered for enhanced resistance against various abiotic stresses.

Gene	Gene action	Species	Phenotype	Reference
Adc	Polyamine synthesis	Rice	Drought resistance	Capell et al., 2004
AtTPS1	Trehalose 6 phosphate synthase	*N. tabacum*	Drought resistance & sustained photosynthesis	Almeida et al., 2007
AhCMO	Synthesis of glycine betaine	Cotton	Improved salt tolerance	Zhang et al., 2007, 2009
CHIT33, CHIT42	Endochitinase synthesis	*N. tabacum*	Salt and metal toxicity resistance	Dana et al., 2006
bet A	Choline dehydrogenase	Maize	Drought resistance	Ruidant et al., 2004
Cod A	Choline oxidase	*Brassica juncea*	Tolerance to stress induced photo inhibition	Prasad and Saradhi 2004
HVA1	LEA protein gene	Oat	Delayed wilting during drought stress	Oraby et al., 2005
WCS19	LEA protein gene	*Arabidopsis*	Freezing tolerance	N dong et al., 2002
OsLEA 3.1	LEA protein gene	Rice	Drought resistance for yield in field	Xiao et al., 2007
P5CS	Osmoprotectant proline	*N. tabacum*	Salt tolerance	Konstantinova et al., 2002; Hong et al., 2000
ProDH	Osmoprotectant proline	*Arabidopsis*	Salt tolerance	Nanjo et al., 1999
Stpd1	Osmoprotectant sorbitol	*N. tabacum*	Oxidative stress tolerance	Sheveleva et al., 1998
CBF1, CBF3, CBF4	Transcription factor	*Arabidopsis*	Freezing, salt & drought tolerance	Liu et al., 1998; Gilmour et al., 2000; Haebe et al., 2002
SCOF1	Transcription factor	Soybean	Tolerance to chilling and freezing	Kim et al., 2001
OsDREB1	Transcription factor	Rice	Drought, salt & freezing tolerance	Dubouzet et al., 2003
MnSOD	AOS metabolism	*Alfa alfa*	increased winter survival	Mckersie et al., 2000
SbwAFP	Anti-Freeze Protein	*N. tabacum*	Freezing tolerance	Holmberg et al., 2001
AnnB1	Ca^{+2} dependent phospholipid & cytoskeleton binding protein	Cotton	Improved salt tolerance, relative water content and dry weight	Divya et al., 2010
Apx3	Ascorbate peroxide	Tomato	Chilling & salt tolerance	Komye et al., 2003
AtMDAR1	Monodehydro ascorbate reductase, Ascorbate regeneration	*N. tabacum*	Ozone, salt & PEG tolerance	Eltayeb et al., 2007
GST	Glutathione S- transferase	Rice	Salt & chilling resistance	Zhao & Zhang 2006

AtNHX1	Vacuolar Na^+/H^+ antiporter	*Arabidopsis*	Salt tolerance, increased biomass	He et al., 2005, 2007
AVP1	Codes Vacuolar Pyrophosphatase	*Arabidopsis*	Drought & salt tolerance	Pasapula et al., 2011
SP1	Hsps and molecular Cheperones	*Populus tremula*	Salt and drought tolerance	Wang et al., 2003

Application of same biotechnological approach has enabled researchers to transform plants for increasing their tolerance against varied biotic stresses as well. Based on the molecular basis of plant pathogen interactions, several strategies have emerged for development of crop varieties resistant to pathogens. The easiest approach in developing genetically engineered plants resistant to fungal diseases entails the constitutive expression of a single or a combination of defense related genes in transgenic plants. The strategies utilized to obtain disease resistant plants include the manipulation of resistance by expression of antifungal peptides (defensins), pathogenesis related (PR) proteins, and manipulation of phytoalexins biosynthesis genes (Sanghera et al. 2011; Tohidfar and Khosravi 2015). The expression of genes for antifungal, insecticidal, antiviral, antibacterial resistance and herbicide detoxification for herbicide resistance are also in practice.

Strategies to achieve fungal resistance exploit plant genes acting at different levels of the plant defense system against pathogens. Glucanase and chitinase genes have been used in several crops and have met with success in some cases by providing significant protection (Jongedijk et al. 1995; Tohidfar et al. 2012). Virus resistance is mostly achieved by introducing gene sequences deduced from pathogenic viruses into the crop genome using gene silencing, antisense RNA and RNAi techniques (Zhang et al. 2005; Ramesh et al. 2007). Other partially successful strategies include genes for toxin detoxifying enzymes. However, the tried approach to enhance resistance against bacteria have not achieved desirable levels of protection yet. The developing knowledge and understanding of plant defense mechanisms are expected to enhance resistance levels in this field in the near future (Swathi Anuradha et al. 2008; Takakura et al. 2008).

Presently, the resistance of transgenic plants to pathogens, pests and herbicides have been achieved in almost 20 different crops including wheat, rice, barley, cotton, maize, oilseed rape, sugar beet, tobacco, potato, squash, soybean, alfalfa, tomato, and papaya. Very high levels of resistance to insect, pests, and viral diseases have been achieved so far while examples of fruitful protection to bacterial and fungal diseases are

still scarce. Table 1.2 represents some of the transgenic crops engineered for enhanced resistance against different pathogens.

Table1.2. Examples of transgenic crops engineered for enhanced resistance against different pathogens.

Gene	Plant	Stress	Reference
Viral coat protein	Squash	Resistance to Cucumber Mosaic Virus	USDA, 2000
Viral coat protein	Papaya	Resistance to Papaya Ring Spot Virus	USDA, 2000
Viral coat protein	Soybean	Soybean dwarf virus	Tougou et al., 2006
Class of *Xa21* genes	Rice	Bacterial blight resistance	Song et al., 1995
Chitinase	Rice	Fungal disease resistance	Itoh et al., 2003
Rps1-k	Soybean	*Phytophthora*	Gao et al., 2005
Potato	Defensins (alfAFP)	Resistance to *Alfalfa Verticillium dalhiae*	Gao *et al* (2000)
Bean chitinase	Cotton	Fungal disease resistance	Tohidfar et al., 2005
Cowpea serin PI	Rice	Stem borer	Duan et al., 1996
cryIIIB (Bt toxin)	Eggplant	*Leptinotarsa decemlineata*	Iannacone et al., 1997
cry1H (Bt toxin)	Maize	European corn borer	Jansens, 1997
Snow drop lectin	Potato	Potato aphid	Gatehouse, 1997
Barley trypsin inhibitor	Rice	Insect resistance	Alfonso-Rubi et al., 2003
cry1A (Bt toxin)	Soybean	Insect resistance	Macrae et al., 2005
cryIAc	Chickpea	Insect resistance	Sanyal et al., 2005
cryIAb (Bt toxin),	Cotton	Cotton bollworm	Tohidfar et al., 2008
cry3a (Bt toxin)	Alfalfa	Insect resistance	Tohidfar et al., 2013
Nitrilase	Maize	Bromoxynil (herbicide) resistance	USDA, 2000
Glufosinate N-Acetyltransferase	Soybean	Herbicide resistance	Castle et al., 2004
Glufosinate N-Acetyltransferase	Soybean	Dicamba (herbicide) resistance	Behrens et al., 2007
Aryloxyalkanoate dioxygenase enzymes (aad-1)	Corn	2,4-D (herbicide) resistance	Peterson et al., 2012
aad-1	Soybean	Dicamba (herbicide) resistance	Davis, 2012

Source :Tohidfar and Khosravi 2015; Biotechnol. Agron. Soc. Environ.

Despite the present issues concerning transgenic plants, the cultivation area of transgenic crops for resistance against biotic stresses is rapidly expanding each year with many of them being commercially

released and produced. Considering the production trend of these crops, it is expected that the production as well as commercialization of genetic modified (GM) crops tolerant to abiotic stresses (salinity, drought, extreme temperatures etc.) will materialize in near future as well.

To set a foot ahead and contribute in this expanding field of transgenic research, in the present study we have selected two genes and their functional characterisation studies has been done. First gene, Late Embryogenesis Abundant gene (*AdLEA*) from wild peanut *Arachis diogoi* is involved in giving protection to plants towards abiotic stresses whereas the second gene, an *in vitro* generated variant of defensin (α-*TvD1*) gene from the weedy legume *Tephrosia villosa* imparts tolerance against various biotic stress inducing pathogens. Both the characterization studies were done *in vivo* by raising transgenic tobacco plants for the respective genes and the detailed observations on the transgenic tobacco plants are reported here. This study was undertaken by framing the following objectives.

Objectives

1. Functional characterization of an atypical group 5C Late Embryogenesis Abundant protein (*AdLEA*) from wild peanut *Arachis diogoi* for manipulation of abiotic stress response in transgenic tobacco.

2. Functional characterization of an *in vitro* generated variant of the defensin *TvD1* from the weedy legume *Tephrosia villosa* and its comparative analysis with native defensin for biotic stress responses in transgenic tobacco.

Chapter 2

Review of Literature

2.1 Introduction

Plants are exposed to multiple environmental stresses throughout their life cycle. Natural selection and survival of the fittest have incorporated well developed multi-pathway, multi-level and multi-scale stress-tolerant strategies in plants involving anatomical, physiological, biophysical, biochemical, genetic and developmental modifications. Water deficit caused by dehydration is the most common abiotic stress to which land plants are exposed which impact plants by directly reducing their survival in the natural environment and their productivity in agriculture (Bray 1997). Water deficit is also triggered by osmotic stress due to high salinity or chilling which often results in increased cellular levels of ABA (Zeevaart and Creelman 1988). These elevated levels of ABA consequently induce the expression of stress-related genes and adaptive physiological responses (Cramer et al. 2011; Raghavendra et al. 2010a). Various dehydration induced genes have been identified in a wide range of plant species; their functions have been predicted from their deduced amino acid sequences. However, the exact function of some genes is still unknown. Since the molecular basis for plant tolerance to water stress is not unraveled completely, functional characterization of the genes induced during dehydration is a next logical step in addressing the molecular mechanism of the plants in response to water stress. Under water-deficit conditions, a typical change in gene expression is the induction of genes involved in the synthesis of osmotically active compounds compatible with metabolism. These are low-molecular weight solutes that accumulate in the intracellular compartment for osmotic adjustment most common examples are the non-reducing disaccharide, trehalose and **L**ate **E**mbryogenesis **A**bundant (LEA) proteins (Ingram and Bartels 1996; Ramanjulu and Bartels 2002). Non-reducing disaccharides function as water replacement molecules and vitrification agents and contribute to the formation of bioglasses. These intracellular bioglasses confer long term protection during dry state. In plants LEA proteins are synthesized and stored during seed maturation and are the major seed proteins involved in bioglass formation (Ingram and Bartels 1996; Bray 1997).

2.2 Late Embryogenesis Abundant (LEA) proteins

LEA proteins are highly hydrophilic proteins, which were first identified in cotton as proteins that accumulate during late maturation stage of embryo development in cotton seeds (Dure et al. 1983). In plants, most of LEA proteins and their mRNAs accumulate to high concentrations in embryo tissues during

the last stages of seed development when desiccation occurs (Baker et al. 1988; Bies-Ethève et al. 2008). In most plants, the final stage of seed development maturation is initiated by a reduction in seed water content, which will eventually drop to about 10% (Goldberg et al. 1989). LEA proteins are accumulated in this final stage, in contrast to storage proteins which appear earlier. Moreover, their mRNAs are maintained at high levels in the dehydrated mature embryos, while transcripts of storage protein genes are completely degraded during the last embryogenesis stage (Goldberg et al. 1989). Since orthodox seeds acquire the ability to withstand severe dehydration at this stage, LEA proteins have been associated with desiccation tolerance (Cuming 1999). Members of the LEA family seem to be ubiquitous in the plant kingdom. Since their first description, LEA proteins have been identified and characterized in different plant species ranging from algae (Honjoh et al. 1995) moss (Saavedra et al. 2006), ferns (Raghavan and Kamalay 1993), angiosperms and gymnosperms. In addition, they have now also been identified in some microorganisms (Garay-Arroyo 2000), fungi (Abba' et al. 2006), protozoa, rotifers, nematodes (Browne et al. 2004), insects and crustacean (Tunnacliffe and Wise 2007). The expression of LEA proteins is not restricted to embryonic tissues but also occurs in vegetative tissues of plants in water deficit conditions during various environmental and physiological stresses. Their expression can also be induced in response to exogenous application of abscisic acid, drought, high salinity, sub-optimal temperatures and osmotic conditions on plants implying their role in abiotic stress tolerance mechanism in plants (Dai et al. 2007). However, their precise functions still remain unclear.

2.3 Classification of LEA proteins based on protein structure and motifs

The traditional criterion to classify the LEA proteins was based on the sequence homology of amino acids and conserved motifs from different plants, which presumably undertake different functions during the periods of water deficit (Dure et al. 1989b). However, with increasing information available on newly described members, differences in expression profiles, description in organisms other that plants and the new bioinformatics tool, different criteria to classify LEA proteins were demonstrated. Presently nomenclature of LEA proteins is different according to different classification methods. Table 2.1 compares classification systems proposed by different groups. According to the classification introduced by Battaglia's group, LEA proteins are classified into seven distinct families (Battaglia et al. 2008). Groups 1,

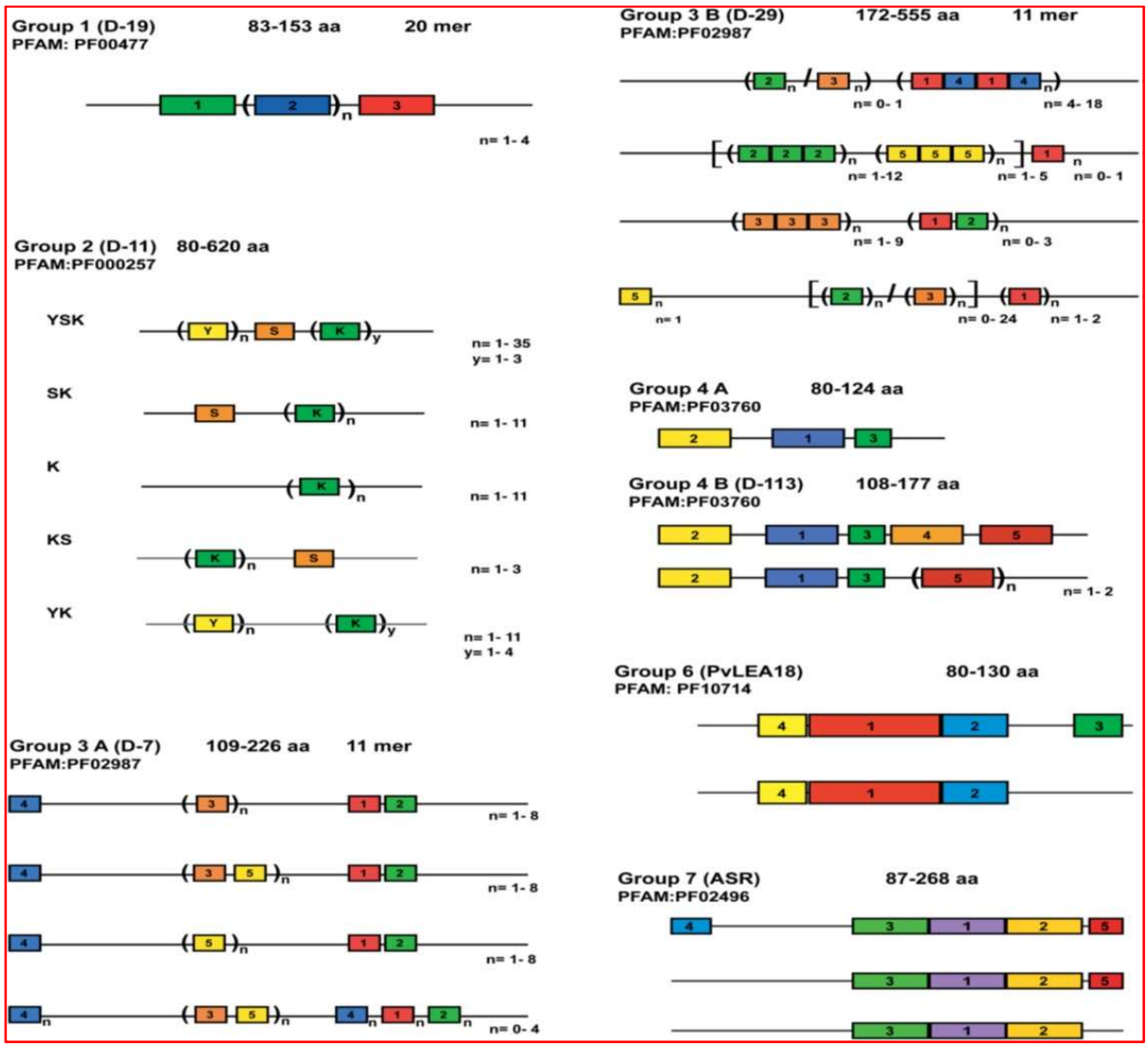
2, 3, 4, 6, and 7 are hydrophilic and have specific motifs within each respective group, hence considered "typical" or genuine LEA proteins. Nevertheless, groups 1, 2 and 3 are considered the major LEA groups containing most members of the protein family and LEA proteins belonging to these groups are collectively called as hydrophilins. Since group 5 lacks a significant motif or consensus sequence and also contain a significantly higher proportion of hydrophobic amino acids, it is considered an "atypical" LEA protein group (Battaglia et al. 2008). Group 5 LEA proteins are non-homologous proteins, which on the basis of sequence similarity are further divided into 3 subgroups corresponding to the first described proteins in this group, 5A (D-34), 5B (D-73) and 5C (D-95) (Cuming 1999; Battaglia et al. 2008). According to Pfam database, this subgroup is classified as 5A (SMP), 5B (LEA_3) and 5C (LEA_2), with corresponding Pfam No's PF04927, PF03242, and PF03168 respectively. The inventory of LEA proteins was first performed in the *Arabidopsis thaliana* genome where fifty LEA genes have been identified (Bies-Ethève et al. 2008). In rice the lea genome comprises 33 genes, 36 in grapevine and 33 in poplar (Hundertmark and Hincha 2008a). LEA protein database (LEAP db) is publicly available at http://forge.info.univ-angers.fr/~gh/Leadb/index.php (Hunault and Jaspard 2010).

Table 1. Different nomenclature of the given LEA protein groups with time-introduction of class nomenclature.

Pfam	Dure et al.,	Bray	Tunnacliffe and Wise	Battaglia et al.,	Bies-Esthève et al.,	Hundermark and Hincha	LEAP db
	1989	1993	2007	2008	2008	2008	2010
PF00257	D11	Group 2	Group 2	Group 2	Group 2	dehydrin	Classes 1 to 4
	D19	Group 1	Group 1	Group 1	Group 1	LEA_5	Classes 5
PF00477	D132						
	D7	Group 3	Group 3	Group 3A	Group 6	LEA_4	Classes 6
PF02987	D29	Group 5		Group 3B			
PF03168	D95			Group 5C	Group 7	LEA_2	Classes 7 and 8
PF03242	D73		LEA_5	Group 5B	Group 6	LEA_3	Classes 9
		Group 4	Group 4	Group 4A	Group 4	LEA_1	Classes 10
PF03760	D113			Group 4B			
PF04927	D34	Group 6	Group 6	Group 5A	Group 5	SMPO	Classes 11
PF03168				Group 6	Group 8	PvLEA18	Classes 12
				Group 5A			

LEA protein amino acid sequence analysis recognizes seven different groups, each one showing distinctive motifs (LEA1–LEA7). In particular, for groups 2, 3 and 4 different protein variants have been found showing a particular organization of their corresponding motifs. Similarity in sequences has not been

detected among the seven LEA protein groups. However, group 5 LEA proteins have higher structural order, high heterogeneity and lack distinct conservative sequence motifs. Schematic representation of motifs distinctive for each LEA protein group are representated in **Figure 2.1** and **Table 2.2**.



Source: Battaglia et al., 2008; Plant Physiology.

Figure2.1. Array of the distinctive motifs in the LEA protein groups. Each block contains a schematic representation of the arrangement of the motifs that distinguish each group of LEA proteins and their corresponding subgroups. Although similar colors and numbers indicate the different motifs for each group, they do not imply any sequence relation among the motifs in the different blocks. The amino acid sequence corresponding to each motif

represented here is shown in Table 2. The range of protein sizes in each group is indicated at the top of each block, in number of amino acid (aa) residues.

Table 2.2. Consensus amino acid sequences of the different motifs characteristic of each LEA protein group

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 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 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/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/R 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 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 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).

Source: Battaglia et al., 2008; Plant Physiology.

2.4 Sub Cellular Localization and Expression Profiles

LEA proteins from different groups show no inclination or preference for a specific subcellular localization in a cell. Subcellular localization studies of LEA proteins implicate that they are not transmembrane proteins rather they were found to be expressed in almost all cellular compartments including the chloroplast (N Dong et al. 2002) mitochondria (Grelet et al. 2005; Tolleter et al. 2010) cytoplasm (Mundy and Chua 1988; Goyal et al. 2005) nucleus (Colmenero-Flores 1999; Gai et al. 2011); (Duan and Cai 2012) endoplasmic reticulum (Ukaji et al. 2001) vacuole (Heyen et al. 2002) and even in

vicinity of plasma membrane (Danyluk et al. 1998). Most LEA proteins from the different groups are accumulated during embryo development in the dry seed.

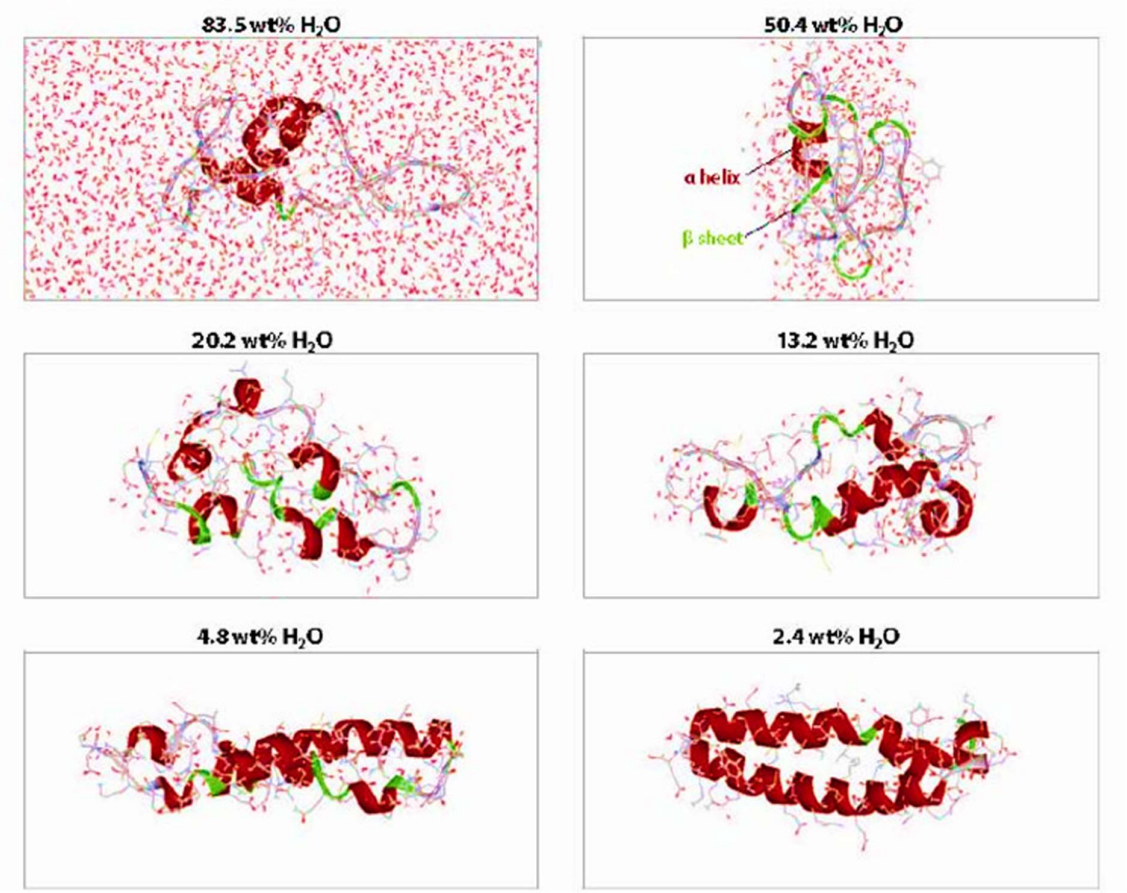
Transcripts from group 5 LEA proteins accumulate during the late stage of seed development and in response to stress conditions, such as drought, UV light, salinity, cold, and wounding (Maitra and Cushman 1994; Stacy et al. 1999; Zegzouti et al. 1999; (Boucher et al. 2010). Localization studies in maize showed localization of Rab28 in the nucleolus of scutellar cells of mature dry embryos (Niogret et al. 1996). RcLEA group 5 LEA protein of *Rosa chinensis* was reported to be a cytoplasm localized protein (Zhang et al. 2014) whereas SiLEA14 from *Foxtail millet* and JcLEA from *Jatropha curcas* were found to be localized in both nucleus and cytosol (Wang et al. 2014; Liang et al. 2013).

2.5 Structure of LEA proteins

The structural characterization study of a LEA protein was initiated by McCubbin et al., way back in 1985, who used a variety of biophysical techniques to study the wheat group 1 protein, Em and reported that this protein lacks expected structural compactness, with an asymmetrical and flexible conformation, and has less secondary structure (i.e. α- helix or β-sheet) with 70% of the protein behaving as random coil. This largely unstructured nature of Em was attributed to its high hydration potential owing to the rare abundance of amino acids Gly, Glu, and Gln (McCubbin et al. 1985). Since then, structural studies of many LEA proteins have been done, most proteins were observed to have an unfolded structure in hydrated state (Goyal et al. 2003; Boudet et al. 2006; Tunnacliffe and Wise 2007).

Lack of conventional secondary structure means that members of the major LEA protein groups are included in the large class of proteins variously called “natively unfolded”, “intrinsically disordered” or “intrinsically unstructured” (Uversky et al. 2000; Tompa 2002). This also explains the reason for failed attempts to crystallize purified LEA proteins for X-ray crystallography (McCubbin et al. 1985; Goyal et al. 2003. [There is just one report of the solution structure of Lea14 from *Arabidopsis thaliana* (Singh et al. 2005), which belongs to atypical group 5 LEA protein]. Such lack of structure clearly has implications for LEA protein function since it is unlikely that they have any catalytic function if they are almost entirely unfolded unless they are induced to fold by co-factor or substrate binding.

Many natively unfolded proteins are known to undergo increased folding under some conditions, usually, when they bind a partner molecule or cation (Uversky et al. 2000). Environmental conditions can also affect folding, and several LEA proteins become more structured when dried. (Wolkers et al. 2001) showed by FTIR that a small (8 kDa) group 3 LEA protein from *Typha latifolia* became largely α-helical when dried rapidly whereas slow drying resulted in intermolecular β-sheet formation, as well as α- helix. Similarly, the group 3 LEA proteins AavLEA1 from nematode *Aphelenchus avenae* (Goyal et al. 2003) and LEAM from pea mitochondria (Tolleter et al. 2007) also gain structure on drying. Li and He (2009) while working with a 66-amino-acid fragment of AavLEA1 reported many of these properties through molecular dynamics simulation technique (Li and He 2009). Water was removed from 83.5 wt % to 2.4 wt % during the course of experiment. As water is removed, the protein progressively assumes a more folded conformation. At 83.5 wt %, LEA protein is completely solvated. At 50% water between 83.5% and 50.4%, the protein is unstructured. In this range and below this point, water molecules no longer are sufficient to fully solubilize the protein. At less than 20% water, the protein becomes more dehydrated and begins to adopt significant secondary structure. α-helical structure is apparent, and hairpin-like structures are formed. At 2.4% water, the structure is very similar to that in the complete absence of water (**Figure 2.2**). The structural changes observed at very low water percentage suggest a functional role for the proteins in dry state rather than in the hydrated state (Goyal et al. 2003; Tolletar er al 2007; Li and He 2009). This property of some LEA proteins to gain structure under some conditions might have an important functional implication in their physiological roles (Olvera-Carrillo et al. 2011).



Source: Hand et al. 2011, modified from Li and He 2009; Biomacromolecules.

Figure 2.2: Representative conformations of the 66-amino acid fragment of a LEA protein (AavLEA1) from nematode *Aphelenchus avenae* at different water contents. The smaller water molecules (grey and red) are depicted in the line style, and the larger LEA protein molecules are denoted using the solid ribbon style (α- helix, red; β- sheet, green; random coil, grey).

2.6 Biochemical properties of LEA proteins

LEA proteins are variable in size, ranging from 5 to 77 kDa among most groups. They can be basic, acid or neutral. Group 1 proteins are acidic to neutral; group 2 comprises proteins with different *p*Is and groups 3 are neutral to basic (Amara et al. 2014). Most of the biochemical properties of LEA proteins are of a consequence and resultant of their hydrophilic nature and unusual amino acid composition. Although significant similarities do not occur between the members of the different groups, a unifying and outstanding feature of most of them is their high hydrophilicity, high content of Gly, abundance of certain amino acid residues such as Glu, Ala, Lys/Arg, and Thr and lack or low proportion of Cys and Trp residues

(Baker et al. 1988; Close et al. 1996; Garay-Arroyo 2000; Oliveira et al. 2007). This high hydrophilicity is likely to be the reason for their lack of conventional secondary structure in the hydrated state. Nevertheless, most of them are intrinsically unstructured proteins and exist prominently as randomly coiled proteins in hydrated state as well as in solutions (Goyal et al. 2003). Since heat-induced proteins aggregation results from their partial denaturation and association via exposed hydrophobic regions, a phenomenon that cannot happen in hydrophilic and natively unfolded proteins in solution (Wise et al. 2003). Hence, the ability of LEA proteins to remain soluble at high temperatures is probably associated with their hydrophilic, unstructured nature as well (Close et al. 1996) Additionally, during SDS- PAGE, they have a tendency to migrate at a higher molecular mass than the one predicted from their deduced amino acid sequences The aberrant mobility of this type of proteins in SDS-PAGE is rather caused by the reduced interaction between SDS and charged amino acid residues due to their unstructured nature (Gentile et al. 2002).

2.7 LEA protein functions

2.7.1 Transgenic studies

Since the discovery of first LEA proteins in cotton, research has been centred on elucidating the role and function of these proteins in plants and there is enough evidence to support the role of LEA proteins in enhancing plant tolerance towards various abiotic as well as biotic stresses. Most attempts to understand their functions had been aimed at over expressing the concerned gene in different plant species, yeast or bacteria. Overexpression of barley (*Hordem vulgare*) HVA1 in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) conferred enhanced drought tolerance (Xu et al. 1996; Sivamani et al. 2000). Protein from tomato (*Solanum lycopersicum*) LE25, improved tolerance against high salinity and freezing when expressed in *Saccharomyces cerevisiae* (Imai et al. 1996). In addition, freezing tolerance of Arabidopsis is increased by overexpression of wheat WCS19 or *Arabidopsis* Cor15A (Shimamura et al. 2006; Artus et al. 1996). Likewise, overexpression of LEA proteins from different species in *Arabidopsis*, wheat, tobacco, rice, lettuce, maize or cabbage produces improved abiotic stress tolerant phenotypes (Hong-Bo et al. 2005; Leprince and Buitink 2010; Amara et al. 2014). However, overexpression of individual genes encoding LEA proteins from *Craterostigma plantagineum* and spinach (*Spinacia oleracea*) in tobacco does not lead to any significant changes in freezing or drought tolerance (Iturriaga et al. 1992; Kaye 1998). These results indicate

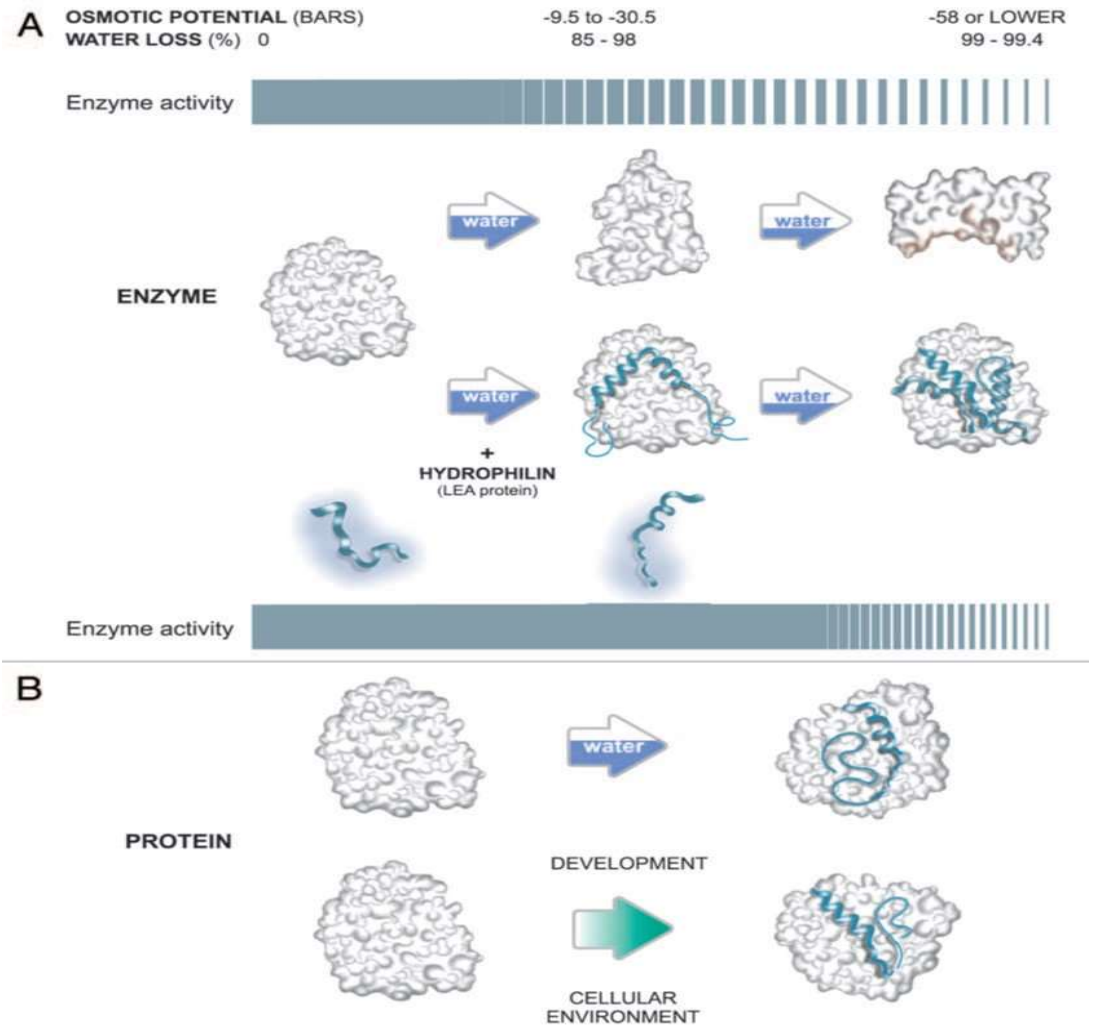
that either all LEA proteins do not have important roles in stress tolerance or that they require other factors for activation (Hundertmark and Hincha 2008). Nevertheless, there are elaborate and extensive studies to correlate the expression of LEA proteins or their genes with stress tolerance. However, the specific molecular functions of LEA proteins are still not clear and by far they have been proposed to act as hydration buffers, membrane protectants and, stabilizers, antioxidants, organic glass formers and/or ion chelators (Tunnacliffe and Wise 2007).

2.7.2 *In vitro* studies; Protein stabilization and molecular shield function

There are several reports which establish the fact that LEA proteins can preserve enzyme activity *in vitro* after freezing, partial dehydration or desiccation (Goyal et al. 2003; Grelet et al. 2005; Reyes et al. 2005). One proposed mechanism of protection is the prevention of protein aggregation, induced by water stress; (Chakrabortee et al. 2007; Hatanaka et al. 2013). Many proteins, including the enzymes like lactate dehydrogenase, citrate synthase, and fumarase form insoluble aggregates when frozen or dried, but in the presence of LEA proteins this aggregation is very much reduced. Additionally, Group 2 LEA proteins can also prevent protein aggregation as a consequence of heat stress (Kovacs et al. 2008a). The anti-aggregation properties of group 3 LEA proteins have been reported in mammalian cells as well, where a LEA protein from nematode *A. avena*, AavLEA1 reduced aggregate formation of overexpressed aggregation-prone proteins **(Figure 2.2)** (Chakrabortee et al. 2007).

A “molecular shield” activity has also been proposed for LEA proteins (Goyal et al. 2003). In the course of gradually increasing dehydrating environment of cytoplasm, LEA proteins could reduce the interaction between partially denatured polypeptides and avert their aggregation. The shield proteins probably have a space-filling activity, which helps in preventing cell collapse as it loose its water content. Another functional hypothesis proposed for LEA proteins is the chaperone activity (Kovacs et al. 2008a; Tunnacliffe and Hincha, 2010). LEA proteins resembles a “holding” molecular chaperone, which functions in the cell by passively stabilizing protein species in a partially unfolded state and preventing aggregation during the stress. Though LEA proteins resemble chaperones, they are distinct from classical folding chaperones in a way that they lack structure and do not form transient complexes with their target proteins through hydrophobic surfaces, owing to their hydrophilicity. Also, they function without ATP, in contrast to classical chaperones which require ATP (Olvera-Carrillo et al. 2010; Reyes et al. 2005). The two functional

mechanisms are not very distinct and both activities might contribute to avoid damage in relation to desiccation tolerance (Tunnacliffe and Hincha, 2010) **Figure 2.3** illustrates a hypothetic model for the function of LEA proteins and other hydrophilins.



Source: Olvera-Carrillo et al. 2011**;** Plant Signaling & Behavior.

Figure 2.3. A hypothetic model for the function of LEA proteins and other hydrophilins. (A) illustrates an example, where an enzyme suffers conformational changes under moderate water deficit that leads to a decrease in its activity and under more severe stress conditions, more critical structural modifications lead to the exposure of hydrophobic residues (red shadowing). The presence of LEA proteins (hydrophilins) (green strand) prevents changes in the conformation of the enzyme, as a result of which the enzyme retains its activity, under water limitation conditions. This effect can be achieved at a 1:1 hydrophilin: enzyme ratio under moderate water stress; however, under severe dehydration, the action of more than one hydrophilin per enzyme molecule could avoid further conformational changes

that may lead to protein aggregation. (B) Under certain environmental conditions, LEA proteins or other hydrophilins could act as molecular "knobs" to modulate the activity of their ligands by inducing light structural modifications upon binding.

2.7.3 Membrane Protection

It is imperative to protect membranes and sustain the cellular and organellar integrity during desiccation. LEA proteins are highly soluble polypeptides owing to their hydrophilic nature, which do not interact with cellular membranes in hydrated conditions. However, some LEA proteins could protect membranes in the dry state by interacting with sugars through H-bonding networking (Hoekstra et al. 2001). It has been proposed that LEA proteins provide protection in the dry state by possibly forming inter- or intra-molecular helix bundles within membranes in a dehydrating cell. Among LEA proteins, group 2 dehydrins like wheat WCOR414, and *Arabidopsis* LTi29 acidic dehydrins were found to be localized in the plasma membrane vicinity during cold acclimation (Danyluk et al. 1998; Puhakainen et al. 2004). It has been proposed that the K-segment, a 15-mer Lys-rich signature sequence of dehydrins might be involved in membrane binding (Close et al. 1996). DHN (Rab17), native and recombinant dehydrins of maize bind to anionic lipid vesicles *in vitro.* This binding results in an increase in amphipathic α-helix (a structural element, which interacts with membranes and proteins). Both binding and enhancement in conformation were attributed to the K-segment (Koag et al. 2003; Koag et al. 2009). Recently, group 2 Lti30 LEA protein has been analyzed *in vitro* and the role of dehydrin K-segments in the binding to membranes and the significance of the flanking His side chains in the interaction between the K-segments and membranes in a pH-dependent manner has been reported (Eriksson et al. 2011). Studies of a group 3 LEA protein (LEAM), found to be localized in pea mitochondria, unfolds another mechanism by which an LEA protein could interact with a membrane and contribute protection in the dry state (Tolleter et al. 2007).

To conclude, although absence of definite structure and lack of hydrophobicity suggest that LEA proteins should act in soluble compartments of cells, still membranes seem to be very important targets for these unusual proteins. It has been anticipated that specific LEA proteins upon folding during desiccation or freezing may contribute to membrane protection (Tunnacliffe and Hincha, 2010).

2.7.4 Ion binding and antioxidant function

During dehydration, there is an increase in the concentration of intracellular components, which include ions as well. Increased ionic concentration can have potentially damaging effects on macromolecular structure and function. It has been suggested that LEA proteins, due to the presence of many charged amino acid residues in their structure might act to sequester ions (Dure 1993; Danyluk et al. 1998) . Ca^{2+} binding property had been proposed for both groups 2b and 3a proteins (Wise and Tunnacliffe 2004). A dehydrin related protein from celery (*Apium graveolens*) had been shown to bind Ca^{2+} when phosphorylated and was located in the vacuole (Heyen et al. 2002). Group 2 acidic proteins, ERD10, ERD14 and, COR47 were also reported to exhibit phosphorylation-dependent Ca^{+2} binding. Mass spectrometry data suggested phosphorylation sites to be located in the serine motif of these proteins (Alsheikh et al. 2005). Studies have shown that group 2 LEA proteins can bind a number of other metal ions as well. His residues, which are present in abundance, in most group 2 LEA proteins are proposed to be the factors responsible for these interactions (Svensson et al. 2000). The antioxidant properties as well as *in vitro* scavenging activity for hydroxyl radicals demonstrated and reported for the citrus CuCOR19 protein might be linked to this metal binding property of these proteins (Hara et al. 2005a). To conclude, LEA proteins might curtail oxidative stress in cells during dehydration by scavenging ROS and/or by sequestering metal ions, the source of ROS generation (Tunnacliffe and Wise 2007).

2.7.5 Other Functions; organic glass formation and hydration buffers

Another important function of LEA proteins might be their contribution to the formation of tight glass matrices (biological glasses) with sugar moieties in desiccated cells. When the water content reduces below 10% on a dry weight basis in a desiccating cell, the cytoplasm vitrifies and enters into the so called “glassy state” (Buitink and Leprince 2004). The formation of intracellular glasses is crucial and imperative for survival of the plants in the dry state. LEA proteins get accumulated to high levels in seeds (Roberts et al. 1993) and increase the density of the sugar glasses by enhancing the hydrogen-bonding of the LEA/ sucrose mixture (Buitink and Leprince 2004). Based on the hydrophilic nature of LEA proteins, hydration buffer function has been proposed for them for reducing the rate of water loss during dehydration. Hydration buffers let on ample water activity for proteins to retain function during osmotic, freezing or, partial drought stress (Garay-Arroyo 2000). Hydration buffer activity for LEA proteins was proposed through functional

studies on group1 LEA protein Atm6 employing an *Arabidopsis* knockout mutant whose seeds exhibited premature dehydration (Manfre et al. 2006).

2.8 The Versatility of LEA Proteins

Interestingly, among LEA proteins, proteins with similar sequences might show different structure and *in vitro* properties, which is to say that a single LEA protein might have more than one function. (Nakayama et al. 2007; Lin and Thomashow 1992). The group 3 mitochondrial protein LEAM can protect, both proteins and membranes. Similarly, the chloroplast LEA-like protein COR15am is also reported to exhibit both functions (Tolleter et al. 2007); a group 2 LEA protein from *Citrus* shows antioxidant, nucleic acid and, ionic binding properties (Hara et al. 2005b). Exhibiting more than one function is a common feature among many proteins and it is more likely to emerge comparatively in unfolded proteins, rather than in folded ones (Kovacs et al. 2008a). This versatility or ambidexterity may be a common element of LEA proteins endowed by their unfolded, unstructured nature in solution, which make them truly multi-talented, multi-functional proteins.

2.9 Biotechnological Applications of LEA Genes

Positive response of LEA proteins in overall plant stress tolerance mechanism is unarguable. Genetic engineering technology allows the overexpression of LEA proteins in plants, which results in developing a variety of plants with improved tolerance against environmental stresses most often related to salinity or drought stress. The LEA transgenic plants with elevated growth rates and decreased wilting of aerial parts under laboratory as well under field trials have been reported demonstrating the potential of these proteins in developing crops more tolerant to varied stresses (Leprince and Buitink 2010). Apart from agronomical purposes, the property of LEA to prevent aggregation of proteins could be exploited for other biotechnological applications as well. The usage of a group 3 LEA protein as a fusion partner helps expression of recombinant recalcitrant proteins in a soluble form in *E. coli* (Singh et al. 2009). Additionally, the anti-aggregation properties had been demonstrated *in vivo* also in another group 3 LEA protein. The co-expression of group 3 AavLEA1 LEA protein along with aggregation-prone proteins having larger polyglutamine (polyQ) or polyalamine (polyA) sequences substantially reduces the expansion of protein

aggregates in mammalian cells. This anti-aggregation property can be further exploited in studies associated with neurodegenerative diseases (Chakrabortee et al. 2010).

2.10 Atypical Group 5 LEA proteins

Since group 5 protein members lack a significant motif or consensus sequence with a significantly higher proportion of hydrophobic amino acids, they are considered as atypical LEA proteins (Battaglia et al. 2008). Because of their hydrophobic nature and higher structural order, these LEA proteins are not classified under "Hydrophilins". A distinct property of group 5 members is that they are insoluble after boiling suggesting that they are probably heat labile and may adopt a globular conformation upon heating (Singh et al. 2005; Hundertmark and Hincha 2008). Moreover, among group 5, group 5C LEA proteins have some distinct properties, which make them unique among the LEA protein family. These proteins are natively folded and contain a larger proportion of β- sheets than α- helices in the hydrated state as opposed to other LEA family proteins, which are intrinsically unstructured (Hundertmark and Hincha 2008b). At present, LEA14-A from *Arabidopsis,* which is a group 5C protein, is the only member from LEA family, which was confirmed to have a defined secondary and tertiary structure in solution (Singh et al. 2005). Other characteristic properties of group 5C LEA proteins include lower instability index, narrow range of GRAVY values and a low proportion of polar (hydrophilic) and smaller amino acids but with a high proportion of non-polar (hydrophobic) amino acids (He et al. 2012). All these differences imply that Group 5C LEA proteins might function differently from other groups of LEA proteins.

Presently, only a small fraction of group 5C LEA proteins have been cloned and characterized. Among them many proteins have been reported to be associated with resistance to multiple stresses as reported for cotton *LEA14-A* (Galau et al. 1986), *Craterostigma plantagineum PcC27- 45* (Hand et al. 2011), soybean *D95-4* (Goldberg et al. 1989), hot pepper *CaLEA6* (Cuming et al. 1999), Arabidopsis *LEA14* (Dure et al. 1989b), *At2g44060* (Bies-Ethève et al. 2008) and sweet potato *IbLEA14* (Park et al. 2011a). Overexpression of maize group 5 LEA gene Rab28 resulted in enhanced water stress tolerance of transgenic maize plants (Amara et al. 2013). JcLEA, a group 5 LEA-Like protein from *Jatropha curcas* conferred a high level of tolerance to dehydration and salinity when overexpressed in *Arabidopsis thaliana* (Liang et al. 2013). Also, SiLEA14, an atypical group 5C LEA protein conferred salt and osmotic stress to foxtail millet (Wang et al.

2014). In addition a Group 5 LEA protein from *Zea mays*, ZmLEA5C enhanced tolerance to osmotic and low-temperature stresses when expressed in tobacco and yeast (Liu et al. 2014). Recently *RcLEA*, a late embryogenesis abundant protein gene isolated from *Rosa chinensis* was reported to, confer tolerance to *Escherichia coli* and *Arabidopsis thaliana* under various abiotic stresses (Zhang et al. 2014). All these studies suggest the close association of subgroup 5C LEA proteins with resistance to multiple abiotic stresses.

2.11 Background Information

Wild relatives of many plant species particularly crops like wheat, maize, potato, tomato, cotton, tobacco and sugar cane (Hawkes 1977; Rao et al. 2003) are extremely important as they carry genes for high levels of resistance to several important biotic and abiotic stresses. Discovery and incorporation of resistant genes from these wild species to less resistant or susceptible species hold a great potential for genetic enhancement and sustaining crop improvement and productivity. *Arachis diogoi* Hoehne (Syn. *A. chacoense*) is a diploid wild relative of cultivated peanut *Arachis hypogaea* L. There are many reports that confirm that *A. diogoi* is highly resistant to Late Leaf Spot (LLS) causing pathogen, *Phaeoisariopsis personata* as well as several other fungal and viral pathogens (Rao et al. 2003; Subrahmanyam et al. 1989). In the previous study from our group, *Arachis diogoi* was challenged with late leaf spot pathogen, *Phaeoisariopsis personata,* and a transcript derived fragment (TDF) corresponding to a LEA gene was identified using differential gene expression study (Kumar and Kirti 2015). In the present study, the full-length cDNA sequence was cloned from this partial fragment by RACE-PCR and the gene was designated as *AdLEA.* Functional characterization studies were further conducted related to *AdLEA* gene, framing the following objectives.

2.12 Objectives for Chapter 4

1. Cloning and isolation of full length sequence of *AdLEA* gene from *Arachis diogoi.*
2. Sequence and phylogenetic analysis of deduced AdLEA proteins across diverse taxa.
3. To study differential expression of *AdLEA* in response to various signalling molecules, temperature, desiccation, osmotic and, oxidative stress inducers.
4. To study sub-cellular localization of *AdLEA* within the plant cells.

5. To generate tobacco transgenic lines with a construct pCAMBIA 2300-*AdLEA* under the transcriptional control of the CaMV 35S promoter using the *Agrobacterium*-mediated leaf disc transformation.
6. Molecular analysis of transgenic plants to confirm the presence, integration, and expression of *AdLEA* transgene.
7. To study the effect of various abiotic stress treatments to transgenic tobacco seedlings and wild-type plants to carry out biochemical studies such as chlorophyll content and lipid peroxidation with transgenic lines and wild-type plants in order to establish the functions of *AdLEA* transgene in plants with respect to abiotic stress.
8. To study plant morphology, proline and chlorophyll content and chlorophyll fluorescence measurements of *AdLEA* transgenic plants using Pulse-Amplitude-Modulation (PAM) chlorophyll fluorometer under progressive drought stress and recovery.
9. Reactive Oxygen Species (ROS) quantification in *AdLEA* transgenic plants using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and nitro blue tetrazolium (NBT) staining against dehydration and salinity stress respectively.
10. Expression analysis of few stress-responsive genes in *AdLEA* transgenic plants before and after drought stress.

2.13 Defensins: Introduction

In nature, plants like all living organisms, repeatedly encounter attack from pathogens, for instance, insects, fungi, and bacteria. Plants have developed a number of defense mechanisms, to resist and survive against these circumstances which include production of defense factors like hydrogen peroxide, phenolics, terpenoids, alkaloids, polyacetylenes, and diversified pathogenesis-related (PR) defense proteins (Osbourn et al. 1999; Van Loon et al. 2006 ; Broekaert et al. 2010) including the production of antimicrobial peptides (AMPs). AMPs are small proteins with antimicrobial activity, which are synthesized by organisms among all kingdoms comprising prokaryotes, lower and higher eukaryotes. These are secreted proteins that can efficiently and conveniently inhibit the growth of viruses, bacteria, fungi and parasites (Broekaert et al. 1995). AMPs in unicellular organisms might successfully provide their hosts with the dominance and

advantage to compete with organisms that require similar ecological and nutritional requirements. On the other hand, in multicellular organisms, AMPs establish a primitive mechanism of innate immunity and form the first line of defense to protect their hosts from microbial attack (Ganz 2003). The innate immunity exemplifies an evolutionarily ancient and broad defense mechanism found in insects, plants and vertebrates (Hegedüs and Marx 2013). As products of single genes, AMPs can be synthesized in a prompt and flexible way, and due of their small size, they can be synthesized by the host with a least possible energy and biomass (Bowles 1990; Broekaert et al. 1995; Heil 2002). AMPs are subdivided into distinct classes, based on their tertiary structure being thionins (Bohlmann and A. 2011), defensins (Lay and Anderson 2005), lipid transfer proteins (Kader 1996), knottins (Cammue et al. 1992), snakins, heveins (Broekaert et al. 1995) and cyclotides (Craik et al. 1999).

2.14 Plant defensins

Plant defensins are the largest groups of AMPs, originally termed γ-thionins because of their similarity in size and cysteine content with thionins. These peptides are cysteine-rich and have varied sequences and structures, that are stabilized by three or four conserved cysteine disulphide bridges, which give them compact shapes. They are small in size ranging from 12 to 50 amino acid residues approximately (2–6 kDa) and have at least two positively charged residues (arginine /lysine) (Ren et al. 2011; Gao and Zhu 2012).

2.15 Tissue and subcellular localization of plant defensins

Most plant defensins initially, were isolated from seeds. However, both gene expression and protein localization studies showed that plant defensins are present in all tissues of the plant including pollen, fruits, flowers, leaves, shoots, roots, cotyledons and, bark (Carvalho and Gomes 2009; de Oliveira Carvalho and Moreira Gomes 2011). Interestingly, some plant defensins are expressed exclusively in very specific or precise parts of a tissue. For example, the maize defensins ZmES1-4, are expressed in the female gametophyte exclusively (Amien et al. 2010), while other defensin from the same species, ZmESR6 is expressed exclusively in the endosperm of immature kernels (Balandín et al. 2005). Other plant defensins are constitutively expressed in diverse tissues. The *A. thaliana* defensin AtPDF2.2, for example, is expressed in seedlings, stems, leaves, roots, flowers, siliques, and even in distinct structures such as syncytia in nematode infected roots (Siddique et al. 2011).

Most plant defensins are proposed to be secreted based on their predicted N-terminal signal peptide and the absence of any known internal retention signal in their primary sequence. The extracellular localization of defensins was confirmed by immunolocalization studies for many plant defensins from seeds along with *Medicago sativa* MsDef1 and *Raphanus sativus* RsAFP2 (Terras 1995; Gao et al. 2000). Another set of experimental evidences proposed that some plant defensins are not transported to the apoplastic region and rather retained intracellularly. The first convincing report about the vacuolar localization of defensins was based on the studies of the flower-specific NaD1 in tobacco (Lay et al. 2003). NaD1 contains an additional C-terminal domain, which has been speculated to contain a vacuolar-sorting determinant (VSD) that guides its localization towards vacuoles.

Recently, it has been suggested by Oomen et al. (2011) that a defensin from *Arabidopsis halleri* AhPDF1.1, is not secreted in the plant. It enters the endomembrane pathway and on its way to the lytic vacuole, it is withheld in intracellular compartments. However, vacuolar localization could not be detected for AhPDF1.1 (Oomen et al. 2011). Since AhPDF1.1 does not contain a C-terminal prodomain, it has been postulated that its redirection to the vacuole requires a VSD, of which two types are presently known. The first is the sequence-specific VSD (ssVSD) essentially characterized by the "NPIR" consensus sequence and the other being the C terminal VSD (ctVSD) that lacks any known consensus sequence but shows predominance of hydrophobic amino acids, and is mainly C-terminally located (Robinson et al. 2005; Zouhar and Rojo 2009). Of the two VSDs, none was found in AhPDF1.1 (Oomen et al., 2011). A possible explanation could be that relatively few VSDs have been studied and characterized till date and new groups need to be explored still. Additionally, Carter et al., based on proteomic analyses, suggested that many proteins are targeted to the vacuole via mechanisms that do not count solely on amino acid sequence or distribution patterns (Carter et al. 2004).

2.16 Structural conformation and structure activity relationships of plant defensins

Plant defensins are peptides consisting of 45–54 amino acid residues and have a well-conserved three-dimensional structure held by a cysteine-stabilized α/β (CSαβ) motif, which forms one α-helix followed by three anti-parallel β-sheets. Further, their amino acid sequence is also conserved due to the presence of six to eight cysteine residues, which form three to four disulphide bridges in the sequence of Cys1-Cys8, Cys2-

Cys5, Cys3-Cys6, and, Cys4-Cys7 (Lay and Anderson,2005). Plant defensins with five disulphide bonds have also been described, i.e., the peptide from *Petunia hybrida* (PhD1), whose cysteine residues interact as follows: Cys1-Cys10, Cys2-Cys5, Cys3-Cys7, Cys4-Cys8, and Cys6-Cys9 (Janssen et al. 2003). This additional disulphide bond does not alter the typical three-dimensional structure of the defensin in any way and is present after the α-helix and the first β-sheet (Janssen et al., 2003).

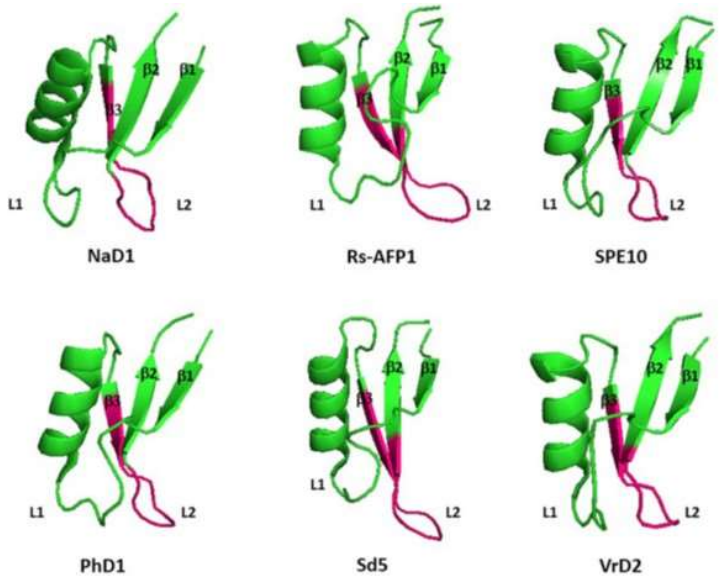
According to the structure of their precursor protein, plant defensins can be subdivided into two groups. The first group encompasses defensins in which the precursor is composed of a signal sequence and a mature defensin domain. The signal sequence targets the protein to the endoplasmic reticulum, where it is folded and subsequently enters the secretory pathway. In a second and less common group, the precursor protein contains an additional acidic C-terminal prodomain that is proteolytically removed during or after transit through the secretory pathway (Lay et al. 2003, 2014). This type of defensins have been identified in Solanaceous plants, such as *Petunia hybrida* and *Nicotiana alata* (Lay et al. 2003). Recently, Lay and co-workers assigned a cytoprotective and subcellular targeting function to this prodomain (Lay et al. 2014).

Extensive structural studies by crystallography and nuclear magnetic resonance (NMR) have been done on defensins during the last few years. The defensins from *Raphanus sativus* (Rs-AFP1), *Pisum sativum* (Psd1), *Petunia hybrida* (PhD1), *Nicotiana alata* (NaD1), *Saccharum officinarum* (Sd5) and *Pachyrrhizus erosus* (SPE10) are among the peptides with antifungal activity, whose structures have been elucidated (Fant et al. 1998; Almeida et al. 2002; Janssen et al. 2003; Lay et al. 2003; de Paula et al. 2011; Song et al. 2011) (**Figure 2.4**). Sequence alignment of amino acids of plant antifungal defensins show that they do not have conservative amino acid sequences, except the cysteine residues and a glycine residue present in the second β-sheet (Pelegrini and Franco 2005; Van der Weerden and Anderson 2013).

According to their structural features, plant defensins show a conserved γ-core signature classified as the dextromeric isoform, which is associated with the conserve amino acid sequence of the NH2. . .[X1−3]-[GXC] = [X3−9]-[C]. . .COOH region (**Figure 2.4 & 2.5**). This conservation in the primary sequence gives them a three- dimensional conformation of γ-core motif, which consists of two antiparallel β-sheets, with an interpolated turn region. In disulfide-stabilized peptides, these γ-core motifis are very important for antimicrobial activity (Yount et al. 2007), not only for their cysteine content but especially due to the presence of positively charged residues at the second β-turn of their structure (Fant et al. 1998). This

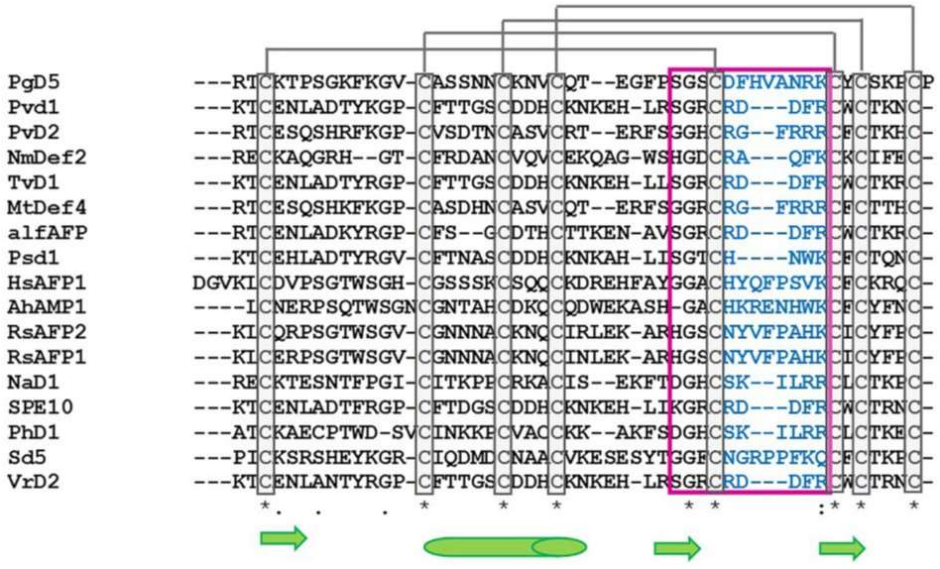
characteristic was first observed in *R. sativus* defensin 1(Rs-AFP1) when its structure was determined and a mutation analysis was also performed using the peptide isoform Rs-AFP2 (De Samblanx et al. 1997; Fant et al. 1998). It was demonstrated that positively-charged aminoacids located at the γ-core motif were essential for the antifungal activity of these peptides, and the substitution of neutral residues inside this γ- core by other positively-charged amino acid residues enhanced their activity towards pathogenic fungi. Similar feature was demonstrated in defensins from *Medicago trunculata*, where it has been verified that the antifungal activity of MtDef1 was due to the presence of four positively-charged amino acids, also located in the γ-core region, which was absent in the structure of the non-antifungal peptide MtDef2 (De Samblanx et al. 1997)

Although, major factors of the antifungal activity and morphogenicity of plant defensins lie in their γ-core motifs, some determinants outside the γ- core motifs are also reported that could contribute to their antifungal activity (De Samblanx et al. 1997; Lay et al. 2003a, b; Sagaram, et al. 2011). More studies are still required to elucidate the structure activity relationships of defensins completely. Nevertheless, the current knowledge about their structural activity will enable a rational design of more potent peptides with a wider spectrum of antifungal activity. For example, it has been feasible to convert morphogenic MsDef1 to near non morphogenic MtDef4 by substituting its own γ-core motif with that of MtDef4 (Sagaram et al. 2011).



Source: Lacerda et al. 2014; Frontiers in microbiology.

Figure 2.4. Three-dimensional structure of six antifungal defensins from plants. Pink region highlights the γ-core motif of each peptide. β1: β-sheet 1; β2: β-sheet 2; β3: β-sheet3; L1: Loop1; L2; Loop2: NaD1: *Nicotiana alata* defensin1; Rs-AFP1: *Raphanus sativus* antifungal peptide 1; SPE10: *Pachyrrihizus erosu* peptide A; PhD1: *Petunia hybrida* defensin1; Sd5: *Saccharum officinarum* defensin 5; VrD2: *Vigna radiata* defensin 2.



Source: Lacerda et al. 2014; Frontiers in microbiology.

Figure 2.5. Alignment of the aminoacid sequence of antifungal plant defensins. PgD5: *Picea glauca* defensin ; Pvd1: *Phaseolus vulgaris* defensin1; PvD2: *Phaseolus vulgaris* defensin2; NmDef1: *Nicotiana megalosiphon* defensin;

TvD1: *Tephrosia villosa* defensin; MtDef4: *Medicago trunculata* defensin4; alfAFP: *Medicago sativa* antifungal peptide1; Psd1: *Pisum sativum* defensin1; HsAFP1: *Heuchera sanguinea* antifungal peptide; AhAMP1: *Aesculus hippocastanum* antimicrobial peptide1; RsAFP1: *Raphanus sativus* antifungal peptide1; RsAFP2: *Raphanus sativus* antifungal peptide2; NaD1: *Nicotiana alata* defensin1; SPE10: *Pachyrrihizus erosus* peptide; PhD1: *Petunia hybrida* defensin1; Sd5: *Saccharum officinarum* defensin5; VrD2: *Vigna radiata* defensin2.Asterisk indicates conserved cysteine aminoacid residues among antifungal defensins (grayboxes). Gray lines represent the disulfide bridges between cysteine aminoacid residues. Pink box and blue amino acid residues correspond to the γ-core region. Green arrows indicate β-sheet region and green cylinder indicate α-helix region.

2.17 Plant defensins as enzyme inhibitors

Defensins are able to inhibit α amylase or other proteinases from insects and in cases can inhibit both trypsin and chymotrypsin (Pelegrini and Franco 2005). Plant defensins constitute one of the six structural classes of proteinaceous amylase inhibitors. Three isoforms from *Sorghum bicolor* (SIa1, SIa2, and SIa3) were first reported α -amylase inhibitory plant defensins. These proteins could inhibit the digestive α -amylases from the gut of locusts and cockroaches strongly but were unable to inhibit mammalian α-amylases (Bloch and Richardson 1991). Subsequently, two other defensins were isolated and identified from barley (BIa1 and BIa2) that inhibited the α-amylase activity of the cereal grain insect pest *Tenebrio molitor* (Mendez et al. 1990). Moreover, defensin from *Vigna radiata* (VrD1) and a peptide similar to plant defensins from *Carica papaya* were shown to inhibit α-amylases from *Tenebrio molitor* and *C. maculatus* respectively (Liu et al. 2006; Farias et al. 2007).

Plant defensins with activity towards plant proteinases have also been demonstrated. Two defensins namely a defensin from *C. fistula* seeds (Wijaya et al. 2000) and a defensin from cowpea (Cp-thionin) have been reported to exhibit trypsin inhibitory activity (Melo, F. R., Rigden, D. J., Franco, O. L., Mello, L. V., Ary, M. B., Grossi de Sá, M. F. and Bloch 2002). Furthermore, a defensin from *N. alata*, NaD1 demonstrated activity towards both trypsin and chymotrypsin from *Helicoperva armigersa* and *H. punctigera*, in addition to its antifungal activity (Lay et al. 2003). The conservative CSαβ motif present in plant defensins might be the key to explain this diversity in function of NaD1 though there is still no hypothesis to explain it fully.

2.18 Plant defensins with antibacterial activity

Contrary to fungi and insect pests, phytopathogenic bacteria are not very common enemies of plants (Thomma et al. 2002). Presumably, for this reason, only few plant defensins exhibit anti-bacterial activity. Accordingly, some anti-bacterial defensins have been reported such as pseudo-thionin (Pth-St1) of *Solanum tuberosum* (Moreno et al. 1994), So-D1-7 of *Spinacia oleracea* (Segura et al. 1998), Pa-AMP-1 of *Phytolacca americana* (Liu et al. 2006), Fa-AMP1 and Fa-AMP2 of *Fagopyrum esculentum* (Fujimura et al. 2005), VaD1 of *Vigna angularis* (Chen et al. 2005) and Cp-thionin II of *Vigna unguiculata* (Franco et al. 2006) Fa-AMPs, belonging to the defensin family, were incorporated in the glycine-rich family because of their primary structural features that exhibit both 10 glycine residues and continuous sequences of glycines (–GGG– and –GG–) characteristic of glycine-rich proteins and, 8 cysteine resides and continuous sequences of cysteines (–CC–), characteristic of defensins. Thus, these proteins were the first to be classified into two families. Some plant defensins appear to be active towards both Gram-positive and Gram-negative bacteria as well as fungi. Additionally, it was also observed that plant defensin So-D1-7 was able to reduce 50% of the development of the bacteria *Clavibacter michiganensis* and *Ralstonia solanacearum*, which causes black rot at 20 µg ml-1 concentration as well as work against some phytopathogenic fungi (*Fusarium culmorum*, *F. solani*, *Bipolaris maydis*, and *Colletotrichum lagenarium*) at 25 µg ml-1 concentration (Segura et al. 1998).

Furthermore, similar data was obtained for VaD1, which inhibits the growth of *Salmonella typhimurium* – the root cause of intragastric infections and *Staphylococcus epidermidis* – a cause of common infections in immune deficient patients, as well as the fungus *F. oxysporum* (Chen et al 2005).

2.19 Plant defensins with antifungal activity

Antimicrobial defensins can also inhibit fungal growth. Most plant defensins inhibit filamentous fungi or yeasts (Thomma et al. 2002) and are usually capable of inhibiting more than one fungal species, such as the defensin isolated from *P. vulgaris*, which showed activity against *F. oxysporum* and *M. arachidicola* (Wang et al. 1999). In addition, a peptide from *Trigonella foenum-graenum* showed activity against both fungi, *R. solani* and *Phaeoisariopsis personata* (Olli and Kirti 2006). However, plant defensins generally present a specific activity towards a unique pathogen, as observed for a peptide from *P. sativum*, which was able to

inhibit the activity of *Neurospora crassa* (Lobo et al. 2007). Another example is defensin from *N. alata* NaD1 that was able to inhibit 56% growth of *F. oxysporum* at a 2 μg ml-1 concentration (Lay et al. 2003). Many of the plant defensins have now been reported to inhibit the growth of a broad range of hemi-biotrophic and necrotrophic fungi at micro molar concentrations *in vitro* while others have no known antifungal activity (Thomma et al. 2002; Lay and Anderson, 2005; Carvalho Ade and Gomes, 2009). It has not been possible to determine the antifungal activity of plant defensins against biotrophic fungi because of the difficulty of culturing these fungi *in vitro*.

Antifungal plant defensins are divided into two different subgroups: morphogenic, which cause reduced hyphal elongation with a concomitant increase in hyphal branching and non morphogenic, which reduce hyphal elongation without causing significant morphological changes (Terras et al. 1992; Broekaert et al. 1995). For example, MsDef1 (previously referred to as AlfAFP, (Gao et al. 2000)) induces prolific hyper branching of hyphae in *Fusarium graminearum,* whereas MtDef4 does not (Ramamoorthy et al. 2007a,b).

2.20 Modes of action of antifungal defensins

Extensive research has provided considerable insight into the modes of antifungal action of plant defensins. Several aspects have been investigated and explored in this context: (i) their interaction with fungal-specific plasma membrane components, (ii) putative uptake of plant defensins and identification of intracellular targets, (iii) downstream signaling pathways activated by plant defensins with emphasis on the induction of apoptosis, and (iv) tolerance mechanisms of susceptible yeast and fungal species against the plant defensin activity (Aerts et al. 2008; De Brucker et al. 2011; Wilmes et al. 2011).

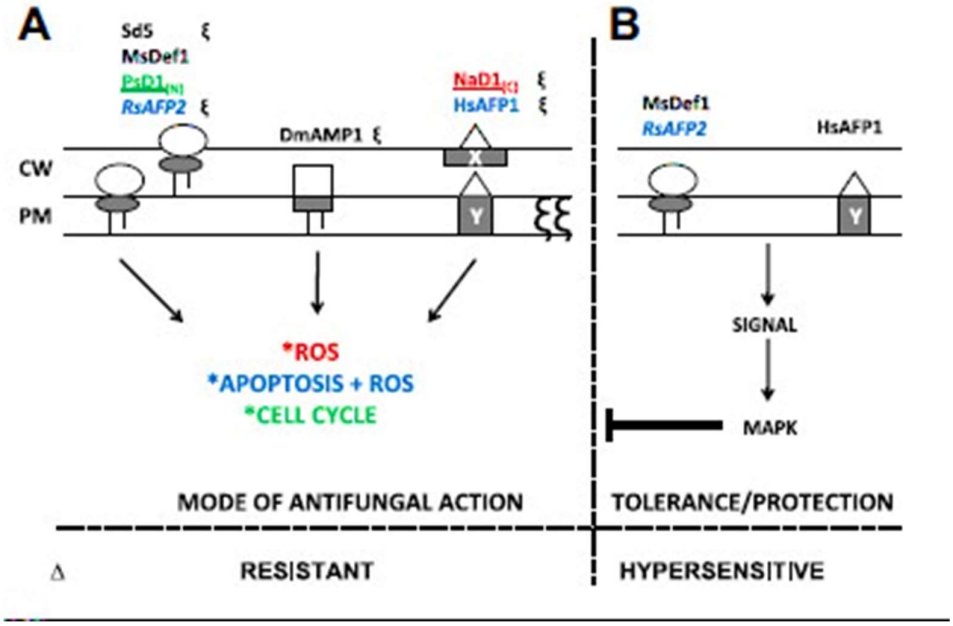
The first studies on the mode of action of plant defensins were reported more than a decade ago which signifies complex sphingolipids such as inositol phosphoryl-containing sphingolipids (M(IP)2C) and glucosylceramides (GlcCer) being interacting partners of fungal-specific membrane components (Thevissen et al. 1997, 2000, 2004). Sphingolipids are very important structural components of eukaryotic membranes and also act as secondary messengers in regulating the subtle balance between cell death and survival (Thevissen et al. 2006). GlcCer is produced by most fungal pathogens (Barreto-Bergter et al. 2004), and was recently reported to be required for virulence in *Candida albicans* (Noble et al. 2010). The important role for sphingolipids in the killing process of fungi by plant defensins has been established for five different

plant defensins, namely DmAMP1 from *Dahlia merckii* (Thevissen et al. 2000), RsAFP2 from *Raphanus sativus* (Thevissen et al. 2004), MsDef1 from *M. sativa* (Ramamoorthy et al. 2007a), Sd5 from *Saccharum officinarum* (de Paula et al. 2008), and Psd1 from pea (de Medeiros et al. 2010). Studies related to backbone dynamics of Psd1 and Sd5 indicated that the dynamic properties of Sd5 were completely different from those of Psd1, demonstrating that although defensins share similar structures, their dynamics can be extremely diverse or distinct. Results of this study proposed that specific regions of the plant defensins are responsible for their ability to interact with GlcCer, ensuring anchorage to fungal membranes (de Medeiros et al. 2010; de Paula et al. 2011). It has been demonstrated recently that the major determinants of the antifungal activity and morphogenicity of MsDef1 and MtDef4 from *M. truncatula* reside in their γ-core motif (Sagaram et al. 2011). Interestingly, the membrane interaction of Psd1 was also found to be mediated in part by this γ-core motif.

Recently, Thevissen et al. demonstrated that RsAFP2 present in the *C. albicans* cell wall interacts with GlcCer, but is not taken up intracellularly (Thevissen et al. 2012). This finding was contrary to the intracellular localization studies of the plant defensin NaD1, where it was shown that NaD1 is taken up inside and localized to the cytoplasm of susceptible fungi, which results in granulation of the cytoplasm and subsequent cell death (van der Weerden et al. 2008). It was proposed that NaD1 permeabilized fungal cells via a novel mechanism, which required the presence of the fungal cell wall (van der Weerden et al. 2010). Later, the authors hypothesized that an unidentified NaD1-receptor might be located in the proteinaceous layer of the cell wall.

Many plant defensins have been shown to induce apoptosis or programmed cell death of susceptible fungal and yeast species. RsAFP2 induces the accumulation of intracellular reactive oxygen species (ROS) and apoptosis in cells of the human pathogenic yeast *C. albicans* (Aerts et al. 2007a, 2007b). This RsAFP2-induced killing of *C. albicans* cells requires caspase or caspase-like proteases, but is independent of metacaspase 1. Additionally, it has been also demonstrated that RsAFP2 induces septin mislocalization and accumulation of apoptosis-inducing molecules, i.e., ceramides, in membranes of *C. albicans* (Thevissen et al. 2012). Similarly, treatment of *C. albicans* cells with other plant defensin HsAFP1 from *Heuchera sanguinea* resulted in ROS accumulation and the induction of apoptosis (Aerts et al. 2011). To defend themselves against the action of plant defensins, susceptible yeast, and fungal species make use of various

tolerance mechanisms. Studies showed that apparently, the MAPK cell wall integrity pathway is involved in protection of the plant pathogenic fungus, *Fusarium graminearum* to the radish RsAFP2 and to the alfalfa MsDef1 defensins (Ramamoorthy et al. 2007b). Recently, direct activation of the *C. albicans* MAPK cell wall integrity pathway by RsAFP2 has been demonstrated by (Thevissen et al. 2012). **Figure 2.6** depicts schematic illustration of mode of action of the plant defensins.



Source: De Coninck et al. 2013; Fungal biology reviews.

Figure 2.6. Schematic representation of the plant defensin modes of antifungal action (A) **and tolerance mechanisms against different plant defensins** (B). Plant defensins interact with various types of receptors, present in the fungal plasma membrane (PM) and/or in the cell wall (CW). Plant defensins that interact with GlcCer (gray ovals) are represented by white ovals; plant defensins that interact with M (IP) 2C (gray rectangle) are represented by white rectangles; plant defensins that interact with as yet unknown receptors (gray rectangles represented by X or Y) are represented by white triangles. Plant defensins that can permeabilize plasma membranes are indicated with X. Upon interaction, some plant defensins stay in the extracellular space and are not taken up by fungal cells (italic), whereas others are taken up intracellularly (underlined), localizing in the nucleus (N) or cytoplasm (C). For the other defensins, uptake by fungal cells has not been proven. Plant defensins that induce reactive oxygen species (ROS) are depicted in red; plant defensins that induce apoptosis and ROS are depicted in blue, and plant defensins that interfere with cell cycle in green. Yeast or fungal deletion mutants in genes encoding for compounds of the above cascades (6) are either

plant defensin resistant, thereby affected in plant defensin targets or components in the signaling cascades leading to killing or inhibitory effect of plant defensins. Alternatively, they are plant defensin hypersensitive, thereby affected in compounds that are part of cascades leading to protection or tolerance mechanisms of the fungus against the action of plant defensins.

2.21 In vivo biological role of plant defensins in defense response

Based on their predominant extracellular localization and in vitro antifungal activity, plant defensins were thought to play an important role in the plant defense response. Defensins were originally and dominantly isolated from seeds (reviewed by Carvalho and Gomes, 2009, 2011) where they were supposed to play a role in defending the vulnerable germinating seed against pathogens (Terras et al. 1995). RsAFP1 and RsAFP2 from radish seeds are preferentially released during seed germination after breaking the seed coat and the amount of released proteins is sufficient to create a microenvironment around the seed in which fungal growth is repressed (Terras et al. 1995).

Plant defensins are also expressed in vegetative tissues where they accumulate in the peripheral cell layers of cotyledons, hypocotyls, endosperms, tubers and floral tissue. These locations are in congruence with their role in first line of defense against potential pathogens (Moreno et al. 1994; Terras et al. 1995; Penninckx et al. 1998; Lay et al. 2003). The accumulation of defensins in the leaves, stomatal cells and in the cell walls surrounding sub stomatal cavities in sugar beet has also been described (Kragh et al. 1995)This is intriguing as stomata are potential entry points for fungal pathogens. NaD1, the floral defensin from *N. alata* is expressed in the epidermal cells of the petals and sepals, in the cortical cells of the style and the connective cells of the anther (Lay et al. 2003). Its expression in these outermost layers that surround, but not in direct contact with the pollen or pollen tubes as they grow through the style, is consistent with a role in protecting the germ cells against damage by potential pathogens (Lay et al. 2003). This is also analogous to the expression of mammalian defensins in reproductive tissues (Rodríguez-Jiménez et al. 2003).

2.22 Induction of defensins in plants

2.22.1 Against Biotic stress

Plant defensins from several plants are induced by a wide range of biotic stresses (reviewed by Lay and Anderson, 2005) and plant hormones involved in stress signaling. First such induction of plant defensins

(RsAFP3 and RsAFP4) was studied in radish leaves after inoculation with fungus *Alternaria brassicicola* Terras et al. 1995). The best-known example of induced plant defensin is AtPDF1.2a from *A. thaliana,* which is induced by necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Penninckx et al. 1996; Manners et al.) and herbivoric insects (Moran et al. 2001; Abe et al., 2008). The signaling pathways leading to pathogen-induced expression of AtPDF1.2a also indicated its induction by the plant hormones ethylene (Et) and methyl jasmonate (MeJa). In *A. thaliana* mutants, AtPDF1.2a transcripts which are involved in methyl jasmonate (coi1) and ethylene signaling (ein2) pathways fail to accumulate after inoculation with *A. brassicicola* (Penninckx et al. 1996, 1998). Hence, AtPDF1.2a is now considered a general marker gene in *A. thaliana* for MeJa/ Et-mediated plant responses. On the contrary, AtPDF1.2a is not induced by the salicylic acid-mediated signaling pathway (Penninckx et al. 1996; Manners et al. 1998) which is generally linked with the plant response to biotrophic pathogens. Recently in a study, MeJa responsiveness of AtPDF1.2a is being correlated in different accessions of *A. thaliana* with enhanced basal resistance against the necrotrophic fungus *Plectosphaerella cucumerina* and the herbivoric cotton leafworm *Spodoptera littoralis*. Interestingly, while AtPDF1.2a was found to be uninduced by nematodes such as *Heterodera schachtii*, another *Arabidopsis* defensin, AtPDF2.1, is highly induced by the cyst nematode in root-specific structures called syncytia (Szakasits et al. 2009; Siddique et al. 2011). The induction studies of these two defensins AtPDF1.2a and AtPDF2.1 from *A. thaliana* exemplifies a possible distinct and specialized regulation of different defensins during various stress conditions.

2.22.2 Environmental stress induction

Additionally, defensins can also be induced by environmental stress. Plant defensins were reported to be induced by a wide variety of stresses including wounding (van den Heuvel 2001; Bahramnejad et al. 2009), cold (Koike et al. 2002; Carvalho et al. 2006), salt and drought stresses (Mee Do et al. 2004). The defensin genes from *N. excelsior* (NeThio1 and NeThio2) and *N. paniculata* (NpThio1) were shown to be expressed in response to salt stress in leaves at 250 mM NaCl (Yamada and Komori 1997). A soybean defensin gene (Dhn8) was identified by Maitra and Cushman (1998) that was induced by artificial drought stress. The level of the Dhn8 transcript was 10-fold higher in the leaves and roots of a drought resistant cultivar than in a drought-sensitive cultivar (Maitra and Cushman 1994). The up-regulation of several defensin transcripts in winter wheat in response to cold induction and acclimation was also reported (Koike et al. 2002). The

induction of these defensins was correlated with the enhanced pathogen resistance during winter hardening and potentially in freezing resistance as well. However, reports on a functional relationship between abiotic stress and plant defensins are restricted to the intracellular defensin AhPDF1.1 from the zinc hyper-accumulating plant *A. halleri* (Mirouze et al. 2006). AhPDF1.1 is induced by ZnCl2 treatment and overexpression of AhPDF1.1 in *A. thaliana* resulted in enhanced tolerance to both Zn and selenite (Mirouze et al. 2006).

2.23 Biotechnological applications and transgenic studies of defensin

Transgenic plants have the potential to provide broad resistance against different pathogens, which could reduce dependence on chemical pesticides. Hence, plant defensins demonstrating antifungal activity have become the first targets for developing transgenic crops resistant to phytopathogens. Many defensins genes have been successfully transformed into tobacco, oilseed rape, tomato, papaya and rice (Lay and Anderson 2005; Thomma et al., 2002; Zhu et al., 2007; Stotz et al. 2014; Carvalho et al. 2006).

The first attempt to evaluate transgenic plants containing foreigner antifungal defensin genes was done in tobacco plants expressing Rs-AFP2, a peptide from radish, which results in high levels of peptide expression in the transformed tobacco plants, with enhanced resistance towards the phytopathogenic fungus *Alterneria longipes* (Terras et al.,1995). Since then, a number of transgenic plants tolerant against varied phytopathogenic fungi were developed expressing this particular gene in various plant species including apple (De Bondt et al. 1998), tomato (Parashina et al. 2000), pea (Lebedev et al. 2002) and the most recent being its introduction into rice (*Oryza sativa* L. cv. *Pusa* basmati 1).The transgenic plants were tested *in vitro* and *in vivo* against fungi *Magnaporthe oryzae* and *Rhizoctonia solani*, the main causes of rice losses in agriculture, demonstrating that overexpression of Rs-AFP2 can control the rice blast and sheath blight diseases (Jha and Chattoo 2010). Similarly, overexpression of wasabi defensin (WT1) in rice, potato and orchid has resulted in increased resistance against *Magnaporthe grisea, Erwinia carotovora* and *Botrytis cinerea* (Kanzaki et al. 2002; Ntui et al. 2010).

The generation of transgenic tomato plants constitutively expressing the chilli defensin (cdef1) gene resulted in enhanced resistance against *Phytophthora infestans* and *Fusarium* sp (Zainal et al. 2009). Expression of Dahlia defensin, Dm-AMP1 in rice directly inhibited the pathogens, *Magnaporthe oryzae,*

and *Rhizoctonia solani.* It was observed that constitutive expression of Dm-AMP1 suppresses the growth of *M. oryzae* and *R. solani* by 84% and 72%, respectively (Jha et al. 2009). Transformation of tobacco and peanut with the mustard defensin BjD further validated the potential of these peptide family members as excellent antifungal agents, as transgenic plants displayed improved resistance towards *F. moniliforme; Phytophtora parasitica* and *Cercospora arachidicola; Pheaoisariopsis personata* respectively (Swathi Anuradha et al. 2008). Also, maize defensin, ZmDEF1 when transformed into tobacco plants, showed enhanced tolerance against *Phytophtora parasitica* (Wang et al. 2013). Recently, it was shown that transgenic banana expressing Petunia floral defensin effectively controled the fungal pathogen *Fusarium oxysporum* (Ghag et al. 2012). Additionally, defensin (TvD1) from *Tephrosia villosa* exhibited strong anti-insect and anti-fungal activities against *Spodoptera litura* and *Rhizoctonia solani* respectively, in transgenic tobacco plants (Vijayan et al. 2013).

Interestingly, overexpression of rabbit and insect defensins in tobacco and a human defensin HBD2 in *A. thaliana* rendered plants more resistant against *Golovinomyces cichoracearum* and *Sclerotinia minor* (Fu et al. 1998; Langen et al. 2006) and *Botrytis cinerea* (Aerts et al. 2007b), respectively. Hence, apart from the known structural homology, additional functional homology also exists between defensins originating from different eukaryotic kingdoms. All these studies suggest that defensin genes have important commercial potential for effective pathogen control specifically against varied fungi, in economically important crops (Gao et al., 2000).

2.24 Concluding remarks

Significant research in the past has helped in elucidating structure activity relationships of antifungal plant defensins. This knowledge will allow development of more potent defensins and expanding the antifungal spectrum of these proteins. Further, significant progress has been made in deciphering their modes of antifungal action. This knowledge is critical for ensuring mammalian and crop safety of these proteins and also useful for engineering durable resistance to fungal pathogens since co-expression of defensins with different modes of action will likely delay or even suppress emergence of fungal resistance to these proteins.

2.25 Previous work; background information

2.25.1 Mutational analysis

Previously from our group, a defensin TvD1 from a weedy legume, *Tephrosia villosa* had been cloned and characterized and it was found to be very effective among the legumes and showed detrimental effects against several plant pathogenic filamentous fungi when checked for *in vitro* functions (Vijayan et al. 2008). Transgenic plants were also raised overexpressing TvD1 which exhibited enhanced anti-fungal and anti-insect activities in transgenic tobacco plants (Vijayan et al. 2013).

The homology modelling showed that TvD1 has 91% similarity with that of *Vigna radiata* defensin (VrD2). Previous reports have demonstrated that changing a non-polar or uncharged aminoacid in the loop 3 of protein structure greatly affects the activity of defensin. In VrD2, the introduction of 5 amino acids in the loop3 by replacing the existing four amino acids through site directed mutagenesis, showed the enhanced α-amylase inhibitory activity against the insect *Tenebrio molitor* and it is more than that of VrD1 (Lin et al. 2007). In line with the previous studies, a mutant of native TvD1 was generated by removing the original -D-D-F-R- sequence in the β2−β 3/loop-3 region of the wild type peptide with the amino acids -G-M-T-R-T- in the mutant peptide through site directed mutagenesis. The mutant TvD1 peptide was called Alpha-TvD1(α-TvD1) and it demonstrated enhanced antifungal and insect α-amylase inhibitory activities *in vitro* when compared to native defensin (Vijayan et al. 2012).

In extension of the previous work, in the present study, we have overexpressed mutant α-*TvD1* gene in tobacco to raise transgenic plants and the *in vivo* functional characterization of mutant α*TvD1* was done against insect and fungal pathogens. Following objectives were framed to achieve this:

2.26 Objectives of chapter 5

1. Cloning of α-*TvD1* gene in the pCAMBIA2300 vector driven by 35S promoter and polyA signal with *npt*II gene as selection marker.
2. Generation of putative α-*TvD1* transgenic plants through *Agrobacterium* mediated transformation.
3. Molecular analysis such as PCR and RT-PCR to confirm the α-*TvD1* transgenic plants.
4. Characterization of α-*TvD1* transgenic plants against fungal pathogens.

4. Comparative analysis of native *TvD1* and α*TvD1* against various fungal pathogens and insect predation.

Chapter 3

General Materials and Methods

3.1 Plant Materials

Two varieties of tobacco *Nicotiana tabacum,* cv. Samsun and *Nicotiana benthamiana* were used in the present study. *Arachis diogoi* seeds (accession number: ICG 8962) were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad.

3.2 Chemicals

The chemicals used in the study are purchased from Clontech, USA; Sigma St. Louis, MO, USA; Promega Life Science, Madison, WI, USA; Amersham Biosciences, UK; Finnzymes, New England BioLabs, Ipswich, MA; Fermentas, Germany; Qualigens fine chemicals, Mumbai, India and Himedia Chemicals, Mumbai, India.

3.3 Restriction enzymes, modifying enzymes and Markers

Restriction enzymes, modifying enzymes like T4 DNA polymerase, T4 DNA ligase, DNA and Protein markers were obtained from Fermentas, Germany.

3.4 Plasmid and DNA vectors

3.4.1 pTZ57R (MBI Fermentas, Germany) This vector has gene encoding β-lactamase, a bacterial selection marker for ampicillin resistance and also allows α-complementation for visual selection based on blue/white colonies The vector was used in the cloning of a number of cDNAs of *AdLEA* and *α-TvD1* genes and also RACE products of *AdLEA* gene.

3.4.2 pRT100 (Topfer et al. 1987) pRT100 is a plant expression vector that has a gene encoding for β-lactamase for the selection in bacteria on ampicillin. This vector was used for cloning of *AdLEA* and *α-TvD1* cDNA's at multiple cloning site which was flanked by CaMV 35S promoter and the polyadenylation signal.

3.4.3 pCAMBIA 2300 (CAMBIA, Australia) pCAMBIA 2300 is a binary vector which has *nptII* gene encoding for kanamycin conferring resistance to bacteria and work as a plant selectable marker also. It was used to clone CaMV 35S-(*AdLEA and α-TvD1*) constructs which were then used for *Agarobacterium tumefaciens* mediated transformation of tobacco.

3.4.4 pEGAD and pCAMBIA1302 vectors (CAMBIA, Australia) These vectors were used for N and C terminal translational fusions of *AdLEA* with GFP respectively for localization studies.

3.5 Preparation of competent cells of *Escherichia coli*

The *E. coli* (DH5-α, Bangalore Genei, India) culture was inoculated in 25 mL of LB (Luria Broth, Himedia, India) and was incubated overnight at 37 ^{0}C on a rotary shaker at 200 rpm. From the overnight culture, 0.5 mL was taken and re inoculated in a fresh batch of 50 mL of LB and incubated again until the OD reached 0.2 at 600 nm. From here onwards, all the steps were performed on ice. Sterile micro-tubes were cooled on ice and 1.5 mL of the chilled bacterial suspension was transferred to each one of them. The cells were centrifuged in a cooling centrifuge (Eppendorf®, Germany) at 4 ^{0}C and 5000 rpm for 10 min. The supernatant was removed and the pellet was suspended in 0.5 mL of ice cold 0.1 M CaCl2 solution and was stored on ice for 10 min. The cells were again centrifuged at 4 ^{0}C and 5000 rpm for 10 min, and the pellet was resuspended in ice cold 0.5 mL of 0.1 M CaCl2. These competent cells were quick frozen and stored at -70 ^{0}C after adding 50% sterile glycerol in batches of 100 µl.

3.6 Transformation of *E. coli* competent cells

Around 50 to 100 ng of the plasmid construct carrying the desired gene was added to *E. coli* competent cells and the cells were incubated on ice for 10 to 30 min. A heat shock was given at 42 ^{0}C in a water bath for 120 seconds and the culture was immediately chilled on ice. The volume was made upto 1.0 mL by adding 400 or 900 µl of sterile LB medium. The tubes were then incubated on a rotary shaker at 37 ^{0}C for 1hr. After recovery and growth of the transformed cells, they were plated on LA medium containing selection antibiotics. The plates were incubated in an oven for overnight at 37 ^{0}C for the appearance of colonies. Plates with visible colonies were stored at 4 ^{0}C in a refrigerator for four weeks as the cells stay viable for one month at this temperature. Plasmid DNA was isolated from the colonies to check and confirm the transformation.

3.7 Plasmid isolation (mini prep) from *E. coli* (Sambrook et al. 1989)

A single colony of *E. coli* cells after transformation was incubated in 10 mL of LB medium with appropriate antibiotics on a rotary shaker at 37 ^{0}C and 200 rpm for 12-16 h. This overnight grown culture was taken in 1.5 mL micro-tube and was centrifuged at 12000 rpm for 60 seconds at 4 ^{0}C. The supernatant was removed and the pellet was suspended in 100 µl sterile ice cold Solution I [25 mM Tris Cl (pH 8.0), 10 mM EDTA [Ethylene Diamine Tetra Acetic Acid (pH 8.0)], 50 mM Glucose, stored at 4 ^{0}C] using a vortex

mixer. To the suspension, 150 µl of Solution II [0.2 N NaOH, 1% SDS, freshly prepared and stored at RT] was added. The contents were mixed thoroughly by inversion till the solution became clear. The tubes were incubated on ice for 10 min. After the lysate got chilled, 200 µl of Solution III [3.0 M potassium acetate (pH 4.8), autoclaved and stored at RT] was added. The solution was mixed thoroughly by gentle inversion. The tubes were incubated on ice for a further 5 min. The contents of the tubes were centrifuged at 4 ^{0}C and 12,000 rpm for 10 min in a cooling centrifuge. The supernatant was transferred to a fresh 1.5 mL microtube. From a stock of 10 mg mL-1 of RNase, 2-3 µl was added to the lysate and was incubated at 37 ^{0}C in a water bath for 1 hr. The lysate was treated with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) successively and was centrifuged after each treatment at RT and 12,000 rpm for 15 min to separate the aqueous phase from the organic layer. The upper aqueous layer was separated to a new tube without disturbing the middle protein layer. The purified dsDNA was precipitated with two volumes of 100% chilled ethanol or an equal volume of isopropyl alcohol. The mixture was allowed to stand at -20 ^{0}C for 30 min and the DNA was collected at the bottom of the tube by centrifuging at 4 ^{0}C and 12,000 rpm for 10 min. The supernatant was decanted completely and the pellet was rinsed with 1.0 mL of 70% ethanol. The pellet was air dried and dissolved in 30 to 50 µl TE buffer [10 mM Tris HCl and 1.0 mM EDTA (pH 8.0)]. The isolated plasmid DNA was stored at -20 ^{0}C. Plasmid isolations were also carried out using kit (Sigma-aldrich, USA) following the manufacturer's instructions.

3.8 *Agrobacterium* competent cell preparation and transformation

Competent cells *of Agrobacterium tumefaciens* (EHA105 and LBA4404) were prepared as described for *E. coli* except that cells were grown at 28 ^{0}C. Freeze thaw method (Holsters et al., 1978) was used for *Agrobacterial* competent cells transformation. It was performed by immediate freezing of competent cells in liquid nitrogen after adding plasmid DNA and then followed by incubating in a 37 ^{0}C water bath for 5 min. To this, upto 1 mL of LB medium was added and incubated at 28 ^{0}C for 3-4 h with shaking. The cells were pelleted at 5000 rpm for 5 min and plated on LB agar medium supplemented with rifampicin and the corresponding selectable marker of the plasmid DNA. For long-term storage of the transformed cells, liquid cultures of the cells were stored at -70 ^{0}C after adding sterile 50% glycerol.

3.9 Agarose gel preparation and electrophoresis:

DNA fragments were resolved by using 0.8% agarose gel, prepared by melting 0.8 g agarose (A-9539, Sigma-Aldrich, St. Louis, USA) in 100 mL of 1x TAE buffer [50x TAE: 2.0 M Tris Cl, 1.0 M Acetate, and 100 mM EDTA (pH 8.0)]. From the stock of ethidium bromide (10 mg mL-1) solution, 2 µl was added in melted agarose and poured in gel casting tray fitted with a proper comb. After the polymerization, they tray was kept inside the electrophoresis tank containing 1x TAE buffer so as to cover the gel. The DNA mixed with 6x loading dye [0.15% bromophenol blue, 0.15% xylene cyanol, 5.0 mM EDTA, 40% sucrose] to a concentration of 1x and was loaded in the wells created by the comb. The gel was electrophoresed at 70 V for 1 h or till the dye front covered almost 3/4th of the length of the gel. A molecular weight marker was loaded along with the samples for reference.

3.10 Purification of DNA fragments from the agarose gel

After the PCR amplification or restriction digestion of plasmid DNA constructs, the identified DNA bands or plasmid inserts were cut out along with the gel slice, weighed and taken in a micro-tube. GenElute Gel Extraction Kit (Sigma, USA) was used for extracting DNA from agarose gel following manufacturer ‘s instructions.

3.11 Dephosphorylation

In order to avoid self-ligation of cohesive/blunt-end termini of the plasmid DNA during DNA recombination, the single digested DNA fragments were dephosphorylated at their 5'-ends with Calf intestine alkaline phosphatase (Fermentas, Germany). The total volume of 50 µl of reaction mixture included 5 µl dephosphorylation buffer (10X), 1 µl (1.0 U) of Calf intestine alkaline phosphatase and appropriate plasmid DNA in µg. The mixture was incubated at 37 ^{0}C for 30 min, followed by heat inactivation at 85 ^{0}C for 15 min.

3.12 Ligation

T4 DNA ligase (Fermentas, Germany) was used in various independent experiments during ligation. The reaction mixture was made up in a total volume of 20 µl comprising 2 µl ligation buffer (10X), appropriate volumes (in µl) each of linear insert DNA and digested plasmid DNA, and finally T4 DNA ligase (1-2 U for

cohesive ends and 5 U for blunt ends). For cohesive ends, the reaction mixture was incubated for 16 h at 16 ^{0}C and blunt end ligation at 22 ^{0}C overnight.

3.13 Transformation of tobacco leaf discs (Horsch et al. 1985)

Tobacco leaves were surface sterilized by rinsing in 400 mL of water containing 5% of commercial bleaching powder and 20 drops of Tween 20. The leaves were rinsed for 5-10 min in distilled water to remove the surfactant and the bleach. The mid ribs of the leaves were cut out and the lamina was cut into uniform squares using sterile blade. The leaf pieces were rinsed for 5 min in sterile distilled water followed by the treatment with $HgCl_2$ (0.01%) for 5 min. Then they were rewashed with sterile water 5 times for 5 min each. The leaf pieces were placed on sterile tissue paper and the wound the leaf edges and were cut out. The pieces were kept in plates containing pre-solidified MS medium with 0.1 mg l^{-1} and NAA 2 mg l^{-1} BAP. The leaf discs were co-cultivated with *Agrobacterium* for a period of 3 d and then they were transferred to regeneration medium containing the antibiotic cefatoxime to kill the bacteria. The leaf disks started expanding and callusing within two wks. The leaf pieces were sub cultured to regeneration medium i.e. MS medium with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. Each piece was cut into 3-4 pieces and those pieces were pressed into the medium in order to ensure a proper contact between the plant tissue and the MS medium. Shoots appeared in about 3 weeks after inoculation and they grew generally from the edge of the disks or from internal wounded areas. When the shoots were around 1-2 cm long, they were excised and placed carefully on the rooting medium i.e. MS medium. Cefotaxime was added in the medium to avoid any *Agrobacterium* growth. The rooted plantlets were transferred from agar medium to a mixture of sterile soil and vermiculite (1: 3). To harden the *in vitro* grown plantlets, which need to develop a cuticle to control water losses, each plantlet was covered with a polythene bag. After one week to 10 d, they were transferred to green house for further growth.

3.14 RNA isolation

Total RNA from different samples were isolated by using the TRI-reagent (Sigma- Aldrich, USA), following the manufacturer's instructions.

3.15 Genomic DNA extraction

CTAB method (Murray and Thompson 1980): Plant genomic DNA isolation was done from the second or third leaf from the shoot tip of young plants. The leaves were freshly collected, frozen in liquid nitrogen and stored at -70 ^{0}C. The leaf tissue (100-500 mg) was homogenized to a fine powder using liquid nitrogen along with a pinch of PVPP (Polyvinyl Polypyrrolidone). About 1.0 mL of CTAB buffer (Cetyl/ Hexadecyltrimethyl Ammonium Bromide) extraction buffer [2% CTAB, 100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 2% β- mercapto ethanol (β-merc)] was taken in 2.0 mL micro tubes and homogenized powder was transferred to the tube, and mixed well to suspend the powder uniformly by repeated inversion of the tubes. The mixtures were incubated at 65 ^{0}C for 1 h with intermittent mixing. After incubation, 0.5 mL of Chloroform: Isoamyl alcohol (24:1) mixture was added and mixed thoroughly by repeated inversion. The two phases were separated by centrifugation at 14,000 rpm for 15 min. The upper aqueous layer was taken in a fresh 2.0 mL tube. The nucleic acid content was precipitated from the aqueous phase by mixing well an equal volume of isopropyl alcohol and incubating the tubes at -20 ^{0}C for a minimum of 30 min. The tubes were centrifuged at 12,000 rpm for 15 min to sediment the nucleic acids. The solution was decanted completely and 1.0 mL of 75% ethanol was added, and incubated for 10 min at RT. The tubes were centrifuged at 12,000 rpm for 5 min and ethanol was decanted. The pellet was air-dried and dissolved in required volume of TE [10 mM Tris HCl and 1.0 mM EDTA (pH 8.0)] buffer. For further purification, DNA was treated with RNase (1mg/mL) for 2 h at 37 ^{0}C. Once again, the sample was treated with phenol: chloroform: isoamyl alcohol (25: 24:1) and twice with chloroform: isoamyl alcohol (24:1) for the removal of any residual protein contamination. Each time the organic phase was mixed thoroughly with the aqueous, centrifuging at 12,000 rpm for 15 min and collecting carefully the upper clear aqueous phase in a fresh tube. Finally, the purified DNA was precipitated by adding 1/10th volume of 3M sodium acetate, (pH 5.2) and one volume of isopropanol followed by centrifugation at 12,000 rpm for 15 min at 4 ^{0}C. The pellet was washed with 70% ethanol, dried and dissolved in TE. Genomic DNA samples were stored at –200C for long-term use.

3.16 Quantification of DNA and RNA

The quality and concentration of DNA and samples were examined by agarose gel electrophoresis (ethidium bromide stained) and Nanodrop spectrophotometer (Thermo scientific).

3.17 Polymerase Chain Reaction (PCR)

Consumables from Sigma-aldrich (USA) and Invitrogen (USA) were used for the PCR reactions. PCR reactions were performed on Biorad thermal cycler, USA or Eppendorf Personal Thermal cycler, Germany. PCR conditions were optimized according to the template and primer combinations.

3.18 5′/ 3′ RACE and isolation of full length cDNA

Rapid amplification of cDNA ends (RACE) was performed to derive full length *AdLEA* gene, by using SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's instructions. In brief, one microgram of total RNA was reverse transcribed using Transcriptor reverse transcriptase provided in the kit. The 5′-RACE cDNA was synthesized using a modified lock-docking oligo (dT) primer and the SMARTer II A oligo. The modified oligo (dT) primer, termed the 5′-RACE CDS Primer A (5′-CDS) has two degenerate nucleotide positions at the 3′ end. These nucleotides position the primer at the start of the poly A+ tail and thus eliminate the 3′ heterogeneity inherent with conventional oligo (dT) priming. The 3′-RACE cDNA was synthesized using a traditional reverse transcription procedure, but with a special oligo (dT) primer. This 3′-RACE CDS Primer A (3′- CDS) primer includes the lock-docking nucleotide. The Universal Primer A Mix (UPM) was used in conjunction with distinct gene-specific primers (GSP) for 5′ and 3′ end during RACE-PCR reactions. All the RACE-PCR reactions were performed using hot-start DNA polymerase provided along with the kit. Genomic DNA served as templates for the amplification of genomic sequences. All PCR products were cloned in to pTZ57R/T vector and sequenced for sequence confirmation.

3.18.1 Primers for first-strand cDNA synthesis for 3'/5' RACE:

SMART II™ A Oligonucleotide for 5' RACE

5'–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3'

5'-RACE CDS Primer A (5'-CDS)

5'–$(T)_{25}$V N–3' (N = A, C, G, or T; V = A, G, or C)

3'-RACE CDS Primer A (3'-CDS)

5'–AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ V N–3'

(N = A, C, G, or T; V = A, G, or C)

Universal primers for 3'/5'-RACE PCR :

10X Universal Primer A Mix (UPM)

Long (0.4 µM):

5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3'

Short (2 µM):

5'–CTAATACGACTCACTATAGGGC-3'

Nested Universal Primer A (NUP; 10 µM)

5'–AAGCAGTGGTATCAACGCAGAGT-3'

3.19 Total Protein Extraction

For determining the presence of AdLEA-GFP fusion proteins in the *N. benthamiana* plants, total protein was extracted. Leaf tissue was homogenized in liquid nitrogen using mortar and pestle adding a pinch of PVPP and 1.0 g of the fine powder was collected in pre-chilled micro tubes. Into the tubes kept on ice, 1.0 ml of chilled extraction buffer [50 mM Tris-Cl and 1.0 mM PMSF (Phenyl Methane Sulphonyl Fluoride)] was added, mixed thoroughly and were made to stand at 4ºC for half an hour with intermittent shaking. The tubes were centrifuged at 4ºC and 15,000 rpm for 20 minutes and the supernatant containing the protein was collected in a fresh tube. All the steps were performed at 4ºC. Isolated protein samples were immediately used or stored at 4ºC. It stayed without degradation for one week.

3.20 Quantification of the leaf-extracted total protein by Bradford method (Bradford 1976)

Bradford dye concentrate was prepared by dissolving 100 mg Coomassie Brilliant blue G250 in 50 ml of 95% ethanol. To this, 100 ml concentrated phosphoric acid was added and the volume was made up to 200 ml with H2O. The Bradford dye concentrate was stable for 6 months at 4ºC. Samples of BSA were prepared with 10, 20, 30, 40, 50, 75 and 100 µg/ 100 µl of BSA (fraction V) in the same buffer solution in which the protein samples were extracted. The Bradford dye concentrate was diluted to 5x with double distilled water and 1.0 ml was added to each sample. The red dye turned blue after binding to the protein. The samples

were allowed to develop color for at least 5 minutes (not longer than 30 minutes). Absorbance at 595 nm was read, and a linear standard curve was prepared to calculate the concentration of protein.

3.21 Polyacrylamide Gel Electrophoresis (PAGE)

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE) with acrylamide: N, N'-bis acrylamide (30:1) was carried out according to the standard protocol (Laemmli, 1970). A 15% (standardized for defensin protein) 7.5 cm gel was prepared with 1 cm stacking gel. Electrode buffer was prepared by dissolving 10 g SDS, 30.3 g Tris and 144.1 g glycine in 800 ml H2O [25 mM Tris and 192 mM glycine with 1% SDS]. The volume is adjusted to 1 liter with H2O and stored at RT. Protein samples are prepared by boiling in a water bath for 3-5 minutes after mixing 50 μg of each sample with one-tenth volume of the sample buffer [0.125 M Tris-HCI (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue]. Electrophoresis is carried out at 90 V in an electrophoresis and electro-transfer unit (Hoefer mini VE, Amersham Pharmacia Biotech, USA) till the dye came out of the gel into the electrode buffer.

3.22 Coomassie Blue staining of the gel

Coomassie Brilliant Blue R250 staining detection limit is 300 to 1000 ng protein and is used in the experiments. The gel with electrophoretically separated proteins is incubated for staining in Coomassie solution (0.025% Coomassie Brilliant blue- R250 in 45% methanol and 10% acetic acid) for 30 minutes. Destaining was done with a destaining solution [45% methanol and 10% acetic acid] to remove background staining. The destaining solution is replaced every 10-15 minutes with a fresh solution, until the protein bands are visible.

3.23 Western Blotting and Immuno staining

After electrophoresis, the protein samples are electro-blotted on to Polyvinylidine fluoride (PVDF)/ nitrocellulose membrane (PALL®, India) using Trans-Blot apparatus (electrophoresis and electro-transfer unit, Hoefer mini VE, Amersham Pharmacia Biotech, USA) according to the manufacturer ‘s instruction using Towbin buffer (Towbin et al. 1979). The gel is first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) and the membrane is kept above the gel towards the positive end of apparatus and is stacked in between Whatman filter papers taking care not to trap any air bubbles. Transfer is conducted for 4 hrs at 25V. The transfer of protein to the membrane was checked by reversible Ponceau S

staining (100 mg Ponceau S in 5% acetic acid). The stain was removed by 3-4 washes with TBST [10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20 (v/v)]. Blocking of the membrane is carrying out using 3% BSA (w/v) in TBS for 1 hr at RT followed by 5 washes with TBS for 10 minutes each. The blot was then incubated overnight with the primary antibody diluted in TBS containing 3% BSA at 4ºC. This is again followed by 5 thorough 58 washes, each of 10 minutes in TBS. Then the blot is incubated in secondary antibody - Goat anti-rabbit IgG (Bangalore GENEi, India) ALP conjugate. The bands are visualized after staining with BCIP/NBT (Bangalore GENEi, India), which is a substrate for ALP conjugate. Proteases are ubiquitous and difficult to separate from proteins. Hence, protease inhibitor, PMSF is used throughout the procedure for successful isolation of total protein from the plants. Protein solutions are stored in aliquots at 2-8ºC or at -70ºC after addition of 20% glycerol and shock freezing in liquid nitrogen. During experiments, proteins are always maintained on ice. Vigorous pipetting and vortexing are avoided. β-merc is avoided in the extraction buffer as it can form disulfide bridges with thiol groups of proteins leading to aggregation and/ or inactivation. Molecular weight of amino acids ranges between 75 and 204 Da. For calculations, the average molecular weight of amino acid (110 Daltons) is considered. Ponceau stain is used only in the initial experiments; later this step is avoided in the western blot for checking the transfer, as the proteins are visible even without using the stain while drying the blot.

Chapter 4

Functional characterization of an atypical group 5C Late Embryogenesis Abundant protein (AdLEA) from wild peanut *Arachis diogoi* for manipulation of abiotic stress response in transgenic tobacco.

4.1 Materials and methods

4.1.1 5'/3' RACE-PCR and isolation of full-length cDNA of *AdLEA*

RACE-PCR was performed to obtain the full length sequence of *AdLEA* gene using SMART^{TM} RACE cDNA Amplification kit (Clontech, USA) following manufacturer's instructions. The 5′ and 3′ RACE products using 5'AdLEA-GSP1 and 3'AdLEA-GSP1 primers respectively were aligned with the partial sequence of *AdLEA* obtained from preliminary differential gene expression study using cDNA-AFLP in *A. diogoi* after infection with *P. personata* (Kumar and Kirti 2015) to obtain full-length sequence of *AdLEA*. Finally, the open reading frame of *AdLEA* was amplified using Phusion® High-Fidelity DNA polymerase (Finnzymes, New England BioLabs, Ipswich, MA) with primers having specific restriction enzyme sites for cloning in pRT100 vector. Primers used in this step are AdLEA-F and AdLEA-R flanked by restriction sites for *Sac*1 and *Kpn*1 respectively. All the primer sequences used in the pare listed in (Table 4.1).

4.1.2 cDNA sequence and protein analysis

Sequence analysis was performed using BLASTx and BLASTp (Altschul et al. 1990). Nucleotide translation, prediction of theoretical molecular mass and the isoelectric point of AdLEA protein were performed using Expasy tool (http://expasy.org/tools/pi_tool.html) (Artimo et al. 2012). SignalP 4.1 was used for predicting the signal peptide within the protein (Petersen et al. 2011). Motif analysis was performed using the Pfam program (http://www.ebi.ac.uk/tools/InterProScan). Multiple sequence alignment was performed by ClustalW (Thompson et al. 1994) and phylogenetic tree construction was done by MEGA 5.1 software (Tamura et al. 2011). The grand value of hydropathy (GRAVY) analysis and instability index for deduced amino acid sequence were predicted with PROTParam (http://au.expasy.org/tools/protparam.html) and PSORT (http://psort.nibb.ac.jp) programs (Yu et al. 2010). Analysis of protein hydropathy was done by constructing hydropathy plot with Kyte and Doolittle algorithm (http://ipsort.hgc.jp/) (Kyte and Doolittle 1982). Subcellular localization predictions were performed using PSORT II (http://psort.hgc.jp/form2.html) (Nakai and Horton 1999); MultiLOC (http://abi.inf.uni-tuebingen.de/Services/Multiloc) (Hoglund et al. 2006); and Y LOC (http://abi.inf.uni-tuebingen.de/Services/Y LOC/webloc.cgi) (Briesemeister et al. 2010).

4.1.3 Plant material and stress treatments in gene expression studies of *AdLEA* in *A. diogoi*

Tobacco (*Nicotiana tabacum* var. Samsun), wild peanut (*A. diogoi*) accession number ICG-8962 (kindly provided by ICRISAT, Patancheru, India) and *Nicotiana benthamiana* plants were used in this study and maintained in separate greenhouses at 24±1°C with a photoperiod of 14/10 h of light /dark with light intensity of 100 µmol $m^{-2}s^{-1}$. Stress and hormonal treatments on *Arachis* plants were done according to protocol standardized by Kumar et al., 2011 (Raja et al. 2011). In brief, twigs from two-month-old plants of *A. diogoi* were cut with a sharp sterilized blade, washed with sterile distilled water and kept in trays lined with moist filter paper. Cut ends of shoots were wrapped properly with water soaked cotton and whole set up was sealed with polythene covers to maintain moist condition. This helps the shoots to get acclimatized in a growth room at 25±1°C (with a photoperiod of 16 h of light and 8 h of dark, light intensity of 60 µmol $m^{-2}s^{-1}$) and subsequently enhance adventitious roots formation at the cut ends of shoots. These shoots with adventitious roots were subjected to different hormonal treatments namely 100µM salicylic acid (SA), 100µM methyl jasmonate (MeJA), 100µM abscisic acid (ABA), 250µM ethephon and 100µM sodium nitroprusside (SNP). The trifoliate compound leaves of *Arachis* were used as a sample in each stress, sufficient enough to isolate 2 µg of RNA (taken in duplicates). This constitutes one biological sample for the experiment. Heat (high-temperature stress) and cold (low-temperature stress) treatments to leaf samples were applied by keeping them at 42°C and 4°C respectively. For other stress treatments, samples were treated with 200 mM sorbitol, 100 mM NaCl, 10% (w/v) polyethylene glycol (PEG-MW6000) and 10 µM methyl viologen (MV). Samples were collected in liquid nitrogen at regular intervals of 0 min, 5 min, 15 min, 30 min, 1 h and 2 h in case of heat, and 0 h, 3 h, 6 h, 12 h and 24 h in rest of the treatments. The samples were stored at –80°C for experimental studies.

4.1.4 Gene expression profile of *AdLEA* in native conditions

Total RNA was extracted from leaf samples (subjected to various treatments) according to Chang et al., (Chang et al. 1993). Two microgram of total RNA was used for first-strand cDNA synthesis by an oligo dT (18 mer) primer using SMART MMLV Reverse Transcriptase (Clontech, Becton Dickinson, USA). Diluted first strand cDNA samples were subjected to qRT-PCR with gene-specific primers for *AdLEA* (Table 4.1) and 10 µl SYBR® Premix Ex Taq with ROX (Takara Bio Inc., Japan). Three independent biological replicates with three technical replicates of each biological replicate for each sample were used for analysis

including three non-templates, which served as negative control (non-template control with just water without cDNA). qRT-PCR was carried out in Realplex (Eppendorf, Germany) amplifier with the following program: 95°C for 5 min; 40 cycles of 95°C for 15 s, 58°C for 20 s, 72°C for 30 s followed by the melting curve to ensure a single product of each amplicon. Alcohol dehydrogenase class III (*adh3*) and polyubiquitin (*UBI1*) were used as internal controls to calculate relative quantification of gene expression (Brand and Hovav 2010). The threshold cycle (C_T) was used to calculate relative fold change (RFC) in RNA expression at each time point of the treated sample compared to control conditions by using the formula, fold change = $2^{-(\Delta Ct\ treated - \Delta Ct\ control)}$ (Livak and Schmittgen 2001).

4.1.5 Sub-cellular localization studies of AdLEA protein

GFP was fused translationally with *AdLEA* at both N-terminal (GFP: *AdLEA*) and C-terminal (*AdLEA*: GFP) in pEGAD and pCAMBIA1302 vectors respectively for AdLEA localization studies (Snapp 2005). *AdLEA* Open Reading Frame (ORF) was amplified with primers AdLEA-pEGAD-F and AdLEA-pEGAD-R having restriction sites for *Eco*RI and *Hin*dIII respectively, (Table 4.1) and cloned into pEGAD vector after digestion with above-mentioned restriction enzymes, for generating the N-terminal fusion with GFP. Similarly, to generate C-terminal GFP fusion, stop -codon- less, coding region of *AdLEA* was amplified with primers AdLEA-pC1302-F and AdLEA-pC1302-R having restriction sites for *Bgl*II and *Spe*I respectively (Table 4.1) and cloned into pCAMBIA1302 vector after digestion with the same set of restriction enzymes. Both fusions were confirmed by sequencing. The recombinant vectors and empty pCAMBIA1302 vector generating free GFP serving as control were mobilized into *Agrobacterium* strain LBA4404 by the freeze-thaw method. Agroinfiltration and microscopic studies were performed according to Yang *et al*. (Yang et al. 2000). The overnight-grown cultures of *Agrobacterium* with both constructs and control vector were pelleted down and resuspended in infiltration medium (10mM MES, 10mM $MgCl_2$, and 200µM acetosyringone) and infiltrated into the adaxial side of *N. benthamiana* leaves after adjusting the OD value to 0.6. Leaves were visualized for GFP expression 72-96 hours post infiltration (hpi) at different time points with laser scanning confocal microscopy (Leica TCS SP2 with AOBS, Heidelberg, GmbH, Germany) with excitation and emission wavelengths at 475-495 nm and 520-560 nm respectively. The experiment was performed in triplicates. Protein extraction and subsequent immunoblot analysis were done to check the

expression and stability of AdLEA: GFP fusion protein in cells following the protocol as described by Kumar and Kirti (Kumar and Kirti 2012). Western Blot analyses was done using, anti-mouse Histidine primary antibody, as GFP was tagged with Histidine in pCAMBIA1302 vector, which was detected with ALP-conjugated goat anti-rabbit secondary antibody using BCIP/NBT as substrate.

4.1.6 Construction of recombinant *AdLEA* binary vector and genetic transformation of tobacco

The *AdLEA* ORF was amplified with primers AdLEA-F and AdLEA-R having restriction sites for *Sac*I and *Kpn*I respectively (Table 4.1) and cloned into corresponding sites of pRT100 vector. The expression cassette that has *AdLEA* flanked by CaMV35S promoter and polyadenylation signal was excised with *Hin*dIII and subsequently sub-cloned into binary vector pCAMBIA2300 at the same site. The recombinant vector pCAMBIA2300-*AdLEA* having kanamycin as marker gene was transformed into *Agrobacterium* strain LBA4404 by freeze-thaw method and used for transformation of tobacco plants (*N. tabacum* cv. Samsun) by leaf disc method according to Horsch et al. (Horsch et al. 1985) as described in **method 3.13**. The transformants were selected on 125 mg L^{-1} kanamycin. Genomic DNA and total RNA were isolated from T_0 *AdLEA* transformants for molecular analysis. RNA was reverse transcribed and PCR with specific primers for the target gene as well marker gene *nptII* was undertaken. High and low expression plants were identified and their progenies were maintained for further analysis. All experimental analyses were conducted in T_2 generation of transgenic plants.

4.1.7 Chlorophyll fluorescence measurements of *AdLEA* transgenic plants using Pulse-Amplitude-Modulation (PAM) chlorophyll fluorometer after drought treatment

Chlorophyll fluorescence measurements were performed in drought-induced two-months-old *AdLEA* transgenic plants to check for the photochemical efficiency under drought stress in greenhouse conditions. Three-week-old seedlings of wild-type (WT) and three high expression transgenic lines (#2, 4 and 9) grown on half strength MS medium without organic nutrients were transferred to soil in plastic cups for further growth of two weeks. WT and transgenic plants of same age and size were then shifted individually to separate pots in the greenhouse and allowed to grow for ten days. After acclimatization, these plants were subjected to drought stress by withholding water for 18 days, after which significant differences in wilting

were observed. Plants were watered on the 18th day and kept for recovery for three days. Chlorophyll fluorescence measurements were performed on leaves at the third to fourth position from shoot apex of drought treated WT and transgenic plants with MINI-PAM (Walz, Effeltrich, Germany). The measurements were taken on D0, D6, D12, and D18 (D–day) after drought treatment followed by a recovery ® period of three days for both WT and transgenic lines. The leaves were first dark adapted for 30 min by fixing leaf clips to ensure that all photosystem-II (PSII) reaction centers (RCs) were open. The potential maximum quantum yield (F_v/F_m) was recorded by illuminating the leaves with a beam of saturating light (intensity – 4500 µmol m^{-2} s^{-1} of 650 nm peak wavelength, an excitation intensity sufficient to ensure closure of all PS-II RCs) focused on the leaf surface using a special leaf clip holder (model 2030-B, Walz) described by Bilger et al. (U. Schreiber, W. Bilger 1994). This leaf clip holder allows the measurement of light and saturating light pulses that fall on a leaf at an angle of 60°. Further, steady-state fluorescence (F_s) measurement was performed after continuous illumination with white actinic light and a second saturating pulse (4500 µmol m^{-2} s^{-1}) was imposed to determine the effective photochemical quantum yield F'/F_m', also known as [Y(II)] in the light-adapted state.

4.1.8 Abiotic stress tolerance assays for transgenic *AdLEA* plants

Stress tolerance assays were performed at the young seedling stage and on mature plants by leaf disc assays following the protocols (Sanan-Mishra et al. 2005);(Sun et al. 2013). We selected two high expression transgenic lines (#2 and 4) and one low expression transgenic line (#7) for different stress tolerance assays. The experiments were performed on the homozygous T_2 generation of transgenic plants and repeated three times. Seeds from all three transgenic lines and WT plants were surface-sterilized by 2% (v/v) sodium hypochlorite solution for 10 min by continuous shaking, rinsed 4 – 5 times with sterile distilled water and were allowed to germinate on half strength MS medium with 125 mg L^{-1} kanamycin (for transgenic) and without kanamycin (for WT), devoid of organic nutrients. Seedlings were maintained in culture room at 25±1°C with a photoperiod of 16/8 h light/dark with light intensity 60 µmol $m^{-2}s^{-1}$ for 11 days and then shifted to corresponding stress medium plates having half strength MS (MSH) without organic nutrients, i.e. 200mM and 300mM NaCl for salt stress, 10% and 12% (w/v) PEG (corresponding to final osmotic potential of - 0.123 and -0.184 MPa) (Money 1989), for dehydration stress and 200 mM and

300 mM sorbitol for osmotic stress. Duration of stress varied for different stress treatments, depending upon the visible phenotypic changes observed between the seedlings of WT and transgenic lines. Oxidative stress was applied by placing the seedlings on MSH medium containing 5 and 10 µM MV for 4 d and then shifted to the stress-free recovery medium. [11 seedlings per plate were kept (in duplicate) and together they made one biological set of experiment. Likewise, three experiments were done].

For leaf disc assays leaf discs of 1 cm radius were punched from healthy, fully distended leaf of two months old transgenics as well as WT plants with the help of cork borer. [15 leaf discs from a single leaf were used as a sample for stress treatment (in duplicate). This constitutes one biological sample. Similar experiments were repeated three times]. Discs were floated on 10 mL solution of 0, 200 and 300mM NaCl for salt stress, 0, 10, 12 and 14% PEG (corresponding to final osmotic potential of -0.123, -0.184, -0.256 and -0.341) for dehydration stress, 0, 300, 400 and 500mM sorbitol for osmotic stress and 0, 5 and 10µM MV for oxidative stress treatments respectively. The treatments were carried out in continuous white light at 25± 1°C until visible differences were observed among the lines.

4.1.9 Biochemical Analysis - total chlorophyll content, lipid peroxidation and proline estimation

Total chlorophyll content was estimated spectrophotometrically after extraction of fresh material in 80% acetone according to Arnon (Arnon 1949). [for seedling assays - 200 mg, for disc senescence assay and drought stress under field conditions - 500 mg of fresh weights was taken]. The extent of lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) (Heath and Packer 1968). Leaf discs (500mg) after different stress treatments were extracted in a solution of 0.5% w/v TBA in 20% trichloroacetic acid and the absorbance was measured at 532 and 600 nm. The malondialdehyde (MDA) levels were estimated using the extinction coefficient 155 mM^{-1} cm^{-1}. The proline content in leaves was estimated according to Bates et al., (Bates et al. 1973). The leaf samples from unstressed (D0) and drought stressed (D18) transgenic and WT plants were collected and frozen in liquid nitrogen. Frozen leaf tissue (100mg) was ground to a fine powder in liquid nitrogen and extracted with 4 ml of 3% sulpho salicylic acid.

The homogenate was filtered through filter paper and incubated at 100°C for 10 min. The absorbance of homogenate was measured spectrophotometrically at 520 nm using toluene as blank. The proline concentration was determined as μmole g^{-1} FW.

4.1.10 Reactive Oxygen Species (ROS) quantification in *AdLEA* transgenic tobacco using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and nitro blue tetrazolium (NBT) staining

Confocal studies were done to detect total cell ROS using H_2DCFDA, against dehydration stress induced with PEG (Konopka-Postupolska et al. 2009). Single leaf from two-months old mature plants was used in one experiment (in duplicates), which was repeated thrice. Briefly, epidermal peels from abaxial leaf surface of transgenic lines and WT plants were treated with H_2DCFDA for 20 min and kept in the dark. The excess dye was removed by three washes with washing buffer (30mM KCl + 10mM MES-KOH). Stress was induced by incubating peels in 10% (w/v) PEG solution for 30 min. Epidermal peels treated with only buffer solution were kept as controls, to detect the basal levels of ROS in the guard cells of the WT and transgenic plants. The fluorescence was captured by Laser Scanning Confocal Microscope (Leica, TCS-SP2 with AOBS, Heidelberg, GmbH, Germany) with excitation and emission wavelengths at 475-495 nm and 517-527 nm respectively. Fluorescence quantification was done using Image-J 1.42 software (NIH, USA) by selecting appropriate areas of pigmentation. For detection of Superoxide anion (O_2^-), histochemical studies using NBT staining was done against salinity stress induced with NaCl according to Driever et al., (Driever et al. 2009) with minor modifications (Grellet Bournonville and Díaz-Ricci 2011). Briefly, petioles of one month old transgenic plants and WT were cut under water to avoid obstructions in the vasculature. Two days before the experiment, all plants were watered or treated with 100mM NaCl solution. Petioles were soaked in 6mM NBT dissolved in 10mM potassium phosphate buffer (pH 7.8) for probe feeding of the leaves. Subsequently, the treated leaves were transferred to continuous white light for 15 min to assess the effect of photosynthesis on superoxide production. Colored leaves were then boiled in 4:1:1 (v/v/v) solution of ethanol, lactic acid, and glycerol to remove chlorophyll. Finally, to quantify formazan formation, leaves were boiled in dimethyl sulfoxide until they were clear and formazan concentration was measured spectrophotometrically at 560 nm.

4.1.11 Expression analysis of stress-related genes in *AdLEA* transgenic tobacco

Relative gene expression studies of some candidate genes expressed in abiotic stress conditions were performed in transgenic lines as compared to WT with or without induced stress. Leaf samples from unstressed (D0) and drought stressed (D18) plants were collected from representative high expression transgenic line 2 and WT plants. Single leaf from each plant constituted one biological sample and the experiment was done in triplicates. Samples were frozen in liquid nitrogen and kept at –80 °C until further use. RNA was isolated from frozen samples using RNeasy Plant Mini Kit (Qiagen, Germany) and subjected to quality check and reverse transcription. Two microgram of total RNA was used for first-strand cDNA synthesis and quantitative real-time PCR was performed as described earlier. Gene-specific primers for various stress-related genes, 18S rRNA, and polyubiquitin (*NtUBI1*), which were used as reference genes to calculate relative quantification of gene expression are listed in (Table 4.2). C_T value was used to calculate RFC by $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

4.1.12 Statistical analysis

The data analysis was done by analysis of variance (one-way ANOVA and two-way ANOVA) using GraphPad Prism ver. 5.0 and the mean values were compared by the student Newman-Keuls analysis and Bonferroni post-tests. All the experiments were performed in triplicates. Details of the individual sample size for each analysis is mentioned in the figure legends.

4.2 Results

4.2.1 Isolation of full-length *AdLEA* cDNA by RACE-PCR and sequence analysis of AdLEA protein

A transcript derived fragment of *LEA* gene was found to be upregulated in wild peanut, *A. diogoi* upon infection with the late leaf spot pathogen, *P. personata* in a study of differential gene expression using cDNA-AFLP, in the previous study from same group (Kumar and Kirti 2015). In the present study, this partial sequence of *LEA* was amplified and the full-length cDNA sequence was obtained by 5' and 3' RACE **(Figure 4.2.1.1)**, which was further confirmed by sequencing. This sequence was designated as *AdLEA* on

the basis of sequence homology it showed with LEA proteins and submitted to GenBank under NCBI accession number GU223575. *AdLEA* cDNA comprises of 1387 bp, which carried an open reading frame of 972 bp flanked by 152 bp 5′ untranslated region and 263 bp 3′ untranslated region. The ORF of *AdLEA* potentially encodes a polypeptide of 323 amino acids **(Figure 4.2.1.2)**. The theoretical *p*I and the molecular mass of AdLEA are 4.74 and 36.071 kDa respectively. AdLEA protein contains "LEA_2" motif (PF03186,1.38e-15), which was classified into subgroup 5C (D-95) according to Battaglia's classification of LEA proteins (Battaglia et al. 2008).

AdLEA does not contain any signal peptide as predicted by signal P4.1 software analysis. Amino acid composition of AdLEA protein showed that it has an abundance of Asp, Lys, Ile, Gly, Leu, and Glu amino acids which constituted 12.6, 10.8, 9.5, 8.3, 7.7 and 7.6% of the total amino acid pool, respectively. Also, it lacks Cys and has just two Trp residues. Hydropathy analysis of AdLEA showed that it is a nonpolar, hydrophobic -neutral protein with a negative GRAVY value of 0.496 **(Figure 4. 2.1.3B)** and has an instability index of 21.45. All these features are in accordance with other group 5C members of LEA proteins (He et al. 2012). The sequence alignment of deduced amino acids of *AdLEA* was carried out with amino acid sequences of LEA proteins of other species. The alignment results by BLAST analysis showed that AdLEA protein shows the maximum similarity of 89% with LEA protein from *Glycine max*, which belongs to the D95 family of LEA proteins. It showed 79% similarity with NtLEA, 80% similarity with MtLEA and 74% similarity with AtLEA, which are LEA proteins from *Nicotiana tabacum, Medicago trancatula* and *Arabidopsis thaliana* respectively. The LEA_2 conserved domain was found in position about 204-299 amino acid of all these proteins **(Figure 4.2.1.3A)**. Phylogenetic analysis of AdLEA showed that it is closely related to *Phaseolus vulgaris* and *Glycine max* LEA proteins, both comprising 320 amino acids as against 323 amino acids sequence of AdLEA **(Figure 4.2.1.4)**. This close similarity might be due to the fact that all three species belong to family Fabaceae.

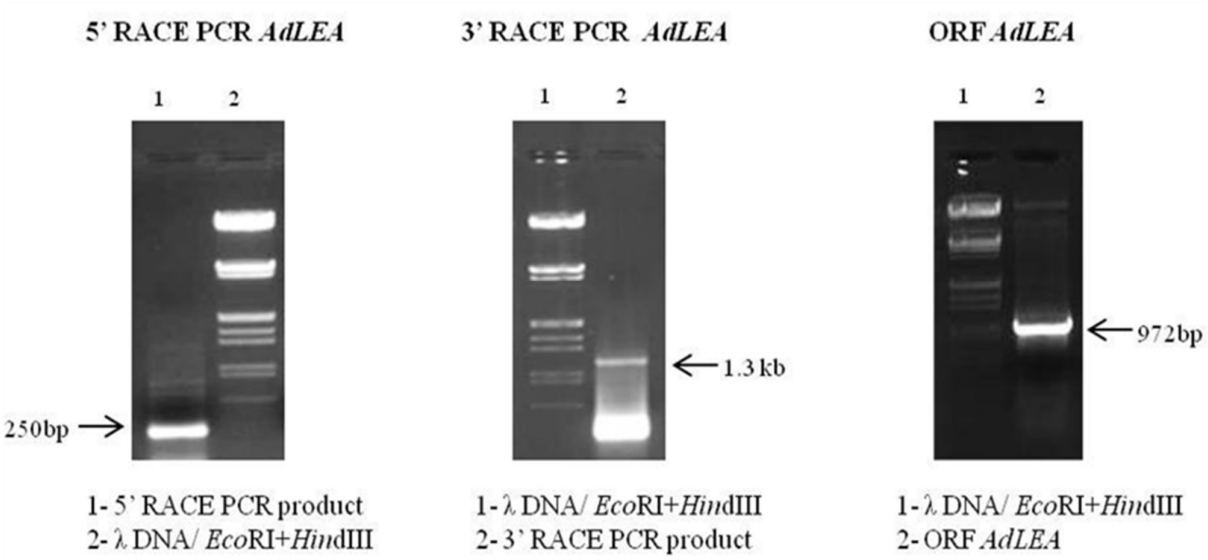


Figure 4.2.1.1. RACE-PCR and cloning of *AdLEA* from *Arachis diogoi.* Representative pictures of 5′ and 3′ RACE PCR products of *AdLEA* and its Open Reading Frame (ORF).

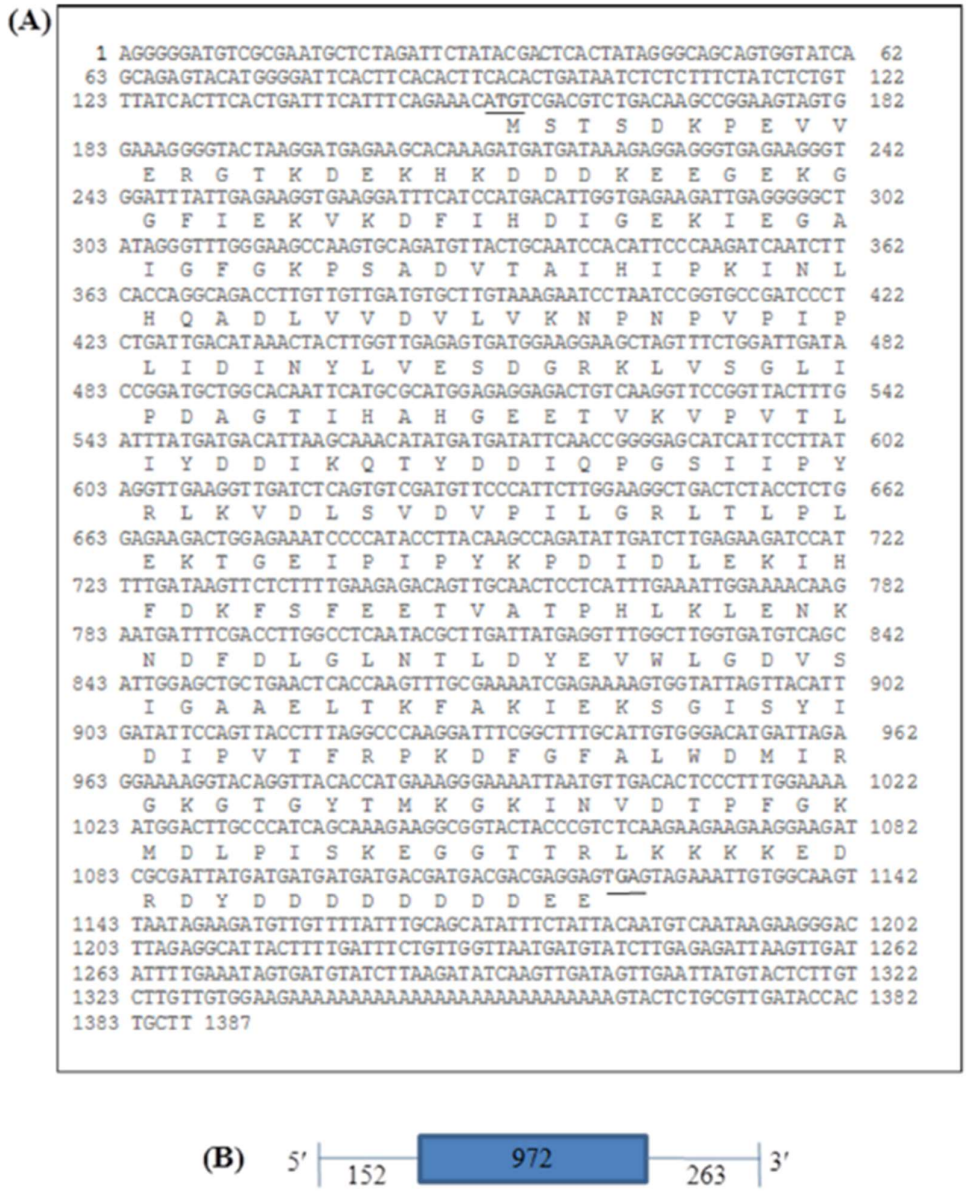
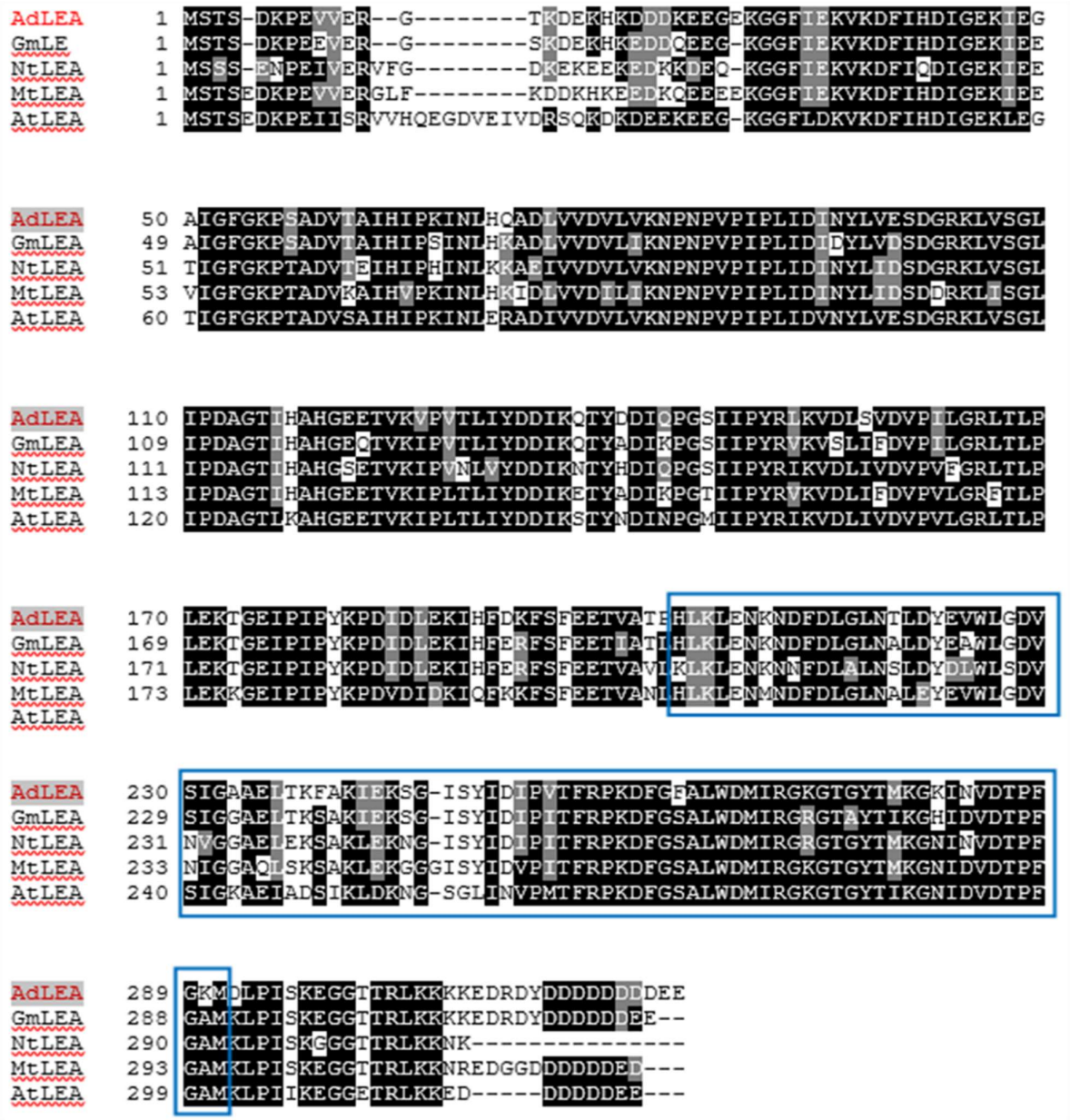
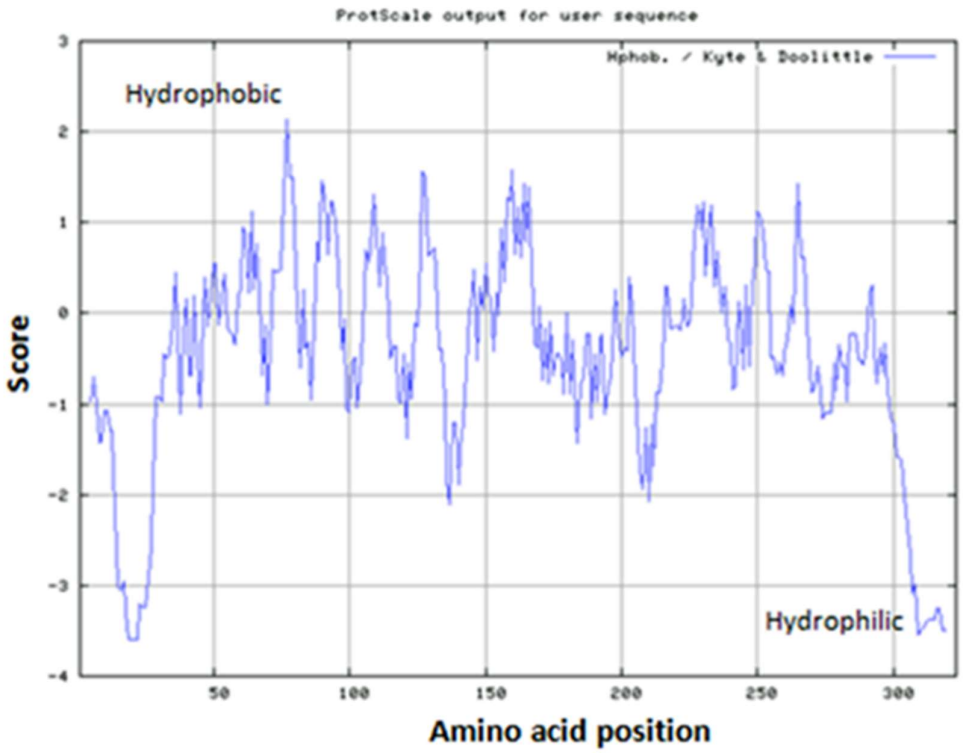


Figure 4.2.1.2. The nucleotide sequence, deduced protein and gene structure of *AdLEA*. (A) Nucleotides are numbered and the start and stop codons are underlined and in bold. (B) The nucleotide length composition of the cDNA is shown; closed box represents the Open Reading Frame (ORF) of *AdLEA* and lines represent 5′ and 3′ UTR.

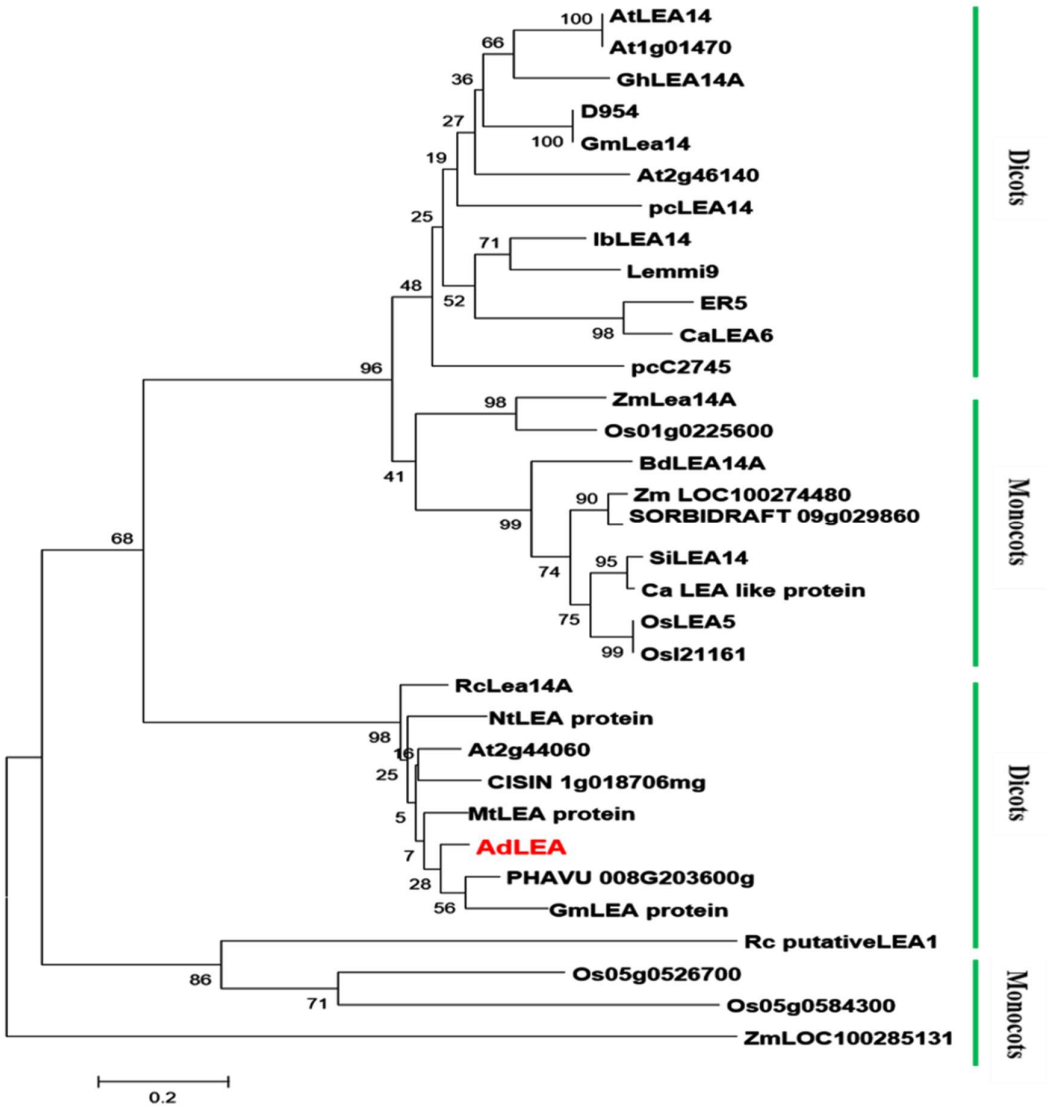

AdLEA 1 MSTS-DKPEVVER--G--------TKDEKHKDDDKEEGEKGGFIEKVKDFIHDIGEKIEG
GmLE 1 MSTS-DKPEEVER--G--------SKDEKHKEDDQEEG-KGGFIEKVKDFIHDIGEKIEE
NtLEA 1 MSSS-ENPEIVERVFG--------DKEKEEKEDKKDEQ-KGGFIEKVKDFIQDIGEKIEE
MtLEA 1 MSTSEDKPEVVERGLF--------KDDKHKEEDKQEEEEKGGFIEKVKDFIHDIGEKIEE
AtLEA 1 MSTSEDKPEIISRVVHQEGDVEIVDRSQKDKDEEKEEG-KGGFLDKVKDFIHDIGEKLEG
AdLEA 50 AIGFGKPSADVTAIHIPKINLHQADLVVDVLVKNPNPVPIPLIDINYLVESDGRKLVSGL
GmLEA 49 AIGFGKPSADVTAIHIPSINLHKADLVVDVLIKNPNPVPIPLIDIDYLVDSDGRKLVSGL
NtLEA 51 TIGFGKPTADVTEIHIPHINLKKAEIVVDVLVKNPNPVPIPLIDINYLIDSDGRKLVSGL
MtLEA 53 VIGFGKPTADVKAIHVPKINLHKIDLVVDILIKNPNPVPIPLIDINYLIDSDDRKLISGL
AtLEA 60 TIGFGKPTADVSAIHIPKINLERADIVVDVLVKNPNPVPIPLIDVNYLVESDGRKLVSGL
AdLEA 110 IPDAGTIHAHGEETVKVPVTLIYDDIKQTYDDIQPGSIIPYRLKVDLSVDVPILGRLTLP
GmLEA 109 IPDAGTIHAHGEQTVKIPVTLIYDDIKQTYADIKPGSIIPYRVKVSLIFDVPILGRLTLP
NtLEA 111 IPDAGTIHAHGSETVKIPVNLVYDDIKNTYHDIQPGSIIPYRIKVDLIVDVPVFGRLTLP
MtLEA 113 IPDAGTIHAHGEETVKIPLTLIYDDIKETYADIKPGTIIPYRVKVDLIFDVPVLGRFTLP
AtLEA 120 IPDAGTIKAHGEETVKIPLTLIYDDIKSTYNDINPGMIIPYRIKVDLIVDVPVLGRLTLP
AdLEA 170 LEKTGEIPIPYKPDIDLEKIHFDKFSFEETVATFHLKLENKNDFDLGLNTLDYEVWLGDV
GmLEA 169 LEKTGEIPIPYKPDIDLEKIHFERFSFEETIATIHLKLENKNDFDLGLNALDYEAWLGDV
NtLEA 171 LEKTGEIPIPYKPDIDLEKIHFERFSFEETVAVIKLKLENKNNFDLALNSLDYDLWLSDV
MtLEA 173 LEKKGEIPIPYKPDVDIDKIQFKKFSFEETVANIHLKLENMNDFDLGLNALEYEVWLGDV
AtLEA
AdLEA 230 SIGAAEITKFAKIEKSG-ISYIDIPVTFRPKDFGFALWDMIRGKGTGYTMKGKINVDTPF
GmLEA 229 SIGGAEITKSAKIEKSG-ISYIDIPITFRPKDFGSALWDMIRGRGTAYTIKGHIDVDTPF
NtLEA 231 NVGGAEIEKSAKLEKNG-ISYIDIPITFRPKDFGSALWDMIRGRGTGYTMKGNINVDTPF
MtLEA 233 NIGGAQISKSAKLEKGGGISYIDVPITFRPKDFGSALWDMIRGKGTGYTMKGNIDVDTPF
AtLEA 240 SIGKAEIADSIKLDKNG-SGLINVPMTFRPKDFGSALWDMIRGKGTGYTIKGNIDVDTPF
AdLEA 289 GKMDLPISKEGGTTRLKKKKEDRDYDDDDDDDEE
GmLEA 288 GAMKLPISKEGGTTRLKKKKEDRDYDDDDDEE--
NtLEA 290 GAMKLPISKGGGTTRLKKNK----------
MtLEA 293 GAMKLPISKEGGTTRLKKNREDGGDDDDDDED---
AtLEA 299 GAMKLPIIKEGGETRLKKED-----DDDDDEE---

(A)



(B)

Figure 4.2.1.3. The sequence alignment and hydropathy curve of AdLEA. (A) Alignment of deduced amino acid sequence of AdLEA with other closely related LEA proteins from other plant species. The conserved "LEA_2" motif (PF03168) is boxed. AdLEA (*Arachis diogoi*, GU223575); GmLEA (*Glycine max*, KHN11020.1) NtLEA (*Nicotiana tabacum*, ABS50432.1) MtLEA (*Medicago truncatula*, XP_013459494) AtLEA (*Arabidopsis thaliana*, BT024723). (B) Hydropathy curve for AdLEA protein.



(C)

Figure 4.2.1.4 Phylogenetic tree analysis of AdLEA with LEA homologues from different plant species. The divergence of the clades between the monocots and dicots is highlighted. Bootstrap values are indicated at the branches. The accession numbers of sequences used for construction of phylogenetic tree are as follows*:* SiLEA14 (*Setaria italica*, KJ767551); AtLEA14 (*Arabidopsis thaliana*, NM_100029); Lea14-A (*Zea mays*, NM_001159174); D95-4 (*Glycine max*, U08108); IbLEA14 (*Ipomoea batatas*, GU369820); ER5 (*Solanum lycopersicum*, U77719); Lemmi9 (Solanum lycopersicum , Z46654); CaLEA6 (*Capsicum annuum*, AF168168); OsLEA5 (*Oryza sativa*, JF776156); pcC27-45 (*Craterostigma plantagineum*, M62990)); pcLEA14 (*Pyrus communis*, AF386513); At1g01470 (*Arabidopsis thaliana*, BT015111); LEA14-A (*Gossypium hirsutum*, M88322); Lea14 homolog (*Glycine max*,

NM_001251780); At2g46140 (*Arabidopsis thaliana*, NM_130176); Os01g0225600 (*Oryza sativa* , NM_001048996); LEA14-A-like (*Brachypodium distachyon*, XM_003567779); LOC100274480 (*Zea mays*, NM_001148839); SORBIDRAFT 09g029860 (*Sorghum bicolour*, XM_002441543); LEA-like protein (*Cenchrus americanus* , AY823547); OsI21161 (*Oryza sativa*, CM000130); Os05g0526700 (*Oryza sativa*, NM_001062639); Os05g0584300 (*Oryza sativa* , NM_001062985); At2g44060 (*Arabidopsis thaliana*, BT024723); LOC100285131 (*Zea mays*, EU970969); CISIN_1g018706mg (*Citrus sinensis*, KDO49745); Th LEA protein (*Tamarix hispida*, AHF21584.1); PHAVU_008G203600g (*Phaseolus vulgaris*, XP_007141526); Lea14-A (*Ricinus communis*, XP_002533345); GmLEA (*Glycine max*, KHN11020.1) Mt LEA protein (*Medicago truncatula*, XP_013459494); chilling-responsive protein (*Nicotiana tabacum*, ABS50432); putative LEA-1 protein (*Rosa chinensis*, AKC88473).

4.2.2 Relative gene expression studies of *AdLEA* in response to phytohormones and various stress treatments in *A. diogoi*

A. diogoi twigs were subjected to various treatments at different time intervals and expression analysis of *AdLEA* in response to these treatments was carried out employing qRT-PCR using the primers RTLEA-F and RTLEA-R (**Table 4.1**). The expression pattern showed that there is a basal level expression of *AdLEA* even in the absence of any treatment (depicted as 0 h in **Figures 4.2.2.1 and 2**), which gradually increased to ~8 folds after 24 h post treatment of ABA. On the other hand, there was an early 6-fold increased expression within 3 h treatment with SA followed by a gradual decrease in transcript level below basal level, 24 h post treatment. Similar patterns were observed with ethephon and SNP where there was an increase in ~10 fold and ~30-fold expression within 3 h post treatment respectively. Then, gradually the expression decreased to basal levels. There was no significant effect of meJA on *AdLEA* expression levels **(Figure 4.2.2.1)**.

The highest expression of *AdLEA* was observed in high-temperature stress treatment where the expression was recorded to be beyond 30 fold within 2 h post treatment. Similarly, significant early accumulation of transcripts up to 15 fold and 8 fold were recorded for dehydration stress with PEG and osmotic stress with sorbitol, respectively within 3 h post treatment which decreased gradually. Also, there was a nearly 4-fold increase in transcript levels within 3 h post treatment with NaCl, which decreased and reached basal level after 24 h treatment. However, there was a gradual increase in expression levels from 2

to 4 fold with MV treatment in 24 h post treatment. There was no significant upregulation of *AdLEA* under low-temperature stress treatment **(Figure 4.2.2.2)**.

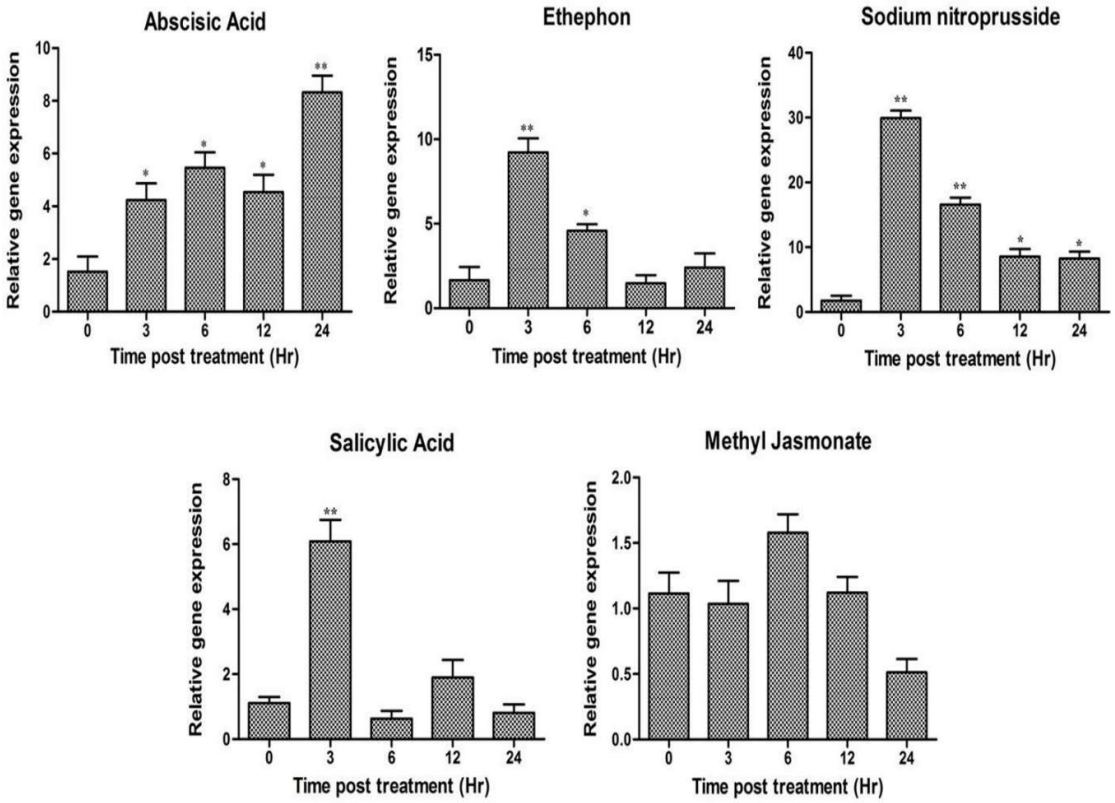


Figure 4.2.2.1. **Expression profile of *AdLEA* gene in response to various phytohormones in *A. diogoi* using qRT-PCR.** *A. diogoi* leaf samples were subjected to treatment with abscisic acid, salicylic acid, ethephon, sodium nitroprusside and methyl jasmonate. The samples were collected at various time intervals (in hours) and qRT-PCR was performed. Data plotted are the mean values ± SD from three independent experiments (n = 3; biological replicates). RNA from two trifoliate leaves of *A. diogoi* represents one biological sample. Statistical analysis was performed with one-way ANOVA (*P<0.05, **P<0.001).

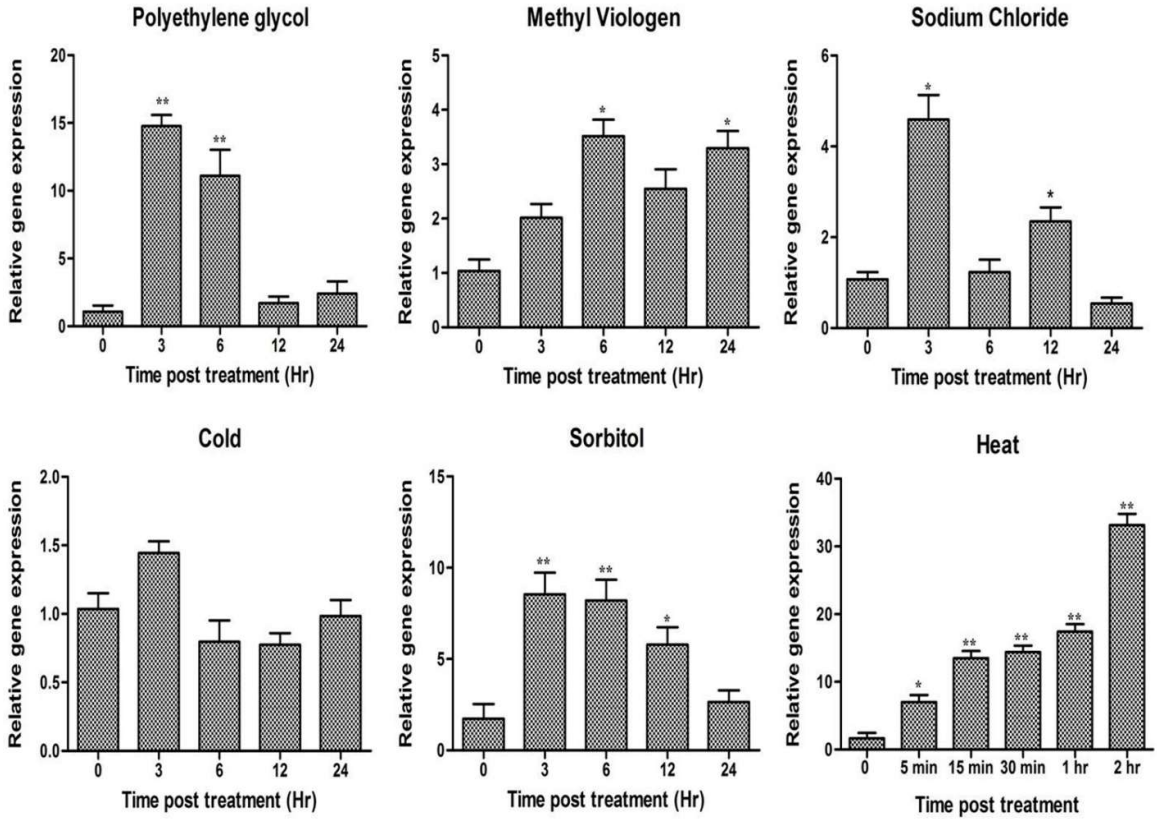
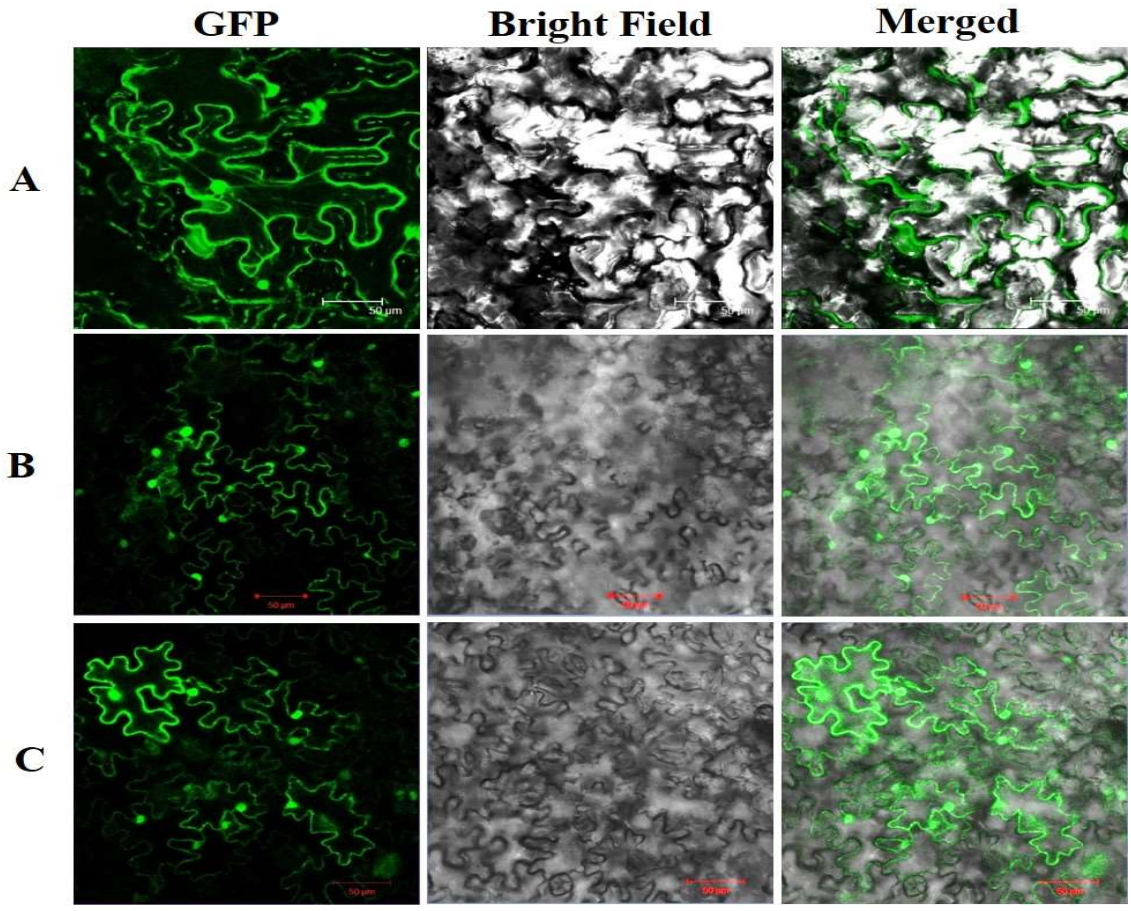


Figure 4.2.2.2. Relative gene expression of *AdLEA* in *A. diogoi* in response to various abiotic stresses. *A. diogoi* samples were treated with extreme temperatures (high and low), polyethylene glycol, sorbitol, sodium chloride and methyl viologen and *AdLEA* expression profile was analyzed by qRT-PCR. Data plotted are the mean values ± SD from three independent experiments (n = 3; biological replicates). RNA from two trifoliate leaves of *A. diogoi* represents one biological sample. Statistical analysis was performed with one-way ANOVA (*P<0.05, **P<0.001).

4.2.3 Sub-Cellular Localization of AdLEA

Subcellular localization predictions of AdLEA protein were done employing many prediction software programs such as PSORTII, MultiLOC and Y LOC. All the programs predict localization of AdLEA to be mainly cytosolic. In order to assess the localization of AdLEA experimentally in plant cells, N and C-terminal GFP translational fusion expression vectors of *AdLEA* were constructed under the control of 35 S promoter and transiently expressed in tobacco leaves by agroinfiltration. Confocal laser fluorescence microscopy results showed that both the N and C-terminal AdLEA: GFP fusion proteins were localized to

the cytosol and nucleus of the cell and there was no impact of either of the fusions on the localization pattern of the fused proteins **(Figure 4.2.3B and C)**. The expression of the fusion protein was subsequently confirmed by Western Blot where anti-mouse Histidine was used as primary antibody which was detected with ALP- conjugated goat anti-rabbit secondary antibody using BCIP/NBT as substrate. Western Blot results also showed that the AdLEA: GFP fusion protein was not degraded and intact in the cells **(Figure 4.2.3D)**.



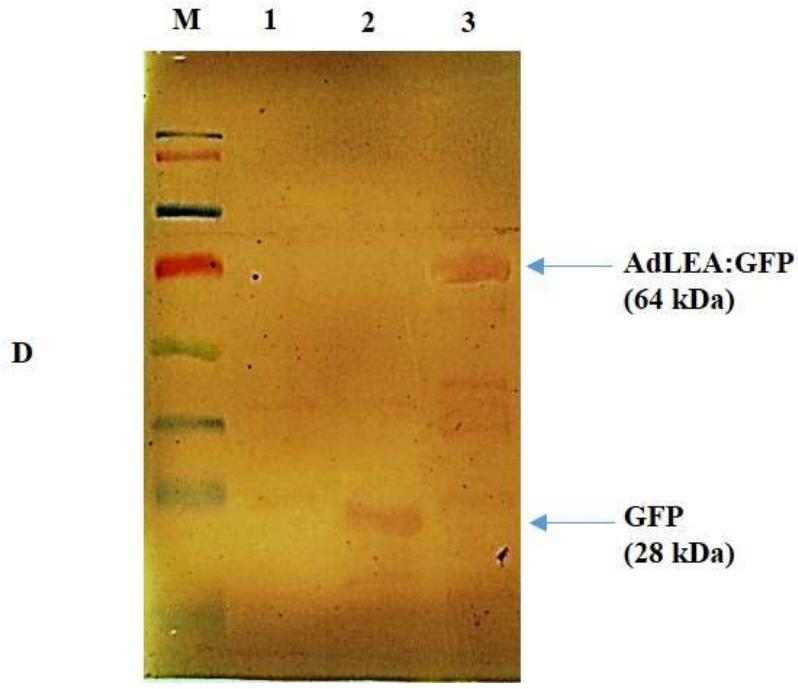
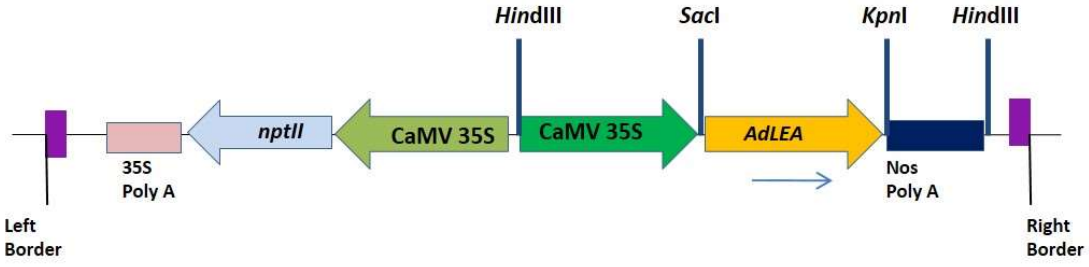


Figure 4.2.3. Subcellular localization of AdLEA protein in tobacco leaves. Empty pCAMBIA1302 vector serving as control and pCAMBIA1302-AdLEA and pEGAD-AdLEA recombinant vectors were transiently expressed in *N. benthamiana* leaves through agroinfiltration and GFP fusion proteins localization was visualized through confocal laser scanning microscope. (A) Control pCAMBIA1302 vector showing GFP throughout the cell. (B) pCAMBIA1302-AdLEA recombinant vector showing GFP in nucleus and cytosol. (C) pEGAD- AdLEA recombinant vector showing GFP in nucleus and cytosol (Bar 50 µm). Experiment was repeated three times and the representative pictures of best result among three is shown in Figure. (D) Western blot analysis of recombinant pCAMBIA1302-AdLEA protein in agro infiltrated area of leaves. Samples were collected at 72 h post agroinfiltration. M- represents pre-stained protein marker, 1- represents negative control, 2- represents a sample from positive control pCAMBIA1302 vector containing GFP-tagged with Histidine, 3- represents samples of plants infiltrated with AdLEA fused with GFP.

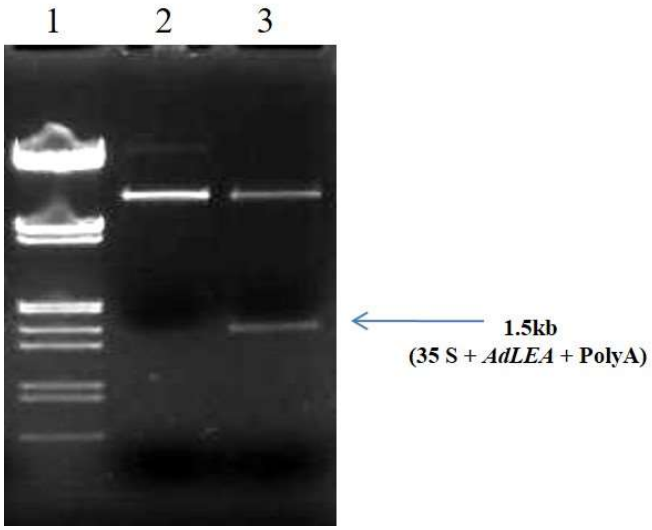
4.2.4 Cloning of *AdLEA* in binary vector, genetic transformation of tobacco and molecular analysis of transgenic *AdLEA* tobacco plants

The coding region of *AdLEA* cDNA was cloned into pRT100 vector in between the sites *Sac*I and *Kpn*I to create the plant expression cassette pRT100- *AdLEA* in which the *AdLEA* gene was flanked by CaMV

35S promoter and the poly-adenylation signal. This expression cassette was sub-cloned further into binary vector pCAMBIA2300 at *Hin*dIII site and the resultant binary vector pCAMBIA2300- *AdLEA* (**Figure 4.2.4.1A and B**) was used to transform *N. tabacum* var. Samsun by *Agrobacterium* mediated leaf-disc transformation (Horsch et al., 1985). MS medium supplemented with 2 mg/l BAP and 0.1 mg/l NAA was used for shoot induction and elongation of transformants respectively which were selected on 125 mg/l kanamycin. Afterwards, the shoots were shifted on full or half strength MS medium without hormones for root induction, which were later transferred in soil in plastic cups to get acclimatized (**Figure 4.2.4.2**). The putative T_0 transgenic plants were screened by PCR amplification for *AdLEA* and marker *nptII* genes from the genomic DNA of plants. Out of 10 plants initially selected on kanamycin, 9 plants showed PCR amplification of 972 bp ORF of *AdLEA* and 739 bp of the *nptII* gene (**Figure 4.2.4.3A and B)**. These plants were further analysed by semi-quantitative RT-PCR to assess the relative expression of *AdLEA.* which demonstrated that plants #2, 4, 5, 6 and 9 showed high expression and plants #1, 7 and 8 relatively low expressions of *AdLEA* transcripts (**Figure 4.2.4.3C**). These high and low expressing plants were further grown to the next generations and T_2 plants were obtained by selfing, which were again verified for expression levels of *AdLEA* and maintained in green house for further analysis (**Figure 4.2.4.3D**). Simultaneously, WT plants (non-transgenic) were also maintained separately as controls.



(A)



(B)

Figure 4.2.4.1. Construct preparation for plant transformation. (A) Pictorial representation of the T-DNA region of the plasmid pCAMBIA2300 that contains *AdLEA* gene driven by CaMV 35S promoter and *NptII* gene. RB, right border; LB, left border. **(B)** Gel picture showing the release of ~ 1.5kb *AdLEA* cassettes with 35S promoter and polyA signal from the pCAMBIA2300 vector after *Hin*dIII restriction digestion.

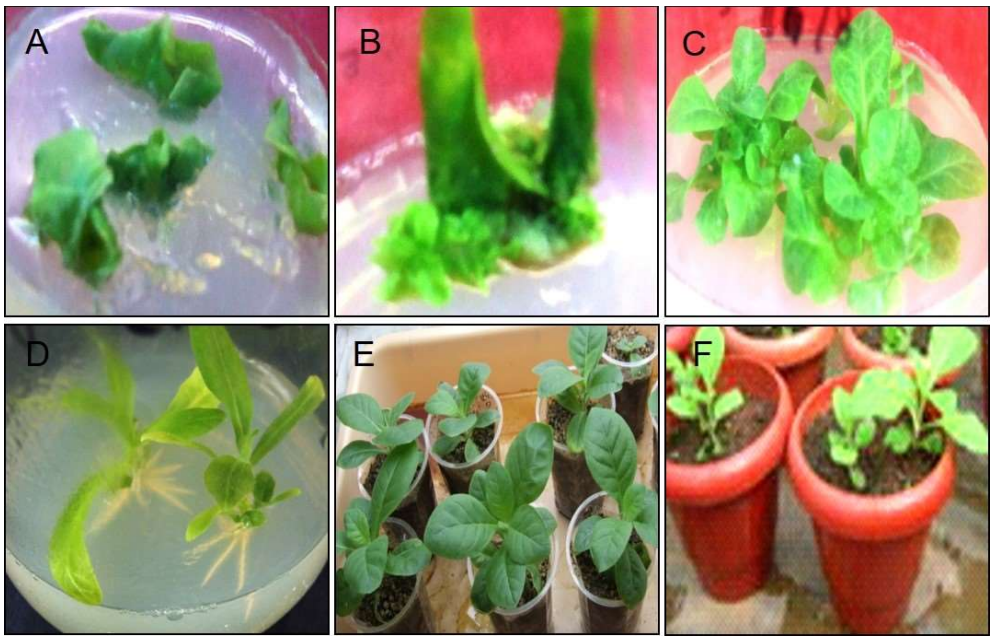


Figure 4.2.4.2. Different stages of *Agarobacterium* mediated transformation of tobacco. (A) Explants forming callus on shoot induction medium. (B) Shoot proliferation on shoot induction medium. (C) Elongation of shoots on

shoot elongation medium. (D) Rooting of putative transgenics on root induction medium. (E) and (F) Hardening and acclimatization of putative transgenic tobacco plants in culture conditions and greenhouse conditions respectively.

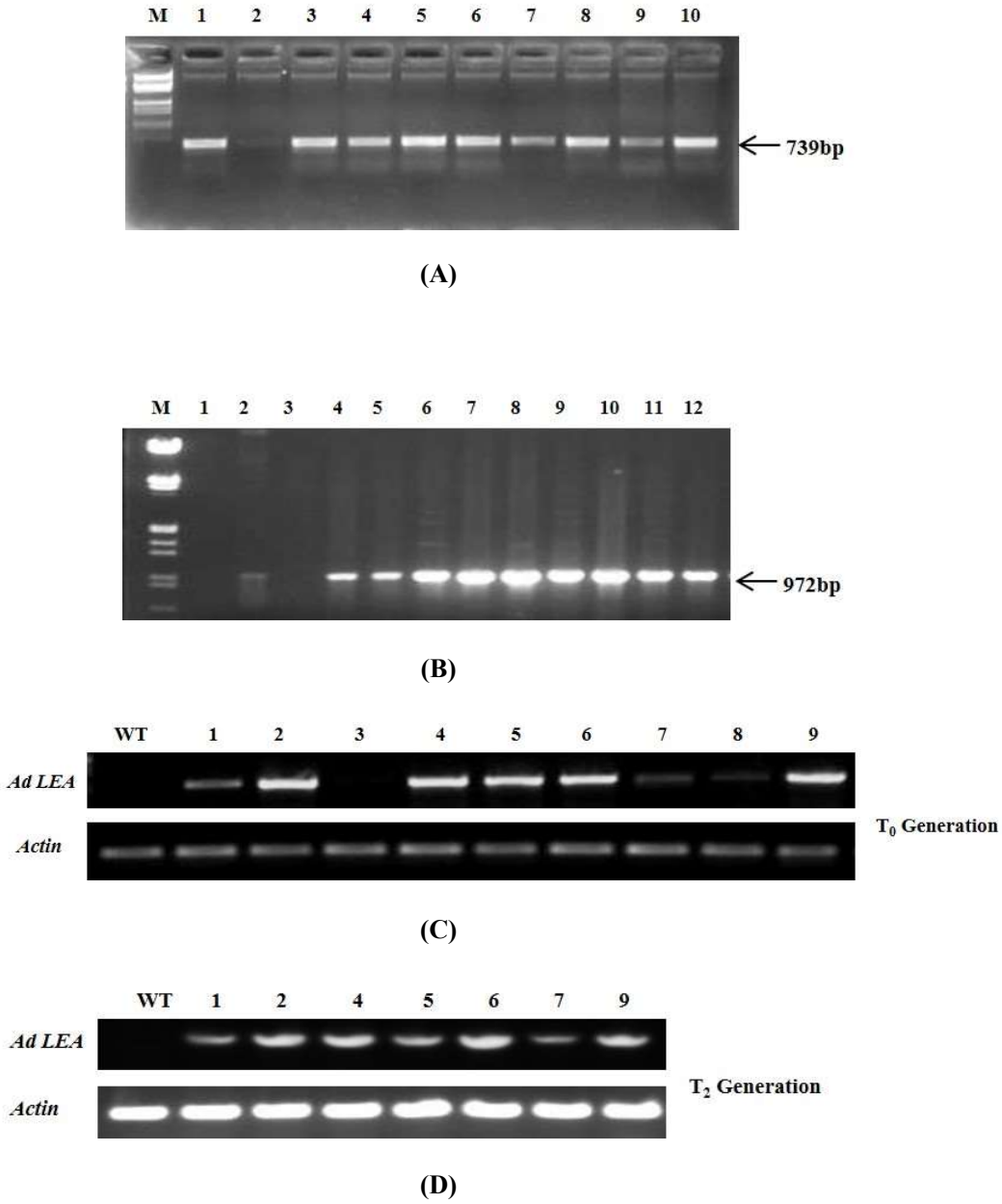


Figure 4.2.4.3. Integration and expression of *AdLEA* in transgenic tobacco plants. (A) PCR analysis of putative T_0 transformants for the *nptII* gene; M- λDNA/*Eco*RI+*Hin*dIII ladder, 2- WT negative control, 1 and 3-10 transgenic plants showing 739 bp amplified PCR product of the *nptII* gene. (B) PCR analysis of putative T_0 transformants for *AdLEA* gene; M- Marker, 1- negative control (without DNA), 2- positive control vector, 3- WT negative control, 4

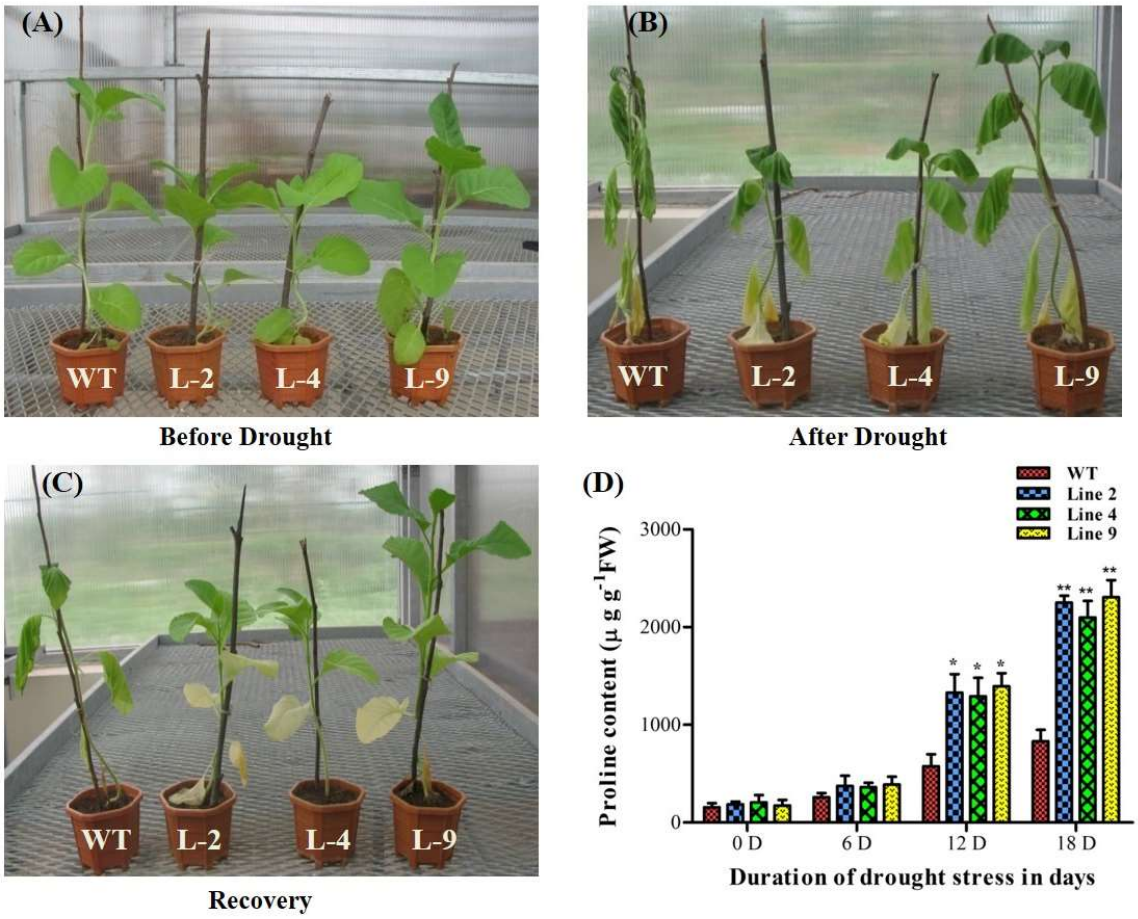
to12 transgenic plants showing 972 bp amplified PCR product of *AdLEA* gene. (C and D) Transcript levels of *AdLEA* in T_0 and T_2 generation. Line 2, 4, 5, 6 and 9 are high expression lines and 1, 7 and 8 are low expression lines.

4.2.5 Plant morphology, PS(II) efficiency and proline and chlorophyll content in *AdLEA* transgenics under progressive drought stress and recovery

Chlorophyll fluorescence measurements were performed in drought induced *AdLEA* transgenic plants to check for the photochemical efficiency under drought stress in green house conditions. The plants were subjected to drought stress by withholding water for 18 days and were watered on the 18th day and kept for recovery for three days. Chlorophyll fluorescence measurements were taken on D0, D6, D12, and D18 (D–day) after drought treatment followed by a recovery (R) period of three days for both WT and transgenic lines. The *AdLEA* expressing tobacco transgenic demonstrated enhanced tolerance under progressive drought stress as evident from the morphological differences recorded during drought and recovery in comparison to WT plants (**Figure 4.2.5.1A, B, and C**). The WT leaves exhibited extensive wilting and bleached-out appearance under progressive drought stress for 18 days in comparison to leaves of transgenic plants, which were less affected. Interestingly, even after withdrawal of drought treatment for 3 d, the leaves of WT plants displayed wilting symptoms, whereas transgenic plants recovered completely and leaves appeared healthy (**Figure 4.2.5.1C**). There was no significant difference in leaf proline content of WT and transgenic plants at the initiation of the treatment (D0). As the duration of stress increased, variations in proline content were recorded between WT and transgenic plants. These differences were more evident on the 12th and 18th day of drought treatment with a significantly higher level of proline on the 18th day in transgenic plants when compared to WT plants (**Figure 4.2.5.1D**).

Drought-induced changes in the maximal and effective photochemical quantum yield of WT and transgenic plants from chlorophyll fluorescence measurements are illustrated in (**Figure 4.2.5.1E and F**). There was no significant difference observed in F_v/F_m on D0 and D6 between WT and transgenic plants. However, the F_v/F_m of WT plants on D12 was significantly decreased by ~31% in comparison to transgenic plants, which were similar to D0 and D6. At D18 (i.e. extreme drought conditions), the F_v/F_m of both WT and transgenic plants significantly decreased with the WT plants exhibiting a steep decline (~66%) in comparison to D12. The F_v/F_m values significantly correlated with the chlorophyll content of the leaves of

AdLEA transgenic plants under progressive drought stress (**Figure 4. V.1.G**). Later, after withdrawal of drought conditions (i.e. recovery) the F_v/F_m recorded for transgenic plants were similar to D0 in comparison to WT which recorded significantly lower F_v/F_m similar to D12 (**Figure 4. V.1E**). A similar pattern was observed in F'/F_m' [Y(II)] for both WT and transgenic plants demonstrating improved PS(II) efficiency under progressive drought stress and recovery (**Figure 4.2.5.1F**).



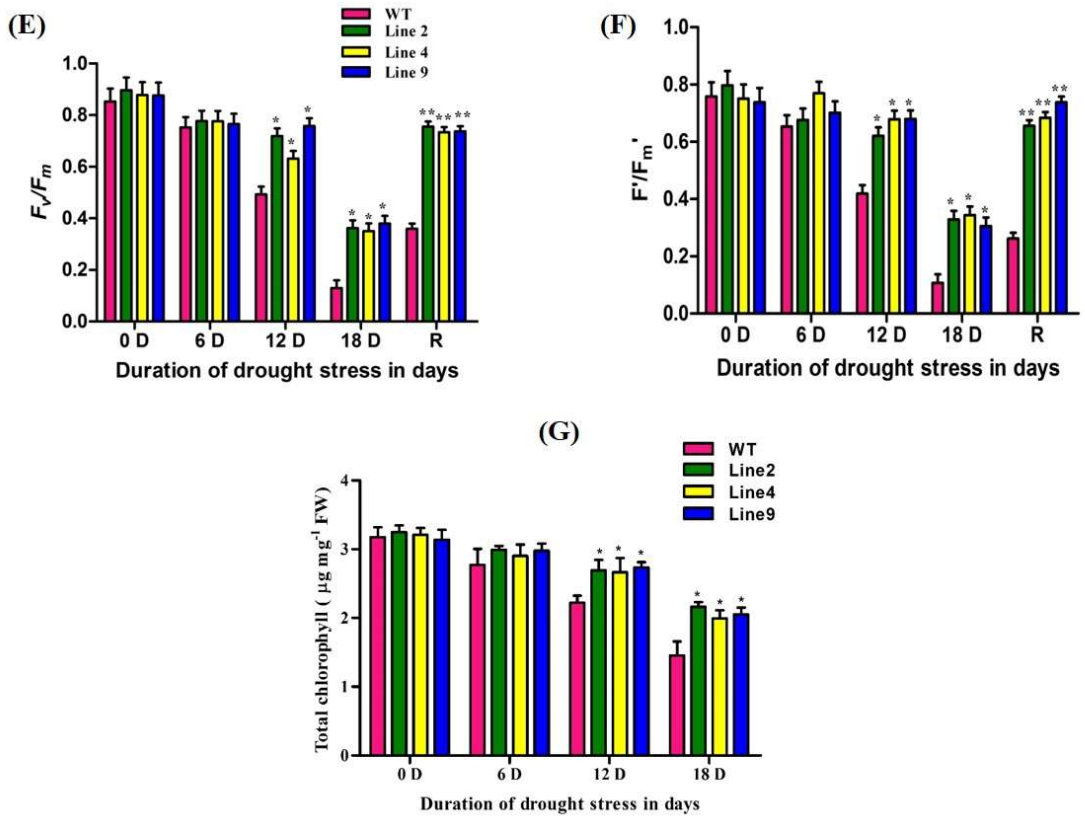


Figure 4.2.5.1. Morphological and physiological variation in WT and *AdLEA* transgenic plants under progressive drought stress. (A) Plants before drought induction; (B) during severe drought; and (C) recovery phase after drought. (D) Estimation of proline content in leaves of WT and transgenic plants during drought stress; (E and F) Chlorophyll fluorescence measurements of plants during drought stress and recovery phase; F_v/F_m and F'/F_m' for both WT and transgenic plants; (G) Chlorophyll estimation in WT and transgenic plants during drought stress. Data plotted are the mean values ± SD from three independent experiments (n = 3; biological replicates). Single leaf from each plant constitute one biological sample. Statistical analysis was performed with two-way ANOVA (*P<0.05, **P<0.001).

4.2.6 Abiotic stress tolerance assays for transgenic *AdLEA* plants

Dehydration stress tolerance

Stress tolerance assays were performed at the young seedling stage and on mature plants by leaf disc assays. PEG and sorbitol were used for mimicking dehydration and osmotic stress conditions in transgenic plants at both seedling stage and in leaf discs from mature plants. Seedlings were allowed to grow for 25 d on 10% PEG containing media, effect of which on seedlings is depicted in (**Figure 4.2.6.1A**). No visible

bleaching of seedlings was observed in WT and transgenic lines but there were prominent differences in the growth of seedlings of WT and transgenic lines. Shoots of WT seedling were stunted with reduced leaf area in comparison to transgenic seedlings. Also, there was a significant difference in the root length between WT and transgenic seedlings with the root length of transgenic plants being ~2-2.5 folds higher than that of WT (**Figure 4.2.6.1B and F**). The difference between WT and transgenic plants started appearing after 20 d of growth in 12% PEG medium. There was a reduction in the overall size of WT seedlings in comparison to transgenic seedlings with further reduced leaf lamina. Few seedlings showed signs of chlorosis as well (**Figure 4.2.6.1C**). The roots of transgenic seedlings were long and slender with well-developed lateral roots and the root length being ~1.5-2 folds more than that of WT seedlings (**Figure 4.2.6.1D and F**). However, there were no significant differences recorded in growth between seedlings of WT and low expression line, signifying the quantitative effect of degree of expression of *AdLEA* in ameliorating stress effects. Leaf discs from fully extended leaves of two-month-old plants were excised and allowed to float over 10 mL solutions of 10, 12 and 14% of PEG and distilled water (control) for 3 d under continuous white light for leaf senescence assay. During 3rd day of incubation, leaf discs from WT demonstrated bleaching with increasing concentration of PEG, while there was very little bleaching observed in leaf discs from transgenic plants even in the maximum concentration of PEG, which stayed green and healthy as control discs in water (**Figure 4.2.6.1E**). The total chlorophyll content of these leaf discs after 3 d of treatment was also in accordance with the phenotype observed in them. The chlorophyll content was significantly higher in the leaf discs of the transgenic plants at all concentration of PEG when compared to WT, which showed dose-dependent loss of chlorophyll pigment. The decrease in chlorophyll content was 47% to 70% with increasing concentration of PEG in WT and 32% to 53% in low expression line when compared to water controls whereas this decrease was 6% to 41% in high expression transgenic lines (**Figure 4.2.6.1G**). The level of oxidative stress as a consequence of dehydration treatment was also determined by measuring TBARS in these discs. WT showed higher TBARS levels, the increase being 120% to 450% with increasing PEG concentration than leaf discs of all three transgenic lines which showed increments within a range of 5% to 117% with respect to control leaf discs indicating lesser lipid peroxidation and more membrane integrity under stress in transgenic plants (**Figure 4.2.6.1H**).

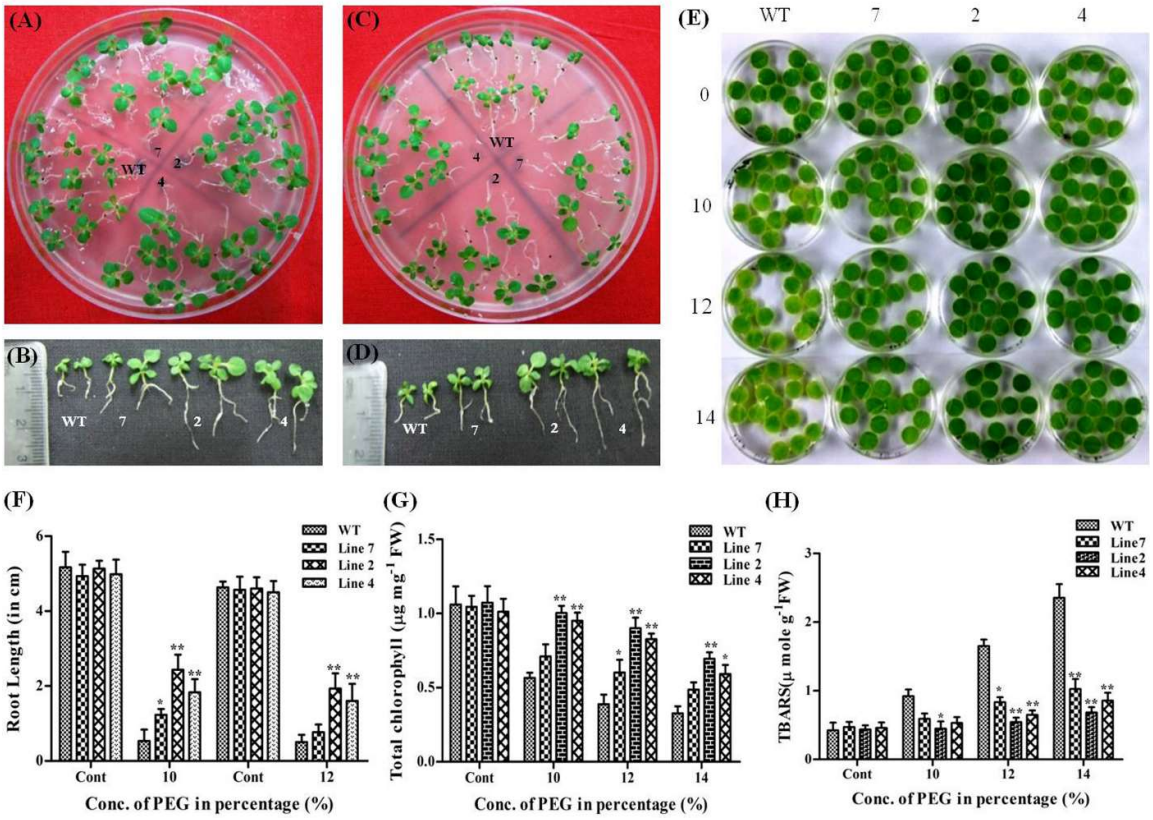


Figure 4.2.6.1. Effect of dehydration stress on *AdLEA* transgenic plants. (A and C) WT and transgenic seedlings after growth on MS medium supplemented with 10% and 12% PEG respectively. (B and D) Morphology of individual seedlings after growth on 10% and 12% PEG respectively depicting differences in root length between the seedlings. (E) Phenotypic differences between WT and transgenic leaf discs floated in dose-dependent concentrations (0, 10, 12 and 14%) of PEG. (F) Graphical representation of root length in PEG-treated seedlings after 25 d and 20 d growth on 10% and 12% PEG respectively. (G) Graphical representation showing the chlorophyll content (µg mg^{-1} FW) in the leaf discs after 72 h of treatment with 10, 12 and 14% PEG. (H) Lipid peroxidation expressed as TBARS content (µmol g^{-1} FW) in leaf discs after 72 h of treatment with 10, 12 and 14% PEG. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). 11 seedlings per plate constitute one biological sample for seedling assay. 15 leaf discs from a single leaf constitute one biological sample for disc senescence assay. Statistical analysis was performed with two-way ANOVA (*P<0.05, **P<0.001).

Osmotic stress tolerance

Similar morphological and growth responses as in dehydration stress were recorded for transgenic plants under osmotic stress with sorbitol at two different concentrations (200 mM and 300 mM). There was

significant bleaching of leaves and retarded shoot and root growth in WT seedlings in comparison to transgenic seedlings in 200 mM sorbitol (**Figure 4.2.6.2A, B, and F**). Interestingly, the transgenic seedlings of high expression lines demonstrated superior growth performance even in 300 mM when compared to WT seedlings (**Figure 4.2.6.2C, D, and F**). This difference was more evident in roots of transgenic and WT seedling. High expression transgenic seedlings had long slender roots with the profuse lateral root system and root length being ~3.5 to 5 folds and ~3 to 4.5 folds higher than that of WT seedlings in the case of 200 mM and 300 mM sorbitol respectively. Similarly, transgenic leaf discs demonstrated relatively green and healthy phenotype even at a higher concentration of sorbitol (500mM) in contrast to the leaf discs of WT after 3 d of incubation. This difference was more pronounced in high expression lines (**Figure 4.2.6.2E**). Chlorophyll and TBARS estimation results were also in agreement with the above-mentioned phenotypic observations with loss of chlorophyll pigment in WT ranging from 54% to 79% whereas it was 7% to 23%, 24% to 52% and 36% to 73% respectively in transgenic lines 2, 4, and 7 in comparison to discs floated on water (**Figure 4.2.6.2G**). TBARS content in leaf discs from transgenic plants was also significantly lower, which was in the range of 5% to 90% in both high expression lines and 95% to 257 % in low expression line compared to the WT (175% to 380%) with increasing concentration of sorbitol (**Figure 4.2.6.2H**).

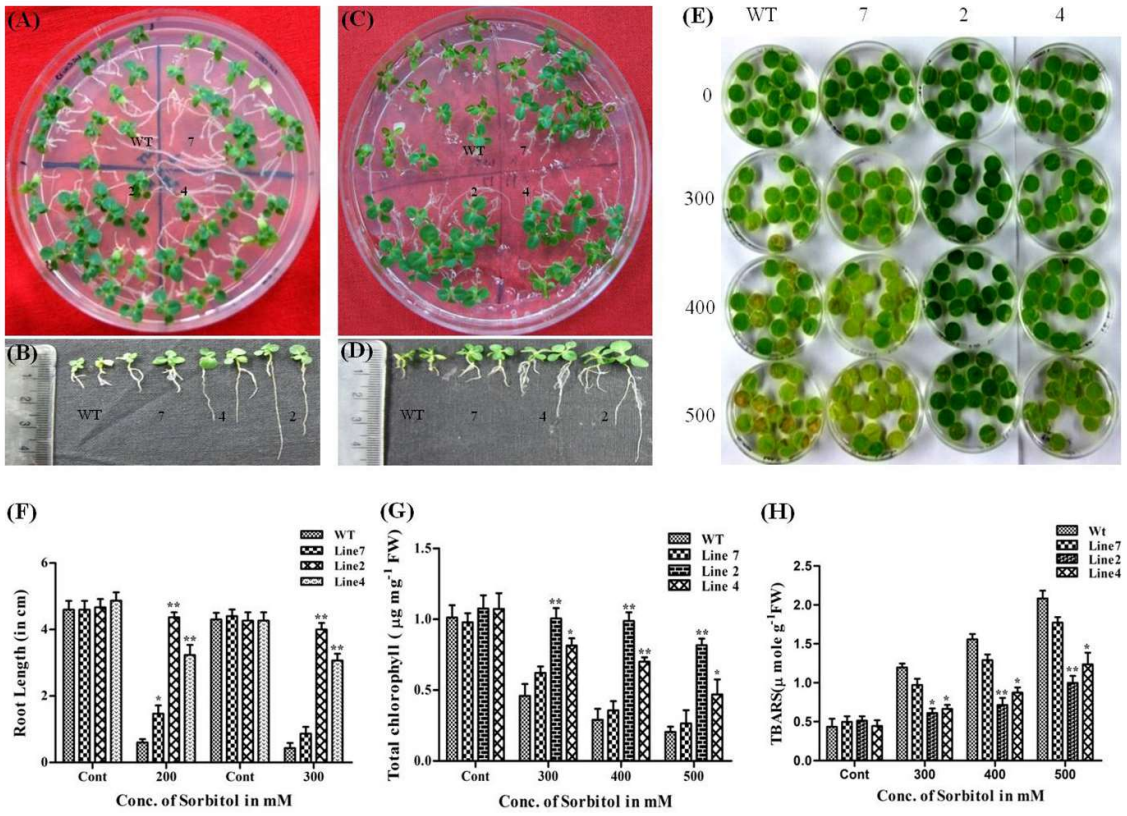


Figure 4.2.6.2. Effect of osmotic stress on *AdLEA* transgenic plants. (A and C) WT and transgenic seedlings after growth on MS medium supplemented with 200 mM and 300 mM sorbitol respectively. (B and D) Seedling morphology after growth on 200 mM and 300 mM sorbitol respectively, depicting difference in root lengths between the seedlings. (E) Phenotypic differences between the leaf discs from WT and transgenic plant lines floated in dose-dependent concentrations (200mM and 300mM) of sorbitol. (F) Graphical representation of root length in sorbitol-treated seedlings after 20d and 16d growth on 200mM and 300mM sorbitol respectively. (G) Chlorophyll content (µg mg^{-1} FW) in the leaf discs after 72h treatment with sorbitol. (H) Lipid peroxidation expressed as TBARS content (µmol g^{-1} FW) in leaf discs after 72h treatment with sorbitol. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). 11 seedlings per plate constitute one biological sample for seedling assay. 15 leaf discs from a single leaf constitute one biological sample for disc senescence assay. Statistical analysis was performed with two-way ANOVA (*P<0.05, **P<0.001).

Salinity stress in transgenic plants

The WT seedlings showed complete growth inhibition and bleaching at 200mM NaCl after 15 d of growth whereas transgenic seedlings maintained near normal growth at this concentration with proper

development of green leaves and long roots with lateral branching (root length being ~5 to 6 folds more than that of WT) (**Figure 4.2.6.3A, B, and F**). This stunted growth in WT was more evident by 8th day in 300mM NaCl when compared to transgenic seedlings of high expression lines which could grow normally with mild signs of chlorosis and developed healthy shoots, leaves, and roots (**Figure 4.2.6.3C, D, and F**). To further investigate salinity tolerance in the growth and morphology of the mature plant, leaf discs from WT and transgenic were treated with 200mM and 300mM NaCl. Severe bleaching was observed in WT leaf discs after 2 d of treatment with 200 mM and 300 mM NaCl. The loss of chlorophyll pigment was 59% to 79% in WT with compared to controls (in water) whereas high expression transgenic lines could withstand the stress and showed tolerance (**Figure 4.2.6.3E**) as observed from total chlorophyll content (**Figure 4.2.6.3G**) and TBARS (**Figure 4.2.6.3H**) in these lines. The low expression line showed similar response as that of WT plants (**Figure 4.2.6.3G and H**).

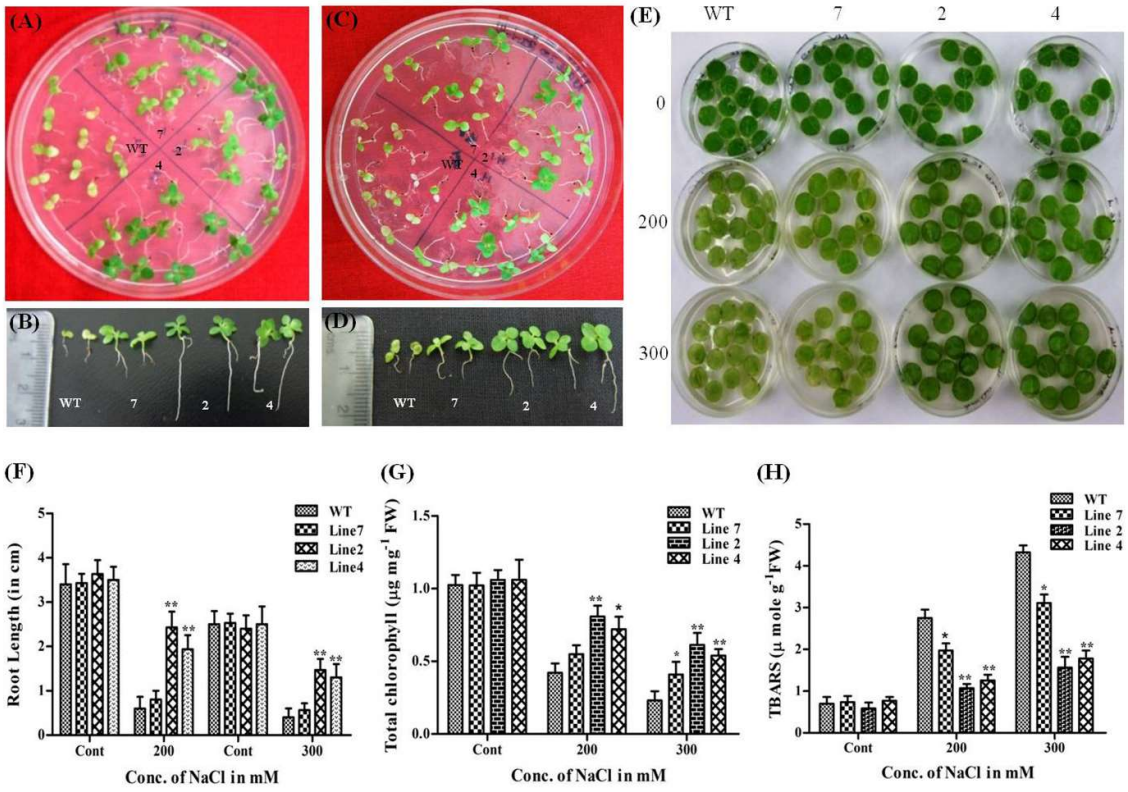


Figure 4.2.6.3. Effect of salinity stress on *AdLEA* transgenic plants. (A and C) WT and transgenic seedlings after growth on MS medium supplemented with 200mM and 300mM NaCl respectively. (B and D) Individual seedlings morphology after growth on 200mM and 300mM NaCl respectively, depicting differences in root length between the

seedlings. (E) Phenotypic differences in the leaf discs from WT and transgenic plant lines floated in dose-dependent concentrations (200mM and 300mM) of NaCl. (F) Graphical representation of root lengths in NaCl-treated seedlings after 15d and 9d growth on 200mM and 300mM NaCl respectively. (G) **C**hlorophyll content (µg mg^{-1} FW) in the leaf discs after 72 h of treatment with NaCl. (H) Lipid peroxidation expressed as TBARS content (µmol g^{-1} FW) in leaf discs after 72 h of treatment with NaCl. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). 11 seedlings per plate constitute one biological sample for seedling assay. 15 leaf discs from a single leaf constitute one biological sample for disc senescence assay. Statistical analysis was performed with two-way ANOVA (*P<0.05, **P<0.001).

Oxidative stress studies in transgenic plants

At 5µM MV, all the seedlings of high expression transgenic lines were healthy with negligible chlorosis whereas 70-80% of WT seedlings bleached out completely after 4 d treatment (**Figure 4.2.6.4A**). This bleached population of WT was unable to recover on MV free medium whereas seedlings of high expression transgenic lines could survive well on recovery medium (**Figure 4.2.6.4B**). Similar responses in growth as in 5µM were observed for WT and transgenic seedlings at 10µM MV (**Figure 4.2.6.4C and D**). The chlorophyll content in WT was recorded to be 66% to 88% less when compared to controls, which was similar to the chlorophyll content in low expression line. However, the relative decrease in chlorophyll content of high expression transgenic lines in comparison to corresponding controls was lesser and recorded between 28% to 42% and 30% to 44% respectively (**Figure 4.2.6.4E**). Leaf senescence assay performed with leaf discs also demonstrated improved performance in transgenic leaf discs in comparison to WT. Severe bleaching was observed in WT discs at both concentrations of MV with 10 µM MV being more detrimental to discs (**Figure 4.2.6.4F**). The treatment resulted in 55% to 72% loss of chlorophyll from WT as against 27% to 44% and 35% to 50% in high expression transgenic lines 2 and 4 respectively, hence recording higher chlorophyll content than WT discs (**Figure 4.2.6.4G**). TBARS content of WT was also considerably higher with a rise from 280% to 505% with increasing concentration of MV whereas this increment in TBARS was within the range of 90% to 345% in high expression transgenic lines (**Figure 4.2.6.4H**). Here also, low expression line showed a response to similar to that of WT plants with a decrease

in chlorophyll and MDA content in the range of 47% to 57% and 266% to 500% respectively (**Figure 4.2.6.4G and F**).

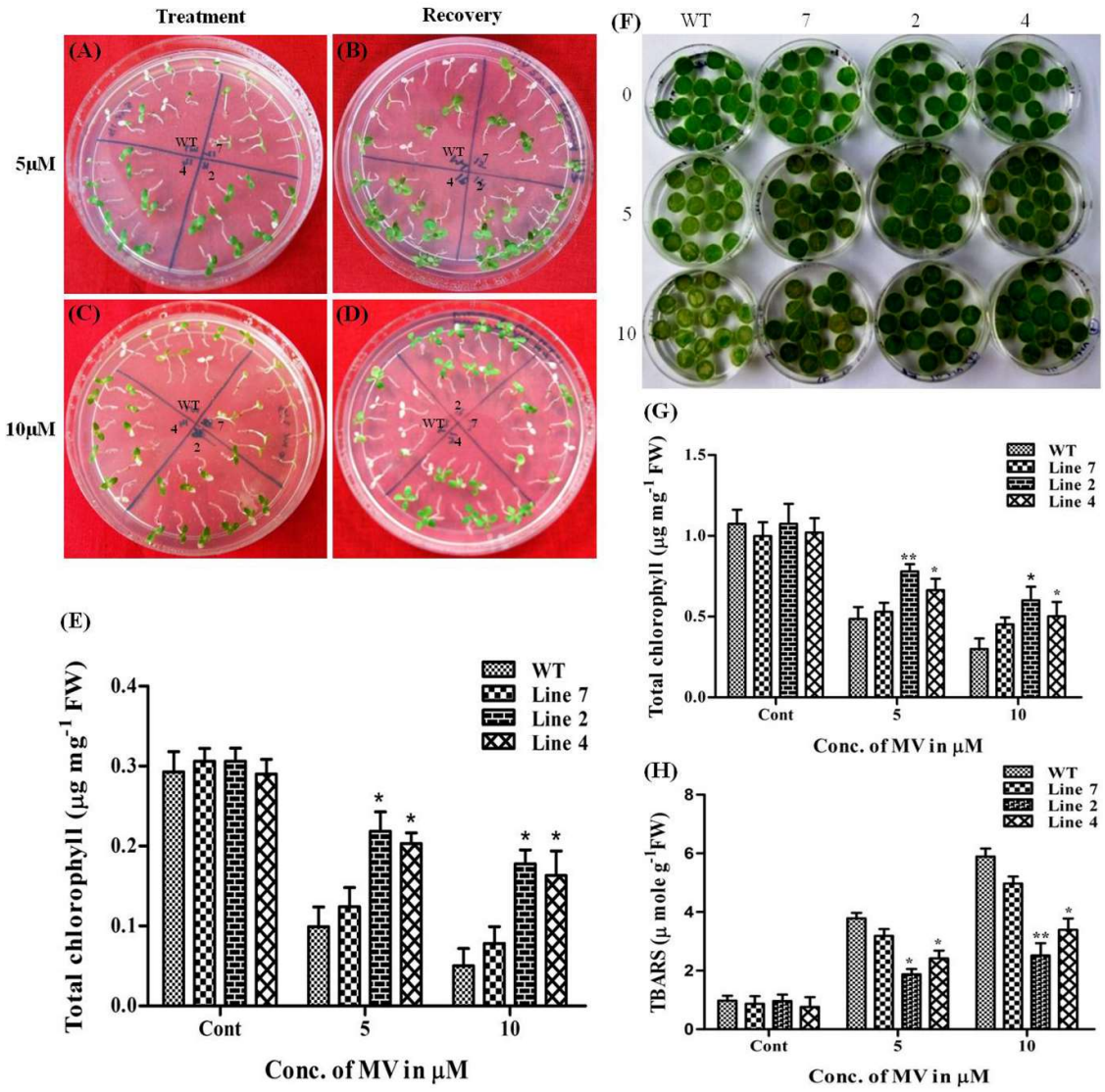


Figure 4.2.6.4. Effect of oxidative stress on *AdLEA* transgenic plants. (A and C) WT and transgenic seedlings after growth on MS medium supplemented with 5 and 10µM MV respectively. (B and D) Recovery of seedlings on MV free medium. (E) Chlorophyll content (µg mg^{-1} FW) in the seedlings after growth on MV supplemented medium. (F) Phenotypic differences in the leaf discs from WT and transgenic plant lines floated in dose-dependent concentrations (5 and 10µM) of MV. (G) Chlorophyll content (µg mg^{-1} FW) in the leaf discs after treatment with MV. (H) Lipid peroxidation expressed as TBARS content (µmol g^{-1} FW) in leaf discs after treatment with MV. All the experiments

were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). 11 seedlings per plate constitute one biological sample for seedling assay. 15 leaf discs from a single leaf constitute one biological sample for disc senescence assay. Statistical analysis was performed with two-way ANOVA (*P<0.05, **P<0.001).

Tolerance of AdLEA transgenics towards phytohormone ABA

Effect of ABA was checked on seedling stage of *AdLEA* transgenics. For seedling assay, seedlings were shifted to a medium containing two different concentrations of ABA (5 and 8 µM) and were allowed to grow for 20 d. There was visible difference in the phenotypes of WT and transgenic lines on 5 µM ABA. Most of the WT seedlings showed clear signs of chlorosis with more than 50 % of population completely bleached out. The size of the WT seedlings was severely repressed with stunted shoot growth and damaged roots. On the other hand, most of the transgenic seedling were green with very less chlorosis and healthy and well formed shoots and roots (**Figure 4.2.6.5A**). Roots of transgenics have lateral branches and they showed 2.5-3 folds more length as compared to WT seedlings (**Figure 4.2.6.5B and C**). The effect of 8 µM ABA was more severe as there were signs of chlorosis even in transgenics seedlings and 50% of them also showed bleached phenotype, the others were healthy and showed normal shoots and roots with lateral branches, length of roots being 2 -2.5 times more than that of WT seedlings (**Figure 4.2.6.5C, D and E**).

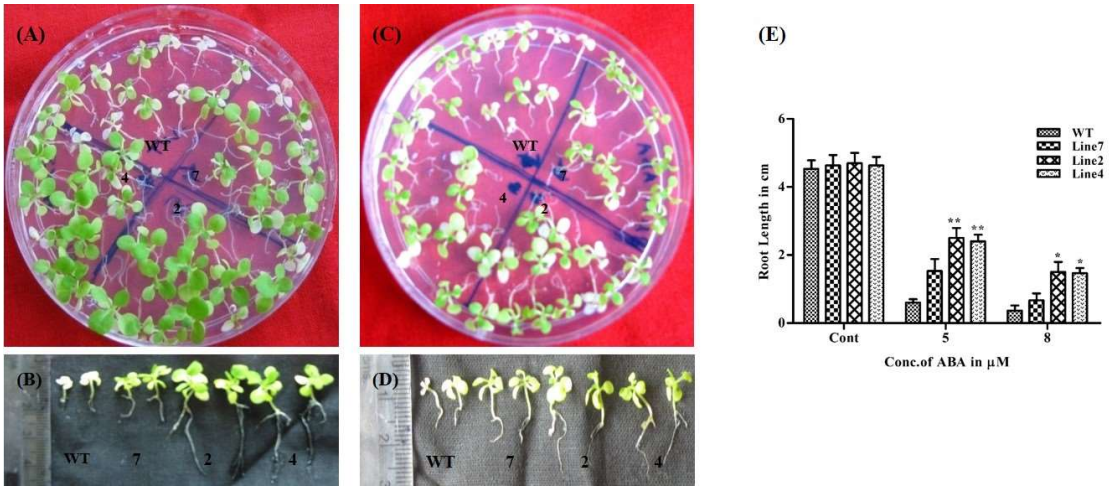


Figure 4.2.6.5. Effect of exogenous ABA on *AdLEA* transgenic plants. (A and D) WT and Transgenic seedlings after growth on MS medium supplemented with 5µM and 8 µM ABA respectively. (B and E) Individual seedling morphology after growth on 5µM and 8 µM ABA respectively, depicting differences in root lengths between the

seedlings. **(**C) Graphical representation of root lengths in ABA treated seedlings after 20 d growth on 5 µM and 8 µM ABA respectively. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). 11 seedlings per plate constitute one biological sample for seedling assay. Statistical analysis was performed with two-way ANOVA (*P<0.05, **P<0.001).

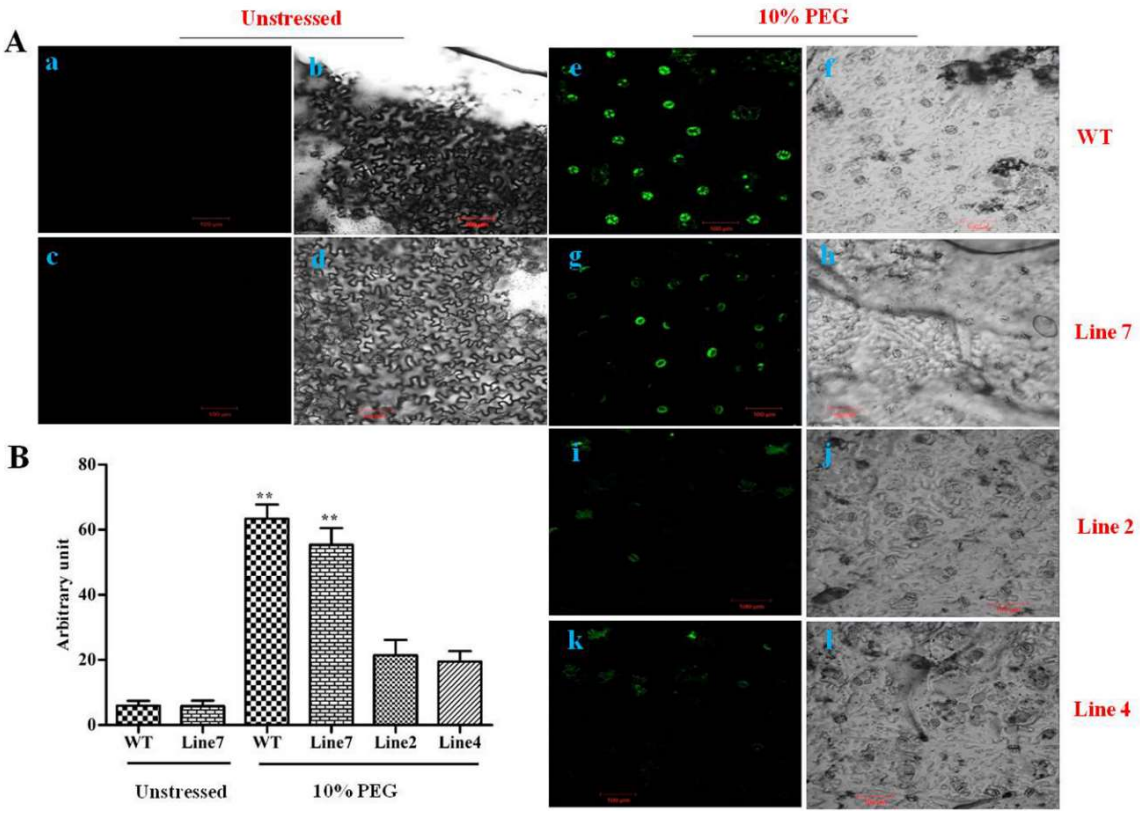
4.2.7 ROS detection in stomatal guard cells by confocal microscopy using H_2DCFDA staining in *AdLEA* transgenic tobacco plants

Confocal studies were done to detect total cell ROS using H_2DCFDA, against dehydration stress induced with PEG in leaf epidermal peels of plants. The level of DCF fluorescence under control condition (non-stress) was almost similar in all three transgenic lines and WT, thus, representative guard cell fluorescence of only one transgenic line was shown in **Figure 4.2.7A (a, b, c, and d)**. There was an increase in florescence signal after treatment with 10% PEG in all the lines including WT; however, this increment was much intense in stomatal guard cells of WT and low expression line in comparison to the high expression transgenic lines. This indicates lower levels of ROS formation in high expression transgenic lines when compared to WT and low expression line under stress [**Figure 4.2.7A (e, f, g, h, i, j, k, and l)**]. There were nearly 10 folds and 8 folds' increase in the fluorescence levels in WT and low expression line respectively under stress conditions as opposed to both high expression transgenic lines 2 and 4, where there is an increment of only 2.5 folds and 2 folds in fluorescence respectively after PEG treatment (**Figure 4.2.7B**).

Detection and quantification of ROS in leaves using NBT staining

For detection of Superoxide anion (O_2^-), histochemical studies using NBT staining was done against salinity stress induced with NaCl in leaf of plants. The extracts from unstressed leaf samples (serving as controls) showed negligible staining of formazan precipitate and also very little absorbance in comparison to the treated samples indicating low levels of superoxide radicles in non-treated samples. Both WT and transgenic leaves treated with 100 mM NaCl showed significantly high levels of absorbance implicating its role in inducing oxidative stress in plants. The WT showed very high levels of formazan precipitate formation as shown by intense blue coloration covering almost entire lamina in comparison to high expression transgenic lines in which only traces of blue formazan staining can be seen (**Figure 4.2.7C**). The

levels of formazan formation in the low expression line were almost similar to WT plant. Similar results were observed when formazan levels were quantified and absorbance was measured (**Figure 4.2.7D**).



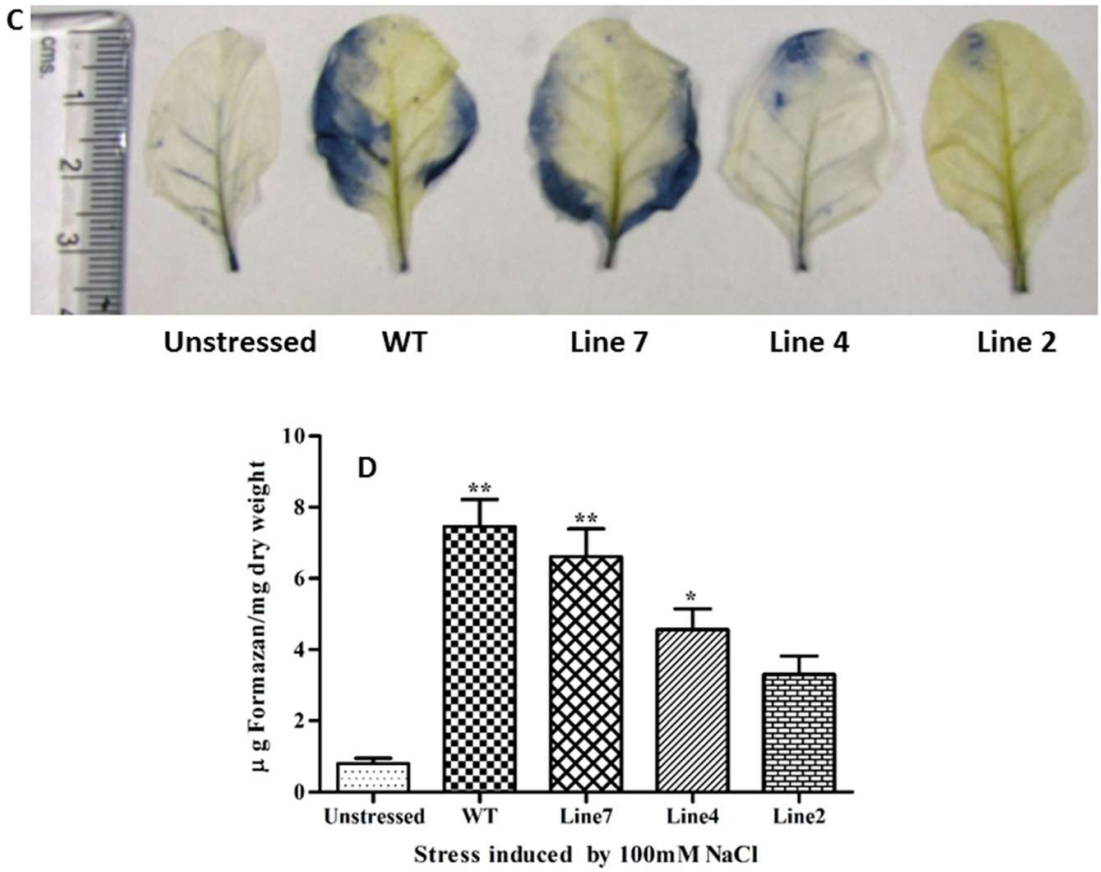


Figure 4.2.7. ROS detection in leaf epidermal cells by confocal microscopy and quantification of ROS in leaves using NBT staining. (A) Fluorescence levels in WT and transgenic plants before and after treatment with PEG as shown in confocal microscopy. Bright field images of WT and transgenic plants are also displayed. Stomata with water treatment. (a and b) WT; (c and d) representative transgenic line 7. Stomata after treatment with PEG. (e and f) WT; (g and h) transgenic line 7; (i and j) transgenic line 2; (k and l) transgenic line 4. The figures are representative confocal images of stomatal guard cells (n < 1000) with three biological repetitions (Bar-100µm). (B) Quantification of ROS production in cells after H_2DCFDA staining using ImageJ software. (C) Unstressed (untreated), WT, and transgenic leaves treated with 100mM NaCl as visualized after NBT staining. (D) Graphical representation of formazan content (µg mg^{-1} dry weight) in leaves of unstressed, WT, and transgenic leaves after treatment with 100mM NaCl. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Single leaf from each plant constitute one biological sample. Statistical analysis was performed with one-way ANOVA (*P<0.05, **P<0.001).

4.2.8 Expression analysis of some stress related genes in *AdLEA* transgenic plants

qRT-PCR was done for some stress-responsive genes in leaves of a representative high expression line #2 in comparison to WT to elucidate their expression before and after drought treatment. There was a ~0.5-fold basal level expression in *NtP5CS* (1-pyrroline-5-carboxylate synthetase) of *AdLEA* transgenic and WT under control conditions. However, the expression of *NtP5CS* was up-regulated under stressed conditions in both WT and transgenic plants. Its expression was ~3 fold higher in transgenic plants in comparison to WT after exposure to drought (**Figure 4.2.8A and B**). There was a trivial change in expression levels of *NtAPX* (ascorbate peroxidase), *NtCAT* (Catalase), and *NtSOD* (Superoxide dismutase) (genes for corresponding ROS scavenging enzymes) from leaves of transgenic and WT plants in the absence of stress. However, there was a significant up-regulation of all three enzyme genes, after drought stress in transgenic leaves with maximum being in *NtSOD* (2.2 folds) followed by *APX* (2.1 folds) and *NtCAT* (1.9 folds) compared to WT expression levels (**Figure 4.2.8A and B**). There was significant up-regulation (2.5 folds) in the expression of *NtNCED* (9-cis-epoxycarotenoid dioxygenase), in transgenic line compared to WT even in control conditions. After drought exposure, the transcript level of *NtNCED* increased manifold in transgenic as well as in WT with ~ 4.2 folds higher in transgenic line (**Figure 4.2.8A and B**). *NtERD10c* (early response to dehydration), encoding a group 2 LEA protein was similarly up-regulated under stress conditions. The transcript levels increased to ~2.5 folds in transgenic plants than WT plants (**Figure 4.2.8A and B**). The result of this study suggested that there were enhanced transcript levels of these abiotic stress responsive genes associated with the expression of *AdLEA* in the transgenic line in response to drought stress.

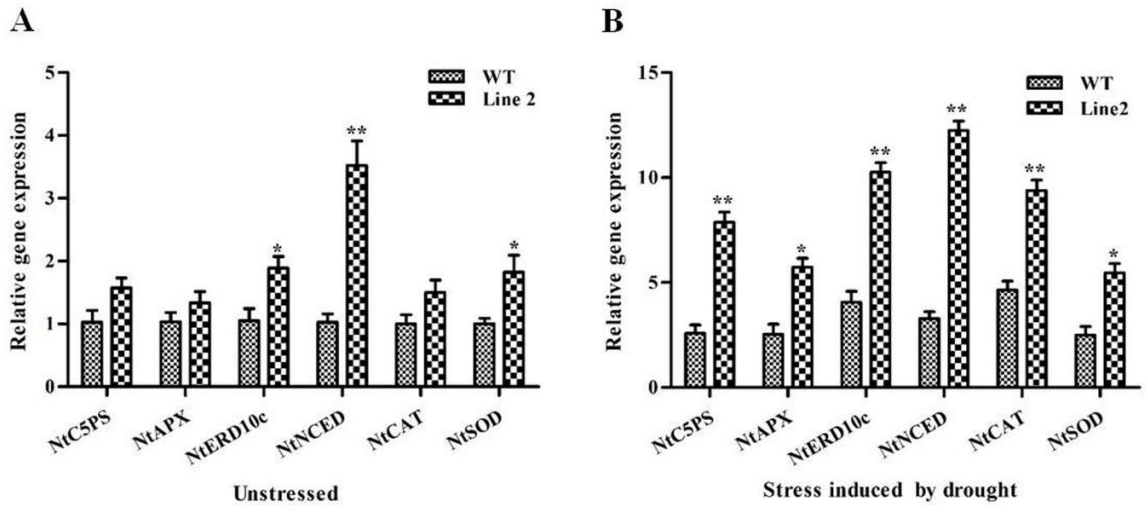


Figure 4.2.8. Expression analysis of stress responsive genes in *AdLEA* transgenic plants. Relative transcript levels of few abiotic stress responsive genes (*NtP5CS*, *NtAPX*, *NtERD10c*, *NtNCED, NtCAT,* and *NtSOD*) in *AdLEA* transgenic line #2 compared with WT were analyzed by qRT-PCR in (A) untreated and (B) drought stress conditions. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). RNA from single leaf from each plant constitute one biological sample. Statistical analysis was performed with two-way ANOVA (*P<0.05, **P<0.001).

4.3 Discussion

LEA proteins are a large family of hydrophilic proteins which have been demonstrated to be associated with tolerance against multiple stresses. LEA family has been classified into seven groups, among which members of group 1, 2 and 3 are considered typical LEA proteins, which have been studied extensively in relation to abiotic stress tolerance in plants. However, “atypical” Group 5 LEA proteins have not been studied well enough and there are fewer studies to characterize their functions related to abiotic stress tolerance mechanism. Here we have reported the functional characterization of an atypical, novel *LEA* gene from *Arachis diogoi*, which was isolated earlier as a transcript derived fragment (TDF) in a differential gene expression analysis in *A. diogoi* challenged with late leaf spot pathogen, *P. personata* (Kumar and Kirti 2015).

In the present study, the full-length cDNA sequence was amplified from this partial fragment and the gene was designated as *AdLEA*. On the basis of sequence similarity, AdLEA protein was grouped under

group 5C LEA proteins and it showed maximum homology with *Glycine max* LEA protein, which belongs to D95 family of LEA proteins as well. In contrast to other LEA proteins, which are very hydrophilic, D95 LEA proteins display a hydropathic character throughout the length of the protein (Espelund et al. 1992). This is evident in AdLEA as well, which is a nonpolar and neutral protein with a GRAVY value of –0.496.

In plants ABA is a key phytohormone involved in plant development and responses to various biotic and abiotic stresses (Raghavendra et al. 2010b). There is an increase in endogenous ABA levels in plant cells in response to various abiotic stresses leading to the expression of stress responsive genes (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006). The stress tolerance mechanisms in the plant can be ABA-dependent or independent and osmotic stress-regulated genes can be activated through both pathways (Abe et al. 2003; Kobayashi et al. 2006). However, there was a report which suggests that for activation of LEA-like proteins, stress signalling pathways independent of ABA might not exist (Xiong et al. 2002). It has been also reported that promoter region of the group 5 LEA genes generally contain an ABA-responsive element (ABRE), which drives the induction of these genes in response to various abiotic conditions (Park et al. 2011b). There are several reports on LEA protein expression induced by various abiotic stresses emphasizing their role in imparting abiotic stress tolerance (Baker et al. 1988; Dure et al. 1989a; Wang et al. 2003). Consistent with the previous reports, *AdLEA* expression was also induced by ABA, high temperature, dehydration, salinity and oxidative stresses in *A. diogoi* indicating the potential role of *AdLEA* in response to these stresses. *AdLEA* transcripts were also induced after treatment with SNP, ethephon and SA suggesting a possible cross-talk between these phytohormones in AdLEA signalling pathway (García-Mata and Lamattina 2001).

Subcellular localization study of LEA proteins may be correlated with understanding different aspects of their functionality and their participation in cellular protection during various stresses. Group 5 LEA proteins are located in different subcellular compartments. The *Arabidopsis thaliana* group 5 protein SAG1/AtLEA5 was localized in mitochondria (Salleh et al. 2012). The maize LEA protein Rab28 was found to be localized in the nucleoli (Amara et al. 2013). RcLEA group 5 LEA protein from *Rosa chinensis* was reported to be localized in the cytoplasm (Zhang et al. 2014). SiLEA14 from *Foxtail millet* and JcLEA from *Jatropha curcas* were found to be localized in the nucleus and cytosol both (Wang et al. 2014; Liang et al. 2013). Similar localization results were observed for AdLEA protein, which was found localized in

nucleus and cytosol both. Previous studies have demonstrated that for nuclear localization of proteins, the presence of a nuclear localization signal (NLS) and its subsequent phosphorylation are essential. The localization of the maize LEA protein RAB17 to the nucleus is dependent on its phosphorylation state (Goday et al. 1994; Jensen et al. 1998). Similarly, the NLS-segment present in the dehydrin WCS120 plays an important role in its import in the nucleus (Houde et al. 2004). Sequence analysis of AdLEA did not reveal any known nuclear localization signal (NLS) and it also lacks the S segment that is involved in nuclear localization (Jensen et al. 1998). Thus, it is possible that an unknown nuclear localization mechanism devoid of NLS and S segment delivers AdLEA to the nucleus (Rorat 2006). Also, the majority of localization prediction programs predicts its localization to be mostly cytosolic. Hence, there is another possibility that the protein is actually cytosolic in origin but diffused passively in the nucleus (Candat et al. 2014). Nevertheless, its localization in these places suggested a comprehensive protective role for AdLEA in plant cells which is consistent with its role of imparting enhanced tolerance against abiotic stresses.

Transgenic approaches have shown that overexpression of LEA proteins from different species in *Arabidopsi*s, Tobacco, wheat, rice, maize and many plants demonstrate better growth and morphology under abiotic stress (Tunnacliffe and Wise 2007). In congruence with these reports, the *AdLEA* transgenic plants also exhibited improved tolerance against phytohormone ABA (in seedling stage) and also towards dehydration, salinity, osmotic, oxidative stress as confirmed by the data on stress assays on seedling and leaf discs from mature plants. Many abiotic stresses induce excessive accumulation of ROS in the cell, that is detrimental to cells at high concentration leading to oxidative damage to membrane lipids, nucleic acids, and proteins. This increased damage to membranes of cell and cell organelles can, in turn, hinder the growth and development of cells (Gill and Tuteja 2010). Damage to chloroplasts leads to depletion in the chlorophyll levels and membrane damage leads to high MDA levels in cells (Gaweł et al. 2004). The leaf disc assays showed higher chlorophyll content and less MDA levels in transgenic *AdLEA* plants in comparison to WT plants under all the stress treatments. This clearly indicated reduced lipid peroxidation and membrane damage in these plants. Similar results were observed when *in situ* H_2O_2 and O_2^- free radicals were measured in WT and *AdLEA* transgenic plants using H_2DCFDA fluorescence and NBT staining under dehydration and salinity stress conditions. Accumulation of ROS, specifically superoxide anion (O_2^-) has been used as an effective index to assess the tolerance to multiple stresses because it directly reflects on the

capability to resist oxidative stress in plants (Gill and Tuteja 2010). In both dehydration and salinity stresses, transgenic plants displayed enhanced antioxidative capacity in the form of decreased accumulation of O_2^- compared to WT. Low levels of O_2^- commonly indicate higher tolerance to abiotic stresses in transgenic plants, which is consistent with the above physiological and biochemical results under different abiotic stress assays, and may also explain the enhanced tolerance to these stresses in transgenic plants.

In plants, the ability to maintain photosynthesis under environmental stress is a fundamental requirement to maintain their growth and development. The potential function of *AdLEA* in stress adaptation was further explored by monitoring the photosynthesis performance of transgenic plants. The chlorophyll a fluorescence studies demonstrated improved PS-II efficiency in *AdLEA* transgenic plants when compared to WT plants under progressive drought stress. F_v/F_m reflects an estimate of the maximum quantum efficiency of PSII photochemistry and has been widely used to detect stress-induced perturbations in the photosynthetic apparatus (Baker et al. 2004). In this study, WT plants showed a significant decrease in F_v/F_m under drought stress when compared to transgenic plants suggesting the development of slowly relaxing quenching processes and photo-damage to PSII reaction centers, both of which reduce the PSII photochemistry. Similarly, F'/F_m' measurement also demonstrated the same trend as for F_v/F_m in WT plants and *AdLEA* transgenic plants. This indicates improved operational quantum efficiency of PSII electron transport and hence, CO_2 assimilation in transgenic plants as both are directly related (Baker 2008). Even after a recovery period of 3 d after extreme drought treatment (D18), the WT plants were unable to recover on re-watering in comparison to transgenic plants as demonstrated by F'/F_m' and F_v/F_m measurements, suggesting irreversible damage to photosynthetic apparatus. The *AdLEA* transgenic plants showed F'/F_m' and F_v/F_m values similar to that of the healthy unstressed plant, which showed the ability of *AdLEA* to partially reverse the stress induced by growth inhibition under drought. The improved CO_2 assimilation of transgenic plants under stress possibly would have allowed the plants to sustain superior growth. This better photosynthesis performance of the transgenics can also be linked to the accumulation of more proline levels and less damage to chlorophyll content in leaves of transgenic plants under drought in comparison to WT. Proline is an organic osmolyte, which works in multiple ways to impart stress tolerance to cells by the protection of cellular structures, detoxification of enzymes, scavenging ROS alone or in combination with other stress-related enzyme system. These compounds also confer integrity to the membrane and keep the

photosynthesis intact under stress. Proline accumulation is a well-known measure adopted for the alleviation of osmotic stress in plants and it has also been documented that leaf proline content is correlated with plant tolerance to abiotic stresses (Matysik et al. ; Ben Ahmed et al. 2010; Karaba et al. 2007).

Further, to understand the possible molecular mechanisms, which help *AdLEA* transgenic plants to combat stress in response to drought, some of the stress responsive genes were analyzed in high expression transgenic plants compared with WT. Plants possess a complex antioxidant defense system of ROS detoxification generated as a result of any induced stress, which consists of an accumulation of non-enzymatic antioxidants like proline and activation of ROS scavenging enzymes (Miller et al. 2010). It has been reported that drought tolerance is positively correlated with the activity of antioxidant enzymes such as SOD, CAT, and APX in plants (Wang et al. 2009). Consistent with these studies, *AdLEA* transgenic plants demonstrated enhanced expression of *NtAPX, NtSOD,* and *Nt*CAT in comparison to WT under drought conditions, which might presumably also explain the lower levels of ROS in leaves of transgenic plants under dehydration stress induced by PEG. Also, there was enhanced expression of *NtP5CS* (a key enzyme involved in the synthesis of amino acid proline in plants) under drought stress in transgenic plants in comparison to WT. This result was in concordance with the data of elevated proline levels in transgenic plants during drought stress. There was enhanced expression of stress responsive genes *NtERD10c* and *NtNCED* in transgenic plants when compared to WT plants. *ERD10c* encodes a group 2 hydrophilic LEA protein that is assumed to play critical roles in combating cellular dehydration by binding water, stabilizing labile enzymes and protecting cellular macromolecular structures (Kovacs et al. 2008b). Greater induction of this gene in transgenic line suggested that plants might synthesize more protective chaperones for protein stabilization providing better defense against water loss in dehydration or drought stress as opposed to WT plants (Chakrabortee et al. 2007). NCED is a rate limiting key enzyme in the biosynthesis of ABA, which plays an essential role in adaptive responses to environmental stresses including drought stress (Seo 2002). Interestingly, there was enhanced expression of *NtNCED* in transgenic plants in comparison to WT plants in control conditions before stress treatment, which became more pronounced after drought stress. This suggests increased *de novo* ABA biosynthesis in transgenic plants, which could have been promoted by the constitutive expression of *AdLEA* in these plants. The assumption sounds reasonable since there are reports where an increase in the endogenous ABA level by expression of *NCED* gene resulted in improved drought

tolerance in various plants (Iuchi et al. 2001). This study on these genes (*NtERD10,* and *NtNCED*) reconfirm the fact that their associated enhanced expression in *AdLEA* transgenic plants appeared to facilitate plant tolerance towards drought stress. Hence, the enhanced tolerance of *AdLEA* transgenic plants against dehydration or drought stress could be attributed to the cumulative effects of up regulation of many genes and effects of their gene products. *AdLEA* transgenic plants demonstrated higher antioxidant activity owing to enhanced expression of antioxidant enzymes under stressed conditions, which might have aided the transgenic plants to scavenge ROS more effectively than WT plants. Similarly, up-regulation of stress-responsive genes involved in proline metabolism and ABA signalling pathways might have played a role in conferring enhanced tolerance to drought stress in *AdLEA* transgenic plants. Upregulation of an LEA homolog of tobacco (*ERD10*) and its functions in imparting stress tolerance can also be associated with increased tolerance of *AdLEA* transgenic plants.

To conclude, overexpression of *AdLEA* imparts abiotic stress tolerance to transgenic tobacco plants most specifically in water limiting conditions by increasing O_2^- scavenging and up-regulation of various stress-related genes.

Table 4.1. Oligo and their sequences used for RACE-PCR, cloning and gene integration studies in *AdLEA* transgenics plants.

Gene	Primer Name	Primer sequence (5′-3′)
AdLEA	5'AdLEAGSP1	CATCTGCACTTGGCTTCCCAAACCCT
	3'AdLEAGSP1	AGGGTTTGGGAAGCCAAGTGCAGATG
	AdLEA-F-SacI	CGAGCTCATGTCGACGTCTGACAAGC
	AdLEA-R-KpnI	AGGTACCTCACTCCTCGTCGTCATCG
	AdLEA-pEGAD-F	AGAATTCAT GTC GAC GTC TGA CAA GC
	AdLEA-pEGAD-R	TAAGCTTTC ACT CCT CGT CGT CAT C
	AdLEA-pC1302-F	CAGATCT ATGTCGACGTCTGACAAGCC
	AdLEA-pC1302-R	CACTAGT CTC CTC GTC GTC ATC GTCAT
	RTLEA-F	GGCTTTGCATTGTGGGACATGA
	RTLEA-R	TCACTCCTCGTCGTCATCGTC
nptII	NptII-F	AGATGGATTGCACGCAGGTTCTC
	NptII-R	ATCGGGAGCGGCGATACCGTA
Adh3	AdRTADH3-F	GACGCTTGGCGAGATCAACA
	AdRTADH3-R	AACCGGACAACCACCACATG
Actin	Actin-F	TGGCATCACACTTTCTACAA
	Actin-R	CAACGGAATCTCTCAGCTCC
UBI1	UBI1RT-F	TCTTGTCCTCCGTCTTAGGG
	UBI1RT-R	AGCAAGGGTCCTTCCATCTT

Underlined bases indicate the recognition sequences for the corresponding restriction enzymes

Table 4.2 Oligo sequences used in the study of drought stress tolerance in *AdLEA* transgenic tobacco plants

Gene	Primer Name	Primer sequence (5′-3′)
NtAPX	NtAPX-F	GTTTGGGCTTTTCTCCTCGAC
	NtAPX-R	GGAGCATAAGAGGAGCGCAA
NtMnSOD	NtMnSOD-F	TCCCCTACGACTATGGAGCA
	NtMnSOD-R	CGGTATGCAATTTGGCGACG
NtERD10C	NtERD10C-F	AAAGCCAACTCATGCCCAAG

	NtERD10C-R	AGAGCTGCTACTTGATCGATGG
NtP5CS	NtP5CS-F	GCTGCTCAACAGGCTGGATA
	NtP5CS-R	CCATCAGCAACCTCCGTTCT
NtCAT	NtCAT-F	GGCCGCTACAACTCTCTCTTT
	NtCAT-R	ACAGGACCTCTTGCACCAAC
NtNCED3	NtNCED3 F	TGTCTGAAATGATCCGGGGC
	NtNCED3 R	AGTTTCCGGCTCTTCCCAAG
Nt18S	Nt18S-F	CCAGGTCCAGACATAGTAAG
	Nt18S-R	GTACAAAGGGCAGGGACGTA
NtUBI1	NtUBI1- F	GAGTCAACCCGTCACCTTGT
	NtUBI1- R	ACATCTTTGAGACCTCAGTAGACA

Chapter 5

Functional characterization of an in vitro generated variant of the defensin TvD1 from the weedy legume *Tephrosia villosa* and its comparative analysis with native defensin for biotic stress responses in transgenic tobacco

5.1 Materials and Methods

5.1.1 Construct preparation and *Agrobacterium* mediated Tobacco transformation

The intron less 231-bp *α-TvD1* open reading frame (ORF) was amplified with primers *α-TvD1*-F and *α-TvD1*-R having restriction sites for *Kpn*I and *Bam*H1 respectively (Table 5.1) and cloned into corresponding sites of pRT100 vector. The expression cassette that has *α-TvD1* flanked by CaMV35S promoter and polyadenylation signal was released by *Pst*I restriction digestion and subsequently sub-cloned into binary vector pCAMBIA2300 at the same site. The recombinant vector pCAMBIA2300- *α-TvD1* having *nptII* as marker gene for kanamycin selection was transformed into *Agrobacterium* strain LBA4404 by freeze-thaw method and used for transformation of tobacco plants (*N. tabacum* cv. Samsun) by leaf disc method as described in **method 3.13** according to Horsch et al. (Horsch et al. 1985). The transformants were selected on 125 mg L^{-1} kanamycin. Genomic DNA was isolated from T_0 *α-TvD1* transformants and PCR with specific primers for the target gene as well marker gene *nptII* was undertaken. Further, total RNA was isolated and reverse transcriptase polymerase chain reaction (RT-PCR) for the target gene (*α-TvD1)* was also undertaken to identify high and low expression plants and subsequently their progenies were maintained for further analysis. All the primer sequence details used in this study are mentioned in Table 5.1.

5.1.2 Detached-leaf anti-fungal bioassay

To evaluate the disease resistance response of transgenic *α-TvD1* plants against fungal pathogen *Phytophthora parasitica* var. *nicotianae* and *Alternaria alternata var. nicotianae,* detached leaf fungal bioassay was performed in T_0 generation. The fungus was cultured on potato dextrose agar (PDA) (Himedia, India) for five to six days at 24 °C. Three fully expanded leaves from two months old low expression (*α*-T1) and two high expression (*α*-T8 and *α*-T16) transgenic plants along with wild type (non-transgenic) controls were used in the assay and the experiment was performed three times. The leaves were washed thoroughly in running tap water, blot dried and were placed on moist filter papers for fungal inoculation. Actively growing mycelia of fungi *Phytophthora parasitica* var. *nicotianae* and *Alternaria alternata var. nicotianae* along with potato dextrose agar block (0.5 cm^2) were kept on the adaxial surface of the leaves after abrasion to promote fungal infection (Tedford et al. 1990). The leaves were kept in growth room at 28° C with 16 h

photoperiod. Disease symptoms and leaf damage was observed and photographed after five and ten days post inoculation (dpi) for *Phytophthora parasitica* var. *nicotianae* and *Alternaria alternata var. nicotianae* respectively.

For comparison analysis studies, homozygous T_2 transgenic plants of both *α-TvD1* and native *TvD1* were used. In earlier study of characterisation of native *TvD1,* high expression transgenic line T-26 showed enhanced resistance against many fungal pathogens and insect pests. Hence, in the present study two progeny plants of the same line T-26; T-26.1 and T-26.2 were used (Na T-26.1 and Na T-26.2). Two high expression T_2 lines of *α-TvD1;* αT-8 and αT-16, which were characterised in T_0 generation were used for comparison studies. The same experimental procedure as described above, was used for comparison studies for detached leaf fungal bioassays with fungi *Phytophthora parasitica* var. *nicotianae, Alternaria alternata var. nicotianae* and *Rhizoctonia solani.* All the experiments were performed three times. The fungal cultures used in study were procured from Indian Type Culture Collection (ITCC), New Delhi.

5.1.3 Whole plant bioassay with *Rhizoctonia solani*

Whole plant assays with *Rhizoctonia solani* were done in soil as well as on media (Anderson 1982). For both bioassays, one month old transgenic native *TvD1* and *α-TvD1* seedlings along with wild type seedlings were used. For assay in soil, actively grown fungal mycelium on potato dextrose agar block (0.5 cm^2) was kept in each plastic cup containing seedling. Before that plastic cups were pretreated with 3% sucrose solution for promoting better growth of fungal mycelium. The seedlings were incubated in growth room at 28° C with 16 h/ 8 h light/ dark photoperiod and symptoms were recorded after ten days post inoculation. For fungal bioassay on media, all the seedlings were shifted to culture bottles having half strength MS media and fungal mycelium on PDA block (0.5 cm^2) was placed in each bottle for infection. The culture bottles were kept in growth room at 24° C with 16 h/ 8 h light/ dark photoperiod and symptoms were recorded after seven days post inoculation. In each experiment ten seedlings in each bottle were kept and the experiment was repeated three times.

5.1.4 Quantitative determination of fungal biomass in necrotic infected leaf tissues of plants

Quantitative determination of fungal biomass in necrotic infected tissues of transgenic and wild type plants was calculated undertaking the development of standard curves. The standard curves were plotted

using different concentrations of fungal genomic DNA ranging from 10 to 10^{-4} ng/µl of *Phytopthora parasitica* pv. *nicotianae, Alternaria alternata* and, *Rhizoctonia solani*, which showed a linear relation (r^2 = 0.986, 0.953 and, 0.994 respectively) between log values of fungal genomic DNA and real-time PCR threshold cycles (Kumar et al. 2015). These standard curves with 200 ng of DNA were used to calculate fungal biomass present in the roots and leaves of infected transgenic lines and wild type plants.

5.1.5 Anti-feedant bioassay with generalist herbivore *Spodoptera litura*

Leaves of two month old, native *TvD1* and *α-TvD1* plants along with wild type plant were tested and compared for insect herbivory against the generalist herbivore, *Spodoptera litura* using the 1st and 2nd instar larvae forms. For assay, the leaf petioles were kept in petriplates with 0.8 % agar which helps in maintaining the moisture content within the leaves. Five 1st and 2nd instar larvae of *Spodoptera litura* were added separately onto the adaxial surface of the leaves and the petriplates were then sealed. The plates were kept for 2 days at 28° C with 16 h/ 8 h light/ dark photoperiod. The leaf area damage (in cm^2) and weight of the larva (in mg) two days post larval feeding was measured in both instar stages. The experiment was repeated three times.

5.1.6 Statistical analysis

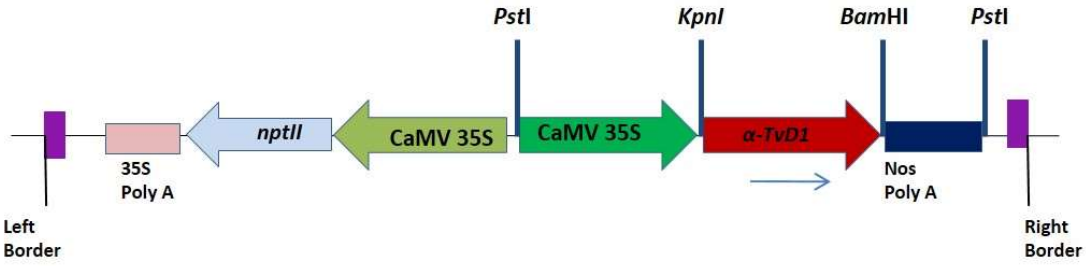
The data analysis was done by analysis of variance (one-way ANOVA) using GraphPad Prism ver. 5.0 and the mean values were compared by the student Newman-Keuls analysis. All the experiments were performed in triplicates.

5.2 Results

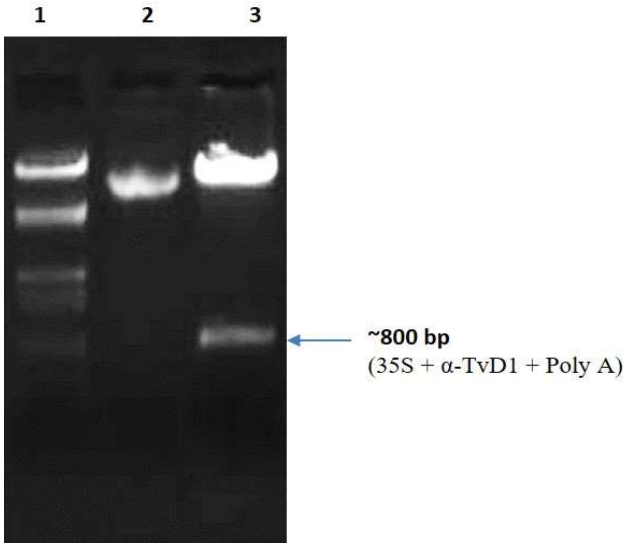
5.2.1 Cloning of α-*TvD1* and generation of transgenic tobacco lines overexpressing α-*TvD1* gene

The coding region of *α-TvD1* cDNA was cloned into pRT100 vector in between the sites *Kpn*I and *Bam*HI to create the plant expression cassette pRT100- *α-TvD1* in which the gene was flanked by CaMV 35S promoter and the termination signal. This expression cassette was sub-cloned further into binary vector pCAMBIA2300 at *Pst*I site with the marker gene *npt*II driven by 35S promoter with t-Nos termination signal **(Figure 5.2.1.1A and B**). The resultant binary vector pCAMBIA2300- *α-TvD1* was used to transform

tobacco plants by *Agrobacterium* mediated leaf-disc transformation (Horsch et al., 1985). The transformants were kept on MS medium supplemented with 2 mg/l BAP and 0.1 mg/l NAA for shoot induction and elongation and selected on 125 mg/l kanamycin. Afterwards, the shoots were shifted to root induction medium for rooting (full or half strength MS medium without hormones), which were later transferred to soil in plastic cups to get acclimatized in growth room conditions and subsequently in greenhouse conditions in pots **(Figure 5.2.2)**.



(A)



(B)

Figure 5.2.1.1. Construct preparation for plant transformation. (A) Representation of the T-DNA region of the plasmid pCAMBIA2300 that contains α-TvD1 gene driven by CaMV 35S promoter and *NptII* gene. RB, right border;

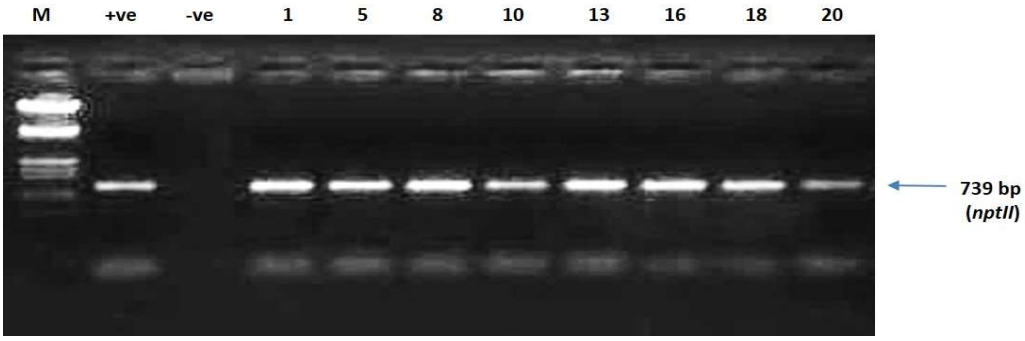
LB, left border. **(B)** Gel picture showing the release of ~ 800 bp α-TvD1 cassettes with 35S promoter and polyA signal from the pCAMBIA2300 vector.

Figure 5.2.1.2. Different stages of *Agarobacterium* mediated transformation of tobacco. (A) Explants forming callus on shoot induction medium. **(B)** Shoot proliferation on shoot induction medium. **(C)** Elongation of shoots on shoot elongation medium. **(D)** Rooting of putative transgenics on root induction medium. **(E)** and **(F)** Hardening and acclimatization of putative transgenic tobacco plants in growth room conditions and greenhouse conditions respectively.

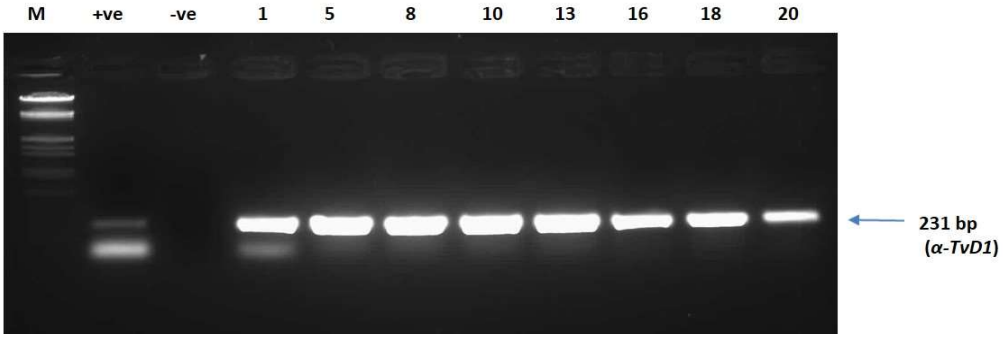
5.2.2 Molecular analysis of putative T_0 and T_2 transgenic plants

Several T_0 putative transgenic *α-TvD1* plants were raised by *Agarobacterium* mediated transformation after different stages of tissue culture selection. Genomic DNA was isolated from some of these putative T_0 transgenic plants and they were screened by PCR amplification for *α-TvD1* and marker *nptII* genes **(Figure 5.2.2A and B)**. Plants # 1, 5, 8, 10, 13, 16, 18 and 20 which showed amplification for both *α-TvD1* and *nptII* genes were further analyzed by semi-quantitative RT-PCR to check the relative expression of *α-TvD1* gene in them. These results demonstrated that plants #5, 8, 16, and 18 showed high expression, plants

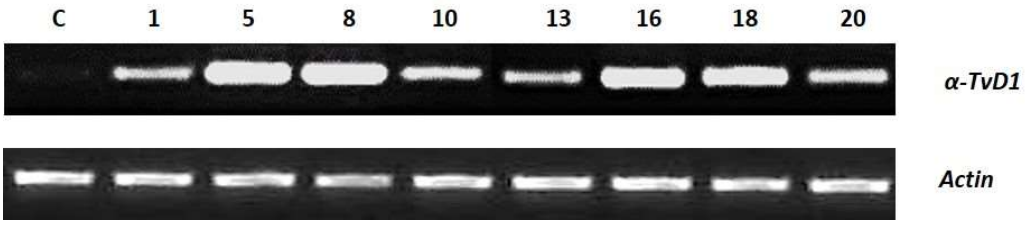
#1, 10 and 13 low expression and plant# 20 showed relatively moderate expression of *α-TvD1* transcript **(Figure 5.2.2C)**. Two high expression plants (# 8 and 16) were selected which were further grown to the T_2 generation through T_1 and plants were obtained by selfing and germination of the seeds on kanamycin containing medium. These T_2 plants were again verified for the expression levels of *α-TvD1* transcripts **(Figure 5.2.2D)** and maintained in green house for further analysis. All the comparison studies were done with these two high expression lines. Simultaneously, WT plants (non-transgenic) serving as controls were also maintained separately.



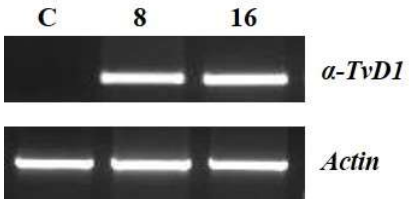
(A)



(B)



(C)



(D)

Figure 5.2.2. Integration and expression of *α-TvD1* in putative T_0 transgenic tobacco plants. (A) PCR analysis of putative T_0 transformants for the *nptII* gene; M, +ve and -ve are λDNA/*Eco*RI+*Hin*dIII ladder, positive control, negative control (WT non transformed plant) respectively; 1, 5, 8, 10, 13, 16, 18, and 20 are transgenic plants showing 739 bp amplified PCR product of the *nptII* gene. **(B)** PCR analysis of putative T_0 transformants for *α-TvD1* gene; M, +ve and -ve are λDNA/*Eco*RI+*Hin*dIII ladder, positive control, negative control (WT non transformed plant) respectively; 1, 5, 8, 10, 13, 16, 18, and 20 are transgenic plants showing 231 bp amplified PCR product of the *α-TvD1* gene. **(C)** Transcript levels of *α-TvD1* in T_0 generation. Lines 5, 8, 16, and 18 are high expression plants, and 1, 10 and 13 are low expression plants and 20 is moderate expression plant; C is negative control (untransformed WT plant). **(D)** Transcript level of *α-TvD1* in T_2 generation. Lines 8 and 16 are high expression lines; C is negative control (untransformed WT plant).

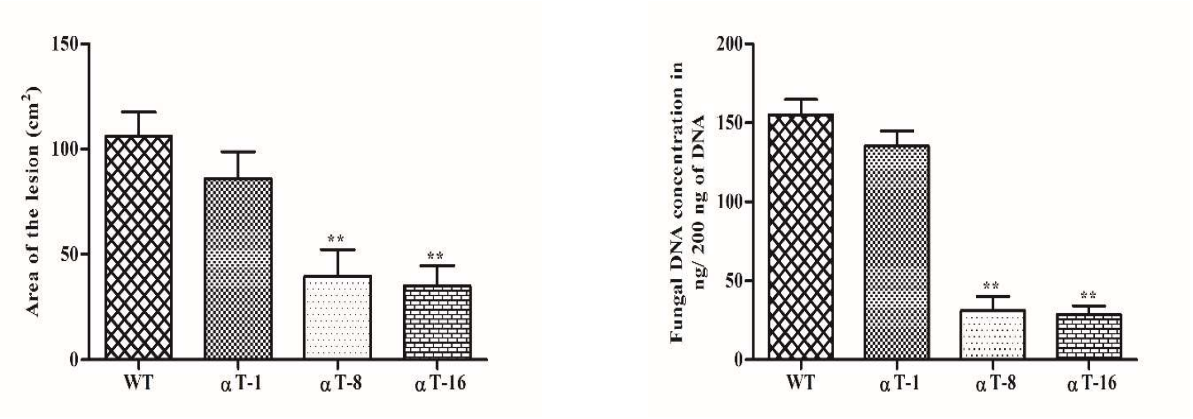
5.2.3 Detached leaf antifungal bio-assay for T_0 transgenic tobacco plants

The T_0 tobacco transgenic plants were analyzed for antifungal activity against the fungal pathogen, *Phytopthora parasitica p.v. nicotiana* that causes black shank disease and fungal pathogen *Alternaria alternata* pv. *nicotianae* that causes brown or leaf spot disease (both fungi are specific for tobacco). These assays also helped us to identify the expression of *α-TvD1* in low and high expression lines in congruence with the expression data of this gene in molecular analysis of T_0 transgenic plants. Leaves from two high

expression lines # 8 (T-8) and, 16 (T-16) and a single low expression line# 1 (T-1) along with wild type leaf (WT) as control were taken for the assays.

The high expression line plants T-8 and T-16 showed very less necrosis of the leaf after fungal disc inoculation over the leaf with *P. parasitica p.v. nicotianae* whereas the leaf damage area was very high in low expression plant (T-1) which was almost comparable to that of the damage in WT control plant. The low expression line as well as control plants showed more than 90% damage across the leaf within 5 days post inoculation (dpi) with the fungus **(Figure 5.2.3.1A and B)**. When fungal DNA was quantified, there was significant reduction in fungal DNA biomass of both high expression lines in comparison to WT and low expression lines. Fungal DNA concentration was almost 79% and 81 % less in transgenic lines T-8 and T-16 respectively in comparison to wild type plants whereas this reduction was only 13% less in low expression line T-1 (**Figure 5.2.3.1C).**

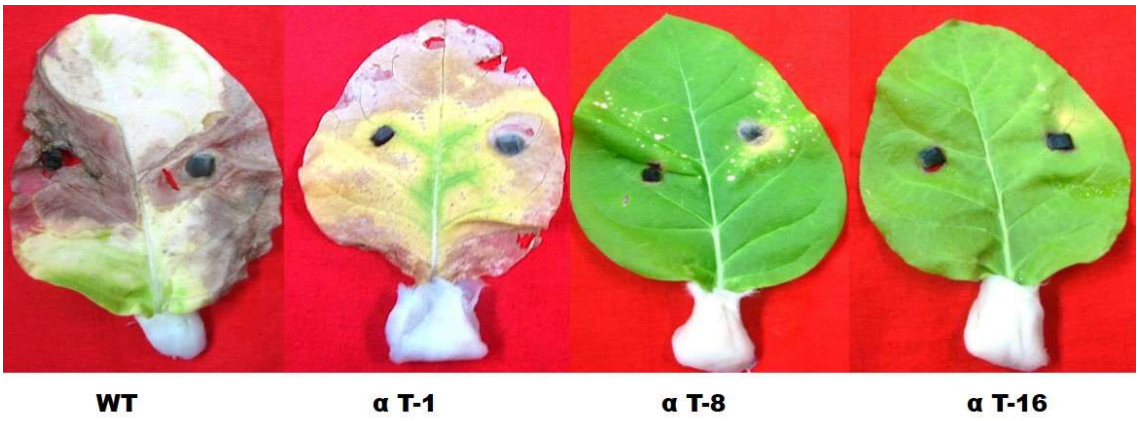
(A)



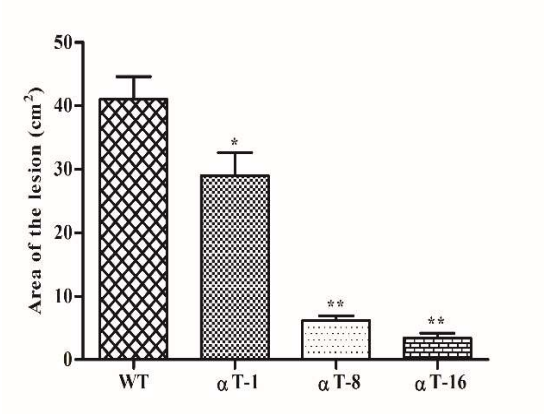
(B) **(C)**

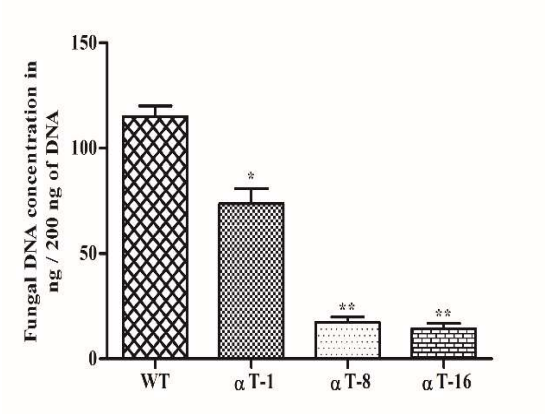
Figure. 5.2.3.1. Detached leaf fungal bioassay using the fungus, *Phytopthora parasitica* p.v. *nicotianae.* (A) Pictorial representation of leaves infected with fungus; T-1, T-8 and, T-16 are low expression and high expression lines respectively. (Photograph was taken after 5 days post inoculation; dpi) (B). Graphical representation of the symptoms that are expressed as diameter (cm^2) of the diseased lesion area on leaves after fungal infection at 5 dpi. (C) Graphical representation of the fungal DNA quantification in infected leaves at 5 dpi. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between WT and *αTvD1* transgenics (**P<0.01).

Similarly, for the fungus *Alternaria alternata* pv. *nicotianae*, the necrosis was prominent in the control plant, which showed almost 85% damage of the leaf after ten days post fungal inoculation (dpi). Low expression line (T-1) showed the same response as wild type with almost 80% leaf damage due to necrosis. However, very less necrosis was observed in the leaves of both the high expression lines (T-8 and T-16) with damage of the leaf between 5-8 % of the total leaf area (**Figure 5.2.3.2A and B)**. As expected, in DNA quantification studies also WT plants showed more fungal DNA in comparison to high expression lines T-8 and T-16. The amount of fungal DNA in lines T-8 and T-16 was almost 85% and 87% less respectively in comparison to WT plants (**Figure 5.2.3.2C)**.



(A)





(B) **(C)**

Figure 5.2.3.2. Detached leaf fungal bioassay using the fungus, *Alternaria alternata* pv. *nicotianae*. (A) Pictorial representation of leaves infected with the fungal pathogen; T-1, T-8 and, T-16 are low expression and high expression lines respectively. (Photograph was taken after 10 days' post inoculation; dpi) (B). Graphical representation of the symptoms that are expressed as diameter (cm^2) of the diseased lesions area on leaves after fungal infection at 10 dpi. (C) Graphical representation of the fungal DNA quantification in infected leaves at 10 dpi. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between WT and *αTvD1* transgenics (*P<0.05, **P<0.01).

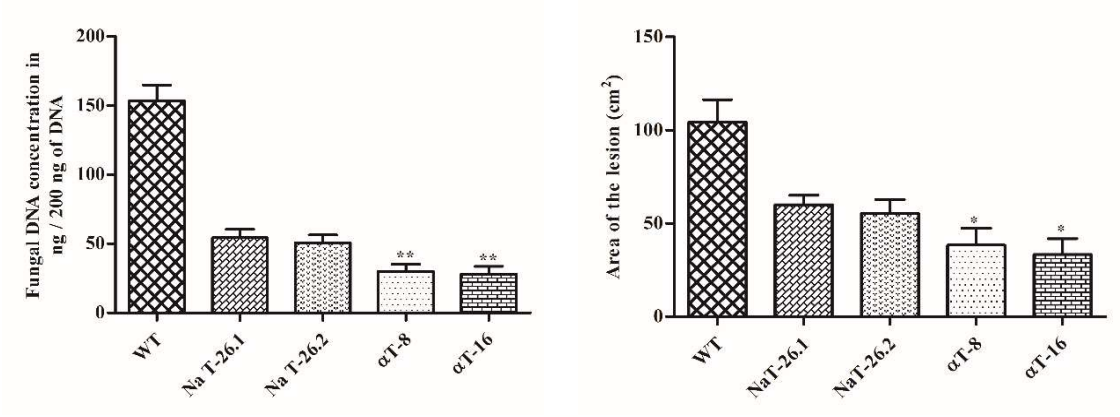
5.2.4 Comparative analysis of *α-TvD1* and native *TvD1* transgenic plants against fungal infection and insect infestation

For comparison analysis studies, homozygous high expression lines of *α-TvD1;* αT-8 and αT-16 and progenies of high expression line of T-26; T-26.1 and T-26.2 (Na T-26.1 and Na T-26) were used.

5.2.4.1 Against fungus *Phytopthora parasitica* pv. *nicotianae*

Leaves of transgenic plants of both *α-TvD1* and native *TvD1* along with WT control were inoculated with fungal medium blocks and were photographed on the 5th day and subsequently the area of disease lesion and fungal DNA quantification were quantified for each of them and the parameters were compared between native *TvD1* and *α-TvD1*. There was significant reduction in the disease lesion area of both αT-8 and αT-16 leaves, which was almost 45% and 38% less respectively, when compared to native *TvD1* leaves infection area (**Figure 5.2.4.1A and B)**. Similar results were observed when fungal DNA biomass of both αT-8 and αT-16 leaves were estimated and compared. Fungal DNA concentration was almost 46% and 35 % less in these lines respectively in comparison to native *TvD1* plant leaves. The results demonstrate enhanced tolerance of *α-TvD1* plants against fungus *Phytopthora parasitica* pv. *nicotianae* as compared to native *TvD1* plants. Statistical analysis in result graphs has been done between *α-TvD1* and native *TvD1* transgenic plants only (not WT) to highlight the significance of statistical parameters between these two in comparison studies.

(A)



(B) **(C)**

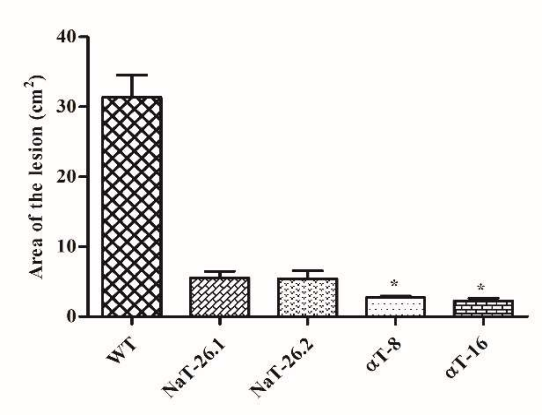
Figure 5.2.4.1. Detached leaf fungal bioassay using the fungal pathogen, *Phytopthora parasitica* p.v. *nicotianae.* (A) Pictorial representation of leaves infected with the fungus; NaT-26.1 and NaT-26.2 are progenies of high expression line T-26 of native *TvD1* and αT-8 and αT-16 are high expression lines of *αTvD1* respectively. (Photograph was taken after 5 days' post inoculation; dpi) (B). Graphical representation of the symptoms that are expressed as diameter (cm^2) of the diseased lesions area on leaves after fungal infection at 5 dpi. (C) Graphical representation of the fungal DNA quantification in infected leaves at 5 dpi. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between native *TvD1* and *αTvD1* transgenics (**P<0.01, *P<0.05).

5.2.4.2 Against the fungus *Rhizoctonia solani*

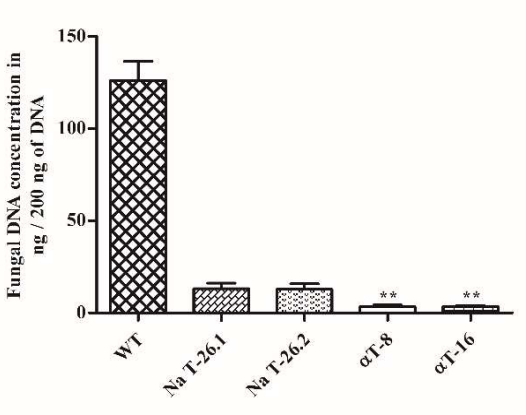
Transgenic plants were compared against soil born, root rot disease causing fungus *Rhizoctonia solani* on leaves as well as in whole plant assays in soil and media. *αTvD1* transgenic lines αT-8 and αT-16 showed enhanced tolerance against fungus with almost 45-50 % reduction in the infected disease area and 55-60 % reduction in fugal DNA concentration in comparison to native transgenic plant leaves at 8 dpi (**Figure 5.2.4.2.1A- C**). Fungal blocks were inoculated on to the one month old transgenic plants in whole plant fungal assay in soil. After 8 days of treatment, fungal mycelia grew and covered the complete upper soil layer in the cups. The fungus *R. solani* infects the root shoot junction of the plants with the symptoms starting from base of the plant and progresses upwards i.e., older leaves get affected early. Even after 10 days post treatment, there were no external disease symptoms observed in both *α-TvD1* and native *TvD1*

plants, but the *α-TvD1* transgenic plants appeared healthier and showed better growth in comparison to native *TvD1* plants (**Figure 5.2.4.2.2A and B)**. Further, to observe the effect of fungus on plants morphology and survivability, the assay was repeated on culture media. Here, the difference between the transgenics was more prominent phenotypically and morphologically with *α-TvD1* transgenic plants showing longer root lengths and better plant survival percentage in comparison to native *TvD1* plants (**Figure 5.2.4.2.3A- D)**. The difference in fungal growth between both *α-TvD1* and native *TvD1* transgenics in roots was also more pronounced on media in comparison to its growth in soil as observed in DNA quantification studies. There was a reduction of approximately 42% fungal growth in soil whereas almost 52% reduction was observed in fungal mass on media of *α-TvD1* plants roots in comparison to native *TvD1* plant roots (**Figure 5.2.4.2.4A and B)**.

(A)



(B)



(C)

Figure 5.2.4.2.1 Detached leaf fungal bioassay with the fungal pathogen *Rhizoctonia solani.* (A) Pictorial representation of leaves infected with fungus; NaT-26.1 and NaT-26.2 are progenies of high expression line T-26 of native *TvD1* and αT-8 and αT-16 are high expression lines of *αTvD1* respectively. (Photograph was taken at 8 dpi) (B). Graphical representation of the symptoms that are expressed as diameter (cm^2) of the diseased lesions area on leaves after fungal infection at 8 dpi. (C) Graphical representation of the fungal DNA quantification in infected leaves at 8 dpi. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between native *TvD1* and *αTvD1* transgenics (**P<0.01, *P<0.05).

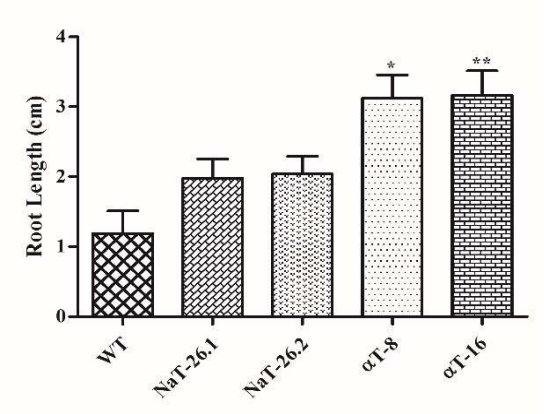
(A)

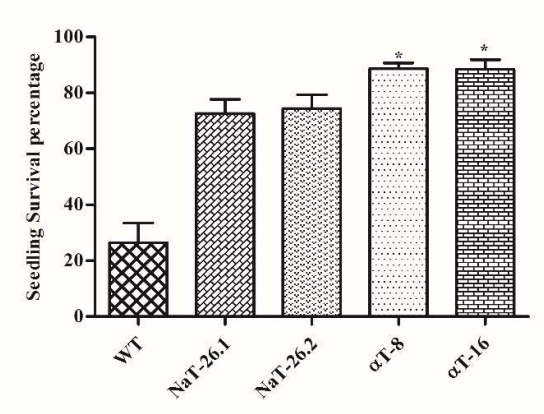
(B)

Figure 5.2.4.2.2. Whole plant fungal bioassay with the fungus *Rhizoctonia solani* in soil. (A) Pictorial representation of one month old plants infected with the fungus; NaT-26.1 and NaT-26.2 are progenies of high expression line T-26 of native *TvD1* and αT-8 and αT-16 are high expression lines of *αTvD1* respectively. (B) Uprooted plants after fungal assay. Arrowheads indicate the site of *R. solani* infection at shoot–root junction. (Photograph was taken at 10 dpi).

(A)

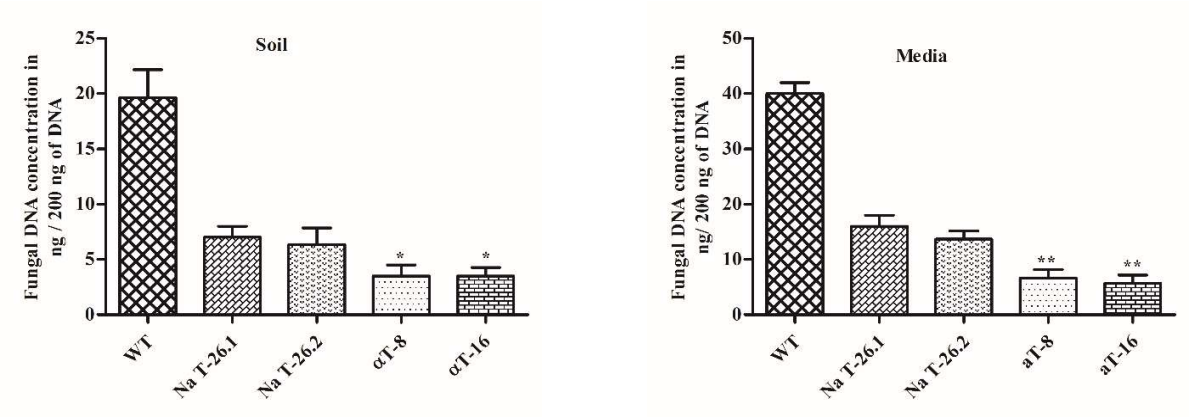
(B)





(C) **(D)**

Figure 5.2.4.2.3. Whole plant fungal bioassay with *Rhizoctonia solani* on media. (A) Pictorial representation of one month old seedlings infected with fungus; NaT-26.1 and NaT-26.2 are progenies of high expression line T-26 of native *TvD1* and αT-8 and αT-16 are the high expression lines of *αTvD1* respectively. (B) Uprooted plants after the fungal assay. Arrowheads indicate the site of *R. solani* infection at shoot–root junction. (Photograph was taken at 7 dpi). (C). Graphical representation of Seedling root lengths after fungal assay. (D) Graphical representation of seedling survival percentage after fungal assay. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between native *TvD1* and *αTvD1* transgenics (**P<0.01, *P<0.05).

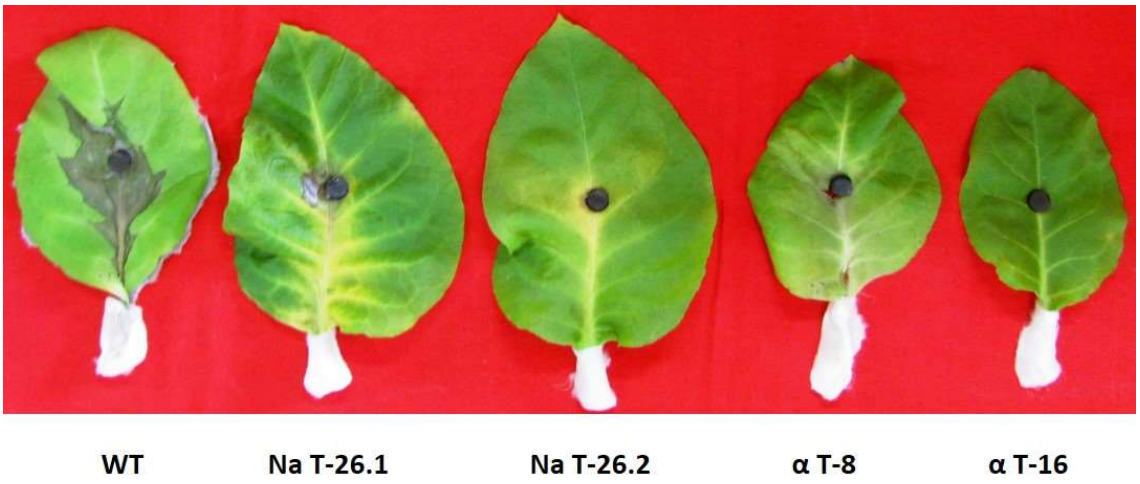


(A) **(B)**

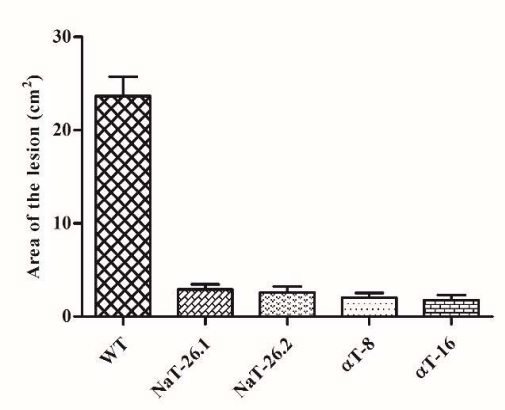
Figure 5.2.4.2.4. Whole plant fungal bioassay with *Rhizoctonia solani* in soil and media. (A) Graphical representation of the fungal DNA quantification in infected roots in soil at 10 dpi. (B) Graphical representation of the fungal DNA quantification in infected roots on media at 7 dpi. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between native *TvD1* and *αTvD1* transgenics (**P<0.01, *P<0.05).

5.2.4.3 Against fungus *Alternaria alternata* var. *nicotianae*

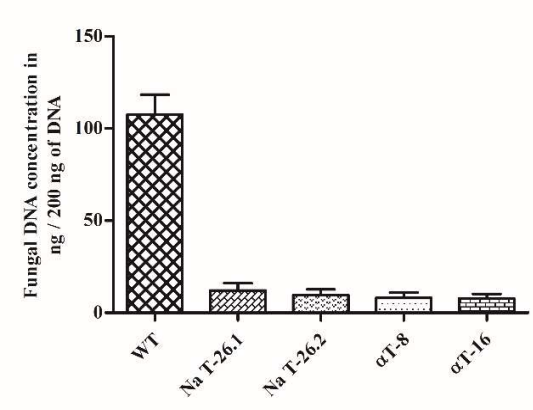
The difference between *α-TvD1* and native TvD1 transgenics was not much significant against fungus *A. alternata var. nicotianae.* Both the transgenics showed more or less similar disease symptoms with similar disease lesion area on leaves and fungal DNA amount in the leaves (**Figure 5.2.4.3)**.



(A)



(A)

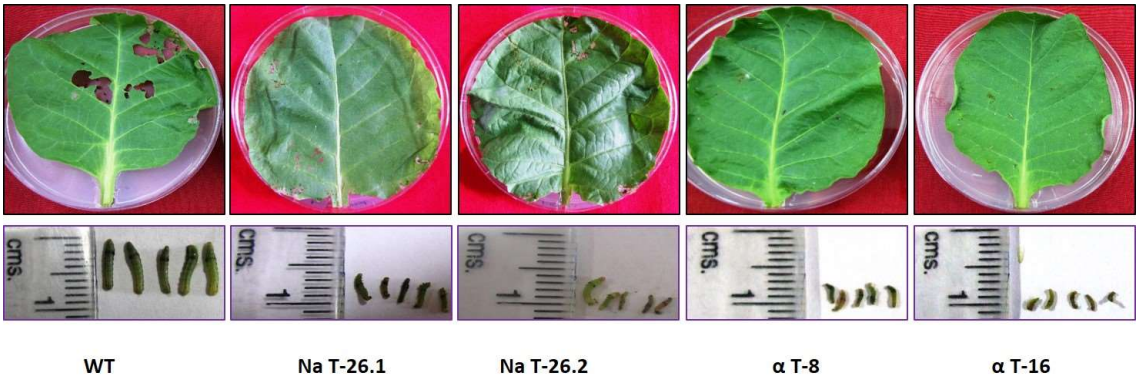


(C)

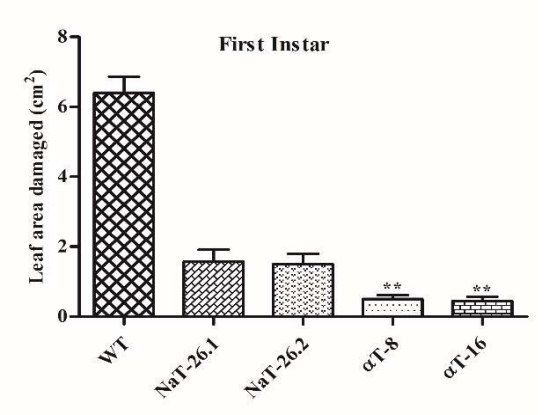
Figure 5.2.4.3. Detached leaf fungal bioassay with the fungus *Alternaria alterneta.* (A) Pictorial representation of leaves infected with the fungus; NaT-26.1 and NaT-26.2 are progenies of high expression line T-26 of native *TvD1* and αT-8 and αT-16 are high expression lines of *αTvD1* respectively. (Photograph was taken at 8 dpi) (B). Graphical representation of the symptoms that are expressed as diameter (cm^2) of the diseased lesion area on leaves after fungal infection at 7 dpi. (C) Graphical representation of the fungal DNA quantification in infected leaves at 7 dpi. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are not significant between native *TvD1* and *αTvD1* transgenics.

5.2.4.4 Anti-insect bioassay against *Spodoptera litura*

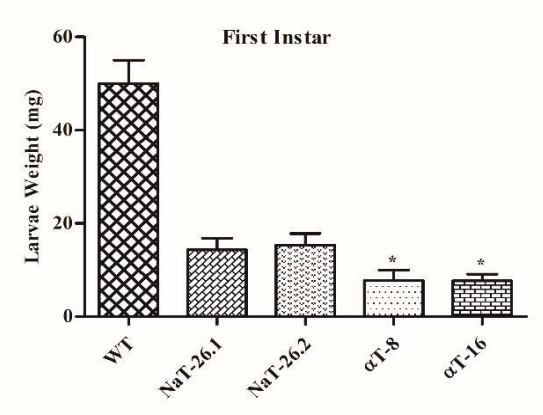
Further, *α-TvD1* and native *TvD1* transgenics plants were tested and compared for insect herbivory against the generalist herbivore, *Spodoptera litura* using the 1st and 2nd instar larvae forms. The 1st instar larvae, feeding on the leaves of *α-TvD1* transgenic lines caused very little damage to the leaves compared to larvae feeding on native *TvD1* leaves with almost 52-55% reduction in leaf damaged area in comparison to native *TvD1* plants within three days of post feeding. These larvae consequently gained less weight (avg. weight of each larvae was 7 mg) when compared to larvae fed on native *TvD1* plants, (avg. weight of larvae was 13 mg) which was almost 42% less as compared to native *TvD1* plants fed larvae. (**Figure 5.2.4.4.1**). 2nd instar larvae were also tested simultaneously, with the same set of plants. Here also the larvae feeding on *α-TvD1* transgenic lines gained less weight with 34-36 % reduction in larval weight and the reduction in damaged area of about 45-48% in comparison to larvae fed on native *TvD1* transgenic plants (**Figure 5.2.4.4.2**). These results showed enhanced resistance or tolerance of *α-TvD1* plants against insect pests in comparison to native *TvD1* plants.



(A)

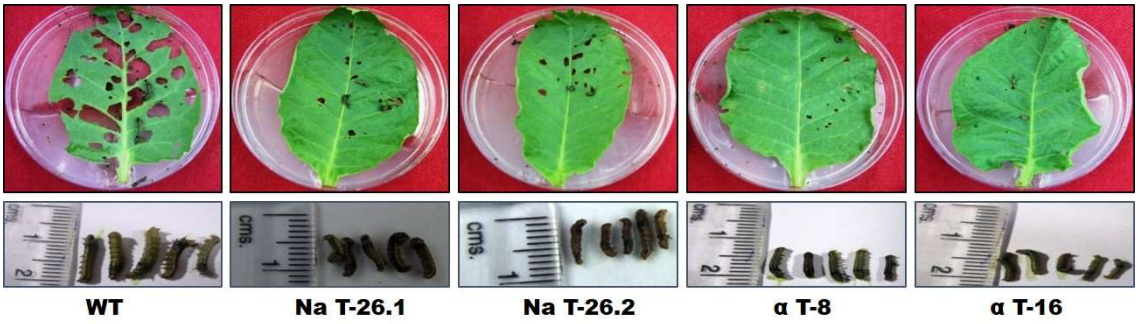


(B)

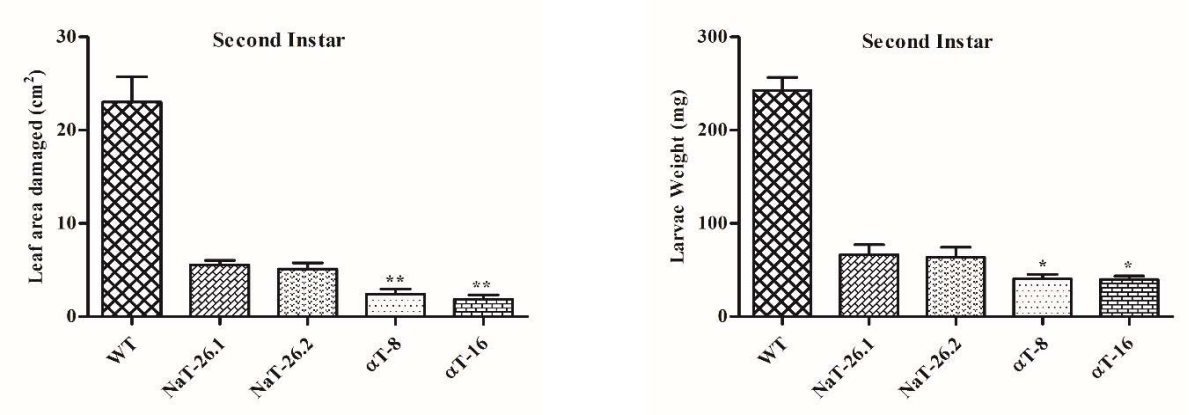


(C)

Figure 5.2.4.4.1. Insect bioassay using the 1st instar larvae of *Spodoptera litura*. (A) Pictorial representation of transgenic leaves and insect larvae after feeding. Photograph was taken after 3 days post feeding. (B) Graphical representation of leaf damage area after insect feeding. (D) Graphical representation of the weight gained by 1st instar larvae of *Spodoptera litura* post feeding. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between native *TvD1* and *αTvD1* transgenics (**P<0.01, *P<0.05).



(A)



(B) **(C)**

Figure 5.2.4.4.2. Insect bioassay using the 2nd instar larvae of *Spodoptera litura*. (A) Pictorial representation of transgenic leaves and insect larvae after feeding. Photograph was taken after 2 days post feeding. (B) Graphical representation of leaf damage area after insect feeding. (D) Graphical representation of the weight gained by 2nd instar larvae of *Spodoptera litura* after post feeding. All the experiments were performed in triplicates and data represented as mean ± SD (n = 3; biological replicates). Statistical analysis was performed with one-way ANOVA. Differences are significant between native *TvD1* and *αTvD1* transgenics (**P<0.01, *P<0.05).

5.3 Discussion

In nature, plants being sessile organisms, repeatedly encounter attack by microbial pathogens all through their life cycle. Over the course of evolution, plants have developed a potent defense mechanism conferring systemic resistance, to resist and survive against these circumstances in which peptides and proteins are the most evolved molecular entities. Among these peptides are antimicrobial peptides defensins. Plant defensins are cysteine-rich, basic peptides consisting of 45–54 amino acid residues and have a three-dimensional well-conserved structure held by a cysteine-stabilized α/β (CSαβ) motif, which forms one α-helix followed by three anti-parallel β-sheets.

Many studies in the past have established that plant defensins exhibit inhibitory activity against broad range of filamentous fungi *in vitro* and *in vivo* when expressed in transgenic plants (Terras et al. 1995; Chen et al. 2006; Anuradha et al. 2008; Jha and Chattoo 2010; Ghag et al. 2012). Due to their potent *in vitro* antifungal activity, plant defensins have the potential to be used as antifungal agents in transgenic crops to

reduce the dependence on chemicals and pesticides for improved crop productivity and cleaner environment.

A defensin *TvD1* from a weedy legume, *Tephrosia villosa* had been cloned and characterized previously from our group, which showed inhibitory effects on several plant pathogenic filamentous fungi when checked for *in vitro* functions (Vijayan et al. 2008). Transgenic *TvD1* tobacco plants also exhibited strong anti-fungal and anti-insect activities (Vijayan et al. 2013). Further, a variant of native *TvD1* was generated by replacing the original -D-D-F-R- sequence in the β2–β 3/loop-3 region of the wild type peptide with the residues -G-M-T-R-T- through site directed mutagenesis in the variant peptide. This *TvD1* variant peptide was called Alpha-*TvD1* (α-*TvD1*) and it demonstrated enhanced antifungal and insect α-amylase inhibitory activities *in vitro* when compared to native defensin (Vijayan et al. 2012). It was also demonstrated that *α-TvD1* did not possess any inhibitory effect on human as well as plant α-amylases but had enhanced α-insect amylase inhibitory activity. Thus it was proposed that, it is safe for plant transformation and overexpression as well as for human consumption.

In extension of the previous work, α-*TvD1* gene was cloned in the binary vector pCAMBIA2300 driven by the promoter 35S with a polyA signal in the present study and was overexpressed in tobacco for *in vivo* functional characterization. Transgenic plants were checked for antifungal activity against fungal pathogens and low and high expression lines were identified. The high expression lines αT-8 and αT-16 exhibited enhanced antifungal activity against black shank disease causing necrotrophic fungal pathogen, *Phytopthora parasitica p.v. nicotianae* and brown or leaf spot disease causing necrotrophic fungus *Alternaria alternata* pv. *nicotianae* in comparison to low expression line αT-1 and wild type nontransformed plants. There was almost 90% necrosis of leaves observed in low expression αT-1 and wild type plants against both the fungal pathogen as compared to the high expression lines where very less leaf necrosis was observed.

DNA quantification studies also demonstrated very less fungal growth in high expression lines in comparison to the low expression and wild type plants. Similar observations were reported in the past where several plant defensins have shown effective tolerance against these fungi along with other fungus. A defensin from Chinese cabbage (*Brassica compestris*) showed tolerance against the pathogen *Phytophthora parasitica* when overexpressed in tobacco (Park et al. 2002). Transformation of tobacco and later peanut with the mustard defensin BjD resulted in developing transgenic plants with improved resistance towards *F.*

moniliforme; Phytophtora parasitica and *Cercospora arachidicola; Pheaoisariopsis personata* respectively (Anuradha et al. 2008). Overexpression of wasabi defensin (WT1) in rice, potato and orchid has resulted in increased resistance against *Magnaporthe grisea, Erwinia carotovora*, *Botrytis cinerea* and *Alternaria solani* (Kanzaki et al. 2002; Ntui et al. 2011). Also, maize defensin, ZmDEF1, when transformed into tobacco plants, showed enhanced tolerance against *Phytophtora parasitica* (Wang et al. 2011).

Previously it has been demonstrated that changing a non-polar or uncharged amino acid in the loop 3 region of defensin protein structure greatly affects its activity. The mutational analysis studies with *Raphanus sativus* defensins (Rs-AFP1 and Rs-AFP2) establish the fact that the substitution of neutral amino acid residues with the positively charged residues located at the γ-core region of a defensin peptide can enhance the antifungal properties of the peptide (De Samblanx et al. 1997; Fant et al. 1998). Same observation was further verified with the studies on *Medicago trunculata* defensins which states that antifungal activity of MtDef1 is apparently due to the presence of four positively-charged amino acids, in the γ-core region, which was found to be absent in the structure of the non-antifungal peptide MtDef2 (Spelbrink et al. 2004). Further, replacing the existing four amino acid residues with five foreign amino acids in the loop 3 region of *Vigna radiata* defensin VirD2, enhanced its α-amylase inhibitory activity against the insect *Tenebrio molitor* (Lin et al. 2007). *In vitro* generated α-*TvD1* peptide also showed enhanced resistance to many fungal pathogens (Vijayan et al. 2012). In comparative analysis studies, the transgenic α-*TvD1* plants showed enhanced activity against fungi *Phytopthora parasitica* pv. *nicotianae* and soil borne fungus *Rhizoctonia solani* in comparison to native *TvD1* plants. There was significant reduction in the leaf disease lesion area of both high expression lines which was almost 40-45% less compared to native *TvD1* leaves infection area after 5 dpi with fungus *Phytopthora parasitica* pv. *nicotianae*.

Rhizoctonia solani, that causes root rot disease and infects the roots and lower parts of the stem, is a serious pathogen affecting a large number of plant species (Ogoshi et al. 1996). The detached leaf antifungal bio-assay and whole seedling assay against *R. solani* also showed enhanced level of resistance of *α-TvD1* transgenic plants against native *TvD1* plants with almost 50% reduction in leaf necrotic area in comparison to native *TvD1* plants. The *α-TvD1* plants showed far better growth in whole plant seedling assay in soil as well. But, the effect of *α-TvD1* was more pronounced in whole plant seedling assay in culture media where *α-TvD1* plants demonstrated longer root lengths and more survival percentage in comparison to native *TvD1*

plants. These results are in congruence with the previous studies, in which many plant defensins showed anti-fungal activity against *Rhizoctonia solani*. Rice plants overexpressing *DmAMP*1 showed significantly improved resistance against the fungal pathogen *Rhizoctonia solani* and *Magnaporthe oryzae* when compared to non-transgenic plants. Also Rs-AFP2 overexpressing rice plants (Pusa Basmati1) showed enhanced tolerance to fungi *M. oryzae* and *R. solani in vitro* and *in vivo* (Jha and Chattoo 2010).

Interestingly, there was no difference in the pathogenicity of fungus *Alternaria alternata* on native and *α-TvD1* plants which showed same level of tolerance against them. This difference might be due the fact that each plant defensin has a specificity factor against different pathogens (Lay et al. 2003) and evidently, they have unique property to target fungal membranes which provide selectivity to their action (Sagaram et al. 2011). Studies have established that plant defensins bind to specific sphingolipids present in the plasma membrane of the sensitive fungi with high affinity. For example, *Raphanus sativa* defensin, RsAFP2 binds to glucosylceramide (GlcCer) of the plasma membrane where as defensin from *Dahlia merckii.* DmAMP1 binds to mannosyl diinositolphosphoryl ceramide (Thevissen et al. 2000). Further, Ramamoorthy et al., also reported that GlcCer is a prerequisite for the antifungal action of MsDef1, but not of MtDef4. (Ramamoorthy et al. 2007b). Thus, specific plasma membrane sphingolipids are essential as receptors of some plant defensins and the resistance of certain fungi to some defensins is most likely due to the inexistence of these sphingolipids in their plasma membranes.

Quantitative estimation of pathogen DNA in infected tissues by qPCR has recently been evolved as a new tool to determine fungal biomass in infected plant tissues (Su'udi et al. 2013; Kumar et al. 2015) and it has been proved effective in quantifying the disease progression in various plant tissues (Ayliffe et al. 2013; Pasche et al. 2013). In the present study, we have performed quantitative estimation of fungal DNA biomass of *P. parasitica* var. *nicotianae*, *A. alternata and R. solani* in the infected tissues. The values were found to be in accordance with our treatment results with fungal pathogens. In characterization studies of *α-TvD1* high expression lines of *α-TvD1* showed very less fungal biomass values in comparison to wild type plants. Also, in comparison studies, *α-TvD1* plants showed lesser value of fungal DNA in the infected tissues which was almost 40-45 % reduced, in comparison to native *TvD1* plants.

In addition to antifungal activities some plant defensins appear to possess insecticidal activity as well. Defensin peptide from *Sorghum bicolor* and *Carica papaya* have been reported previously to show *α-*

amylase activities (Bloch and Richardson 1991; Farias et al. 2007). Additionally, a defensin VrD1 from *Vigna radiata* and *V. unguiculata* defensin (VuD1) were reported to inhibit *T. moliter* α-amylase (Lin et al. 2007; Pelegrini et al. 2008). Further, *α-TvD1* peptide was also reported to show enhanced α-amylase activity in comparison to native *TvD1* peptide *in vitro* (Vijayan et al. 2012). Lin et al., relate the loop length and electrostatic distribution as essential factors in determining the activity of plant defensins on insect a-amylase (Lin et al. 2007). To check the anti-insect activity *in vivo*, comparative analysis of *α-TvD1* with native *TvD1* against the generalist herbivore *Spodoptera litura* larvae was undertaken. As expected, *α-TvD1* transgenics showed enhanced anti-insect activity against both 1st and 2nd larval stages, with plants showing approximately 50-60% less damage to leaves and larvae having reduced weight in the range of 38-42% after feeding in comparison to native *TvD1* plants.

To the best of our knowledge this is the first report on the *in vivo* characterization of any defensin mutant or variant exhibiting enhanced antifungal and insecticidal property as compared to its native counterpart. Hence *α-TvD1* could be a good option in pest management programs for developing transgenic plants resistant to insect pests as well imparting resistance to fungal pathogens at the same time. Also it is safe for human consumption which makes it a more desirable gene for crop transformations.

Table 5.1. Oligo and their sequences used for cloning and gene integration studies in *α-TvD1* transgenics plants.

Gene	Primer Name	Primer sequence (5′-3′)
nptII	NptII-F	AGATGGATTGCACGCAGGTTCTC
	NptII-R	ATCGGGAGCGGCGATACCGTA
α-TvD1	α-TvD1-F- *Kpn*I	GGGTACCATGGAGAAGAAATCACTAGC
	α-TvD1-R-*Bam*HI	GGGATCCTTTAACATCTTTTAGTACACCA
Actin	Actin-F	TGGCATCACACTTTCTACAA
	Actin-R	CAACGGAATCTCTCAGCTCC

Underlined bases indicate the recognition sequences for the corresponding restriction enzymes

Chapter 6

Summary of Work

Present study attempts to characterize two different genes functionally, which are involved in imparting varied level of tolerance to plants against many environmental stress conditions. First gene, Late Embryogenesis Abundant gene (*AdLEA*) from wild peanut *Arachis diogoi* is involved in giving protection to plants towards abiotic stresses whereas the second gene, an *in vitro* generated variant of defensin (α-*TvD1*) gene from the weedy legume *Tephrosia villosa* provide tolerance against various biotic stress inducing pathogens. The study helps in understanding and further validating their role in plant stress tolerance mechanism. This knowledge can be applied to develop improved crops tolerant towards many environmental stress conditions.

The first part of study characterizes a novel, atypical group 5 *LEA* gene from *A. diogoi.* In native plant *Arachis, AdLEA* is responsive towards ABA, PEG, NaCl, sorbitol, high temperature and MV and its expression increased to different levels against these treatments. Localization studies showed that AdLEA protein is distributed equally in cytosol and nucleus. To investigate further the biological role of *AdLEA* in plant responses to different stresses, several independent transgenic tobacco plants were developed by *Agarobacterium* mediated leaf disc transformation. The integration and expression of the transgene was confirmed by PCR analysis and three independent high expression transgenic lines (#2, 4, and 9) and single low expression line (#7) were identified which were used for stress tolerance studies.

The chlorophyll a fluorescence studies demonstrated improved PS-II efficiency in *AdLEA* transgenic plants when compared to WT plants under progressive drought stress. Additionally, the transgenic plants showed elevated proline and chlorophyll levels compared to WT plants under such stress conditions. In abiotic stress tolerance assays, *AdLEA* transgenics showed enhanced tolerance towards dehydration, osmotic, salinity, and oxidative stresses in seedling and mature plant stages and also toward phytohormones ABA in seedling stage, with transgenic plants retaining higher chlorophyll content and reduced lipid peroxidation in comparison to WT controls. ROS quantification studies with hydrogen sensitive dye (H_2DCFDA) and O_2^- sensitive dye (NBT) showed the detoxification property of *AdLEA* in plants. Also, *AdLEA* transgenic plants showed enhanced expression of few stress responsive genes under whole plant drought assays.

We have also characterized a defensin variant *α-TvD1* from legume *T. villosa.* For *in* vivo characterization, the gene *α-TvD1* was cloned in the binary vector pCAMBIA2300 driven by 35S promoter

with t-Nos terminator and transgenic plants were raised through *Agarobacterium* mediated transformation. Expression analysis of putative transformants was performed through RT-PCR and high expression as well as low expression plants were identified. T_0 transgenic plants were characterised against the fungal pathogens such as *Phytophthora parasitica var. nicotianae* and *Alternaria alternata pv. nicotianae* in detached leaf antifungal bio-assay. The high expression plants αT-8 and αT-16 showed significantly enhanced resistance in these assays in comparison to the low expression line αT-1 and wild type plants.

Comparative analysis of *α-TvD1* T_2 transgenic plants with native *TvD1* plants demonstrated enhanced resistance of the former against phytopathogenic fungus *Phytophthora parasitica var. nicotianae* and *Rhizoctonia solani* with transgenic *α-TvD1* plants showing lesser leaf damage and fungal growth in both cases. In whole plant bioassay with *Rhizoctonia solani*, *α-TvD1* transgenic plants showed lesser damage to the roots with increased root length, reduced fungal growth and enhanced survival percentage of seedlings in comparison to native *TvD1* plants. There was not much difference in the resistance of *α-TvD1* and native *TvD1* plants against the fungus *Alternaria alternata pv. nicotianae.*

In insect bio assay *α-TvD1* transgenic plants showed enhanced resistance against 1st and 2nd instar larvae of the generalist herbivore, *Spodoptera litura*. These transgenic plants had lesser damage of leaves, with larvae gaining less weight after feeding in comparison to native *TvD1*.

Major conclusions of the study

- Overexpression of *AdLEA* imparts abiotic stress tolerance to transgenic tobacco plants most specifically in water limiting conditions.
- *AdLEA* can be a good candidate gene in crop improvement management program for imparting enhanced tolerance to plants towards multiple abiotic stresses.
- Overexpression of the variant defensin gene *α-TvD1* imparts enhanced biotic stress tolerance to transgenic tobacco plants against fungal pathogen and a generalist herbivore.
- The *α-TvD1* appears to be a potent candidate gene and can be used for crop plant transformation.

Bibliography

Abba' S, Ghignone S, Bonfante P (2006) A dehydration-inducible gene in the truffle *Tuber borchii* identifies a novel group of dehydrins. BMC Genomics 7:39. doi: 10.1186/1471-2164-7-39

Abe H, Urao T, Ito T, et al (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78.

Aerts AM, Bammens L, Govaert G, et al (2011) The Antifungal Plant Defensin HsAFP1 from *Heuchera Sanguinea* Induces Apoptosis in Candida Albicans. Front Microbiol 2:1–9. doi: 10.3389/fmicb.2011.00047

Aerts AM, François IEJA, Cammue BPA, Thevissen K (2008) The mode of antifungal action of plant, insect and human defensins. Cell Mol Life Sci 65:2069–79. doi: 10.1007/s00018-008-8035-0

Aerts AM, François IEJA, Meert EMK, et al (2007a) The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. J Mol Microbiol Biotechnol 13:243–7. doi: 10.1159/000104753

Aerts AM, Thevissen K, Bresseleers SM, et al (2007b) *Arabidopsis thaliana* plants expressing human beta-defensin-2 are more resistant to fungal attack: functional homology between plant and human defensins. Plant Cell Rep 26:1391–8. doi: 10.1007/s00299-007-0329-4

Agarwal PK, Shukla PS, Gupta K, Jha B (2013) Bioengineering for salinity tolerance in plants: state of the art. Mol Biotechnol 54:102–23. doi: 10.1007/s12033-012-9538-3

Allan AC, Fluhr R (1997) Two Distinct Sources of Elicited Reactive Oxygen Species in Tobacco Epidermal Cells. Plant Cell 9:1559–1572. doi: 10.1105/tpc.9.9.1559

Almeida MS, Cabral KMS, Kurtenbach E, et al (2002) Solution structure of *Pisum sativum* defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action. J Mol Biol 315:749–57. doi: 10.1006/jmbi.2001.5252

Alsheikh MK, Svensson JT, Randall SK (2005) Phosphorylation regulated ion-binding is a property shared by the acidic subclass dehydrins. Plant, Cell Environ 28:1114–1122. doi: 10.1111/j.1365-3040.2005.01348.x

Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410. doi: 10.1016/S0022-2836(05)80360-2

Amara I, Capellades M, Ludevid MD, et al (2013) Enhanced water stress tolerance of transgenic maize plants over-expressing LEA Rab28 gene. J Plant Physiol 170:864–873. doi: 10.1016/j.jplph.2013.01.004

Amara I, Zaidi I, Masmoudi K, et al (2014) Insights into Late Embryogenesis Abundant (LEA) Proteins in Plants: From Structure to the Functions. Am J Plant Sci 05:3440–3455. doi: 10.4236/ajps.2014.522360

Amien S, Kliwer I, Márton ML, et al (2010) Defensin-like ZmES4 mediates pollen tube burst in maize via opening of the potassium channel KZM1. PLoS Biol 8:e1000388. doi: 10.1371/journal.pbio.1000388

Arimura G-I, Maffei ME (2010) Calcium and secondary CPK signaling in plants in response to herbivore attack. Biochem Biophys Res Commun 400:455–60. doi: 10.1016/j.bbrc.2010.08.134

Arnon DI (1949) Copper enzymes in isolated chloroplasts. polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15.

Artimo P, Jonnalagedda M, Arnold K, et al (2012) ExPASy: SIB bioinformatics resource portal. Nucleic Acids Res 40:597–603. doi: 10.1093/nar/gks400

Artus NN, Uemura M, Steponkus PL, et al (1996) Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. Proc Natl Acad Sci 93:13404–13409. doi: 10.1073/pnas.93.23.13404

Atkinson NJ, Urwin PE (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. J Exp Bot 63:3523–43. doi: 10.1093/jxb/ers100

Ayliffe M, Periyannan SK, Feechan A, et al (2013) A simple method for comparing fungal biomass in infected plant tissues. Mol Plant Microbe Interact 26:658–67. doi: 10.1094/MPMI-12-12-0291-R

Bahramnejad B, Erickson LR, Atnaseo C, et al (2009) Differential expression of eight defensin genes of *N. benthamiana* following biotic stress, wounding, ethylene, and benzothiadiazole treatments. Plant Cell Rep 28:703–17. doi: 10.1007/s00299-009-0672-8

Baker J, Van Dennsteele C, Dure L (1988) Sequence and characterization of 6 Lea proteins and their genes from cotton. Plant Mol Biol 11:277–91. doi: 10.1007/BF00027385

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol 59:89–113. doi: 10.1146/annurev.arplant.59.032607.092759

Baker NR, Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: An examination of future possibilities. J Exp Bot 55:1607–1621. doi: 10.1093/jxb/erh196

Balandín M, Royo J, Gómez E, et al (2005) A protective role for the embryo surrounding region of the maize endosperm, as evidenced by the characterisation of ZmESR-6, a defensin gene specifically expressed in this region. Plant Mol Biol 58:269–82. doi: 10.1007/s11103-005-3479-1

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69:473–88. doi: 10.1007/s11103-008-9435-0

Barreto-Bergter E, Pinto MR, Rodrigues ML (2004) Structure and biological functions of fungal cerebrosides. An Acad Bras Cienc 76:67–84. doi: 10.1590/S0001-37652004000100007

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207. doi: 10.1007/BF00018060

Battaglia M, Olvera-Carrillo Y, Garciarrubio A, et al (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol 148:6–24. doi: 10.1104/pp.108.120725

Ben Ahmed C, Ben Rouina B, Sensoy S, et al (2010) Exogenous proline effects on photosynthetic performance and antioxidant defense system of young olive tree. J Agric Food Chem 58:4216–22. doi: 10.1021/jf9041479

Bies-Ethève N, Gaubier-Comella P, Debures A, et al (2008) Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in *Arabidopsis thaliana*. Plant Mol Biol 67:107–24. doi: 10.1007/s11103-008-9304-x

Bloch C, Richardson M (1991) A new family of small (5 kDa) protein inhibitors of insect α-amylases from seeds or sorghum (*Sorghum bicolor* (L) Moench) have sequence homologies with wheat γ-purothionins.

FEBS Lett 279:101–104. doi: 10.1016/0014-5793(91)80261-Z

Bohlmann H, A. DWBF (2011) The Role of Thionins in Plant Protection. Critical Reviews in Plant Sciences 13: 1-16.

Bohnert HJ, Sheveleva E (1998) Plant stress adaptations — making metabolism move. Curr Opin Plant Biol 1:267–274. doi: 10.1016/S1369-5266(98)80115-5

Bondt A, Zaman S, Broekaert, W Cammue, B. and Keulemans J (1998). Genetic transformation of apple (*Malus pumila* mill.) for increased fungal resistance: in vitro antifungal activity in protein extracts of transgenic apple expressing RS-AFP2 or ACE-AMP1. Acta Hortic. 484, 565-570.

Boucher V, Buitink J, Lin X, et al (2010) MtPM25 is an atypical hydrophobic late embryogenesis-abundant protein that dissociates cold and desiccation-aggregated proteins. Plant Cell Environ 33:418–30. doi: 10.1111/j.1365-3040.2009.02093.x

Boudet J, Buitink J, Hoekstra FA, 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 Physiol 140:1418–36. doi: 10.1104/pp.105.074039

Bowles DJ (1990) Defense-related proteins in higher plants. Annu Rev Biochem 59:873–907. doi: 10.1146/annurev.bi.59.070190.004301

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi: 10.1016/0003-2697(76)90527-3

Brand Y, Hovav R (2010) Identification of Suitable Internal Control Genes for Quantitative Real-Time PCR Expression Analyses in Peanut (*Arachis hypogaea*). Peanut Sci 37:12–19. doi: 10.3146/PS09-014.1

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2:48–54. doi: 10.1016/S1360-1385(97)82562-9

Briesemeister S, Rahnenfuhrer J, Kohlbacher O (2010) YLoc--an interpretable web server for predicting subcellular localization. Nucleic Acids Res 38:W497–W502. doi: 10.1093/nar/gkq477

Broekaert Willem F. et al. "Antimicrobial peptides from plants." *Critical Reviews in Plant Sciences* 16.3 (1997): 297-323.

Broekaert WF, Terras FR, Cammue BP, Osborn RW (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol 108:1353–8.

Browne JA, Dolan KM, Tyson T, et al (2004) Dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode *Aphelenchus avenae*. Eukaryot Cell 3:966–75. doi: 10.1128/EC.3.4.966-975.2004

Buitink J, Leprince O (2004) Glass formation in plant anhydrobiotes: survival in the dry state. Cryobiology 48:215–28. doi: 10.1016/j.cryobiol.2004.02.011

Cammue B, De Bolle M, Terras F, et al (1992) Isolation and characterization of a novel class of plant antimicrobial peptides form *Mirabilis jalapa* L. seeds. J Biol Chem 267:2228–2233.

Candat A, Paszkiewicz G, Neveu M, et al (2014) The Ubiquitous Distribution of Late Embryogenesis

Abundant Proteins across Cell Compartments in *Arabidopsis* Offers Tailored Protection against Abiotic Stress. Plant Cell 26:3148–3166. doi: 10.1105/tpc.114.127316

Carter C, Pan S, Zouhar J, et al (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16:3285–303. doi: 10.1105/tpc.104.027078

Carvalho A de O, Gomes VM (2009) Plant defensins--prospects for the biological functions and biotechnological properties. Peptides 30:1007–20. doi: 10.1016/j.peptides.2009.01.018

Carvalho A, S. Filho G, Ferreira B, et al (2006) Cloning and Characterization of a cDNA Encoding a Cowpea Seed Defensin and Analysis of its Expression. Protein Pept Lett 13:1029–1036. doi: 10.2174/092986606778777515

Chakrabortee S, Boschetti C, Walton LJ, et al (2007) Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. Proc Natl Acad Sci U S A 104:18073–8. doi: 10.1073/pnas.0706964104

Chakrabortee S, Meersman F, Kaminski Schierle GS, et al (2010) Catalytic and chaperone-like functions in an intrinsically disordered protein associated with desiccation tolerance. Proc Natl Acad Sci U S A 107:16084–9. doi: 10.1073/pnas.1006276107

Chakraborty S, Newton AC (2011) Climate change, plant diseases and food security: an overview. Plant Pathol 60:2–14. doi: 10.1111/j.1365-3059.2010.02411.x

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Report 11:113–116. doi: 10.1007/BF02670468

Chen G-H, Hsu M-P, Tan C-H, et al (2005) Cloning and characterization of a plant defensin VaD1 from azuki bean. J Agric Food Chem 53:982–8. doi: 10.1021/jf0402227

Close TJ (1996) Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plant 97:795–803. doi: 10.1111/j.1399-3054.1996.tb00546.x

Colmenero-Flores JM (1999) Pvlea-18, a Member of a New Late-Embryogenesis-Abundant Protein Family That Accumulates during Water Stress and in the Growing Regions of Well-Irrigated Bean Seedlings. Plant Physiol 120:93–104. doi: 10.1104/pp.120.1.93

Craik DJ, Daly NL, Bond T, Waine C (1999) Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. J Mol Biol 294:1327–36. doi: 10.1006/jmbi.1999.3383

Cramer GR, Urano K, Delrot S, et al (2011) Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biol 11:163. doi: 10.1186/1471-2229-11-163

Cuming AC (1999) LEA proteins. In: Casey, R., Shewry, P.R. (Eds.), Seed Proteins. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp.753-780 .

Dai X, Xu Y, Ma Q, et al (2007) Overexpression of an R1R2R3 MYB gene, OsMYB3R-2, increases tolerance to freezing, drought, and salt stress in transgenic *Arabidopsis*. Plant Physiol 143:1739–1751. doi: 10.1104/pp.106.094532

Danyluk J, Perron A, Houde M, et al (1998) Accumulation of an acidic dehydrin in the vicinity of the

plasma membrane during cold acclimation of wheat. Plant Cell 10:623–38.

De Brucker K, Cammue BPA, Thevissen K (2011) Apoptosis-inducing antifungal peptides and proteins. Biochem Soc Trans 39:1527–32. doi: 10.1042/BST0391527

De Coninck B, Cammue BP a, Thevissen K (2013) Modes of antifungal action and in planta functions of plant defensins and defensin-like peptides. Fungal Biol Rev 26:109–120. doi: 10.1016/j.fbr.2012.10.002

De Oliveira Carvalho A, Moreira Gomes V (2011) Plant Defensins and Defensin-Like Peptides - Biological Activities and Biotechnological Applications. Curr Pharm Des 17:4270–4293. doi: 10.2174/138161211798999447

De Paula VS, Razzera G, Barreto-Bergter E, et al (2011) Portrayal of complex dynamic properties of sugarcane defensin 5 by NMR: multiple motions associated with membrane interaction. Structure 19:26–36. doi: 10.1016/j.str.2010.11.011

De Samblanx GW, Goderis IJ, Thevissen K, et al (1997) Mutational Analysis of a Plant Defensin from Radish (*Raphanus sativus* L.) Reveals Two Adjacent Sites Important for Antifungal Activity. J Biol Chem 272:1171–1179. doi: 10.1074/jbc.272.2.1171

DeVilliers S, Hoisington D (2013) The trends and future of biotechnology crops for insect pest control. African J. Biotechnol. 10:4677–4681.

Dey S, Ghose K, Basu D (2009) Fusarium elicitor-dependent calcium influx and associated ros generation in tomato is independent of cell death. Eur J Plant Pathol 126:217–228. doi: 10.1007/s10658-009-9534-z

Driever SM, Fryer MJ, Mullineaux PM, Baker NR (2009) Imaging of reactive oxygen species in vivo. Methods Mol Biol 479:109–16. doi: 10.1007/978-1-59745-289-2_7

Duan J, Cai W (2012) OsLEA3-2, an abiotic stress induced gene of rice plays a key role in salt and drought tolerance. PLoS One 7:e45117. doi: 10.1371/journal.pone.0045117

Dure L (1993) A repeating 11-mer amino acid motif and plant desiccation. Plant J 3:363–369. doi: 10.1046/j.1365-313X.1993.t01-19-00999.x

Dure L, Crouch M, Harada J, et al (1989a) Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol Biol 12:475–86. doi: 10.1007/BF00036962

Dure L, Crouch M, Harada J, et al (1989b) Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol Biol 12:475–486. doi: 10.1007/BF00036962

Dure L, Pyle JB, Chlan CA, et al (1983) Developmental biochemistry of cottonseed embryogenesis and germination : XVII. Developmental expression of genes for the principal storage proteins. Plant Mol Biol 2:199–206. doi: 10.1007/BF01578379

Eriksson SK, Kutzer M, Procek J, et al (2011) Tunable membrane binding of the intrinsically disordered dehydrin Lti30, a cold-induced plant stress protein. Plant Cell 23:2391–404. doi: 10.1105/tpc.111.085183

Errakhi R, Dauphin A, Meimoun P, et al (2008) An early Ca2+ influx is a prerequisite to thaxtomin A-induced cell death in *Arabidopsis thaliana* cells. J Exp Bot 59:4259–70. doi: 10.1093/jxb/ern267

Espelund M, Saebøe-Larssen S, Hughes DW, et al (1992) Late embryogenesis-abundant genes encoding proteins with different numbers of hydrophilic repeats are regulated differentially by abscisic acid and

osmotic stress. Plant J 2:241–52.

Fant F, Vranken W, Broekaert W, Borremans F (1998) Determination of the three-dimensional solution structure of *Raphanus sativus* antifungal protein 1 by 1H NMR. J Mol Biol 279:257–70. doi: 10.1006/jmbi.1998.1767

Farias LR, Costa FT, Souza LA, et al (2007) Isolation of a novel Carica papaya α-amylase inhibitor with deleterious activity toward *Callosobruchus maculatus*. Pestic Biochem Physiol 87:255–260. doi: 10.1016/j.pestbp.2006.08.004

Flowers T, Yeo A (1995) Breeding for Salinity Resistance in Crop Plants: Where Next? Aust J Plant Physiol 22:875. doi: 10.1071/PP9950875

Franco OL, Murad AM, Leite JR, et al (2006) Identification of a cowpea gamma-thionin with bactericidal activity. FEBS J 273:3489–97. doi: 10.1111/j.1742-4658.2006.05349.x

Frova C, Krajewski P, di Fonzo N, et al (1999) Genetic analysis of drought tolerance in maize by molecular markers. TAG Theor Appl Genet 99:280–288. doi: 10.1007/s001220051233

Fujimura M, Ideguchi M, Minami Y, et al (2005) Amino acid sequence and antimicrobial activity of chitin-binding peptides, Pp-AMP 1 and Pp-AMP 2, from Japanese bamboo shoots (*Phyllostachys pubescens*). Biosci Biotechnol Biochem 69:642–5. doi: 10.1271/bbb.69.642

Fujita M, Fujita Y, Noutoshi Y, et al (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol 9:436–442. doi: 10.1016/j.pbi.2006.05.014

Gai Y-P, Ji X-L, Lu W, et al (2011) A novel late embryogenesis abundant like protein associated with chilling stress in *Nicotiana tabacum* cv. bright yellow-2 cell suspension culture. Mol Cell Proteomics 10:M111.010363. doi: 10.1074/mcp.M111.010363

Galau G a., Hughes DW, Dure L (1986) Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. Plant Mol Biol 7:155–170. doi: 10.1007/BF00021327

Galletti R, Denoux C, Gambetta S, et al (2008) The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in *Arabidopsis* is dispensable for the activation of defense responses effective against *Botrytis cinerea*. Plant Physiol 148:1695–706. doi: 10.1104/pp.108.127845

Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 3:710–20. doi: 10.1038/nri1180

Gao AG, Hakimi SM, Mittanck CA, et al (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat Biotechnol 18:1307–10. doi: 10.1038/82436

Gao B, Zhu S (2012) Alteration of the mode of antibacterial action of a defensin by the amino-terminal loop substitution. Biochem Biophys Res Commun 426:630–5. doi: 10.1016/j.bbrc.2012.08.143

Garay-Arroyo A (2000) Highly Hydrophilic Proteins in Prokaryotes and Eukaryotes Are Common during Conditions of Water Deficit. J Biol Chem 275:5668–5674. doi: 10.1074/jbc.275.8.5668

García-Mata C, Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol 126:1196–1204. doi: 10.1104/pp.126.3.1196

Gaweł S, Wardas M, Niedworok E, Wardas P (2004) [Malondialdehyde (MDA) as a lipid peroxidation marker]. Wiad Lek 57:453–5.

Gentile F, Amodeo P, Febbraio F, et al (2002) SDS-resistant active and thermostable dimers are obtained from the dissociation of homotetrameric beta-glycosidase from hyperthermophilic Sulfolobus solfataricus in SDS. Stabilizing role of the A-C intermonomeric interface. J Biol Chem 277:44050–60. doi: 10.1074/jbc.M206761200

Ghag SB, Shekhawat UKS, Ganapathi TR (2012) Petunia Floral Defensins with Unique Prodomains as Novel Candidates for Development of Fusarium Wilt Resistance in Transgenic Banana Plants. PLoS One 7:e39557. doi: 10.1371/journal.pone.0039557

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930. doi: 10.1016/j.plaphy.2010.08.016

Goday A, Jensen AB, Culiáñez-Macià FA, et al (1994) The maize abscisic acid-responsive protein Rab17 is located in the nucleus and interacts with nuclear localization signals. Plant Cell 6:351–60.

Goldberg RB, Barker SJ, Perez-Grau L (1989) Regulation of gene expression during plant embryogenesis. Cell 56:149–160. doi: 10.1016/0092-8674(89)90888-X

Goyal K, Pinelli C, Maslen SL, et al (2005) Dehydration-regulated processing of late embryogenesis abundant protein in a desiccation-tolerant nematode. FEBS Lett 579:4093–8. doi: 10.1016/j.febslet.2005.06.036

Goyal K, Tisi L, Basran A, et al (2003) Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein. J Biol Chem 278:12977–84. doi: 10.1074/jbc.M212007200

Grant M, Lamb C (2006) Systemic immunity. Curr Opin Plant Biol 9:414–20. doi: 10.1016/j.pbi.2006.05.013

Grelet J, Benamar A, Teyssier E, et al (2005) Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. Plant Physiol 137:157–67. doi: 10.1104/pp.104.052480

Grellet Bournonville CF, Díaz-Ricci JC (2011) Quantitative determination of superoxide in plant leaves using a modified NBT staining method. Phytochem Anal 22:268–271. doi: 10.1002/pca.1275

Hammond-Kosack, Kim and JDJ (2000) Responses to plant pathogens. Biochem Mol Biol plants 1102–1156.

Hammond-Kosack KE, Jones JD (1996) Resistance gene-dependent plant defense responses. Plant Cell 8:1773–91.

Hand SC, Menze MA, Toner M, et al (2011) LEA proteins during water stress: not just for plants anymore. Annu Rev Physiol 73:115–34. doi: 10.1146/annurev-physiol-012110-142203

Hara M, Fujinaga M, Kuboi T (2005a) Metal binding by citrus dehydrin with histidine-rich domains. J Exp Bot 56:2695–703. doi: 10.1093/jxb/eri262

Hatanaka R, Hagiwara-Komoda Y, Furuki T, et al (2013) An abundant LEA protein in the anhydrobiotic

midge, PvLEA4, acts as a molecular shield by limiting growth of aggregating protein particles. Insect Biochem Mol Biol 43:1055–67. doi: 10.1016/j.ibmb.2013.08.004

Hawkes JG (1977) The importance of wild germplasm in plant breeding. Euphytica 26:615–621. doi: 10.1007/BF00021686

He S, Tan L, Hu Z, et al (2012) Molecular characterization and functional analysis by heterologous expression in *E. coli* under diverse abiotic stresses for OsLEA5, the atypical hydrophobic LEA protein from *Oryza sativa* L. Mol Genet Genomics 287:39–54. doi: 10.1007/s00438-011-0660-x

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. Arch Biochem Biophys 125:189–198. doi: 10.1016/0003-9861(68)90654-1

Hegedüs N, Marx F (2013) Antifungal proteins: More than antimicrobials? Fungal Biol Rev 26:132–145. doi: 10.1016/j.fbr.2012.07.002

Heil M (2002) Induced Systemic Resistance (ISR) Against Pathogens in the Context of Induced Plant Defences. Ann Bot 89:503–512. doi: 10.1093/aob/mcf076

Herms, Daniel A. and WJM (1992) The Dilemma of Plants: To Grow or Defend. Q Rev Biol 67 (3) Univ Chicago Press 283–335.

Heyen BJ, Alsheikh MK, Smith EA, et al (2002) The calcium-binding activity of a vacuole-associated, dehydrin-like protein is regulated by phosphorylation. Plant Physiol 130:675–87. doi: 10.1104/pp.002550

Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. Trends Plant Sci 6:431–438. doi: 10.1016/S1360-1385(01)02052-0

Hoglund A, Donnes P, Blum T, et al (2006) MultiLoc: prediction of protein subcellular localization using N-terminal targeting sequences, sequence motifs and amino acid composition. Bioinformatics 22:1158–1165. doi: 10.1093/bioinformatics/btl002

Holmberg N, Bülow L (1998) Improving stress tolerance in plants by gene transfer. Trends Plant Sci 3:61–66. doi: 10.1016/S1360-1385(97)01163-1

Hong-Bo S, Zong-Suo L, Ming-An S (2005) LEA proteins in higher plants: Structure, function, gene expression and regulation. Colloids Surfaces B Biointerfaces 45:131–135. doi: 10.1016/j.colsurfb.2005.07.017

Honjoh K, Yoshimoto M, Joh T, et al (1995) Isolation and Characterization of Hardening-Induced Proteins in *Chlorella vulgaris* C-27: Identification of Late Embryogenesis Abundant Proteins. Plant Cell Physiol 36:1421–1430.

Horsch RB, Fry JE, Hoffmann NL, et al (1985) A simple and general method for transferring genes into plants. Science 227:1229–31. doi: 10.1126/science.227.4691.1229

Houde M, Dallaire S, N'Dong D, Sarhan F (2004) Overexpression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves. Plant Biotechnol J 2:381–7. doi: 10.1111/j.1467-7652.2004.00082.x

Hunault G, Jaspard E (2010) LEAPdb: a database for the late embryogenesis abundant proteins. BMC

Genomics 11:221. doi: 10.1186/1471-2164-11-221

Hundertmark M, Hincha DK (2008a) LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana.* BMC Genomics 9:118. doi: 10.1186/1471-2164-9-118

Imai R, Chang L, Ohta A, et al (1996) A lea-class gene of tomato confers salt and freezing tolerance when expressed in *Saccharomyces cerevisiae*. Gene 170:243–248. doi: 10.1016/0378-1119(95)00868-3

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47:377–403. doi: 10.1146/annurev.arplant.47.1.377

Iturriaga G, Schneider K, Salamini F, Bartels D (1992) Expression of desiccation-related proteins from the resurrection plant *Craterostigma plantagineum* in transgenic tobacco. Plant Mol Biol 20:555–558. doi: 10.1007/BF00040614

Iuchi S, Kobayashi M, Taji T, et al (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. Plant J 27:325–333. doi: 10.1046/j.1365-313x.2001.01096.x

Janssen BJC, Schirra HJ, Lay FT, et al (2003) Structure of *Petunia hybrida* defensin 1, a novel plant defensin with five disulfide bonds. Biochemistry 42:8214–22. doi: 10.1021/bi034379o

Jensen AB, Goday A, Figueras M, et al (1998) Phosphorylation mediates the nuclear targeting of the maize Rab17 protein. Plant J 13:691–7.

Jha S, Chattoo BB (2010) Expression of a plant defensin in rice confers resistance to fungal phytopathogens. Transgenic Res 19:373–84. doi: 10.1007/s11248-009-9315-7

Jha S, Tank HG, Prasad BD, Chattoo BB (2009) Expression of Dm-AMP1 in rice confers resistance to *Magnaporthe oryzae* and *Rhizoctonia solani.* Transgenic Res 18:59–69. doi: 10.1007/s11248-008-9196-1

Jongedijk E, Tigelaar H, van Roekel JSC, et al (1995) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173–180. doi: 10.1007/BF00023946

Kader JC (1996) Lipid-transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 47:627–654. doi: 10.1146/annurev.arplant.47.1.627

Kanzaki H, Nirasawa S, Saitoh H (2002) Overexpression of the wasabi defensin gene confers enhanced resistance to blast fungus (*Magnaporthe grisea*) in transgenic rice.

Karaba A, Dixit S, Greco R, et al (2007) Improvement of water use efficiency in rice by expression of HARDY, an *Arabidopsis* drought and salt tolerance gene. Proc Natl Acad Sci U S A 104:15270–5. doi: 10.1073/pnas.0707294104

Kaye C (1998) Characterization of a Gene for Spinach CAP160 and Expression of Two Spinach Cold-Acclimation Proteins in Tobacco. PLANT Physiol 116:1367–1377. doi: 10.1104/pp.116.4.1367

Kazan K, Manners JM (2008) Jasmonate signaling: toward an integrated view. Plant Physiol 146:1459–68. doi: 10.1104/pp.107.115717

Koag M-C, Fenton RD, Wilkens S, Close TJ (2003) The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity. Plant Physiol 131:309–16. doi: 10.1104/pp.011171

Koag M-C, Wilkens S, Fenton RD, et al (2009) The K-segment of maize DHN1 mediates binding to anionic phospholipid vesicles and concomitant structural changes. Plant Physiol 150:1503–14. doi: 10.1104/pp.109.136697

Kobayashi F, Takumi S, Egawa C, et al (2006) Expression patterns of low temperature responsive genes in a dominant ABA-less-sensitive mutant line of common wheat. Physiol Plant 127:612–623. doi: 10.1111/j.1399-3054.2006.00689.x

Koike M, Okamoto T, Tsuda S, Imai R (2002) A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation. Biochem Biophys Res Commun 298:46–53. doi: 10.1016/S0006-291X(02)02391-4

Konopka-Postupolska D, Clark G, Goch G, et al (2009) The role of annexin 1 in drought stress in *Arabidopsis.* Plant Physiol 150:1394–410. doi: 10.1104/pp.109.135228

Kovacs D, Agoston B, Tompa P (2008a) Disordered plant LEA proteins as molecular chaperones. Plant Signal Behav 3:710–3.

Kovacs D, Kalmar E, Torok Z, Tompa P (2008b) Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins. Plant Physiol 147:381–90. doi: 10.1104/pp.108.118208

Kragh KM, Nielsen JE, Nielsen KK, et al (1995) Characterization and localization of new antifungal cysteine-rich proteins from *Beta vulgaris*. Mol. plant-microbe Interact. 8:424–34.

Kumar D, Kirti PB (2015) Transcriptomic and Proteomic Analyses of Resistant Host Responses in *Arachis diogoi* Challenged with Late Leaf Spot Pathogen, *Phaeoisariopsis personata.* PLoS One 10:e0117559. doi: 10.1371/journal.pone.0117559

Kumar D, Rampuria S, Singh NK, et al (2015) Characterization of a vacuolar processing enzyme expressed in *Arachis diogoi* in resistance responses against late leaf spot pathogen, *Phaeoisariopsis personata*. Plant Mol Biol. doi: 10.1007/s11103-015-0318-x

Kumar KRR, Kirti PB (2012) Novel role for a serine/arginine-rich splicing factor, AdRSZ21 in plant defense and HR-like cell death. Plant Mol Biol 80:461–476. doi: 10.1007/s11103-012-9960-8

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132. doi: 10.1016/0022-2836(82)90515-0

Lacerda AF, Vasconcelos EAR, Pelegrini PB, Grossi de Sa MF (2014) Antifungal defensins and their role in plant defense. Front Microbiol 5:116. doi: 10.3389/fmicb.2014.00116

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275. doi: 10.1146/annurev.arplant.48.1.251

Lay F, Anderson M (2005) Defensins - Components of the Innate Immune System in Plants. Curr Protein Pept Sci 6:85–101. doi: 10.2174/1389203053027575

Lay FT, Brugliera F, Anderson MA (2003) Isolation and properties of floral defensins from ornamental tobacco and petunia. Plant Physiol 131:1283–93. doi: 10.1104/pp.102.016626

Lay FT, Poon S, McKenna JA, et al (2014) The C-terminal propeptide of a plant defensin confers cytoprotective and subcellular targeting functions. BMC Plant Biol 14:41. doi: 10.1186/1471-2229-14-41

Lebedev, V.G., Dolgov, S.V., Lavrova, N., Lunin, V.G. and Naroditski BS (2002). Plant-defensin genes introduction for improvement of pear phytopathogen resistance. Acta Hortic. 596, 167-172 DOI: 10.17660/ActaHortic.2002.596.21.

Leprince O, Buitink J (2010) Desiccation tolerance: From genomics to the field. Plant Sci 179:554–564. doi: 10.1016/j.plantsci.2010.02.011

Li D, He X (2009) Desiccation induced structural alterations in a 66-amino acid fragment of an anhydrobiotic nematode late embryogenesis abundant (LEA) protein. Biomacromolecules 10:1469–77. doi: 10.1021/bm9002688

Liang J, Zhou M, Zhou X, 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 8:1–13. doi: 10.1371/journal.pone.0083056

Lin C, Thomashow MF (1992) DNA Sequence Analysis of a Complementary DNA for Cold-Regulated *Arabidopsis* Gene cor15 and Characterization of the COR 15 Polypeptide. PLANT Physiol 99:519–525. doi: 10.1104/pp.99.2.519

Lin K-F, Lee T-R, Tsai P-H, et al (2007) Structure-based protein engineering for alpha-amylase inhibitory activity of plant defensin. Proteins 68:530–40. doi: 10.1002/prot.21378

Liu Y, Wang L, Jiang S, et al (2014) Group 5 LEA protein, ZmLEA5C, enhance tolerance to osmotic and low temperature stresses in transgenic tobacco and yeast. Plant Physiol Biochem 84:22–31. doi: 10.1016/j.plaphy.2014.08.016

Liu Y-J, Cheng C-S, Lai S-M, et al (2006) Solution structure of the plant defensin VrD1 from mung bean and its possible role in insecticidal activity against bruchids. Proteins 63:777–86. doi: 10.1002/prot.20962

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408. doi: 10.1006/meth.2001.1262

Lobo DS, Pereira IB, Fragel-Madeira L, et al (2007) Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle. Biochemistry 46:987–96. doi: 10.1021/bi061441j

Maitra N, Cushman JC (1994) Isolation and characterization of a drought-induced soybean cDNA encoding a D95 family late-embryogenesis-abundant protein. Plant Physiol 106:805–6.

Manfre AJ, Lanni LM, Marcotte WR (2006) The *Arabidopsis* group 1 LATE EMBRYOGENESIS ABUNDANT protein ATEM6 is required for normal seed development. Plant Physiol 140:140–9. doi: 10.1104/pp.105.072967

Manners JM, Penninckx IAMA, Vermaere K, et al (1998) The promoter of the plant defensin gene PDF1.2 from *Arabidopsis* is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. Plant Mol Biol 38:1071–1080. doi: 10.1023/A:1006070413843

Matysik J, Alia, Bhalu B, Mohanty P (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Curr Sci 82:525–532.

McCubbin WD, Kay CM, Lane BG (1985) Hydrodynamic and optical properties of the wheat germ Em

protein. Canadian Journal of Biochemistry and Cell Biology 63.8: 803-811

Mee Do H, Chul Lee S, Won Jung H, et al (2004) Differential expression and in situ localization of a pepper defensin (CADEF1) gene in response to pathogen infection, abiotic elicitors and environmental stresses in *Capsicum annuum*. Plant Sci 166:1297–1305. doi: 10.1016/j.plantsci.2004.01.008

Melo, F. R., Rigden, D. J., Franco, et al (2002) Inhibition of trypsin by cowpea thionin: characterization, molecular modeling, and docking. Proteins 48:311–9. doi: 10.1002/prot.10142

Mendez E, Moreno A, Colilla F, et al (1990) Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, gamma-hordothionin, from barley endosperm. Eur J Biochem 194:533–539. doi: 10.1111/j.1432-1033.1990.tb15649.x

Mengiste T, Chen X, Salmeron J, Dietrich R (2003) The BOTRYTIS SUSCEPTIBLE1 gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. Plant Cell 15:2551–65. doi: 10.1105/tpc.014167

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33:453–67. doi: 10.1111/j.1365-3040.2009.02041.x

Mirouze M, Sels J, Richard O, et al (2006) A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance. Plant J 47:329–42. doi: 10.1111/j.1365-313X.2006.02788.x

Money NP (1989) Osmotic Pressure of Aqueous Polyethylene Glycols 1 Relationship between Molecular Weight and Vapor Pressure Deficit. Plant Physiol 91:766–769.

Moran PJ (2001) Molecular Responses to Aphid Feeding in *Arabidopsis* in Relation to Plant Defense Pathways. PLANT Physiol 125:1074–1085. doi: 10.1104/pp.125.2.1074

Moreno M, Segura A, Garcia-olmedo F (1994) Pseudothionin-St1, a potato peptide active against potato pathogens. Eur J Biochem 223:135–139. doi: 10.1111/j.1432-1033.1994.tb18974.x

Mundy J, Chua NH (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J 7:2279–86.

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4326. doi: 10.1093/nar/8.19.4321

Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci 24:34–6.

Nakayama K, Okawa K, Kakizaki T, et al (2007) *Arabidopsis* Cor15am is a chloroplast stromal protein that has cryoprotective activity and forms oligomers. Plant Physiol 144:513–23. doi: 10.1104/pp.106.094581

NDong C, Danyluk J, Wilson KE, et al (2002) Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. Plant Physiol 129:1368–81. doi: 10.1104/pp.001925

Niogret MF, Culianez-Macia FA, Goday A, et al (1996) Expression and cellular localization of rab28 mRNA and Rab28 protein during maize embryogenesis. Plant J 9:549–557. doi: 10.1046/j.1365-

313X.1996.09040549.x

Noble SM, French S, Kohn LA, et al (2010) Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat Genet 42:590–8. doi: 10.1038/ng.605

Ntui VO, Thirukkumaran G, Azadi P, et al (2010) Stable integration and expression of wasabi defensin gene in "Egusi" melon (*Colocynthis citrullus* L.) confers resistance to Fusarium wilt and Alternaria leaf spot. Plant Cell Rep 29:943–54. doi: 10.1007/s00299-010-0880-2

Ogoshi A (1996) *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Springer Netherlands, Dordrecht

Oliveira E, Amara I, Bellido D, et al (2007) LC-MSMS identification of *Arabidopsis thaliana* heat-stable seed proteins: enriching for LEA-type proteins by acid treatment. J Mass Spectrom 42:1485–95. doi: 10.1002/jms.1292

Olli S, Kirti PB (2006) Cloning, Characterization and Antifungal Activity of Defensin Tfgd1 from *Trigonella foenumgraecum* L. J Biochem Mol Biol 39:278–283. doi: 10.5483/BMBRep.2006.39.3.278

Olvera-Carrillo Y, Campos F, Reyes JL, et al (2010) Functional analysis of the group 4 late embryogenesis abundant proteins reveals their relevance in the adaptive response during water deficit in *Arabidopsis.* Plant Physiol 154:373–90. doi: 10.1104/pp.110.158964

Olvera-Carrillo Y, Luis Reyes J, Covarrubias AA (2011) Late embryogenesis abundant proteins: versatile players in the plant adaptation to water limiting environments. Plant Signal Behav 6:586–9.

Oomen RJFJ, Séveno-Carpentier E, Ricodeau N, et al (2011) Plant defensin AhPDF1.1 is not secreted in leaves but it accumulates in intracellular compartments. New Phytol 192:140–50. doi: 10.1111/j.1469-8137.2011.03792.x

Osbourn AE (1999) Antimicrobial phytoprotectants and fungal pathogens: a commentary. Fungal Genet Biol 26:163–8. doi: 10.1006/fgbi.1999.1133

Parashina E V., Serdobinskii LA, Kalle EG, et al (2000) Genetic engineering of oilseed rape and tomato plants expressing a radish defensin gene. Russ J Plant Physiol 47:417–423.

Park HC, Kang YH, Chun HJ, et al (2002) Characterization of a stamen-specific cDNA encoding a novel plant defensin in Chinese cabbage. Plant Mol Biol 50:57–68. doi: 10.1023/A:1016005231852

Park S-C, Kim Y-H, Jeong JC, et al (2011a) Sweetpotato late embryogenesis abundant 14 (IbLEA14) gene influences lignification and increases osmotic- and salt stress-tolerance of transgenic calli. Planta 233:621–34. doi: 10.1007/s00425-010-1326-3

Pasche JS, Mallik I, Anderson NR, Gudmestad NC (2013) Development and Validation of a Real-Time PCR Assay for the Quantification of *Verticillium dahliae* in Potato. Plant Dis 97:608–618. doi: 10.1094/PDIS-06-12-0554-RE

Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. Curr Opin Plant Biol 14:290–295. doi: 10.1016/j.pbi.2011.02.001

Pelegrini PB, Franco OL (2005) Plant gamma-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins. Int J Biochem Cell Biol 37:2239–53. doi:

10.1016/j.biocel.2005.06.011

Penninckx I a, Thomma BP, Buchala a, et al (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell 10:2103–2113. doi: 10.1105/tpc.10.12.2103

Penninckx IAMA (1998) Concomitant Activation of Jasmonate and Ethylene Response Pathways Is Required for Induction of a Plant Defensin Gene in *Arabidopsis.* PLANT CELL ONLINE 10:2103–2114. doi: 10.1105/tpc.10.12.2103

Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786. doi: 10.1038/nmeth.1701

Puhakainen T, Hess MW, Mäkelä P, et al (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis*. Plant Mol Biol 54:743–53. doi: 10.1023/B:PLAN.0000040903.66496.a4

QIU Y, YU D (2009) Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in *Arabidopsis*. Environ Exp Bot 65:35–47. doi: 10.1016/j.envexpbot.2008.07.002

Raghavan V, Kamalay JC (1993) Expression of two cloned mRNA sequences during development and germination of spores of the sensitive fern, *Onoclea sensibilis* L. Planta 189:1–9.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E (2010a) ABA perception and signalling. Trends Plant Sci 15:395–401. doi: 10.1016/j.tplants.2010.04.006

Raja K, Kumar R, Kirti PB (2011) Differential gene expression in *Arachis diogoi* upon interaction with peanut late leaf spot pathogen, *Phaeoisariopsis personata* and characterization of a pathogen induced cyclophilin. Plant molecular biology 497–513. doi: 10.1007/s11103-011-9747-3

Ramamoorthy V, Cahoon EB, Li J, et al (2007a) Glucosylceramide synthase is essential for alfalfa defensin-mediated growth inhibition but not for pathogenicity of *Fusarium graminearum*. Mol Microbiol 66:771–86. doi: 10.1111/j.1365-2958.2007.05955.x

Ramamoorthy V, Zhao X, Snyder AK, et al (2007b) Two mitogen-activated protein kinase signalling cascades mediate basal resistance to antifungal plant defensins in *Fusarium graminearum*. Cell Microbiol 9:1491–506. doi: 10.1111/j.1462-5822.2006.00887.x

Ramanjulu S, Bartels D (2002) Drought- and desiccation-induced modulation of gene expression in plants. Plant, Cell Environ 25:141–151. doi: 10.1046/j.0016-8025.2001.00764.x

Ramesh SV, Mishra AK, Praveen S (2007) Hairpin RNA-Mediated Strategies for Silencing of Tomato Leaf Curl Virus AC1 and AC4 Genes for Effective Resistance in Plants.

Rao NK, Reddy LJ, Bramel PJ (2003) Potential of wild species for genetic enhancement of some semi-arid food crops. Genet Resour Crop Evol 50:707–721. doi: 10.1023/A:1025055018954

Reguera M, Peleg Z, Blumwald E (2012) Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops. Biochim Biophys Acta 1819:186–94. doi: 10.1016/j.bbagrm.2011.08.005

Ren Q, Li M, Zhang C-Y, Chen K-P (2011) Six defensins from the triangle-shell pearl mussel *Hyriopsis cumingii*. Fish Shellfish Immunol 31:1232–8. doi: 10.1016/j.fsi.2011.07.020

Reyes JL, Rodrigo M-J, Colmenero-Flores JM, et al (2005) Hydrophilins from distant organisms can protect enzymatic activities from water limitation effects in vitro. Plant, Cell Environ 28:709–718. doi: 10.1111/j.1365-3040.2005.01317.x

Ribaut JM, Hoisington DA, Deutsch JA, et al (1996) Identification of quantitative trait loci under drought conditions in tropical maize. 1. Flowering parameters and the anthesis-silking interval. Theor Appl Genet 92:905–14. doi: 10.1007/BF00221905

Ribaut J-M, Jiang C, Gonzalez-de-Leon D, et al (1997) Identification of quantitative trait loci under drought conditions in tropical maize. TAG Theor Appl Genet 94:887–896. doi: 10.1007/s001220050492

Roberts JK, DeSimone NA, Lingle WL, Dure L (1993) Cellular Concentrations and Uniformity of Cell-Type Accumulation of Two Lea Proteins in Cotton Embryos. Plant Cell 5:769–780. doi: 10.1105/tpc.5.7.769

Robinson DG, Oliviusson P, Hinz G (2005) Protein sorting to the storage vacuoles of plants: a critical appraisal. Traffic 6:615–25. doi: 10.1111/j.1600-0854.2005.00303.x

Rodríguez-Jiménez F-J, Krause A, Schulz S, et al (2003) Distribution of new human β-defensin genes clustered on chromosome 20 in functionally different segments of epididymis. Genomics 81:175–183. doi: 10.1016/S0888-7543(02)00034-4

Rorat T (2006) Plant dehydrins--tissue location, structure and function. Cell Mol Biol Lett 11:536–56. doi: 10.2478/s11658-006-0044-0

Roy SJ, Tucker EJ, Tester M (2011) Genetic analysis of abiotic stress tolerance in crops. Curr Opin Plant Biol 14:232–9. doi: 10.1016/j.pbi.2011.03.002

Rushton PJ, Somssich IE, Ringler P, Shen QJ (2010) WRKY transcription factors. Trends Plant Sci 15:247–58. doi: 10.1016/j.tplants.2010.02.006

Saavedra L, Svensson J, Carballo V, et al (2006) A dehydrin gene in *Physcomitrella patens* is required for salt and osmotic stress tolerance. Plant J 45:237–49. doi: 10.1111/j.1365-313X.2005.02603.x

Sagaram, Uma Shankar et al. (2011) "Structure-activity determinants in antifungal plant defensins MsDef1 and MtDef4 with different modes of action against Fusarium graminearum." PLoS One 6.4 (2011): e18550.

Salleh FM, Evans K, Goodall B, et al (2012) A novel function for a redox-related LEA protein (SAG21/AtLEA5) in root development and biotic stress responses. Plant Cell Environ 35:418–29. doi: 10.1111/j.1365-3040.2011.02394.x

Sambrook, Joseph, E. F. Fritsch and TM (1989) "Molecular cloning: A laboratory manual+ Cold Spring Harbor.": 9.

Sanan-Mishra N, Pham XH, Sopory SK, Tuteja N (2005) Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. Proc Natl Acad Sci U S A 102:509–14. doi: 10.1073/pnas.0406485102

Sanghera GS, Wani SH, Singh G, et al (2011) Designing crop plants for biotic stresses using transgenic approach. Vegetos 24:1–25.

Segura A, Moreno M, Molina A, García-Olmedo F (1998) Novel defensin subfamily from spinach (*Spinacia oleracea*). FEBS Lett 435:159–162. doi: 10.1016/S0014-5793(98)01060-6

Seo M (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7:41–48. doi: 10.1016/S1360-1385(01)02187-2

Shimamura C, Ohno R, Nakamura C, Takumi S (2006) Improvement of freezing tolerance in tobacco plants expressing a cold-responsive and chloroplast-targeting protein WCOR15 of wheat. J Plant Physiol 163:213–9. doi: 10.1016/j.jplph.2005.06.008

Siddique S, Wieczorek K, Szakasits D, et al (2011) The promoter of a plant defensin gene directs specific expression in nematode-induced syncytia in Arabidopsis roots. Plant Physiol Biochem PPB / Société Fr Physiol végétale 49:1100–7. doi: 10.1016/j.plaphy.2011.07.005

Singh J, Whitwill S, Lacroix G, et al (2009) The use of Group 3 LEA proteins as fusion partners in facilitating recombinant expression of recalcitrant proteins in *E. coli.* Protein Expr Purif 67:15–22. doi: 10.1016/j.pep.2009.04.003

Singh S, Cornilescu CC, Tyler RC, et al (2005) Solution structure of a late embryogenesis abundant protein (LEA14) from *Arabidopsis thaliana*, a cellular stress-related protein. Protein Sci 14:2601–2609. doi: 10.1110/ps.051579205

Sivamani E, Bahieldin1 A, Wraith J, et al (2000) Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. Plant Sci 155:1–9.

Smirnoff N (1998) Plant resistance to environmental stress. Curr Opin Biotechnol 9:214–219. doi: 10.1016/S0958-1669(98)80118-3

Snapp E (2005) Design and use of fluorescent fusion proteins in cell biology. Curr Protoc Cell Biol Chapter 21:Unit 21.4. doi: 10.1002/0471143030.cb2104s27

Song X, Zhang M, Zhou Z, Gong W (2011) Ultra-high resolution crystal structure of a dimeric defensin SPE10. FEBS Lett 585:300–6. doi: 10.1016/j.febslet.2010.12.039

Stacy RAP, Nordeng TW, Culianez-Macia FA, Aalen RB (1999) The dormancy-related peroxiredoxin anti-oxidant, PER1, is localized to the nucleus of barley embryo and aleurone cells. Plant J 19:1–8. doi: 10.1046/j.1365-313X.1999.00488.x

Stotz HU, Thomson J, Wang Y (2014) Plant defensins. Plant Signal Behav 4:1010–1012. doi: 10.4161/psb.4.11.9755

Su'udi M, Park J-M, Park S-R, et al (2013) Quantification of *Alternaria brassicicola* infection in the *Arabidopsis thaliana* and *Brassica rapa* subsp. pekinensis. Microbiology 159:1946–55. doi: 10.1099/mic.0.068205-0

Subrahmanyam P, Rao VR, McDonald D, et al (1989) Origins of resistances to rust and late leaf spot in peanut (*Arachis hypogaea*, Fabaceae). Econ Bot 43:444–455. doi: 10.1007/BF02935917

Sun YG, Wang B, Jin SH, et al (2013) Ectopic Expression of Arabidopsis Glycosyltransferase UGT85A5 Enhances Salt Stress Tolerance in Tobacco. PLoS One. doi: 10.1371/journal.pone.0059924

Svensson J, Palva ET, Welin B (2000) Purification of recombinant *Arabidopsis thaliana* dehydrins by metal ion affinity chromatography. Protein Expr Purif 20:169–178. doi: 10.1006/prep.2000.1297

Swathi Anuradha T, Divya K, Jami SK, Kirti PB (2008) Transgenic tobacco and peanut plants expressing a mustard defensin show resistance to fungal pathogens. Plant Cell Rep 27:1777–86. doi: 10.1007/s00299-008-0596-8

Szakasits D, Heinen P, Wieczorek K, et al (2009) The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in *Arabidopsis* roots. Plant J 57:771–84. doi: 10.1111/j.1365-313X.2008.03727.x

Takahashi F, Mizoguchi T, Yoshida R, et al (2011) Calmodulin-dependent activation of MAP kinase for ROS homeostasis in *Arabidopsis*. Mol Cell 41:649–60. doi: 10.1016/j.molcel.2011.02.029

Takakura Y, Che F-S, Ishida Y, et al (2008) Expression of a bacterial flagellin gene triggers plant immune responses and confers disease resistance in transgenic rice plants. Mol Plant Pathol 9:525–9. doi: 10.1111/j.1364-3703.2008.00477.x

Tamura K, Peterson D, Peterson N, et al (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–9. doi: 10.1093/molbev/msr121

Tedford EC MT and NM (1990) A detached-leaves technique for detecting *Phytophthora parasitica* var. *nicotianae* in tobacco. Plant Disease, 74 313–316.

Terras F (1995) Small Cysteine-Rich Antifungal Proteins from Radish: Their Role in Host Defense. PLANT CELL ONLINE 7:573–588. doi: 10.1105/tpc.7.5.573

Thevissen K, Cammue BP, Lemaire K, et al (2000) A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of *Saccharomyces cerevisiae* to an antifungal plant defensin from dahlia (*Dahlia merckii*). Proc Natl Acad Sci U S A 97:9531–6. doi: 10.1073/pnas.160077797

Thevissen K, de Mello Tavares P, Xu D, et al (2012) The plant defensin RsAFP2 induces cell wall stress, septin mislocalization and accumulation of ceramides in *Candida albicans*. Mol Microbiol 84:166–80. doi: 10.1111/j.1365-2958.2012.08017.x

Thevissen K, Francois I, Winderickx J, et al (2006) Ceramide Involvement in Apoptosis and Apoptotic Diseases. Mini-Reviews Med Chem 6:699–709. doi: 10.2174/138955706777435643

Thevissen K, Osborn RW, Acland DP, Broekaert WF (1997) Specific, High Affinity Binding Sites for an Antifungal Plant Defensin on Neurospora crassa Hyphae and Microsomal Membranes. J Biol Chem 272:32176–32181. doi: 10.1074/jbc.272.51.32176

Thomma BP, Penninckx IA, Cammue BP, Broekaert WF (2001) The complexity of disease signaling in *Arabidopsis*. Curr Opin Immunol 13:63–68. doi: 10.1016/S0952-7915(00)00183-7

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680. doi: 10.1093/nar/22.22.4673

Tohidfar M, Hossaini R, Shokhandan Bashir N, Meisam T (2012) Enhanced resistance to *Verticillium*

dahliae in transgenic cotton expressing an endochitinase gene from *Phaseolus vulgaris*. Czech J Genet Plant Breed 48:33–41.

Tohidfar M, Khosravi S (2015) Transgenic crops with an improved resistance to biotic stresses. A review. Base 19:62–70.

Tolleter D, Hincha DK, Macherel D (2010) A mitochondrial late embryogenesis abundant protein stabilizes model membranes in the dry state. Biochim Biophys Acta 1798:1926–33. doi: 10.1016/j.bbamem.2010.06.029

Tolleter D, Jaquinod M, Mangavel C, et al (2007) Structure and function of a mitochondrial late embryogenesis abundant protein are revealed by desiccation. Plant Cell 19:1580–9. doi: 10.1105/tpc.107.050104

Tompa P (2002) Intrinsically unstructured proteins. Trends Biochem Sci 27:527–533. doi: 10.1016/S0968-0004(02)02169-2

Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol 8:397–403. doi: 10.1016/j.pbi.2005.05.014

Tunnacliffe, A., Hincha, D., Leprince, O. and Macherel D (2010) LEA Proteins: Versatility of Form and Function. ***Dormancy and resistance in harsh environments***. Springer Berlin Heidelberg,. 91-108

Tunnacliffe A, Wise MJ (2007) The continuing conundrum of the LEA proteins. Naturwissenschaften 94:791–812. doi: 10.1007/s00114-007-0254-y

U. Schreiber, W. Bilger CN (1994) Ecophysiology of Photosynthesis. Springer Berlin Heidelberg, Berlin, Heidelberg

Ukaji N, Kuwabara C, Takezawa D, et al (2001) Cold acclimation-induced WAP27 localized in endoplasmic reticulum in cortical parenchyma cells of mulberry tree was homologous to group 3 late-embryogenesis abundant proteins. Plant Physiol 126:1588–97.

Uversky VN, Gillespie JR, Fink AL (2000) Why are "natively unfolded" proteins unstructured under physiologic conditions? Proteins: Structure, Function, and Bioinformatics, 41(3), 415-427.

Van den Heuvel KJPT (2001) The expression of tgas118, encoding a defensin in *Lycopersicon esculentum*, is regulated by gibberellin. J Exp Bot 52:1427–1436. doi: 10.1093/jexbot/52.360.1427

Van der Weerden NL, Anderson MA (2013) Plant defensins: Common fold, multiple functions. Fungal Biol Rev 26:121–131. doi: 10.1016/j.fbr.2012.08.004

Van der Weerden NL, Hancock REW, Anderson MA (2010) Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process. J Biol Chem 285:37513–20. doi: 10.1074/jbc.M110.134882

Van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44:135–62. doi: 10.1146/annurev.phyto.44.070505.143425

Vijayan S, Guruprasad L, Kirti PB (2008) Prokaryotic expression of a constitutively expressed *Tephrosia villosa* defensin and its potent antifungal activity. Appl Microbiol Biotechnol 80:1023–32. doi: 10.1007/s00253-008-1648-2

Vijayan S, Imani J, Tanneeru K, et al (2012) Enhanced antifungal and insect α-amylase inhibitory activities of Alpha-TvD1, a peptide variant of *Tephrosia villosa* defensin (TvD1) generated through in vitro mutagenesis. Peptides 33:220–229. doi: 10.1016/j.peptides.2011.12.020

Vijayan S, Singh NK, Shukla P, Kirti PB (2013) Defensin (TvD1) from *Tephrosia villosa* exhibited strong anti-insect and anti-fungal activities in transgenic tobacco plants. J Pest Sci (2004) 86:337–344. doi: 10.1007/s10340-012-0467-5

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic Acid, a multifaceted hormone to combat disease. Annu Rev Phytopathol 47:177–206. doi: 10.1146/annurev.phyto.050908.135202

Wang B, Yu J, Zhu D, Zhao Q (2013) Maize defensin ZmDEF1 is involved in plant response to fungal phytopathogens. African J. Biotechnol. 10:16128–16137.

Wang M, Li P, Li C, et al (2014) SiLEA14, a novel atypical LEA protein, confers abiotic stress resistance in foxtail millet. BMC Plant Biol 14:290. doi: 10.1186/s12870-014-0290-7

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. Planta 218:1–14. doi: 10.1007/s00425-003-1105-5

Wang W-B, Kim Y-H, Lee H-S, et al (2009) Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. Plant Physiol Biochem 47:570–7. doi: 10.1016/j.plaphy.2009.02.009

Wang Y, Nowak G, Culley D, et al (1999) Constitutive Expression of Pea Defense Gene DRR206 Confers Resistance to Blackleg (*Leptosphaeria maculans*) Disease in Transgenic Canola (*Brassica napus*). Mol Plant-Microbe Interact 12:410–418. doi: 10.1094/MPMI.1999.12.5.410

Wasilewska A, Vlad F, Sirichandra C, et al (2008) An update on abscisic acid signaling in plants and more... Mol Plant 1:198–217. doi: 10.1093/mp/ssm022

Wijaya R, Neumann GM, Condron R, et al (2000) Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. Plant Sci 159:243–255. doi: 10.1016/S0168-9452(00)00348-4

Wilmes M, Cammue BPA, Sahl H-G, Thevissen K (2011) Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. Nat Prod Rep 28:1350–8. doi: 10.1039/c1np00022e

Wise MJ (2003) LEAping to conclusions: a computational reanalysis of late embryogenesis abundant proteins and their possible roles. BMC Bioinformatics 4:52. doi: 10.1186/1471-2105-4-52

Wolkers WF, McCready S, Brandt WF, et al (2001) Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses in vitro. Biochim Biophys Acta - Protein Struct Mol Enzymol 1544:196–206. doi: 10.1016/S0167-4838(00)00220-X

Wu Y, Deng Z, Lai J, et al (2009) Dual function of *Arabidopsis* ATAF1 in abiotic and biotic stress responses. Cell Res 19:1279–90. doi: 10.1038/cr.2009.108

Xiong L, Schumaker KS, Zhu J-K (2002) Cell Signaling during Cold, Drought, and Salt Stress. Plant Cell 14:S165–183. doi: 10.1105/tpc.000596

Xu D, Duan X, Wang B, 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 Physiol 110:249–257. doi: 110/1/249 [pii]

Yamada, Shigehiro, T. Komori and HI (1997) "cDNA cloning of-thionin from *Nicotiana excelsior* (accession no. AB005266; PGR97-131)." Plant Physiol 115 (1997): 314.

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803. doi: 10.1146/annurev.arplant.57.032905.105444

Yang Y, Li R, Qi M (2000) In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. Plant J 22:543–51.

Yount NY, Andrés MT, Fierro JF, Yeaman MR (2007) The gamma-core motif correlates with antimicrobial activity in cysteine-containing kaliocin-1 originating from transferrins. Biochim Biophys Acta 1768:2862–72. doi: 10.1016/j.bbamem.2007.07.024

Yu L, Guo Y, Li Y, et al (2010) SecretP: identifying bacterial secreted proteins by fusing new features into Chou's pseudo-amino acid composition. J Theor Biol 267:1–6. doi: 10.1016/j.jtbi.2010.08.001

Zainal Z, Marouf E, Ismail I, Fei CK (2009) Expression of the *Capsicuum annum* (Chili) Defensin Gene in Transgenic Tomatoes Confers Enhanced Resistance to Fungal Pathogens. Am J Plant Physiol 4:70–79. doi: 10.3923/ajpp.2009.70.79

Zeevaart J a D, Creelman R a (1988) Metabolism and Physiology of Abscisic Acid. Annu Rev Plant Physiol Plant Mol Biol 39:439–473. doi: 10.1146/annurev.pp.39.060188.002255

Zegzouti H, Jones B, Frasse P, et al (1999) Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display. Plant J 18:589–600. doi: 10.1046/j.1365-313x.1999.00483.x

Zhang P, Vanderschuren H, Fütterer J, Gruissem W (2005) Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. Plant Biotechnol J 3:385–97. doi: 10.1111/j.1467-7652.2005.00132.x

Zhang X, Lu S, Jiang C, et al (2014) RcLEA, a late embryogenesis abundant protein gene isolated from *Rosa chinensis*, confers tolerance to *Escherichia coli* and *Arabidopsis thaliana* and stabilizes enzyme activity under diverse stresses. Plant Mol Biol 85:333–347. doi: 10.1007/s11103-014-0192-y

Zhu J-K (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273. doi: 10.1146/annurev.arplant.53.091401.143329

Zouhar J, Rojo E (2009) Plant vacuoles: where did they come from and where are they heading? Curr Opin Plant Biol 12:677–84. doi: 10.1016/j.pbi.2009.08.004

Publication

- **Sharma A**, Kumar D, Kumar S, et al (2016) Ectopic Expression of an Atypical Hydrophobic Group 5 LEA Protein from Wild Peanut, *Arachis diogoi* Confers Abiotic Stress Tolerance in Tobacco. PLoS One 11:e0150609. doi: 10.1371/journal.pone.0150609