Characterization of some pathogen induced genes from a wild Peanut, *Arachis diogoi*

Thesis submitted to the University of Hyderabad for the award of

DOCTOR OF PHILOSOPHY

By

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HYDERABAD – 500 046, INDIA

"DECLARATION"

I, **Naveen Kumar Singh** hereby declare that this thesis entitled **"Characterization of some pathogen induced genes from a wild Peanut, *Arachis diogoi*"** 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:

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"CERTIFICATE"

This is to certify that this thesis entitled **"Characterization of some pathogen induced genes from a wild Peanut, *Arachis diogoi*"** is based on the results of the work done by **Mr. Naveen Kumar Singh**, 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
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Dedicated to my family and friends

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Naveen Kumar Singh

Abbreviations

µg	Microgram
µl	Microliter
µM	Micromolar
ABA	Abscisic acid
ATP	Adenosine triphosphate
BAP	Benzyl amino purine
bp	Base pairs
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
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
ESTs	Expressed sequence tags
g	Gram
GFP	Green fluorescent protein
GSP	Gene specific primers
h	Hours
H_2DCFDA	2', 7'- dichlorodihydroflourescien diacetate
HPI	Hours post inoculation
HR	Hypersensitive responese
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobases
KDa	Kilodalton
LB	Luria Bertani

M	Molar
MAPKs	Mitogen-activated protein kinase
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

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Chapter 1:

General Introduction

Plant Stresses

Plants are important to the balance of nature and human's lives. They are the ultimate source of food and metabolic energy for animals, which cannot manufacture their own food. Plants, being sessile, are often exposed to different stress factors in combination. In general, stresses are the external conditions that adversely affect development, growth or productivity of plants. They can be also termed as the elements changing the genotype potential of plants. Depending on the stress conditions, plants trigger a wide range of signaling responses by altering the gene expression and cellular metabolism to minimize the damage. These stress conditions can be divided into biotic (imposed by other organism) or abiotic (due to the excess or deficit in the chemical and physical environment) stresses. The examples of biotic stresses are bacteria, virus, fungi and insect predators whereas abiotic stress includes water logging, high or low temperature, inadequate minerals in soil, excess soil salinity, drought etc. Plant protection has an obvious role in meeting the growing demand for food quality and quantity. Roughly, direct yield losses caused by animals, weeds and pathogens are altogether responsible for losses ranging between 20 and 40% of global agricultural productivity (Savary et al., 2012). However abiotic stresses, which usually cause primary crop losses worldwide lead to an average yield loss of >50% in most major crop plants (Qin et al., 2011). Furthermore, world food production needs to be doubled by the end of year 2050 to meet the ever-growing demands of the population (Tilman et al., 2002). Hence, understanding the mechanisms of plant stress responses and the generation of stress-tolerant plants need much attention for the improvement of crop performance.

1.1 Abiotic stress

Breeders and farmers have long known that most of the crop loses are due to the simultaneous occurrence of many abiotic stresses together, rather than a specific stress condition. Basically, plants need water, carbon, mineral nutrients and energy (light) for growth and development. Abiotic stresses cause the reduced plant growth and decrease the yield below optimum levels. Plant adjustment to a specific abiotic stress condition needs a particular response that is tailored to the definite environmental conditions the plant confronts. Thus, biochemical, molecular, and physiological processes set in motion by a definite stress condition might vary from those activated by a marginally different

composition of environmental conditions. Each different stress condition prompts a somewhat unique acclimation response with little overlapping in transcript expression and could be found during as drought, cold, heat, salt, high light or mechanical stresses (Cheong et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Rizhsky et al., 2004; Rossel et al., 2002).

1.1.1 Hormones signaling response to abiotic stress

Plant hormones are essential for their ability to adapt different abiotic stresses by dealing with a wide range of adaptive responses. They include the five classical phytohormones: gibberellins (GA), jasmonate (JA), abscisic acid (ABA), ethylene, cytokinin (CK) and auxin (IAA) as well as nitric oxide (NO), brassinosteroids (BR), salicylic acid (SA), and strigolactone (SL) (Peleg and Blumwald, 2011). Abiotic stresses in plants are widely controlled by phytohormone ABA which is also referred to as 'stress hormone'. Its synthesis is one of the fastest responses of plants to abiotic stresses activating ABA-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006). Various stress conditions can be mimicked by the exogenous application of ABA to plant. Many abiotic stresses, in general, ultimately cause the osmotic imbalance and desiccation of the cell. There is an overlap of different stress related gene expression during drought, high salt, cold or ABA application. This suggests the presence of common elements during the stress and ABA signaling cascades and possibly involved in cross talk to maintain the cellular homeostasis (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999). Apart from this, ABA also plays roles in other physiological processes such as development of seeds, leaf senescence, synthesis of storage proteins and lipids, promotion of stomatal closure, embryo seed dormancy and late germination, morphogenesis, and also in pathogen defense (Tuteja, 2007). A Model of ABA signaling was presented through the PYR/RCAR ABA receptor, SnRK2 kinases and PP2C. It was shown that in the absence of ABA, protein phosphatase 2Cs (ABI1 and ABI2) dephosphorylate the SnRK2 kinases and keep them in in active form. During stress conditions the ABA binds with PYR/RCAR in nuclei and causes the activation of SnRK2 kinases **(Figure 1.1)**. These activated kinases subsequently phosphorylates the AREB/ABF transcription factors, which bind with ABREs located in the promoter regions of ABA-responsive genes (Qin et al., 2011).

Other hormones like SA, ethylene, BR, CK, and JA also play direct or indirect roles in the plant responses to abiotic stress. It was observed that exposure of plants to water limiting conditions results in decreased levels of CK (Peleg and Blumwald, 2011). Microarray analysis data showed that numerous genes associated with CK signaling pathway were differentially affected in *Arabidopsis thaliana* by various abiotic stresses (Argueso et al., 2009).

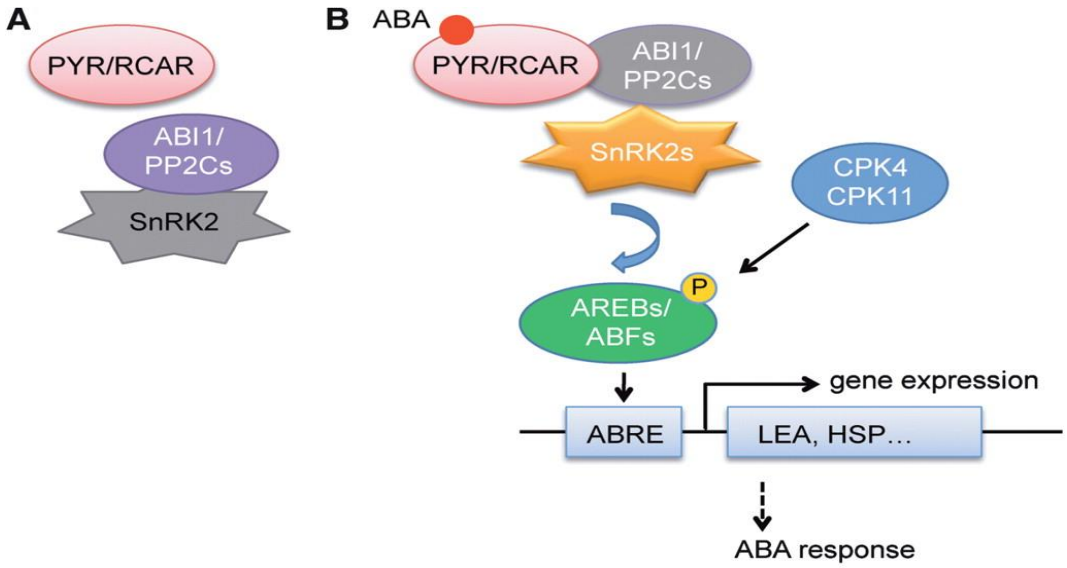

Figure 1.1 A model of ABA signal reception and transduction through the PYR/RCAR ABA receptor and the PP2C and SnRK2 kinases (Qin et al., 2011).

BR were involved in inducing the different stress related genes for the maintenance of photosynthesis activity, the accumulation of osmoprotectants, the activation of antioxidant enzymes and the induction of other hormone responses (Divi and Krishna, 2009). Another hormone, ethylene was termed as “stress ethylene” due to its accelerated biosynthesis associated with biological and environmental stresses experienced by plants (Morgan and Drew, 1997). It is a gaseous hormone involved in many stress responses like ozone, drought, flooding (hypoxia and anoxia), chilling, heat, UV-B light, wounding and also involved in mechanisms like, fruit ripening and bud dormancy by interacting with ABA (Cramer et al., 2011). The role of SA is well studied in biotic stress conditions. However, it is also involved in protection of plants against abiotic stress factors like heat, low temperature, salt and osmotic stress by inducing a wide range of stress tolerance mechanisms (Horváth et al., 2007). Similarly, jasmonates (JA and methyl jasmonate) are

important plant regulators involved in diverse cellular mechanisms like seed germination to fruit ripening, senescence along with biotic and abiotic stresses (Wasternack and Hause, 2002). Salt, drought, and heat stress are the abiotic conditions where JA is believed to be playing some role in plant responses (Brossa et al., 2011; Clarke et al., 2009; Yoon et al., 2009).

1.1.2 MAP kinases and transcription factors

In plants, signal transduction pathways are very developed as well as extremely complex due to their ability to cross talk under different circumstances. The mitogen activated protein kinase (MAPK) cascade is one of the major signaling pathways involved in plant abiotic stress responses. The molecules involved in signaling cascades are conserved among eukaryotic system and essential for transducing the developmental and environmental cues into the cellular responses (Sinha et al., 2011). The MAPK cascades are minimally composed of MAP kinase kinase kinases (MAP3Ks), MAP kinase kinases (MAP2Ks) and MAP kinases (MAPKs) (Mishra et al., 2006) . Under stress condition, the stimulated plasma membrane activates MAP3Ks or MAP kinase kinase kinase kinases (MAP4Ks). The MAP4Ks may act as connecting link or adaptors for upstream signaling steps to the core MAPK cascades. MAP3Ks kinases phosphorylate the activation loop of MAP2K and activated MAP2Ks phosphorylate MAPKs on threonine and tyrosine residues at a conserved T-X-Y motif (Chang and Karin, 2001). MAPKs are serine/threonine kinases and can phosphorylate a wide range of substrates, including transcription factors and/or other kinases. Generally the term “cross talk” is used to refer the situations where different signaling pathways share either one or more intermediate components or some common output product. During the stress conditions, plants can show some common as well as specific tolerance mechanisms suggesting the presence of cross talk of different pathways during stress perception and their signal transduction. The MAPK kinase signaling cascade perhaps provides some of the strongest evidences for cross-talk during abiotic stress signaling in plant. The Arabidopsis genome has approximately 80 MAPKKKs, 10 MAPKKs and 20 MAPKs with significant scope of cross talk between different stress signaling mechanism (Sinha et al., 2011). The MAPK family members can be activated by more than one type of signal suggesting them as points of convergence in stress signaling mechanisms **(Figure 1.2)**.

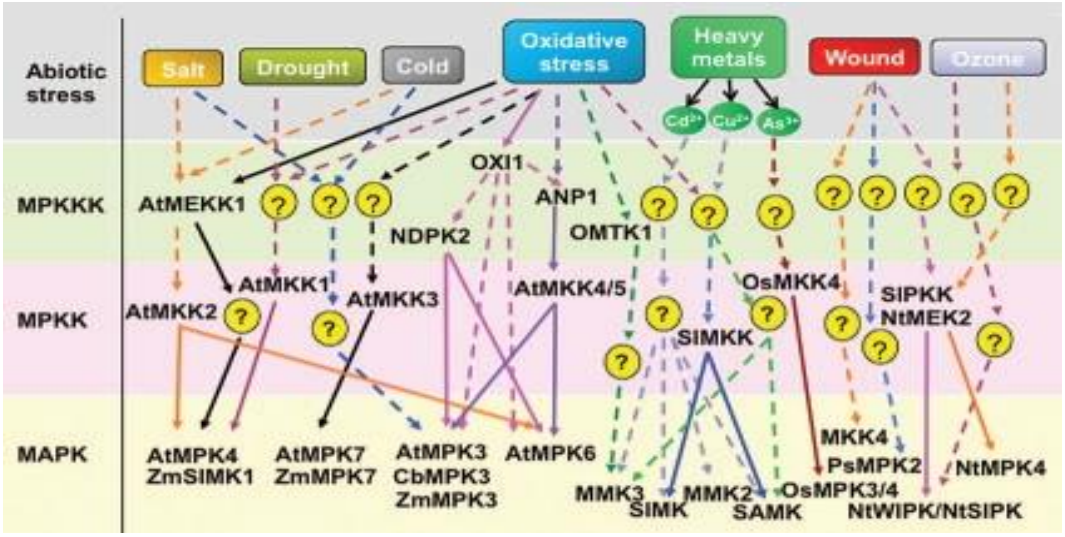

Figure 1.2. Representation of cross-talk among different plant MAP kinase signaling components. The scheme of general signal transduction is shown on the left. The homologs in Arabidopsis (At), tobacco (Nt), maize (Zm), pea (Ps) and *Chorispora bungeana* (Cb) are shown. Solid arrows show proven pathways; dashed arrows indicate postulated pathways; question marks indicate unknown cascade components (Sinha et al., 2011).

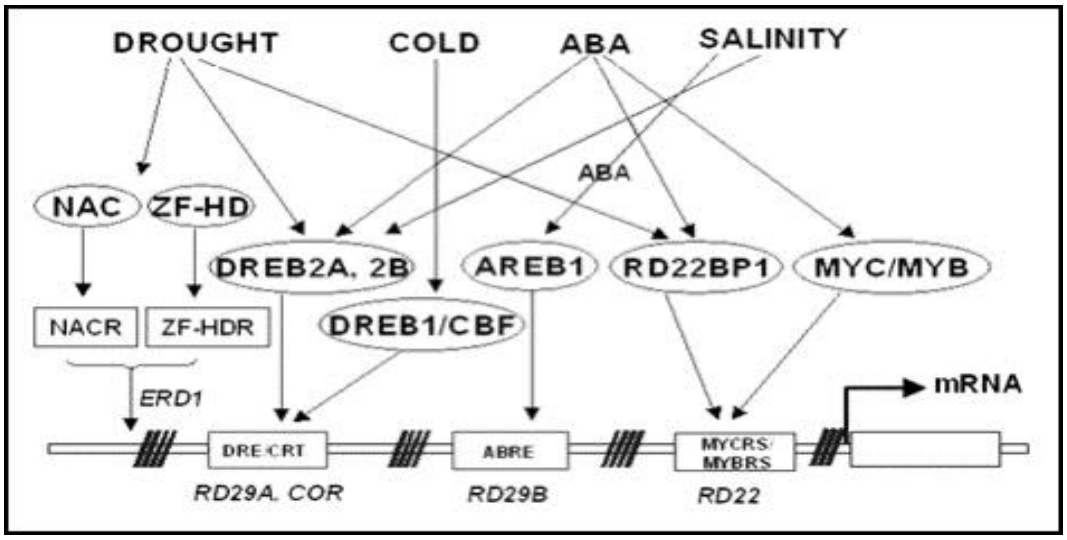

Figure 1.3 Transcriptional regulatory network of cis-acting elements and ABA-dependent transcription factors involved in drought, cold and salinity stress gene expression (Tuteja, 2007).

The promoter regions of various stress-induced genes from both ABA dependent or independent pathways contain cis-regulatory elements like DRE/CRT, ABRE, MYB and MYC recognition sequences, which are regulated by various upstream transcriptional factors

(Figure 1.3). A basic leucine zipper transcription factor AREB activates the ABA-dependent stress signaling mechanism by binding to ABRE element of stress inducible *RD29B* gene, which encodes a LEA-like (late embryogenesis abundant) protein (Uno et al., 2000). Transcription factors like DREBA2A and DREBA2B bind with DRE elements during osmotic stress and are involved in maintaining the osmotic balance of cell (Mahajan and Tuteja, 2005). DREB1D expression was drought inducible and depends on ABA accumulation. The MYC and MYB are found to be synthesized only after the accumulation of endogenous level of ABA (Mahajan and Tuteja, 2005). Overexpression of both NAC and ZF-HD proteins caused the activation of early responsive to dehydration gene, *ERD1* in transgenic plants (Tuteja, 2007). These transcription factors may also coordinate with each other by cross talk and help the plant to give the maximum level of response during stress condition.

1.1.3 Biotechnological application and future perspective

A number of genes associated with ABA synthesis and downstream signaling pathway have been characterized in Arabidopsis (Cutler et al., 2010). Some of these genes have been used in crop plants for enhancing abiotic stress tolerance. Overexpression of *ABA3/LOS5* gene, involved in conversion of abscisic aldehyde to ABA under the constitutive or drought-inducible promoters resulted in a significant increase in transgenic rice yield during drought conditions (Xiao et al., 2009). *NCED* is responsible for the conversion of neoxanthin to xanthoxin, a rate-limiting step in the ABA synthesis pathway. Its overexpression enhanced the salt and drought tolerance level in transgenic plants (Thompson et al., 2007; Zhang et al., 2008). Another gene *ERA1* codes for β-subunit of farnesyltransferase, an enzyme which is associated with the ABA-dependent pathway (Pei et al., 1998). An *era1* antisense gene under drought-inducible *rd29A* promoter from *A. thaliana* displayed higher yield under a mild drought condition in transgenic canola (*Brassica napus* L) plant (Wang et al., 2005). ABA and CK are antagonistic to each other and the exposure of plants to drought conditions result in decreased CK levels. During water stress condition elevated CK level help plants by inhibiting leaf senescence and inducing higher proline levels (Alvarez et al., 2008). The constitutive overexpression of *IPT* gene increased endogenous CK level up to 150-fold and resulted in decreased root growth during water stress (Smigocki and Owens, 1989). The overexpression of *AtDWF4*, a gene involved in BR biosynthesis, under the control of a seed-specific *oleosin* promoter resulted

in improved germination of seeds that were previously treated with ABA, which suggests an antagonic effect of BR on ABA-regulated processes. Additionally transgenic seedlings were more tolerant than wild-type during cold stress condition (Divi and Krishna, 2010).

Significant advances in understanding of molecular mechanism involving hormone synthesis, signaling and their responses during abiotic stresses have occurred in the past few years. These finding facilitate crop improvement. However, there are still many challenges ahead in both basic research and field application. For example, existence of additional ABA receptor remains to be resolved. GTG1/GTG2 proteins have been identified as membrane-bound ABA receptors, but their biological roles in ABA downstream signal transduction remains unclear. In addition, during the stress conditions, the mechanism related with ABA biosynthesis, transportation, storage and its turnover are still not fully understood (Qin et al., 2011). Also studies on interaction and cross talk between ABA and other phytohormone need better understanding. To achieve the combination of high yield and stress tolerance in a crop variety, we need to focus on development of individual traits and their interaction with other each other. Possibly, the intimate collaboration among plant breeders, physiologist and molecular biologist can answer some of the questions and help in fulfilling the growing food demand.

1.2 Biotic stress

Crops encounter a wide range of biotic invaders that include insect pests, viruses and other microbial pathogens. These pathogens can be broadly divided into two those that require a living host to complete their life cycle (biotrophs) and those that kill the host and feed on the contents (necrotrophs). They aim to withdraw host nutrients and thus severely impact crop yield. To overcome host resistance, they use diverse strategies. For example, pathogenic bacteria can increase rapidly in number in intercellular spaces of plant cells after entering through gas or water pores or via wounds (Alfano and Collmer, 1996). Sap feeding insects and nematodes feed by directly inserting a stylet into a plant cell. Many insects cause damage directly by chewing leaf or other organs. Additionally such wound responses release volatile compounds, which attract insect feeding or to deposit eggs into (Wu and Baldwin, 2010). Fungi can grow by extending hyphae on the top of, between or

inside the plant cell. Some fungi can invaginate a feeding structure called haustoria into the host cell plasma membrane for the nutrients (Knogge, 1996; Kolattukudy, 1985).

Green plants, being autotrophs are the only biological system capable of converting solar energy into chemical energy and hence, the prime target for exploitation at all levels by all means. Unlike animals, plants lack an immune system. However, their coexistence with pathogens led them to develop a stunning array of chemical, structural and protein-based defenses for pathogen detection and stopping them from causing extensive damage. They also retain several naturally occurring preexisting defense barriers (physical and chemical) to resist the pathogen penetration (Guest and Brown, 1997). For example, the cuticle covers the epidermal layer of plant cells and consists of pectin layer, a cutinized layer and a wax layer (Metraux et al., 2014). Cutin is the best-known structural component of the cuticular membrane and composed of fatty acids whereas waxes are mixtures of long chain aliphatic compounds which prevent water retention on plant surface and hence stopping fungal spore germination (Martin, 1964; Metraux et al., 2014). A negative charge developed on leaf surfaces due to fatty acids repels the air-borne spores. Only a few pathogens can dissolve the cutin enzymatically (Van Kan, 2006). Similarly, epidermis is the first layer of host cells that comes in contact with attacking pathogens but the presence of polymers of lignin mineral substances, cellulose, hemicelluloses and other polymerized organic compounds makes the conditions tough for pathogen attack and entry (Guest and Brown, 1997).

1.2.1 Molecular mechanism in plant defense response

The innate immune system of plants consists of two branches (Jones and Dangl, 2006). Initial recognition of microbes by host plants is done by using transmembrane pattern recognition receptors (PRRs). They detect the presence of microbial or pathogen-associated molecular patterns (MAMPs or PAMPs) on the surface of the host cell. Theses MAMPs and PAMPs represent small motifs of larger molecules that are essential for microbial survival, e.g. flagellin (Janeway, 1989; Zipfel and Felix, 2005). PAMP-triggered immunity (PTI) activates a many processes like activation of MAPK cascades, production of reactive oxygen species (ROS), gene expression and hormone signaling (Schwessinger and Zipfel, 2008). At this stage, pathogens are stopped. However, successful pathogens suppress PTI and try to invade the host cells by delivering the virulence effector proteins.

The second branch of immune system resides largely inside the cell. Genetic analysis has shown that this branch of induced protection is often determined by complementary pairs of pathogen avirulence (*avr*) genes and host resistance (*R*) gene products (Flor, 1971). When corresponding *R* and *avr* genes are present in both host and invading pathogen, the result is disease resistance and the interaction is called incompatible. The absence of the *avr* gene and/or *R* gene makes host susceptible for pathogen attack and the interaction is called compatible. The *R* gene mediated resistance is effective against only biotrophic or hemi-biotrophic pathogens, but not necrotrophs. Although some R proteins may act as primary receptors of *avr* gene product or pathogen effector proteins, most appear to play indirect roles in this process (Martin et al., 2003). Single peptides, and in some cases even their subregions, which play a role in recognition by R proteins, have been characterized in pathogenic bacteria, viruses and fungi and their presence is suspected in insects and nematodes as well. Based on their sequences, pathogen effectors are extremely diverse and difficult to classify. For example, in different viruses, either the replicase, the movement protein or the coat protein has been found to function as recognition determinants for R proteins (Martin et al., 2003). In fungi, various effectors have been identified from several species and, although none has proven biochemical activities, AVR-Pita from *Magnaporthe grisea* is a putative metalloprotease (Orbach et al., 2000). Similarily, among bacteria, diverse effectors are identified both from screening of proteins that are delivered by the type III secretion system (TTSS) and from bioinformatics approaches (Fouts et al., 2002; Guttman et al., 2002).

R gene products are designed to fulfill two tasks: first to recognize a pathogen signal: and second to initiate a coordinated plant defense reaction. Functional *R* genes isolated so far encode resistance to viral, bacterial, fungal, oomycete and even nematode and insect pathogens with very different lifestyles. Despite this wide range of pathogen taxa and their pathogenicity effector molecules, *R* genes encode only five classes of proteins (Dangl and Jones, 2001). The largest class of *R* genes encodes a 'nucleotide binding site plus leucine-rich repeat' (NB-LRR) class of proteins. NB site is critical for ATP and GTP binding whereas LRR motifs would be involved in interactions like protein-protein, peptide-ligand and protein-carbohydrate. Effectors that enable pathogens to overcome PTI are recognized by specific disease resistance (R) genes. Once the released effector is

recognized by a corresponding NB-LRR protein, effector triggered immunity (ETI) ensues (Jones and Dangl, 2006). ETI is a faster and stronger version of PTI that often culminates in hypersensitive response (HR) leading programmed cell death (Greenberg and Yao, 2004). HR typically does not extend beyond the infected cell; it may retard pathogen growth in some interactions, but is not always observed.

1.2.2 Systemic acquired resistance (SAR) and Induced systemic resistance (ISR)

Plants actually have a number of defense mechanisms and one of these is systemic acquired resistance (SAR). SAR is the induction of broad-spectrum disease resistance in uninfected distal tissues activated by local pathogen attack. It is associated with accumulation of pathogenesis-related (PR) proteins and hypersensitive response, which are thought to contribute to plant resistance against bacteria, fungi and viruses (Ward et al., 1991). These PR proteins, some of which have been proven to have anti-microbial activity, remain within the plant to help protect it from further infection. After the PTI suppression by successful pathogens, ETI triggers salicylic acid (SA) biosynthesis and signaling, leading to local and systemic acquired resistance against pathogens (Delaney et al., 1994; Métraux et al., 1990). It appears that salicylic acid (SA) plays a key role in the long distance signaling, which occurs via the nutrient transport system or phloem and is also believed to activate the plant genes specific to the SAR response.

It was observed that nonpathogenic organisms like rhizobacteria can also induce a systemic resistance in plants that is phenotypically similar to pathogen induced SAR and is known as induced systemic resistance (ISR) (van Loon et al., 1998). It is independent of salicylic acid, but involves jasmonic acid and ethylene signaling and also accompanied by the expression of sets of genes distinct from the PR genes **(Figure 1.4)**. Despite these differences, both SAR and ISR depend on NPR1 (Pieterse et al., 1998). ISR is found to be effective under field conditions and offers a natural mechanism for biological control of plant diseases.

1.2.3 Hormonal interplay in biotic stress

Different hormone signaling pathways are triggered by PTI and ETI separately. As discussed earlier, ETI induces SA biosynthesis and associated signaling pathways. Conversely, PTI stimulates ethylene (ET) biosynthesis (Felix et al., 1999). Jasmonic acid and

ethylene synergistically act against the necrotrophic pathogens in plant defense (Thomma et al., 1998). These JA and ET inducible defense responses might be similar to non-necrotizing pathogens or ISR (Choudhary et al., 2007). It was observed that ethylene and jasmonic acid have a positive effect on the action of salicylic acid, whereas salicylic acid seems to have a negative effect on jasmonic acid and ethylene-inducible defenses (Pieterse and van Loon, 1999) **(Figure 1.5)**. Wounding also triggers the JA and ET inducible defense response. However, the composition varies from that of induced pathogen infections. During the wound response, JA and ET related signals dominate, while the level of SA does not rise (O'Donnell et al., 1996; Vignutelli et al., 1998; Wasternack and Parthier, 1997). Unlike pathogen attack and wounding, the ISR response seems to be associated with an increase in sensitivity to jasmonic acid and/or ethylene rather than an increase in their production, which might lead to the activation of a different set of defense genes (Choudhary et al., 2007).

Pathogens play smart and produce small effector molecule that mimic plant hormones. Some *Pseudomonas syringae* strains make coronatine, a jasmonic acid mimic that suppresses SA-mediated defense response to biotrophic pathogens (Brooks et al., 2005; Melotto et al., 2006) and induces stomatal opening helping pathogenic bacteria gain access to the apoplast (Navarro, 2006). PTI involves repression of auxin responses, mediated in part by a micro-RNA that is also induced during ABA-mediated stress responses (Navarro, 2006). Fungal pathogen *Gibberella fujikuroi* produces Gibberellin by the leading to ‘foolish seedling’ syndrome, and cytokinin produced by many pathogens can promote pathogen success through retardation of senescence in infected leaf tissue. The interplay between normal hormone and PTI signaling, and pathogen mimics that influence it, is just beginning to be unraveled.

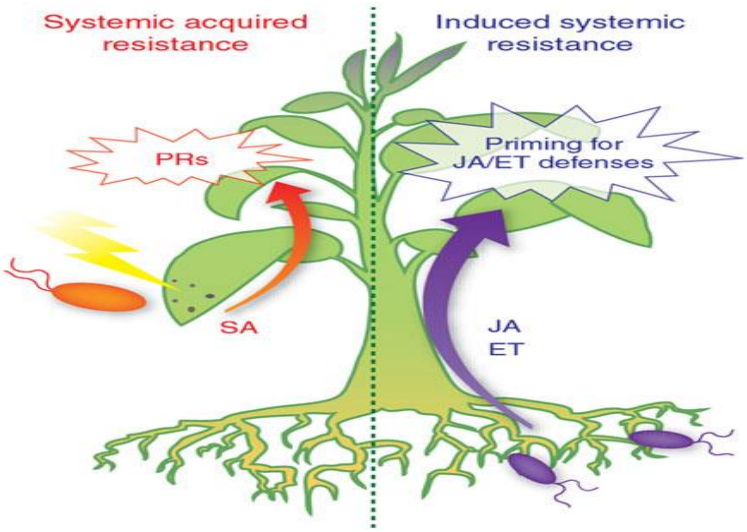

Figure 1.4 Schematic representation of SAR and ISR in plants. (Pieterse et al., 2009)

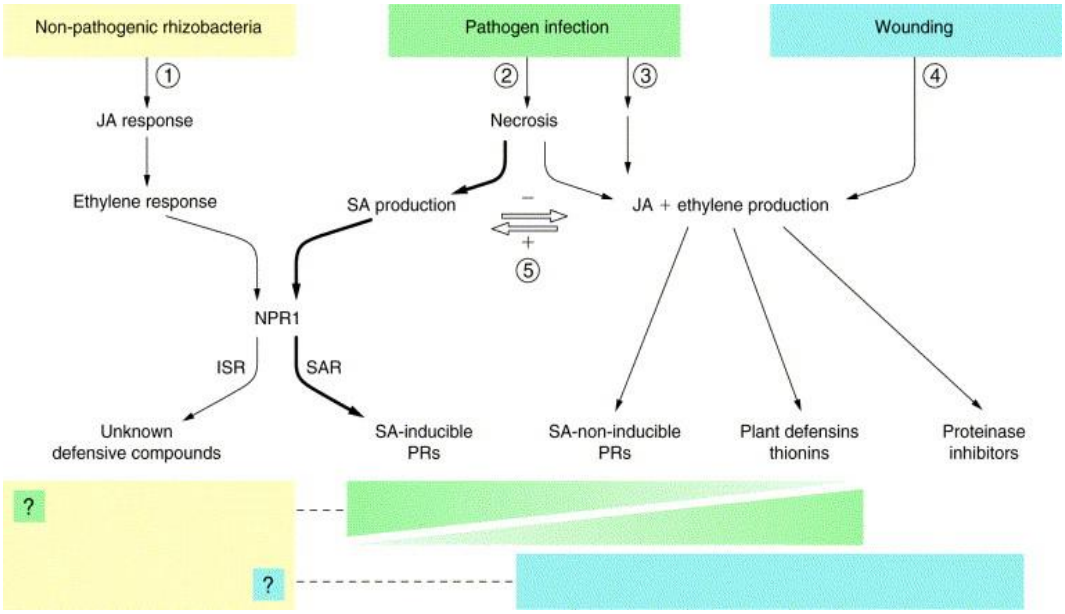

Figure 1.5 Model showing systemic signalling pathways that can be induced in plants by non-pathogenic rhizobacteria, pathogen infection or wounding, such as caused by foraging insects (Pieterse and van Loon, 1999).

1.3 Phenylpropanoid pathway and its role in plant defense

There is a clear distinction between basic and secondary metabolism in plants (Kliebenstein, 2004). Basic metabolism is related to the anabolic and catabolic processes

required for respiration, growth/development and nutrient assimilation, namely those processes required for cell proliferation and maintenance. In contrast, secondary metabolism refers to metabolic intermediates or products, present in specialized cells, found as a differentiation product in restricted taxonomic groups and biosynthesis from one or more general metabolites by a wider variety of pathways than is available in general metabolism. They are not essential for cells survival, but are thought to be important for the plant survival in environment. Highly diverse biological activities have led plants to accumulate a vast number of secondary compounds. The catalogue in vascular plants is at least several hundred thousand secondary metabolites (Wink, 1988).

A large variety of plant secondary metabolites are the compounds containing a phenol group. These phenolic compounds are synthesized via the shikimate and the acetate-malonate pathway. The shikimate pathway participates in the synthesis of most plant phenolics, whereas the malonate pathway is of lesser significance in higher plants, although it is an important source of phenolic products in bacteria and fungi. Most classes of plant phenolic compounds are derived from tyrosine and phenylalanine, and in most plant species the key step of the biosynthesis is the conversion of phenylalanine to cinnamic acid by the elimination of an ammonia molecule. The reaction is catalyzed by an important regulatory enzyme of secondary metabolism, phenylalanine ammonia lyase or PAL (Yao et al., 1995). The functions of phenylpropanoid compounds in plant defense range from preformed or inducible physical and chemical barriers against infection to signal molecules involved in local and systemic signaling for defense gene induction. The relationships among different classes of phenylpropanoids during different stress conditions are outlined **(Figure 1.6)**. Many phenylpropanoids act against broad-spectrum of microbes. Such type of compounds are classified as preformed 'phytoanticipins' or inducible 'phytoalexins' (VanEtten et al., 1994). The best-characterized phenylpropanoid-derived phytoalexins are the isoflavans, pterocarpan, and isoflavanones of legume, including bean, alfalfa, pea, and soybean. Lupin, a prenylated isoflavone which is synthesized during seedling development, is a good example of phytoanticipins (Dixon et al., 2002).

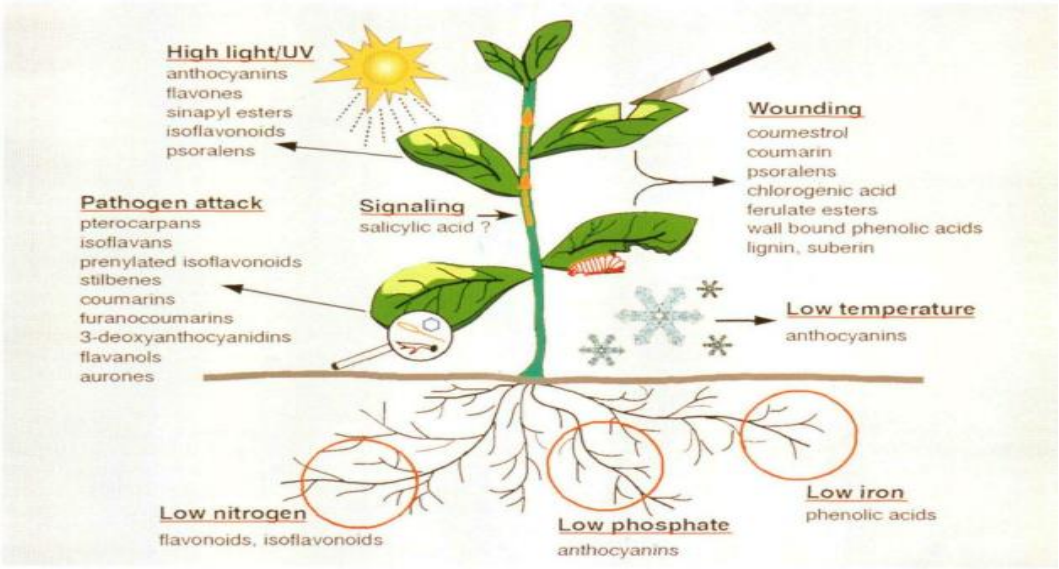

Figure 1.6 Examples of Stress-Induced Phenylpropanoids. (Dixon and Paiva, 1995)

Apart from their defensive functions, phenylpropanoid pathway intermediates can also regulate the expression of the pathways at the transcriptional and posttranslational levels. For example, the biosynthesis of phenylpropanoid compounds increase in cultured pine cells after treatment with a fungal elicitor and have been correlated with an increase in PAL activity (Campbell and Ellis, 1992). Similarly, they also play regulatory roles during the activities of dehydrodiconiferyl glucosides (dimeric monolignol derivatives) and flavonoid glycosides as potential modulators of cell division (Teutonico et al., 1991; Woo et al., 1999). In contrast, plant pathogens have also developed various mechanisms to avoid or destroy these defensive methods used by plants. For example, several plant pathogenic fungi detoxify the host phenylpropanoid derivatives by utilizing cytochrome P450 enzymes that are encoded by genes on supernumerary or 'dispensable' chromosomes (Covert et al., 1996; Wasmann and VanEtten, 1996). These observations direct towards the need for further discussion about plant-microbe interaction and their importance in plant protection.

1.4 Legume crops and their significance

Legumes are among the three largest families of flowering plants, with a long history of use in agriculture. They are the Fabaceae or Leguminosae family members, which

include all types of peas and beans as well as peanuts, soybeans, alfalfa, and clover. It is a large and widely distributed family and also includes various trees and ornamentals such as black locust, wisteria, lupine, and the Texas bluebonnet. The Fabaceae family has been mainly divided into three subfamilies: Caesalpinieae, Mimosoideae, and Papilionoideae (Doyle and Luckow, 2003). The latter exhibits four important clades, which include most of the economically important food and feed legumes. Examining the reproductive structure is the most dependable way to identify and recognize these subfamilies. By whatever criteria are used to measure evolutionary success, the legume family with 670 to 750 genera and 18,000 to 19,000 species of legumes is one of the most successful lineages of flowering plants (Pohill et al., 1981).

In general, legumes have unusual flower structure, podded fruit, and the ability of 88% of the species to form nodules with rhizobia (De Faria et al., 1989) and are second only to the Graminiae in their importance to human uses. Grain legumes provide about one third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Graham and Vance, 2003). The seeds of grain legumes contain around 20% to 40% of protein. In many places of the world, legumes complement cereals or root crops, the primary source of carbohydrates, in terms of amino acid composition. Whereas cereal seed proteins are deficient in Lys, legume seed proteins are deficient in sulfur-containing amino acids and Trp (Wang et al., 2003). Probably, this is the condition why in most places of crop domestication, legumes and cereals have been domesticated together (Gepts, 2010). Legumes are sources of essential minerals required by humans and also produce health promoting secondary metabolite compounds that can protect against human cancers (Gepts et al., 2005). Legume compounds like isoflavans, isoflavanones and nitrogenous metabolites such as alkaloids, terpenoids etc. serve as defense molecules against the pathogens and pests (Dixon et al., 2002; Ndakidemi and Dakora, 2003).

The yield potential of legume crops is seldom achieved due to major constraints like diseases, insect pests, and weeds (parasitic and non-parasitic) along with abiotic stresses. A trademark trait of legumes is their ability to develop root nodules and to fix N_2 in symbiosis with rhizobia. It has an amazing effect on natural and agricultural ecosystems. Many aspects of plant-bacterium recognition, nodule formation, nitrogen fixation, and ammonia assimilation have been explained. However, still less well understood is the mechanisms of

bacterial colonization without triggering plant defense responses. There are a few reports indicating that both rhizobia and successful pathogens are able to suppress plant defenses to establish an infection. But the compounds like isoflavonoid are important in stopping the infection by the pathogens and also involved in controlling nodule number (Samac and Graham, 2007). Apart from that, it has also been suggested that LysM domains and GRAS transcription factors are important in a legume plant for the recognition of both friends and foes (Kaku et al., 2006; Kalo et al., 2005). We are on the verge of understanding how these two complex pathways interact and possibly more efforts towards the functional characterization of the candidate genes may unravel the better picture in future.

1.5 Peanut

Human diet is a mixture of a variety of macro and micronutrients with several types of vitamins, antioxidants, minerals and other beneficial phytochemicals. They all are necessary for maintaining a proper healthy life. The macronutrients are sources of different kinds of proteins, fats (lipids) and carbohydrates. However, a healthy supply of macronutrients would also contain the necessary micronutrients. Lipid is an undesirable part of the diet with some important physical, chemical, and nutritional properties. It remains an essential requirement. Awareness that both the quantity and the quality of the fat consumed are important elements of a healthy diet is the main challenge of this highly developed world.

Arachis hypogaea, also known as peanut, is an important food crop of the tropical and sub-tropical world. This genus is endemic to South America (Bertioli et al., 2011). The species of this genus are diverse in habitat, including open patches of forest, grasslands and in temporarily flooded areas. They produce fruit below the ground which is unusual when compared with other legumes. As a rich source of energy (564 kcal 100 g^{-1}), peanut contains about 48–50% oil, 25–28% proteins and 20–26% carbohydrates. The kernels of peanut also contain many health nutrients such as seven of the 20 essential minerals; 13 essential vitamins particularly vitamin E, niacin, folic acid and antioxidants (Bishi et al., 2015). Dietary biologically active compounds like polyphenolics, flavonoids, and isoflavones (p-coumaric acid and resveratrol) are also present in peanut. The useful effects of phenolic compounds have been attributed to their antioxidant capacity (Heim et al.,

2002). The interest in the naturally occurring antioxidants is increasing as they are natural compounds and due to the plant origin, they are presumed to be safe for human consumption. In addition to that, peanuts are rich source of mono-unsaturated fatty acids and do not contains trans-fatty acids, one of the reasons for obesity (Gunstone, 2011; Sanders, 2001). Apart from eliciting several biological effects such as weight-loss, consumption of peanuts is also helpful in prevention of cardiovascular diseases by lowering blood pressure and blood cholesterol levels, protection against Alzheimer disease, anti-inflammatory effects, and inhibition of cancer (Heim et al., 2002). Hence, peanut is gaining importance as a functional food besides being an oilseed crop. In North and South Americas as well as in Europe, about 75% of the peanut production is used as a foodstuff (Birthal et al., 2010). In India too, due to availability of other edible oils at economical prices, the direct consumption of peanut has been growing and its food value is being increasingly realized. During the 1980s, only 6% of the total peanut produced was used for direct consumption whereas 81% was crushed for oil expulsion. In recent years, however, nearly half of the produce in India is used for direct consumption as value-added products like roasted peanuts, roasted and salted peanuts, boiled peanuts, peanut-butter, peanut-candy (Govindaraj and Jain, 2011).

1.5.1 Constraints and improvement approaches of Peanut production

High temperature and drought are the most important abiotic stresses in peanut-growing areas. Apart from this, peanut is also attacked by pathogens like fungi, bacteria, viruses, nematodes, and a mycoplasma, which affect its potential yield (Wynne et al., 1991). Diseases of the peanut now occur throughout the growing season and in the postharvest period, and also attack all parts of the plant. Among the fungal diseases, early leaf spot (ELS) caused by *Cercospora arachidicola*, late leaf spot (LLS) caused by *Phaeoisariopsis personata*, and rust caused by *Puccinia arachidis* have worldwide occurrence and are of great economic importance. Another fungus *Sclerotium rolfsii,* causes stem and pod rot and is a serious threat to peanut production in many warm and humid areas. Though peanut is also a host to many virus diseases, only a few of them are economically important, tomato spotted wilt virus (TSWV) in the USA, peanut bud necrosis disease (PBND) in India, peanut stem necrosis disease (PSND) in pockets in Southern India, peanut stripe potyvirus (PStV) in East and South East Asia and peanut clump virus disease

(PCVD) in West Africab (Nigam et al., 2012). Among bacterial diseases, Bacterial wilt, caused by *Ralstonia solanacearum*, is predominant. Globally around 11.8% yield loss in peanut caused by nematodes. The lesion nematodes, *Pratylenchus* spp. and root-knot nematodes, *Meloidogyne* spp. are important in peanut (Sharma and McDonald, 1990). Aphids (*Aphis craccivora* Koch), several species of thrips (*Frankliniella schultzei*, *Thrips palmi*, and *F. fusca*), leaf miner (*Aproaerema modicella*), jassids (*Empoasca kerri* and *E. fabae*), red hairy caterpillar (*Amsacta albistriga*) and *Spodoptera* are the major insect pests in peanut, among which aphids, thrips, and *Spodoptera* have global distribution and can cause serious crop losses (Wightman and Amin, 1988). These aphids and thrips are also vectors of important viral diseases. White grubs, termites, and storage pests also cause damage to peanut. Rust-red flour beetle (*Tribolium castaneum*) and peanut borer or weevil (*Caryedon serratus*) are the major storage insect pests in peanut.

Based on sexual compatibilities and morphology, the genus *Arachis* has been subdivided into 80 species and 9 infrageneric taxonomic sections. Cultivated peanut is an allotetraploid (2n = 2x = 40) with “AA” and “BB” genomes. The diploid progenitors, *A. duranensis* and *A. ipaensis* contributed “AA” and “BB” genomes, respectively to the cultivated peanut (Kochert et al., 1996). Some 3500 years ago, a single hybridization event between the diploid progenitors followed by chromosome doubling lead to the origin of cultivated peanut (Kochert et al., 1996). Molecular analysis has revealed that this crop has a narrow genetic base (Halward et al., 1992; Hopkins et al., 1999) and lack of variability in some important traits.

Wild relatives exhibit high level of resistance/tolerance to the various stresses compared to the highly susceptible cultivated peanuts (Pande and Rao, 2001; Rao et al., 2003). Hence, they are assumed to be an important source of genes for resistance to the stresses for peanut improvement. Most of the wild species are diploid however, they are usually agronomically inferior to the modern cultivars (Dwivedi et al., 2003). Due to the difference in ploidy level, the cultivated peanut is sexually isolated from its wild relative and hence, the introgression of genes from wild relatives is only possible through complex breeding procedures or genetic transformation. Breeding procedures are highly laborious and time taking. Apart from that, many of the wild varieties are not cross compatible with

cultivated ones causing the interspecific progenies to carry a lot of unwanted characters as linkage drag.

Several of the wild varieties of *Arachis* show high level of resistance to ELS and LLS, but the progress of resistance breeding in cultivated peanut has been limited due to the linkage of undesirable traits. Transgenic techniques are well established now; hence genetic transformation of cultivated peanut would be the best option to incorporate the resistance genes from wild species for crop improvement. *Arachis diogoi* (syn. *Arachis chacoense*) is a diploid wild relative of *Arachis hypogea* L. It has been confirmed that *A.diogoi* is highly resistance to several fungal and viral pathogens **(Table 1.1)** and an important source of genes peanut improvement (Rao et al., 2003; Subrahmanyam et al., 2001; Subrahmanyam et al., 1985). Several genes were identified from *A. diogoi* during its interaction with LLS pathogen *Phaeoisariopsis personata* by using DDRT-PCR (Kumar and Kirti, 2011) and AFLP method (Kumar and Kirti, unpublished). During the DDRT-PCR study, a total 60 upregulated cDNAs were cloned and sequenced in early response to fungal inoculation whereas during the AFLP study a total 233 differentially expressed TDFs were selected from both early and late response to fungus, of which 125 were upregulated, 64 downregulated and 44 point expressed.

Table 1.1 Resistance of *A. diogoi* against various pathogens (Rao et al., 2003).

Resistance (+) to pathogens and pest identified in wild *Arachis* species.

Species	Pathogen or pest													
	RUS	LLS	ELS	PSV	GRV	PMV	TSWV	PBNV	PBV	APH	MIT	THR	JAS	RN
A. appressipila	+	+	+		+			+						
A. batizocoi	+													
A. benthamii				+										
A. benensis								+						
A. cardenasii	+	+			+	+	+	+				+	+	
A. correntina	+					+	+			+	+	+	+	
A. diogoi	+	+			+	+	+			+		+		
A. dardani			+											
A. decora					+									
A. duranensis	+			+								+	+	
A. glabrata	+	+		+		+			+	+		+	+	
A. hagenbeckii	+	+											+	
A. helodes														+
A. hoehnei					+									
A. kuhlmannii					+									+
A. kretschmeri					+									+
A. magna			+											
A. paraguariensis		+										+		
A. pintoi					+									
A. pusilla	+		+			+	+					+	+	
A. repens		+		+								+	+	

RUS = Rust, LLS = late leaf spot, ELS = Early leaf spot, PSV = Peanut Stunt Virus, GRV = Groundnut rosette virus, PMV = Peanut Mottle virus, TSWV = Tomato spotted wilt virus, PBNV = Peanut Bud Necrosis Virus, PBV = Peanut web blotch, THR = Thrips, APH = Aphids, MIT = Mites, JAS = Jassids.

1.6 Objectives of present thesis work

In the present study, based on DDRT-PCR and AFLP results, we have selected for characterization of three different genes of *A. diogoi*, which were found to be upregulated during the *Phaeoisariopsis personata* infection. These three different genes were named based on and their sequence similarity with other homologs. They are;

1. Thaumatin-like protein (*AdTLP*).
2. CBL interacting protein kinase (*AdCIPK*).
3. Cytochrome P450 monooxygenase (*AdCYP97A3*).

Chapter 2:

General Materials and Methods

2.1 Plant Materials

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

2.2 Chemicals

All the chemicals used in the present study were procured from Sigma-aldrich, USA; Fermentas, Germany, Himedia chemicals, India and Qualigens fine chemicals, India.

2.3 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.

2.4 Plasmid and DNA vectors

- pTZ57R/T (MBI Fermentas, Germany) used for cloning all PCR products.
- pRT100 is a plant expression vector for cloning complete ORFs, to obtain ORF flanked by 35S promoter and poly-adenylation signal.
- pCAMBIA2300 is a binary vector for plant transformation.
- pEGAD and pCAMBIA1302 vector are used for translational fusion with GFP.
- pET32a (Novagen, USA), a bacterial T7 polymerase expression vector is used for expressing the recombinant protein as a fusion to Thioredoxin-Histidine (TRXHIS) tag.

2.5 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 ^{o}C on a rotary shaker at 200 rpm. From the overnight culture, 0.5 mL was taken and reinoculated 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 ^{o}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 $CaCl_2$ solution and was stored on ice for 10 min. The cells were again centrifuged at 4 ^{o}C and 5000 rpm for 10 min, and the pellet was resuspended in ice cold 0.5 mL of 0.1 M $CaCl_2$. These competent cells were quick frozen and stored at -70 ^{o}C after adding 50% sterile glycerol in batches of 100 µl.

2.6 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 ^{o}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 ^{o}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 ^{o}C for the appearance of colonies. Plates with visible colonies were stored at 4 ^{o}C in a refrigerator for four wks as the cells stay viable for one month at this temperature. Plasmid DNA was isolated from the colonies to check and confirm the transformation.

2.7 Plasmid isolation (miniprep) from *E. coli* (Sambrook and Russell, 2001)

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 ^{o}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 ^{o}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 ^{o}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 ^{o}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 ^{o}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 ^{o}C for 30 min and the DNA was collected at the bottom of the tube by centrifuging at 4 ^{o}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 ^{o}C. Plasmid isolations were also carried out using kit (Sigma-aldrich, USA) following the manufacturer's instructions.

2.8 *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 ^{o}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 ^{o}C water bath for 5 min. To this, upto 1 mL of LB medium was added and incubated at 28 ^{o}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 ^{o}C after adding sterile 50% glycerol.

2.9 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 $3/4^{th}$ of the length of the gel. A molecular weight marker was loaded along with the samples for reference.

2.10 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.

2.11 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 ^{o}C for 30 min, followed by heat inactivation at 85 ^{o}C for 15 min.

2.12 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 ^{o}C and blunt end ligation at 22 ^{o}C overnight.

2.13 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 wk to 10 d, they were transferred to green house for further growth.

2.14 RNA isolation

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

2.15 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 ^{o}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 ^{o}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 ^{o}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 ^{o}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 ^{o}C. The pellet was washed with 70% ethanol, dried and dissolved in TE. Genomic DNA samples were stored at –20^{0}C for long-term use.

2.16 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).

2.17 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.

Chapter 3:

Thaumatin-Like Protein (*AdTLP*)

3.1 Background

Plants defend themselves against different kinds of environmental stresses by well-established defense mechanisms. Activation of different pathogenesis related (PR) protein genes is one of them. These PR proteins have been classified into 17 different families based on their structure, amino acid sequences and mode of action (Van Loon et al., 2006).

Members of PR-5 class proteins are also called thaumatin-like proteins (TLPs) because of their sequence similarity with the sweet tasting protein thaumatin from *Thaumatococcus danielli* (Cornelissen et al., 1986). TLPs are reported to be widely distributed PR proteins across kingdoms including gymnosperm, angiosperm, animal and fungal systems (Liu et al., 2010). Molecular mass of TLPs ranges between 21 to 26 kDa with a thaumatin family signature G-X- [GF]-X-C-X-T- [GA]-D-C-X- (1, 2)-G-X-(2, 3)-C (Tachi et al., 2009). In general, TLPs have 16 conserved cysteine residues that are involved in the formation of eight disulfide linkages, which impart stability to the protein under varied thermal and pH conditions (Fierens et al., 2009; Velazhahan et al., 1999).

In vitro antifungal activity of TLPs has been reported against a variety of filamentous fungal pathogen such as *Botrytis cinerea, Fusarium oxysporum*, *Mycosphaerella arachidicola*, *Trichoderma viride* (Chu and Ng, 2003; Garcia-Casado et al., 2000; Ho et al., 2007). Osmotin like protein from black nightshade, *Solanum nigrum* also showed inhibitory effect on the growth of *Rhizoctonia batiticola* and *Sclerotinia sclerotiorum* (Jami et al., 2007). TLPs possess the capacity to rupture the fungal membrane by pore formation (Roberts and Selitrennikoff, 1990). In addition, they can also affect the fungus growth with β -glucanase and xylanase inhibitor activities (Fierens et al., 2007; Grenier et al., 1999). Transgenic plants overexpressing TLP genes have shown enhanced resistance and protection against different fungal pathogens (Fu et al., 2005; Mackintosh et al., 2007). Some TLPs have also been reported to be involved in anti-freeze activity (Hon et al., 1995) and developmental processes like fruit ripening (Pressey, 1997).

Through a differential display approach (DDRT-PCR), we have earlier identified several genes that get significantly upregulated in *Arachis diogoi* upon treatment with *Phaeoisariopsis personata* (Kumar and Kirti, 2011) . One of the upregulate genes, designated as AdDR-11 in the wild peanut was identified to encode a putative thaumatin-like protein and named as *AdTLP*. Full length gene was amplified, cloned and sequenced

(Kumar, 2008). In the present study, we analyzed the transcriptional regulation of *AdTLP* in response to various treatments and its subcellular localization by translational fusion with GFP. *In vitro* and *in vivo* antifungal activity of AdTLP protein was analyzed against different fungal pathogens, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium solani and Rhizoctonia solani*. Abiotic stress assays were also carried out to observe the efficacy of AdTLP in alleviating stress caused high NaCl and H_2O_2 conditions. In addition to this, transcript levels of various defense responsive genes were checked in wild type and transgenic plants.

3.2 Materials and methods

3.2.1 Plant treatments

For different treatments, detached leaves of *A. diogoi* were utilized and the experiments were performed essentially as described earlier (Kumar and Kirti, 2011). In brief, for hormone treatments, concentrations of 500 µM salicylic acid (SA), 100 µM methyl jasmonate (MeJA), 100 µM abscisic acid (ABA) and 250 µM ethephon were utilized. Samples were collected at regular intervals, quick-frozen in liquid nitrogen, and stored at -80 ^{o}C till further use.

3.2.2 Sequence analysis

The cDNA sequence data were analyzed using BLASTn and BLASTp at NCBI website (http://www.ncbi.nlm.nih.gov). Nucleotide translations were performed using translate tool at ExPASy (http://www.expasy.ch/). Multiple sequence alignment and phylogenetic tree were done using ClustalW (www.ebi.ac.uk) and MEGA 4.0.2 software respectively.

3.2.3 Semi-quantitative RT-PCR

First strand cDNA was synthesized by reverse transcribing RNA (500 ng) using MMLV reverse transcriptase (Sigma-Aldrich). Subsequent PCR reactions were performed using the first strand cDNA as template in the presence of gene specific primers ORF-F and ORF-R. PCR reactions were standardized empirically to keep the amplification in linear range. For all PCR reactions *Taq* DNA polymerase (Sigma-Aldrich) was used. Actin was used as internal control. All the primer details were provided in **Table 3.1.**

3.2.4 Homology modeling

A BlastP search of the TLP protein against Protein Structure Databank (PDB) available at www.rcsb.org/ was carried out to identify all proteins in PDB that share sequence similarity and therefore would have similar structures. The sequence alignment thus produced was used to build the 3-D model structure of the TLP protein using "build homology model" protocol under "protein modeling" module in Discovery Studio-2.1 (Accelerys Inc, USA) that implements the methodology described in MODELLER (Šali and Blundell, 1993). MODELLER is a homology or comparative modeling program for constructing the 3-D model of a protein structure from its amino acid sequence. It is based on the alignment between the sequences to be modeled with the sequence of known template structure. The models were validated using Ramachandran plot (Ramachandran et al., 1963) available at http://nihserver.mbi.ucla.edu/SAVES/ and Verify_3D (Liithy et al., 1992) available at http://nihserver.mbi.ucla.edu/SAVES/. Ramachandran plot evaluates the stereochemical geometry and quality of a protein structure model using the automated method PROCHECK (Laskowski et al., 1993). Verify_3D measures the compatibility of the protein 3-D structure with its own amino acid sequence

3.2.5 AdTLP subcellular localization

The ORF of *AdTLP* was amplified using gene specific primers ORF-F1 and ORF-R1 harboring *Apa*I and *Bam*HI restriction sites respectively with a proofreading polymerase. The amplification product was digested with *Apa*I and *Bam*HI and cloned into pRT-GFP vector (Kumar and Kirti, 2011) using the same set of enzymes to generate 35S:AdTLP:GFP. The AdTLP:GFP expression cassette was released using *Sph*I and further sub-cloned into *Hin*dIII site of binary vector pCAMBIA1300 after end filling both the sites, which resulted in AdTLP:GFP:1300. The recombinant binary vector was mobilized into *Agrobacterium* strain LBA4404 using freeze thaw method. Similarly, empty vector pEGAD for the expression of free GFP was also mobilized into the *Agrobacterium* strain.

3.2.6 Agroinfiltration and microscopy

Agroinfiltration was performed essentially as described earlier (Kumar and Kirti, 2011). In brief, agrobacterial strains harboring the free GFP and recombinant AdTLP-GFP vectors were grown in LB medium in the presence of appropriate antibiotics. The

agrobacterial cultures were pelleted and resuspended in the infiltration medium (10 mM MES, 10 mM $MgCl_2$ and 200 µM acetosyringone) adjusted to an OD_{600} of 0.8. Agrobacterial suspensions were then infiltrated into the adaxial side of the tobacco leaves using a needless syringe. GFP expression was visualized in infiltrated leaves 48-96 h post infiltration (hpi) at different time intervals with laser scanning confocal microscopy (Leica TCS SP2 with Leica DM6000 microscope).

3.2.7 Expression and purification of AdTLP protein

The 726 bp open reading frame was amplified using ORF-F and ORF-R primers with *Bam*HI and *Xho*I sites, digested and cloned into the corresponding sites of pET32a(+) expression vector (Novagen Corporation, USA). The cloned vector was transformed into cells of *E.coli* Rosetta gami-2 (DE3) strain and the transformed cells were grown at 37 ^{o}C in Luria and Bertani (LB) broth (Himedia, India) with antibiotics chloramphenicol (25 µg mL^{-1}) and ampicillin (100 µg mL^{-1}) overnight at 200 rpm in an orbital shaker. A 10 mL aliquot from the overnight grown culture was added to 1 L of fresh LB medium and grown further at 37 ^{o}C until the OD_{600} reached 0.5-0.6. Then, these cells were induced to express recombinant protein by adding 1 mM IPTG (Fermentas, Germany) for 4 h. Following the induction, the induced cells were harvested by centrifugation at 5000 rpm for 10 min at 4 ^{o}C. Recombinant proteins were exclusively found in inclusion bodies, which was confirmed by resuspending 2 mL culture pellet in lysis buffer (200 mM Tris-cl pH 7.5, 10 mM EDTA, 1% Triton X-100) followed by sonication and SDS-PAGE analysis. To purify the protein from inclusion bodies, pelleted cells from 1 L culture were resuspended in lysis buffer (8 M urea, 10 mM imidazole, 0.1 M $NaHPO_4$, 0.01 M Tris base pH 8) and sonicated. The lysate was centrifuged at 12,000 rpm for 20 min to remove the debris. The expression of the fusion protein with the N-terminal fusion partners, Thioredoxin-His-S-tag linked to the target peptide (AdTLP) and purification was done by IMAC (immobilized metal ion affinity chromatography) using the His-Tag of the fusion protein. Supernatant was mixed with 2 mL of equilibrated Nickel-NTA resin and added into the column. The flow through was collected and washed with 10 volumes of wash buffer (8 M urea, 50 mM imidazole, 0.1 M $NaHPO_4$, 0.01 M Tris base, pH 6.3). Finally the recombinant peptide was collected with elution buffer (8 M urea, 250 mM imidazole, 0.1 M $NaHPO_4$, 0.01 M Tris base, pH 4.5). Dialysis was conducted for protein renaturation by gradually adding renaturation buffer

(Urea, 0.1 M NaH_2PO_4, 0.01 M Tris base, pH 7.0) containing decreasing concentration of urea from 6 M to 1 mM. Then, Tris buffer (0.01 M) alone was used five times with an interval of 1 h. Finally, the sample was dialysed overnight in the same buffer at 4 ^oC. The purified protein was resolved to 12% SDS-PAGE. Protein concentration was measured using Bradford method.

3.2.8 Antifungal activity assay

Antifungal activity of the recombinant AdTLP (rAdTLP) protein was tested by microspectrophotometry as well as in vitro plate assay with the fungal pathogens, *Fusarium oxysporum, Fusarium solani, Botrytis cinerea, Rhizoctonia solani.* For microspectrometry analysis, a standard method as described earlier (Song et al., 2005; Vijayan et al., 2008) was followed. Briefly, 10 µl of protein, diluted to different concentrations was pipette into the wells of a 96-well microtiter plate containing 140 µl of test fungal spore suspension (~3.0× 10^4 spores/ mL) in potato dextrose broth, which was placed in an incubator at 28^o C. Antifungal activity of each concentration of protein was performed in triplicates. Fungal spore germination was observed microscopically, whereas optical density at 595 nm wavelength was measured to check the spore growth after inoculation for 30 min and 48 h. Controls that were devoid of the test protein were tested for comparing the antifungal activity of the rAdTLP. Values of growth inhibition less than 10% were not considered as significant. Growth inhibition is defined as the ratio of the corrected absorbance at 595 nm of the control minus the corrected absorbance of the test sample, divided by the corrected absorbance of the control. The corrected absorbance is defined as the absorbance at 48 h minus that at 30 min. IC_{50} is defined as the protein concentration at which 50% inhibition was reached (Vijayan et al., 2008).

For the *in vitro* plate assay, fungal discs of uniform size were inoculated at the centre of the Potato Dextrose agar media and incubated at 28^o C. When the mycelial spread reached 4 cm in diameter, four sterile Whatman no.1 filter paper discs of equal size were placed at equal distance from centre. Purified protein was added at various concentrations (ranging from 10-50 µg/ mL) at the centre of disc on the plate. The elution buffer served as control and the plates were incubated at 28 ^oC. Growth of mycelia was observed periodically till they covered the control discs. A graph was plotted showing

percentage inhibition of fungal growth against the concentration of protein to determine the IC_{50} for *Rhizoctonia solani*.

3.2.9 β-1, 3 Glucanase assay

β-1, 3 Glucanase assay was done as described earlier (Looze et al., 2009) with some modifications. The activity of rAdTLP proteins was analyzed by mixing 50 µg of protein sample with 100 µL of 50 mM acetate buffer (pH 5.0) containing 1% Laminarin (Sigma). The mixture was incubated at 37 ^{o}C for different time periods ranging from 30 min to 3 d. Absorbance was measured at 595 nm. Increase in absorbance indicates β-1, 3 glucanase activity.

3.2.10 Agrobacterium mediated Tobacco transformation with 35s-AdTLP construct

The *AdTLP* ORF was reamplified using ORF-F2 and ORF-R2 primers having *Apa*I and *Sma*I sites respectively, digested and cloned in pRT100 vector. Due to presence of internal sites, partial digestion was performed with *Hin*dIII enzyme for releasing ~1.5 Kb cassette containing the CaMV35S promoter and polyadenylation signal from pRT100 vector along with the *AdTLP*. The *AdTLP* expression cassette was further cloned in the binary vector pCAMBIA2300 at the *Hin*dIII site in the multiple cloning site region. Tobacco (*Nicotiana tabacum* cv Xanthi) was transformed as described in **Method 2.13**, using *Agrobacterium tumefaciens* strain EHA105 carrying the binary vector pCAMBIA2300-AdTLP and the transformants were selected on 125mgL^{-1} kanamycin. T_0 putative transgenic were analyzed by polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) for the target gene. Among seven different transgenic plants analyzed, progenies of the primary transgenic plants #4 (low expression *AdTLP* plant) and #7 (high expression *AdTLP* plant) were taken for further analysis. T_1 and T_2 seeds were raised via self-pollination and T_2 seeds were used in functional characterization.

3.2.11 Evaluation of transgenic plants for resistance against fungal pathogen

Root bioassay using the fungal pathogen, *Rhizoctonia solani* was carried out to check the resistance in the transgenic plants expressing *AdTLP* constitutively against the root rot causing fungal pathogen. At maturity, seeds from transgenic plants were collected and germinated for T_2 generation analysis. Seeds from line #4 and #7 were surface sterilized and grown on half strength MS medium (MSH). After germination, they were transferred

to the cups filled with sterilized vermiculite and soil in 3:1 ratio. Four seedlings were transferred to each cup and allowed to grow further for another 25 d. Control seedlings were transferred simultaneously. Ten sclerotia of equal size were added to each cup and they were maintained under humid condition by covering with polyethylene covers in the growth room. Symptoms started appearing after 5 d and observations were taken after 8 d and 10 d of post infection (dpi).

3.2.12 Abiotic stress assays

Seedlings of transgenic and wild type plants (WT) were selected to analyse the salt (sodium chloride) and oxidative stress tolerance. T_2 transgenic seedlings were grown on half MSH medium (without organics) containing 125mgL^{-1} kanamycin and simultaneously, the WT plants were grown on MSH medium without kanamycin. Twenty seedlings each of WT and kanamycin resistant seedling in T_2 generation (plants #4 and #7) were transferred to different stress treatment plates for each experiment. For salt stress, seedlings were transferred to 100-300 mM NaCl in MSH. For oxidative stress treatment, 2% H_2O_2 in MSH medium was used. Total chlorophyll content was measured spectrophotometrically as described by Arnon (Arnon, 1949). Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) as described by Heath and Packer (Heath and Packer, 1968). In the case of NaCl treatment, both the chlorophyll and TBARS estimation were done after 12 d of treatment and for H_2O_2, after 10 d of treatment.

3.2.13 Expression analysis of other stress related genes

Transcript accumulation for some defense responsive genes was monitored in WT and transgenic plants, using semi quantitative RT-PCR. Leaf samples were collected from two months old plants, quick frozen and stored in -80 ^{o}C. Primer sequences used in this study were provided in **Table 3.2.**

3.3 Results

3.3.1 Multiple sequence alignment and phylogenetic tree

Sequence alignment of deduced amino acid AdTLP with TLPs from other plants showed that there were sixteen cysteine residues involved in the formation of eight disulfide bridges and are conserved across the species **(Figure 3.1)**. The AdTLP protein exhibited 68% sequence similarity with MtTLP from *Medicago trancatula*, 66% to GmTLP from *Glycine max*, and 62% to PpTLP from *Pyrus pyrifolia* resepectively. Among well characterized TLPs, *Arabidopsis thaliana* TLP (AtTLP) and *Nicotiana tabacum* TLP (NtTLP) shared 50% and 47% similarity with AdTLP respectively. Phylogenetic analysis showed that the AdTLP was closely related to a non-leguminous MdTLP (*Malus domestica*), as well as leguminous MtTLP (*Medicago trancatula*) and GmTLP (*Glycine max*) **(Figure 3.2)**.

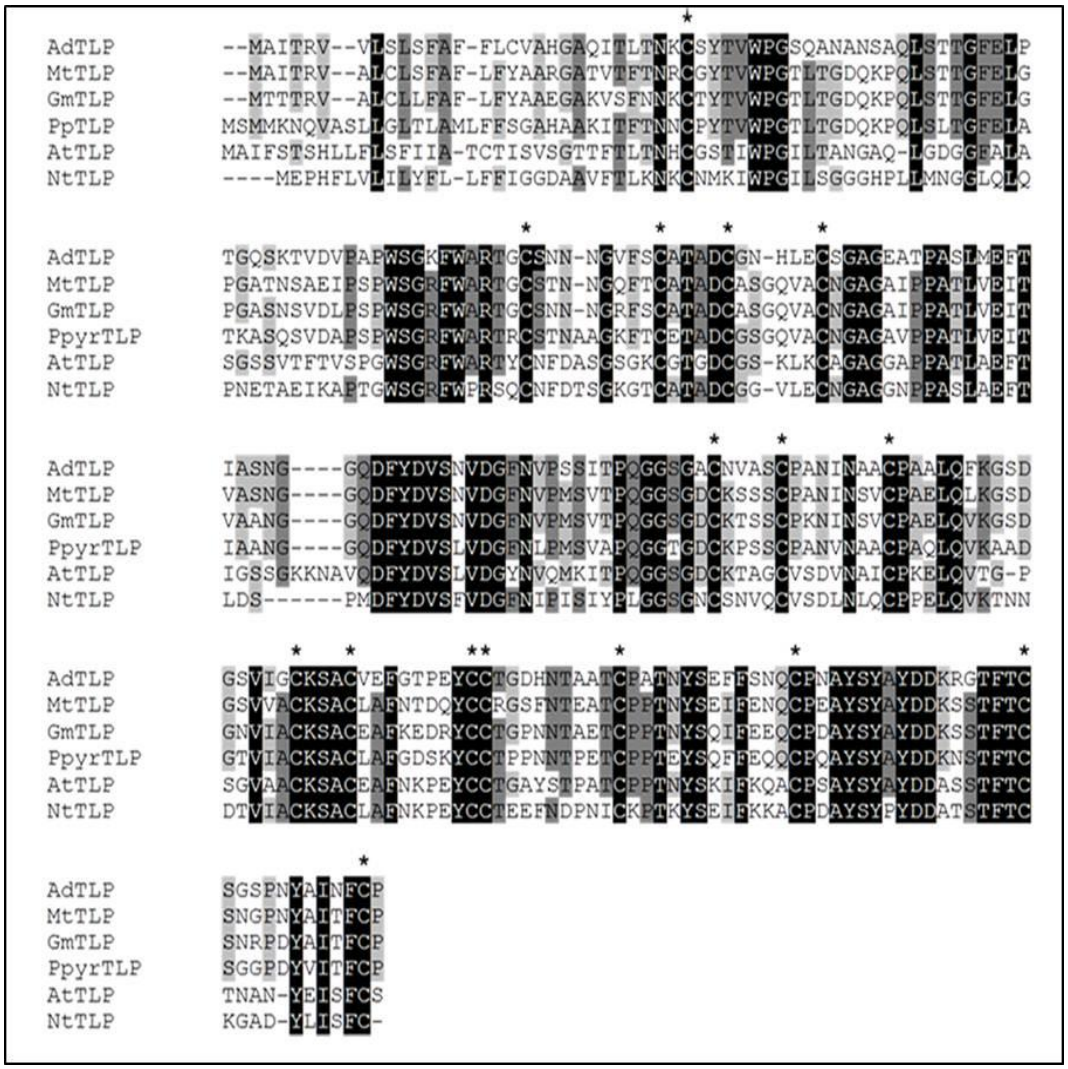

Figure 3.1 Multiple sequence alignment of AdTLP with TLPs from other plant species. The sixteen cysteine residues required for the formation of eight disulfide bridges are conserved in *AdTLP* also and are indicated by asterisk (*). Mt: *Medicago truncatula,* Gm: *Gycine max*, Pp: *Pyrus pyrifolia*, At: *Arabidopsis thaliana*, Nt:*Nicotiana tabacum*.

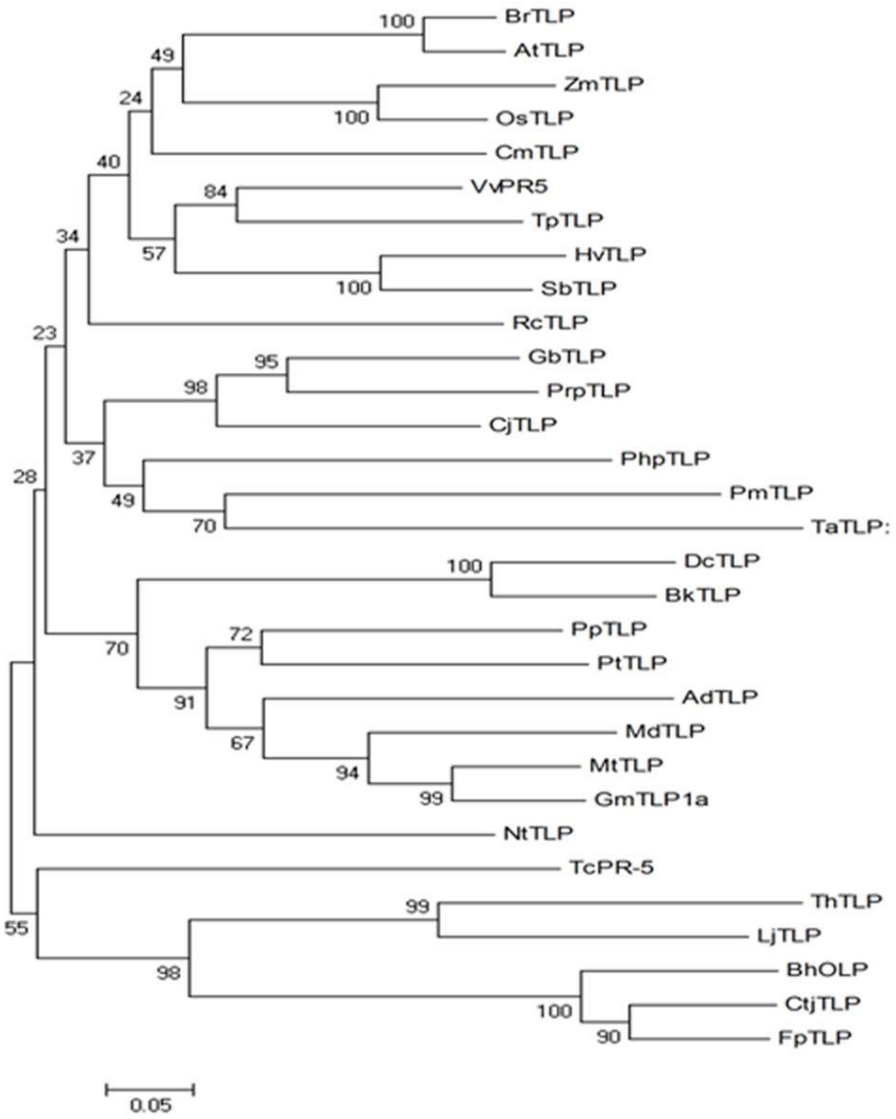

Figure 3.2 Phylogenetic analysis of *AdTLP* with other TLPs. A phylogenetic tree based on genetic distance of the protein sequences was constructed using MEGA 4.0.2 software. Bootstrap values are indicated at the branches. The TLP members used for construction of the tree are listed in the GenBank database under the following accession numbers: *AtTLP* (AAD02499.1)*; BhOLP* (AAD53089.1); *BkTLP* (CBJ55937.1); *BrTLP* (ABV89616.1); *CjTLP* (BAD90814.1); *CmTLP* (ADN33945.1); *CtjTLP* (BAI63297.1); *DcTLP* (AAL47574.1); *FpTLP* (ABB86299.1); *GbTLP* (ABL86687.1); *GmTLP1a* (XP_003535214.1); *HvTLP* (BAJ96850.1); *LjTLP* (AFK33451.1); *MdTLP* (AAC36740.1); *MtTLP* (AFK34461.1); *NtTLP* (BAA74546.2); *OsTLP* (BAD34224.1); *PhpTLP* (XP_001784610.1); *PpTLP* (BAC78212.1); *PmTLP* (ADB97928.1); *PrpTLP* (AEV57470.1); *PtTLP* (XP_002330973.1); *RcTLP* (XP_002519620.1); *SbTLP* (XP_002465570.1); *TaTLP* (AAM15877.1); *ThTLP* (BAJ34394.1); *TpTLP* (BAE71242.1) *VvPR5* (XP_002277548.1); *ZmTLP*(NP_001142502.1)

3.3.2 Transcript expression analysis

Transcript levels of *AdTLP* were analyzed using semi-quantitative RT-PCR in response to pathogen infections and different stress hormones. *AdTLP* ORF-F and ORF-R primers were used to amplify *AdTLP*. Early upregulation of transcripts were observed during SA treatment, which was persistent till 12 hpi whereas they got upregulated at later stages during JA and ABA treatment **(Figure 3.3).**

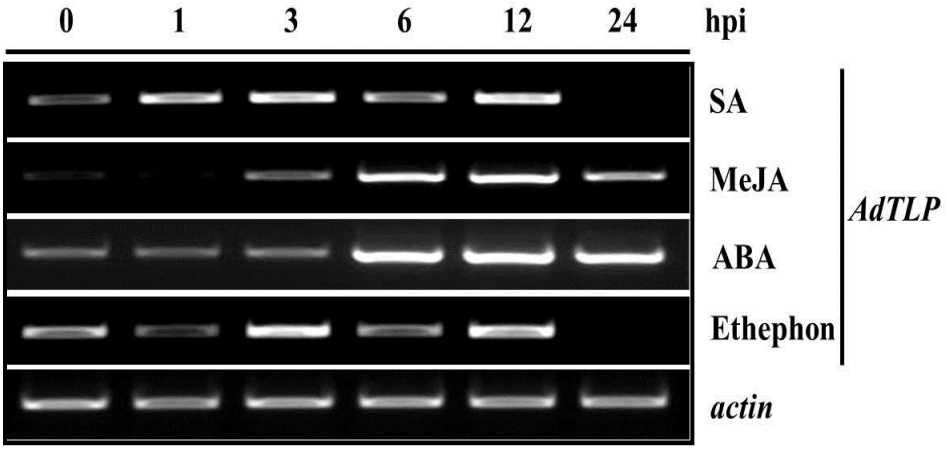

Figure 3.3 Transcript level analysis of *AdTLP* in *A.diogoi*. Transcript level of *AdTLP* in *A.diogoi* was analyzed using semi-quantitative RT-PCR, during various hormones treatments.

3.3.3 Analysis of predicted three-dimensional structure of the AdTLP protein

The BlastP search of the TLP query protein against PDB identified the crystal structure of an allergenic thaumatin-like protein, Pru av 2 with PDB_ID: 2AHN (Dall'Antonia et al., 2005). The homology model constructed using MODELLER is similar to the template structure and superimposes with a root mean square deviation (RMSD) of 0.37 Å indicating high structural similarity. The PROCHECK results indicated that 99.5% residues are in the most favoured and additionally allowed regions of the Ramachandran plot indicating a very good quality of the AdTLP model. The Verify_3D indicated that 99.55% of residues had an averaged 3D-1D score greater than 0.2. This indicated a good compatibility of AdTLP 3-D model structure with its 1-D amino acid sequence. The AdTLP 3-D model comprises three domains **(Figure 3.4A)**. The central flattened region corresponds to domain I and is flanked on either side by two smaller domains. The domain I contains a beta-sandwich made up of two beta sheets with five and five anti-parallel strands. The domain II comprises several short helices and a haiprin segment. The domain III consists of a hairpin segment of two short strands of beta-sheet linked to an extended loop. The disulfide bridges formed by the

conserved cysteines in AdTLPs are crucial to their characteristic 3-D structures and the three domains of the protein are stabilized by 8 disulfide bridges **(Figure 3.4B)**. The domain I and domain II formed a negatively charged cleft consisting acidic amino acid residues with D124, D129, E103, D222 and E111 **(Figure 3.4C)**. The electropositive residue R224 shileds the acidic cleft and the side chain of K71 forms an ion pair interaction with the side chain of E111.

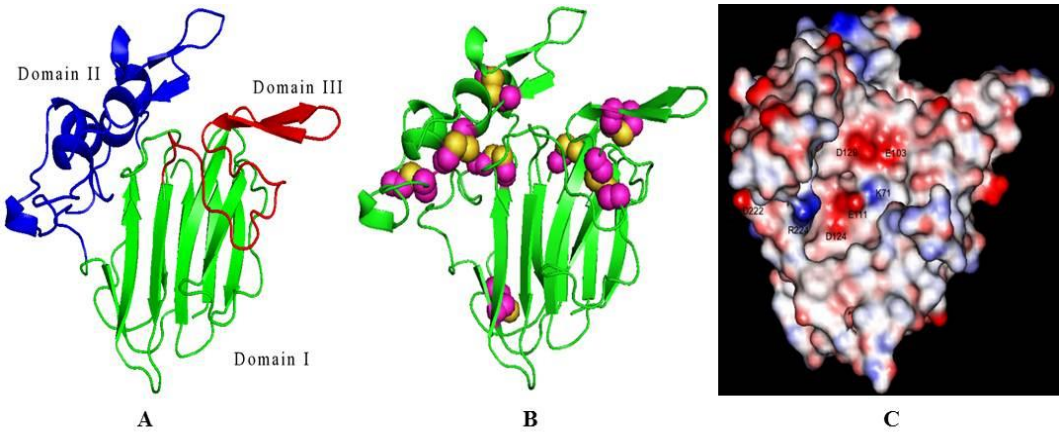

Figure 3.4 Predicted three-dimensional structure of AdTLP. **(A)** A ribbon diagram of AdTLP showing the three domains in green (I), blue (II) and red (III). **(B)** The eight disulphide bridges in structure. **(C)** Representation of molecular surface of AdTLP.

3.3.4 Localization of AdTLP

To study the subcellular distribution of AdTLP, a C-terminal translational fusion with GFP was constructed. When expressed transiently in the epidermal cells of tobacco leaves, the free GFP was found to be accumulated in both cytosol and nucleus **(Figure 3.5A)**. The recombinant AdTLP-GFP protein exhibited predominant localization in extracellular spaces with some presence in nuclear boundaries as well **(Figure 3.5D)**. When the AdTLP-GFP expression cells were visualised at different planes, the distribution of AdTLP-GFP was also observed in subcellular structures, possibly endoplasmic reticulum **(Figure 3.5G)**.

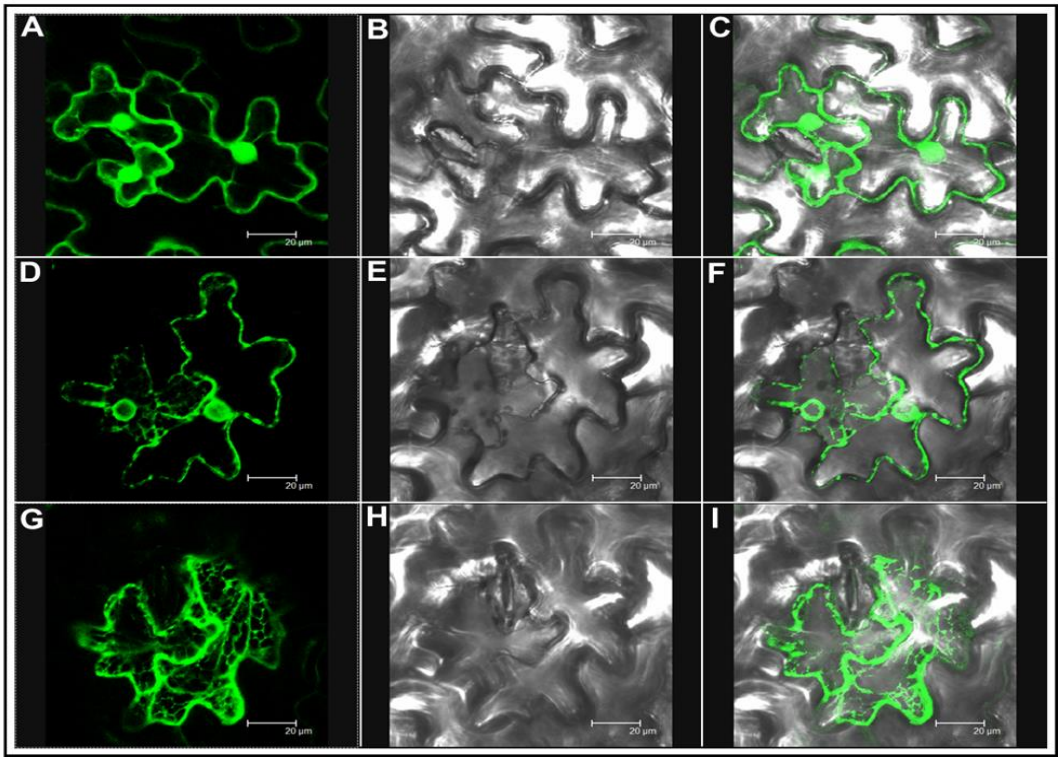

Figure 3.5 Subcellular localization of *AdTLP* by transient expression in tobacco leaves using agroinfiltration. Empty vector pEGAD expressing free GFP (A, B, C). AdTLP:GFP:1300 expressing translationally fused AdTLP-GFP and cells were visualized at different planes (D- I). Expression of free GFP and AdTLP-GFP in epidermal cells (A, D, G), corresponding bright field image (B, E, H), and overlay of GFP signal onto bright field image (C, F, I).

3.3.5 Prokaryotic expression and purification

The *AdTLP* is supposed to code for a protein with 241 amino acids with a protein mass of ~25 kDa. The recombinant protein of 41 kDa, including 16 kDa tag region of pET32a vector was expressed upon induction with 1mM IPTG **(Figure 3.6A)**. The protein was exclusively found in insoluble fraction in the inclusion bodies. Low temperature treatment and various concentration of IPTG did not show any effect on protein solubility. The recombinant protein was isolated from the inclusion bodies and solubilised using a buffer containing urea. Further it was refolded by using dialysis. This purified protein was used for *in vitro* fungal assay against different fungal pathogens **(Figure 3.6B)**.

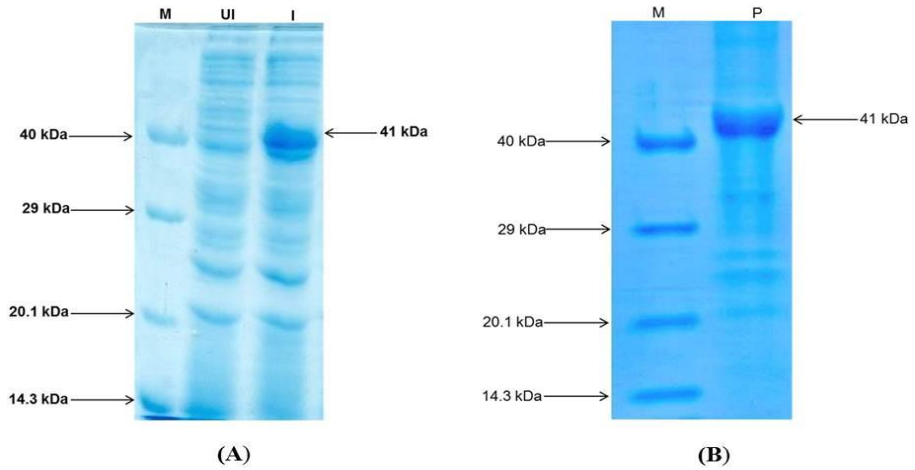

Figure 3.6 12% SDS-PAGE analysis showing total protein profiles (A) and purified protein (B). (M) Protein marker, (UI) Uninduced culture, (I) Induced culture (P) purified recombinant AdTLP protein

3.3.6 In vitro antifungal activity and endo β-1, 3 Glucanase assay

Antifungal activity of *AdTLP* protein was investigated using spore germination and plate assays. During spore germination assays with *F. oxysporum, F. solani* and *B. cinerea,* drastic decrease in hyphal growth was observed even in protein concentration as low as 1 µg/ mL of the recombinant protein **(Figure 3.7**). For these three fungal species, the calculated IC_{50} values were less than 1 µg/ mL. Hyperbranching of mycelium was also observed, which was very much distinct in the case of *B. cinerea*. A 5µg/ mL concentration of proteins was sufficient to stop the fungal spore germination completely.

Plate assay of protein was also performed with *Rhizoctonia solani*. There were varied zones of inhibition in the test fungus depending on the protein concentration applied. A graph was plotted between percentage growth inhibition and protein concentration (data not shown) and the IC_{50} value was calculated to be 38 µg/ mL for this pathogen **(Figure 3.8)**. To observe whether AdTLP exhibits any β-1, 3 glucanase activity, 50 µg of recombinant protein was incubated with Laminarin for 30 min to 3 d. No detectable absorbance was observed at 595 nm indicating that AdTLP did not possess any identifiable glucanase activity.

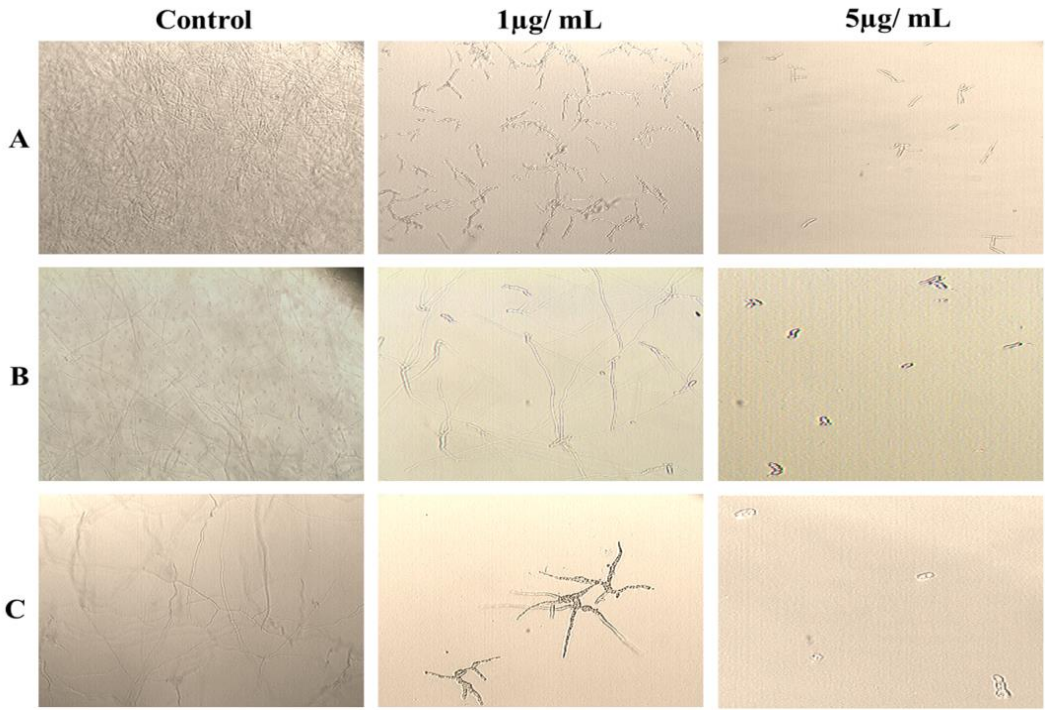

Figure 3.7 Fungal spore germination assay in the presence of different concentrations of AdTLP protein. A. *Fusarium oxysporum* B. *Fusarium solani* C. *Botrytis cinerea.*

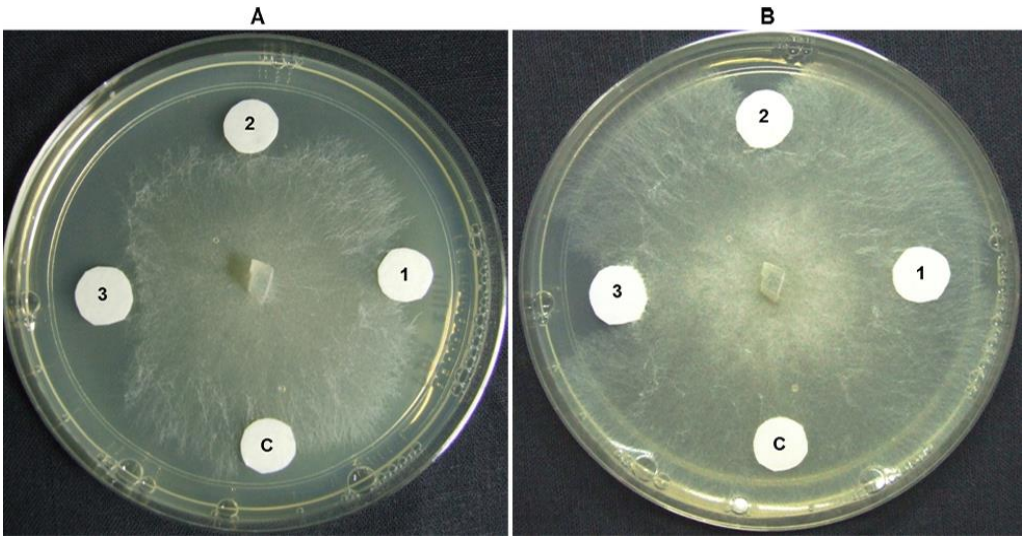

Figure 3.8 Effect of recombinant AdTLP protein on the growth of *Rhizoctonia solani.* C, 1, 2 & 3 represents control, 10µg/ mL, 25µg/ mL, and 50µg/ mL, respectively. Photographs were taken after 24 and 36 h of fungal growth (A and B).

3.3.7 Genomic DNA PCR and RT-PCR analysis on putative transgenic plants

To identify the primary transgenic plants with high and low level expression of the target gene, *AdTLP*, RT-PCR analysis was performed. Genomic DNA was isolated from ten different kanamycin positive plants and PCR reaction was performed by using primers for the marker gene *npt*II and *AdTLP*. Out of ten plants analyzed, seven plants gave expected amplification of 700 bp and 726 bp fragments respectively for *npt*II and *AdTLP* sequences. This was followed by RNA isolation and semi-quantitative RT-PCR analysis for determining the expression of *AdTLP* gene in transgenic plants **(Figure 3.9A)**. Actin amplification served as internal control. This analysis showed that the putative transgenic plants #7 and #4 were conferring highest and lowest level of expression respectively. The primary transgenic plants #7 and #4 were selfed to obtain seeds for subsequent generations of the plants after reconfirmation using PCR and RT-PCR **(Figure 3.9B)**.

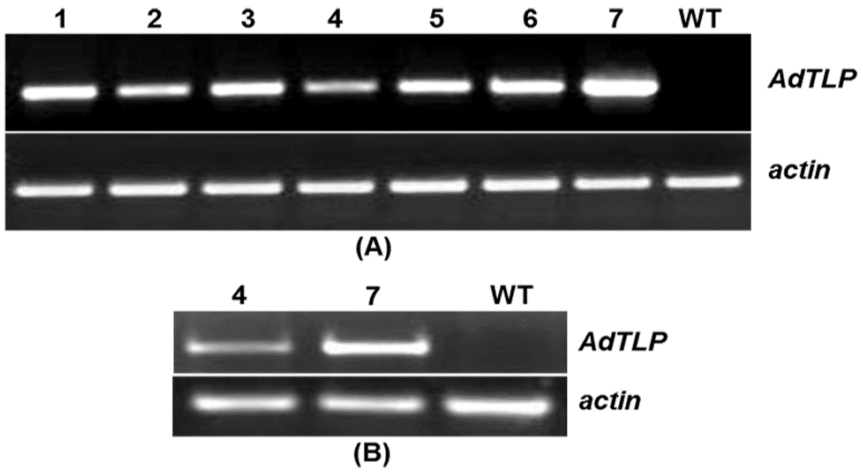

Figure 3.9 Semi-quantitative RT-PCR analysis of transgenic plants. Transcript levels of *AdTLP* were checked in T_0 (A) and T_2 (B) transgenic plants. Lane #7 is high expression line and lane #4 represents low expression line. Actin served as control to demonstrate equal loading.

3.3.8 Rhizoctonia root rot assay

Antifungal resistance in transgenic plants was checked using the broad host range fungal pathogen, *Rhizoctonia solani* that causes seedling rot disease in several crop plants. T_2 generation progeny of the low expression *AdTLP* plant, #4 and the high expression plant, #7 were tested for fungal resistance along with control non-transformed plants in the assay. After 2 d of treatment using the sclerotia of the pathogen, fungal mycelia grew and covered the complete upper soil layer in the cups. The symptoms of infection started

appearing in the wild type (WT) after 5 dpi. Fungal infection was severe on the control WT plants compared with the transgenic plantlets. After 10 dpi, WT plants became completely wilted and turned brownish black **(Figure 3.10)**. Infection symptoms were also prominent in the progeny of the low expression plant #4, but the progeny plants of the high expression transgenic plant #7 were completely healthy. To check and compare the infection at root shoot junction, plants were uprooted from the cups and compared **(Figure 3.11)**. Root growth of WT was completely retarded and the whole plant turned brownish indicating complete necrosis. The progeny plants of the transgenic #7 did not show any symptoms of infection whereas, some symptoms of wilting, necrosis and browning were observed in root and at root-shoot junction in the progeny of the plant #4. These observations showed that the high expression transgenic plant #7 exhibited enhanced levels of resistance against the root rot pathogen *R.solani* and *AdTLP* is a good candidate gene for imparting resistance against some fungal pathogens in crop plants as root rot is a very important disease in several crops.

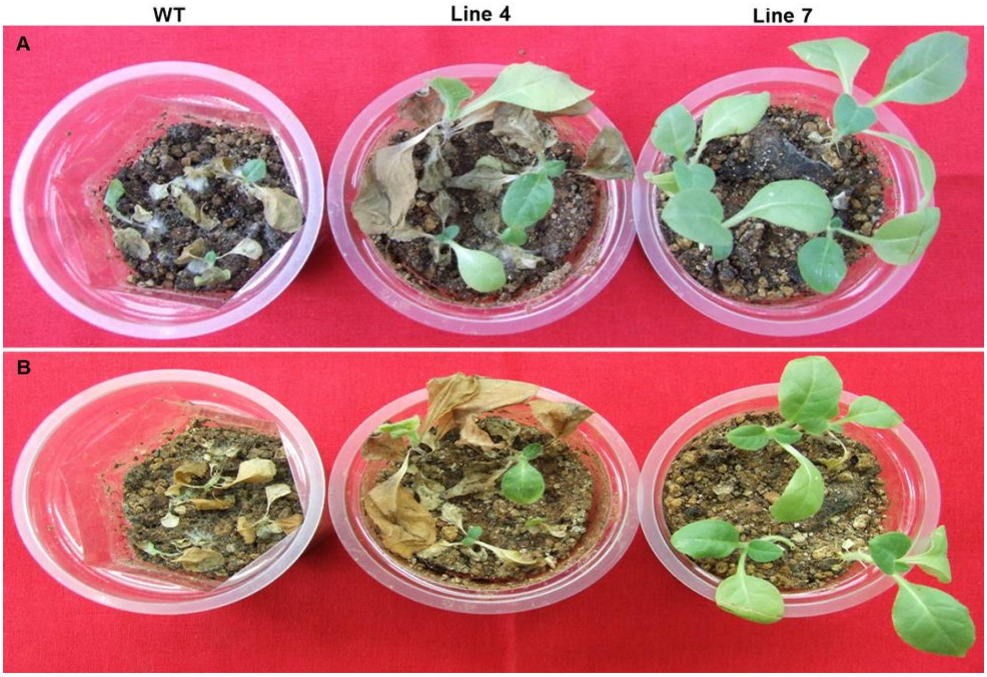

Figure 3.10 *Rhizoctonia solani* wilt bioassay with T_2 transgenic and the non-transformed control. Fungal resistance was checked in control and transgenic plants using phytopathogenic fungus *R. solani.* Control plants were seriously affected whereas high expression line appeared completely healthy. Photographs were taken after 8 (A) and 10 d (B) post inoculation of fungus.

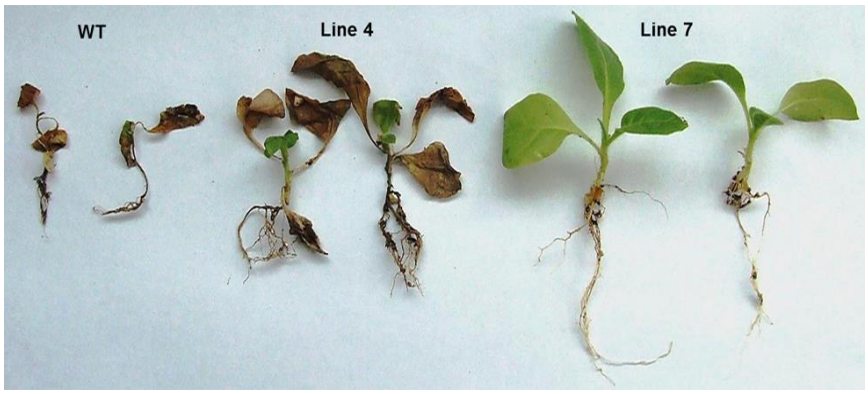

Figure 3.11 Condition of root, root and shoot junction and complete plant after 10 d of post inoculation of fungus. Two plants were taken from each line. Wild type plants became completely wilted. Infection symptoms also appeared in the roots of the low expression line plants whereas high expression line plants were completely healthy.

3.3.9 Salinity and oxidative stress tolerance

To assess the tolerance of transgenic plants to salt stress, 10 d old seedlings of T_2 generation of transgenics and WT were exposed to 100-300 mM NaCl in half MS-medium (MSH) without organic nutrients. The 100 mM concentration of NaCl did not show any discernible differences between WT and transgenic as all seedlings were healthy and green even after 14 d exposure (data not shown). On 200 mM NaCl medium, both the lines of transgenic did not appear to be sensitive after 14 d of exposure, whereas better growth was observed in the progeny of the plant #7 compared to the progeny plants of plant #4 **(Figure 3.12A)**. At the same time, chlorosis appeared with growth inhibition in WT seedlings. On a medium containing 300 mM NaCl, high level of bleaching was observed in the WT seedlings and almost 50% seedlings of plant #4 also got bleached out **(Figure 3.13A)**. Only seedlings of the transgenic plant #7 showed better growth with very little chlorosis. To check the oxidative stress tolerance, seedlings were treated with 2% H_2O_2 in MSH medium without organic nutrients. Some chlorosis appeared in the newly grown leaves of transgenic seedlings, whereas WT seedlings became completely bleached out after 12 d exposure **(Figure 3.14A)**. Total chlorophyll content and TBARS analysis showed that both salt and oxidative stress treated transgenic seedlings exhibited significantly higher chlorophyll **(Figure 3.15A, C)** content with low TBARS value **(Figure 3.15B, D)** compared to WT.

3.3.10 Seedling recovery after stress treatment

To assess the recovery response of the WT and transgenic seedlings treated with various concentrations of sodium chloride and 2% H_2O_2, they were transferred to NaCl and H_2O_2 free media. Since there were no significant differences between WT and transgenic seedlings grown on 100 mM NaCl medium, only the seedlings subjected 200-300 mM NaCl medium were transferred to the recovery medium. After 10 d, progeny seedlings of the transgenic plants #7 and #4 from 200 mM NaCl medium recovered completely and manifested near normal growth in comparison to WT seedlings, which were completely bleached out and did not recover **(Figure 3.12B)**. From 300 mM NaCl medium, progeny seedlings of the plant #7 were only able to recover properly with true leaf formation and well-developed root system. The WT seedlings never displayed any signs of recovery **(Figure 3.13B)**. Similarly, among the seedlings from 2% H_2O_2 medium, only transgenic seedlings of the high expression plant #7 were able to recover well and give healthy appearance. Only very few seedlings from the plant #4 were able to recover, while the WT seedlings remained totally bleached **(Figure 3.14B)**.

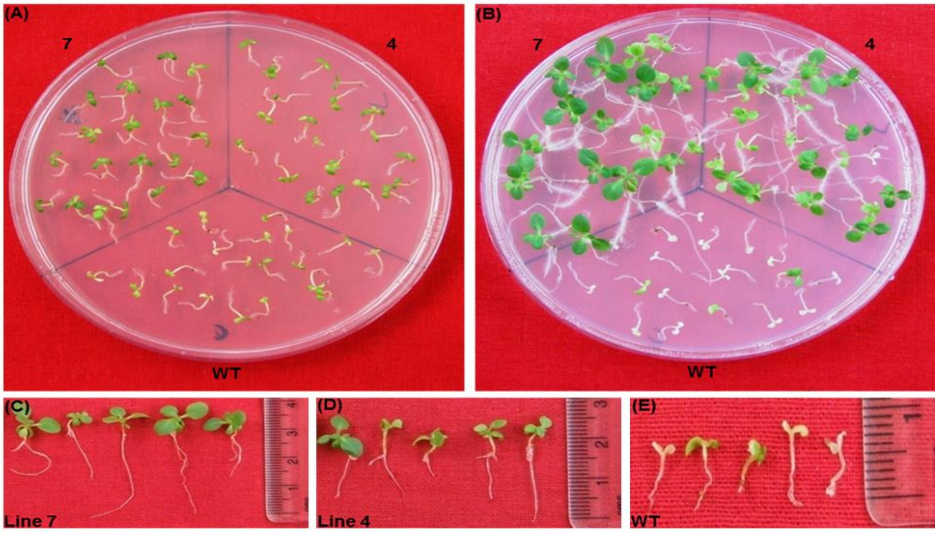

Figure 3.12 Seedling assay with 200 mM NaCl. Seedlings were transferred on 200 mM NaCl medium for 14 d (A). Seedlings on recovery medium (B). Seedlings condition after 10 d of recovery period of line #7 (C), line #4 (D) and the wild type (E).

Figure 3.13 Seedling assay with 300 mM Nacl. Seedlings were transferred on to 300mM NaCl medium for 14 d (A). Seedlings on recovery medium (B). Seedlings condition after 10 d of recovery period of line #7 (C), line #4 (D) and the wild type (E).

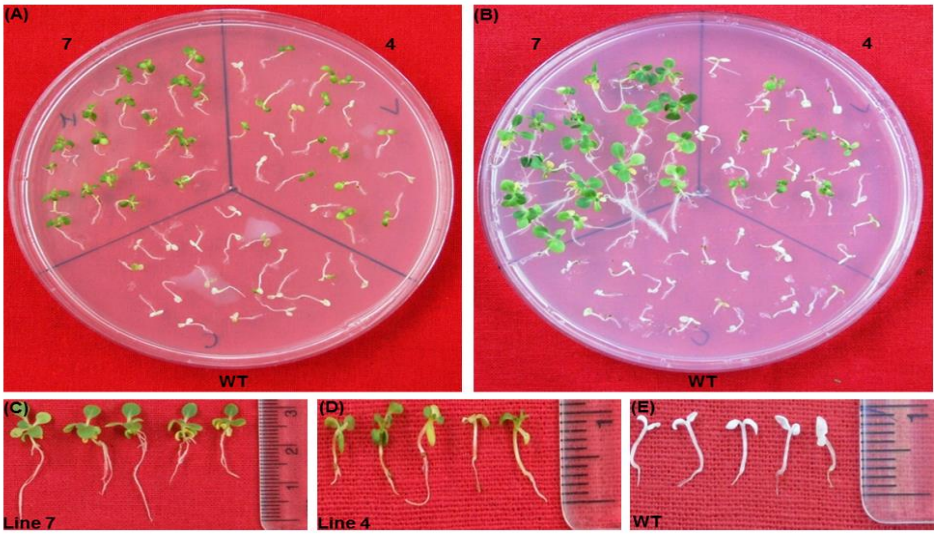

Figure 3.14 Seedling assay with 2% H_2O_2. Seedlings were transferred on 2% H_2O_2 medium for 12 d (A). Seedlings on recovery medium (B). Seedlings condition after 10 d of recovery period of line #7 (C), line #4 (D) and wild type (E)

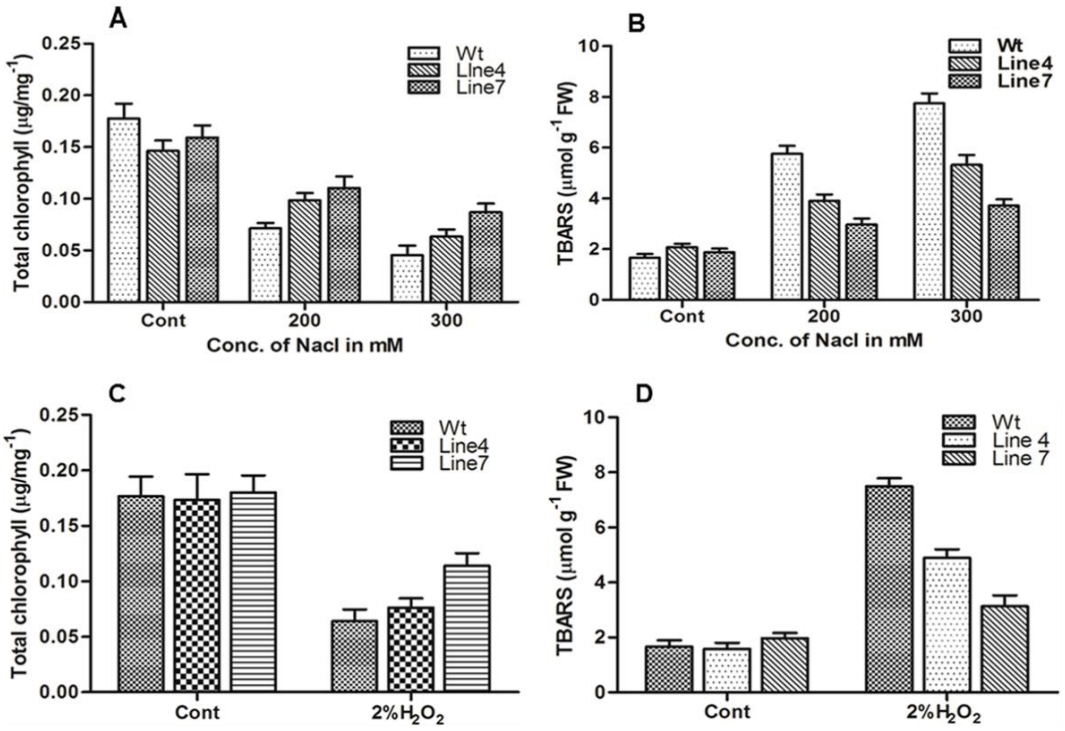

Figure 3.15. Total chlorophyll and TBARS measurement. Total chlorophyll and TBARS were measured in seedlings after 12 d of NaCl treatment (A and B) and after 10d of H_2O_2 treatment (C and D). Note the significantly increased total chlorophyll content and reduced TRABS in the transgenic seedlings after stress treatments. Experiments were repeated three times and means ±SE were plotted (*P < 0.05, n = 3*).

3.3.11 Transcript level analysis of defense responsive genes in transgenic plants

Transcript level of various defense related genes were analyzed in the transgenic plants using semi-quantitative RT-PCR **(Figure 3.16)**. Constitutively higher transcript level of Pathogenesis related protein 1a (*PR-1a*), Protease inhibitor 1 (*PI-1*) and Protease inhibitor 2 (*PI-II*) were displayed by the transgenic plants compared to the WT plants. The transcript level of *ICS, Lox3* and *ACS3a*, which code for the key enzymes in SA, JA and Ethylene biosynthesis pathway respectively were unaffected. Transcript level of wound and Jasmonic acid responsive gene i.e. allene oxide synthase (*AOS*) and allene oxide cyclase (*AOC*) were almost similar to the WT. The Basic PR-5 (osmotin) and defensin genes that are synergistically regulated by ethylene and JA were also unaffected at the transcript level.

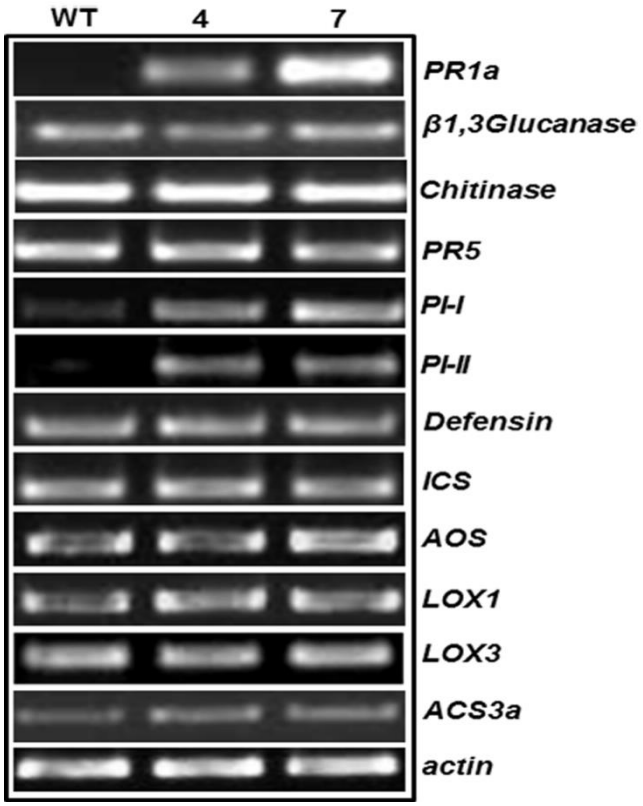

Figure 3.16 Transcript profile of defense responsive genes. Semi-quantitative RT-PCR was performed for transcript profiling of defense response genes in WT and transgenic plants. *PR*: Pathogenesis related proteins, *PI*: Protease inhibitor, *Lox*: Lipoxygenase, *AOS*: allene oxide synthase, *ICS*: isochorismate synthase, *ACS*: 1-aminocyclopropane-1-carboxylic acid synthase.

3.4 Discussion

Thaumatin-like proteins have been isolated and characterized from different plants and tissues. They are classified under the PR-5 proteins and are shown to be involved effectively in alleviating both biotic and abiotic stress tolerance (Das et al., 2011; Datta et al., 1999; Goel et al., 2010). A pathogen induced thaumatin-like protein gene was amplified and cloned from a wild peanut, *Arachis diogoi* and named as *AdTLP* (Kumar and Kirti, 2011). Sequence analysis revealed that *AdTLP* encodes a predicted protein of 241 amino acids which exhibited a 21 amino acid long N-terminal signal peptide with 25.01 kDa molecular weight and 4.71 theoretical pI (Kumar, 2008). The induction of TLP upon pathogen treatment was reported in several plant-microbe interactions(Ferreira et al., 2007; Van Loon et al., 2006). The transcript level of *AdTLP* got upregulated during

Phaeseoropsis personata treatment and reached to the highest level after 72 hpi possibly showing its activity during the later stages of infection (Kumar, 2008).

In the present study, phylogeneitc analysis revealed that the leguminous MtTLP and GmTLP showed closest similarity to AdTLP with 68% and 66% respectively. The stress hormones involved in the signalling of biotic and abiotic stress responses like SA, JA and ABA had a positive induction effect on the *AdTLP* transcript levels suggesting a possible role of *AdTLP* in different stress responses with a significant cross talk. The 3-D structure prediction suggested the presence of a strong electronegative cleft in AdTLP protein, due to the acidic amino acid residues like D124, D129, E103, D222 and E111. This electronegative cleft formed by domain I and II, is a structural requirement for antifungal activity of TLP proteins (Batalia et al., 1996). The lack of antifungal activity in some thaumatin like proteins was attributed to the electro positive cleft between domains I and II (Leone et al., 2006). The structural analysis of various TLPs from PDB indicated that domain I is conserved in all structures. In the domain II, the TLP from zeamatin (PDB_ID: 1DU5) and banana fruit (PDB_ID:1Z3Q) have fewer helices and the hairpin segment is missing.

The recombinant TLP proteins like TaPR5-GFP (Wang et al., 2010) and CkTLP-GFP (Wang et al., 2011) were mainly identified as extracellular proteins during transient expression. Other TLPs like RlemTLP and CsTL1, despite being predicted as extracellular, were found predominantly localized to both periphery of plasma membrane and cytoplasm and involved in anti-fungal activities as well (Kim et al., 2009). The localization analysis of recombinant AdTLP-GFP protein also showed extracellular localization and the observation was consistent with the prediction of secretory signal peptide of 21 amino acids. However, the AdTLP-GFP protein expression in subcellular structures, possibly ER regions, needs further investigation for confirmation. The presence of AdTLP-GFP protein in the nuclear boundaries could be due to the extension of ER from plasma membrane to nucleus or due to the overexpression of GFP fusion protein under 35S promoter.

The antifungal activity of the recombinant AdTLP that was purified after induction in the *E.coli* prokaryotic system was checked using spore germination and plate assays with different filamentous fungal pathogens that attack various crop plants. During spore germination assay, various concentrations of protein were used and it was observed that 5µg/ mL of recombinant protein was sufficient to inhibit spore germination completely in

the case of *F. oxysporum, F. solani* and *B. cinerea* for which the calculated IC_{50} values were less than 1 µg/ mL. These values were significantly low compared to a legume TLPs (Vitali et al., 2006; Ye et al., 1999) as well as osmotins and TLPs from other plants (Jami et al., 2007; Kim et al., 2009). Apart from growth inhibitory activity, the AdTLP also induced morphogenic changes like hyperbranching in the mycelium of fungal pathogen, *B.cinerea* in a way similar to the legume defensins (Vijayan et al., 2008) showing it to be a potent antifungal protein. The IC_{50} value of *R. solani* was 38 µg/ mL. These results suggested better efficiency antimicrobial activity and broad spectrum of AdTLP protein against fungal infection.

β-1, 3 glucan is a common component of fungal cell wall. Some TLPs have been reported to display endo-β-1, 3 glucanase activity (Grenier et al., 2000; Grenier et al., 1999), which could be one of the possible mechanisms for their antifungal activity. However, when the recombinant AdTLP protein was incubated with laminarin as substrate, no detectable absorbance was observed even after 72 h indicating that this protein did not exhibiting any glucanase activity.

It was reported that transgenic tobacco plants overexpressing rice thaumatin-like protein showed enhanced resistance to *Alternaria alternate* (Velazhahan and Muthukrishnan, 2003). Similarly, transgenic tobacco plants overexpressing thaumatin-like protein gene from cotton fibre showed enhanced resistance against *Verticillium dahliae* (Munis et al., 2010). For this reason, the antifungal activity of AdTLP in tobacco transgenic plants was checked against a wide range of plant pathogenic fungi. *Rhizoctonia solani,* which infects the roots and lower parts of the stem, is a serious pathogen affecting a large number of plant species (Ogoshi, 1996) and one of the test pathogens used in the present study. Our results showed that the progeny plants of the primary transgenic plant #7 exhibited superior tolerance with no signs of any infection even after 10 dpi, whereas infection symptoms developed late in progeny of the plant #4. WT plants were seriously affected and damaged completely after infection. Difference in the level of resistance between plants #7 and #4 could be correlated with expression level of *AdTLP* gene. The experiment showed *in vivo* effectiveness of AdTLP protein during fungal attack.

Earlier studies suggested the involvement of TLPs in enhancing tolerance to various abiotic stresses in plants with heterologous/ constitutive/ enhanced expression of TLPs.

For instance, the transcript level of wheat TLP increased remarkably after the treatment with ABA and elicitors (Kuwabara et al., 2002). Similarly, Wang et al. showed that the expression levels of CkTLP from *Cynanchum komarovii* seeds got upregulated during ABA, NaCl, drought, MeJA and SA treatments indicating that CkTLP might play an important role in response to abiotic stresses also (Wang et al., 2011). Transgenic approaches also confirmed their role in enhanced tolerance in transgenic plants expressing TLPs. Rajam et al. reported higher percentage of transgenic seed germination and survival during salt and drought stress conditions in transgenic tobacco plants expressing *Thaumatococcus daniellii* TLP (Rajam et al., 2007). A cotton fibre TLP gene, as discussed above, was also involved in tolerance during salt, drought and oxidative stress (Munis et al., 2010).

The present set of tobacco transgenic plants expressing AdTLP also exhibited tolerance to sodium chloride (osmotic/oxidative stress) and hydrogen peroxide (oxidative stress) when checked *in vitro*. Treatment with 200 and 300mM NaCl showed that transgenic plants #7 and #4 tolerated 200mM salt stress treatment, whereas the high expression plant #7 was better able to tolerate 300 mM salt treatment.

Accumulation of reactive oxygen species occurs during the salt stress condition causing oxidative damage to the membrane proteins, lipids and nucleic acid (Chinnusamy et al., 2005). It leads to the chloroplast damage and depletion in the chlorophyll levels. Membrane damage results in higher TBARS levels in plants under stress. In case of *AdTLP*, the higher chlorophyll contents and lower TBARS suggested that the transgenic lines were more salt tolerant compared to WT with significantly lower membrane damage. The transgenic tobacco plants overexpressing *GbTLP* were able to tolerate H_2O_2 stress condition upto 2% and maintain higher chlorophyll content (Munis et al., 2010). Hence, direct effect of oxidative stress was also checked by transferring WT and transgenic seedlings on 2% H_2O_2 media. Despite chlorosis that appeared after 12 d of treatment, transgenic seedlings showed higher chlorophyll content with low TBARS value compared to WT and indicated their higher tolerance level with less chloroplast damage and better membrane integrity. Based on the recovery results, it appears that the constitutive expression of *AdTLP* can partially reverse the stress induced growth inhibition.

Recent studies have shown that PR5 proteins are not the eventual component of signal transduction cascade. They might indirectly contribute to other defense regulatory

mechanisms in plants. A TLP protein from *Prunus domestica* was involved in activating the genes of phenylpropanoid and phytoalexin pathways in Arabidopsis along with antifungal activity (El-Kereamy et al., 2011). Similarly CsTLP from *Camellia sinensis* was assosiated with the activation of *LOX* and phenylpropanoid pathway in potato (Acharya et al., 2013) . In addition, PR5 overexpression has been found to increase H^+-ATPase activity, improved seed germination and involved during senescence in other species (Ladyzhenskaia and Korableva, 2005; Sakamoto et al., 2006; Seo et al., 2008). In order to verify the involvement of AdTLP in defense responses, we studied the transcript levels of various defense related genes in WT and transgenic tobacco plants using semi-quantitative RT-PCR. The transgenic lines showed higher transcript levels of *PR1a*, *PI-I* and *PI-II* genes compared to WT. *PI-I* and *PI-II* encode protease inhibitors and are believed to be the components of insect defense mechanism in plants. Various TLPs have been reported to be induced during wounding and insect attack (Gao et al., 2007; Kempema et al., 2007; Zarate et al., 2007). Microarray analysis of maize seedling during insect attack had shown differential expression of thaumatin-like protein genes along with other genes (Johnson et al., 2011). Significant increase in TLP expression occurred in poplar phloem during wound treatment and the protein presence was confirmed by immunolocalization studies (Dafoe et al., 2009). The exact reason of TLPs involvement during such stress events has not yet been clearly elucidated. Though α-amylase and protease inhibitor activities are major modes of action of plant resistance proteins to insect attack, these possibilities have been ruled out for PR5 proteins (Gómez-Leyva and Blanco-Labra, 2001). Our observation during this study indicated that AdTLP protein might play some role in plant defense during insect attack by inducing *PI-I* and *PI-II* genes in transgenic lines. However, due to lack of full understanding about the mode of action of TLPs (Franco et al., 2002; Liu et al., 2010), it is difficult at present to predict the exact mechanism behind the induction of other genes in *AdTLP* transgenic plants.

There are several examples suggesting that TLPs can interact with a variety of ligands and proteins including actin, cytokinin and viral proteins (Kim et al., 2005; Kobayashi et al., 2000; Takemoto et al., 1997). The reason behind their involvement in various functions can be understood based on their ability to adopt functional diversification despite their conserved nature. Probably TLPs might exhibit through

multiple functions due to the mutations in appropriate amino acid residues, which might have occurred during the course of evolution (Liu et al., 2010). Not only TLPs but also other PR proteins like PR3 and PR14 (Doxey et al., 2006; Yeh et al., 2000) have also been reported in diversified functions. Finally, our observations suggest that further in depth study is needed for *AdTLP* to explain the mode of action and their involvement in induction of other genes, which ultimately could help unravel a better picture in understanding the functional aspects of TLPs.

3.5 Conclusion

In summary, we have studied the different aspects of a *AdTLP* gene from *Arachis diogoi* (a wild species related to the economically important legume crop peanut), which was expressed in its interaction with the pathogen, *Phaeoisariopsis personata*. The *in vivo* and *in vitro* activities of a thaumatin-like protein have been analyzed. The protein has imparted significant resistance against fungal pathogens, salt and oxidative stress. Apart from this, the transgenic plants also displayed higher transcript level of *PR1a*, *PI-I* and *PI-II* gene compared to WT. Taken together, these observations suggest that the *AdTLP* can be a good candidate gene for deployment in transgenic plants for enhancing their tolerance against various biotic and abiotic stresses.

Table 3.1 Sequences of the oligonucleotides used in the study (see text for details).

Name of the primers	Primer sequences (5'-3')
TLP ORF-F	GGGATCCATGGCGATTACTCGTGTTGT
TLP ORF-R	CCTCGAGTCATGGACAGAAGTTGATAGC
TLP ORF-F1	GGGGCCCATGGCGATTACTCGTGTTGT
TLP ORF-R1	GGGATCCTCATGGACAGAAGTTGATAGC
TLP ORF-F2	GGGGCCCATGGCGATTACTCGTGTTGT
TLP ORF-R2	CCCCGGGTCATGGACAGAAGTTGATAGC
Actin-F	TGGCATCACACTTTCTACAA
Actin-R	CAACGGAATCTCTCAGCTCC

Table 3.2 Sequences of gene-specific primers used in RT-PCR for amplification of defense related gene transcripts.

Oligo name	Forward	Reverse
PR1a	CTTCTTGTCTCTACACTTCTC	GCAAGAGACAACATATCCTC
Chitinase	CTGAAGAATAGGAACGACGGTAG	ATACCTCCTGTAGTATCCAATTCG
Glucanase	ATGGCTTTATGCATTAAAAATGGC	AGCATTGAAGACATTTGTTTCTGG
Defensin	GAGGCGAGAACTTGTGAGTC	AAGCCGAAACCATTATTCATAAC
PR5	CTTGAGATCTTCTTTTGTTTTCTTC	ACTTCCAGGCATTTCCAAGGGAAA
PI-I	ATGGTGAAGTTTGCTCACGT	AATCCCTTAGCCAACCTGG
PI-II	GTTAGTTTCGTCGCTCATCT	CTGCGTTACAACAGTTGATG
ICS	TGCATATCAGTTCTGTTTGCAAC	CCAGCATACATTCCTCGGTCA
AOS	CTTGGTCTTCCGAAGGTTC	GACGTCGATATCCAACGTG
ACS3a	ATAGTTATGAGTGGAGGAGC	CCGTGTCTTTTCCCTAGTCT
LOX1	CACTTCCTACTGATCTCATC	CTCATCGACATTCATCTGCA
LOX3	AATGACAGAGAACTCCAAGC	TAGAACGCTTCGACAATCTC

Chapter 4:

CBL Interacting Protein Kinase (*AdCIPK*)

4.1 Background

A network of signal transduction pathways is used by all the organisms to cope up with the problems in the environment, to realize their developmental programs and to control their metabolism. In plants, Ca^{2+} ion is involved in almost every biological process. It serves as a ubiquitous second messenger and regulates a multitude of physiological and developmental processes, including responses to abiotic stress, pathogen defense and adjustment of ion homeostasis (Kolukisaoglu et al., 2004). In addition, physiological processes like root hair elongation, guard cell regulation and pollen tube growth are accompanied by distinct spatio-temporal changes in calcium concentration (Evans et al., 2001). The fact that Ca^{2+} can serve simultaneously as a regulator and a messenger in so many different processes raises the question of how specificity in information processing and output determination can be achieved (Weinl and Kudla, 2009). Studies on Ca^{2+} dynamics in plant cells have shown that the spatial and temporal composition of Ca^{2+} transients display stimulus-specific characteristics and that this distinctiveness is critical for proper coupling of defined stimuli with the corresponding specific responses (Allen et al., 2001; Allen et al., 2000; Sanders et al., 2002). Such stimulus-specific elevations in cytosolic Ca^{2+} concentration are referred to as 'calcium signatures'. The specific calcium signatures can encode information and contribute to the specificity required for efficient stimulus response coupling. To decode the Ca^{2+} signatures, each cell must be equipped with the mechanisms for interpreting these codes for specific responses. This decoding process starts with the Ca^{2+} sensors that bind Ca^{2+} and thereby alter their own structural properties. These structural changes either result in functional changes in the sensor proteins or trigger interaction with the downstream target proteins of the sensors (Luan, 2009). Ultimately, the sensors or their targets often modulate the function of other proteins (such as membrane transporters or transcriptional factors) and elicit changes in cellular processes (such as gene expression or ionic fluxes). This general mechanism of Ca^{2+} signaling applies to all pathways, but the sensors and effectors in different organisms can be either evolutionarily conserved or significantly diverged.

Plant calcium-dependent protein kinases (CDPKs) harbor both a calcium-binding domain as well as a catalytic Ser/Thr kinase domain within the protein. They are also known as 'sensor-responder' due to the presence of both activities and hence, can sense

calcium signals and transmit them as a single protein (Harmon et al., 2000; Sanders et al., 2002). Further the functional analyses of different plant CDPKs have provided evidence for crucial functions of these protein kinases in divergent processes like hormone and stress signaling as well as pathogen response (Kolukisaoglu et al., 2004). Other known calcium sensors, for example, calmodulin (CaM) and CaM-like proteins do not have an enzymatic activity on their own and also known as 'sensor-relay'.

4.1.1 Calcium sensor relay- Calcineurin B-like protein (CBL) family

Apart from regular ones, plants have also acquired a large number of other Ca^{2+} sensor proteins that are not present in fungal and animal systems. Thus Ca^{2+} signaling mechanism in plants is more complex compared to other organisms. Calcineurin B-like proteins (CBLs) are one of the recently discovered classes of Ca^{2+} sensor-relay type of proteins which are specific to the plants (Luan et al., 2002). SOS3/CBL4 was the first CBL protein which was identified in Arabidopsis (Kudla et al., 1999; Liu and Zhu, 1998). They were originally identified based on their significant sequence similarity to the calcineurin B (CNB) and neural calcium sensor (NCS) from animal cells and hence, designated as Calcineurin B-like proteins. Recent studies have identified several CBLs from different plants, e.g. ten CBLs each from Arabidopsis, rice and poplar, eight CBLs from both maize and grape and six CBLs from sorghum (Yu et al., 2014). These CBLs are identified or characterized by the presence of common helix loop helix structure motif, also known as EF hands. These EF hands act as Ca^{2+} binding sites during the signaling processes. Each CBL harbour four EF hands and each EF hand domain is a loop of 12 amino acids flanked by two alpha-helices, in which the amino acids at positions 1(X), 3(Y), 5(Z), 7(-Y), 9(-X) and 12(-Z) are responsible for Ca^{2+} binding (Batistic and Kudla, 2004).

Subcellular localization of Ca^{2+} sensors is an important criterion towards maintaining response specificity. By using green fluorescent protein (GFP) as fusion protein, it was observed that different Arabidopsis CBLs were found to be localized in different subcellular compartments of cell (Weinl and Kudla, 2009). CBL proteins harbouring a short N-terminal domain (AtCBL1, AtCBL4, AtCBL5 and AtCBL9) were detected at the plasma membrane. CBLs with extended N-terminal domains (AtCBL2, AtCBL3 and AtCBL6) were exclusively localized at tonoplast. However, AtCBL7 and AtCBL8 with evolutionary changes of their N-terminal sequences were present in both cytoplasm and

nucleus. AtCBL10 with unique N-terminal region was detected in endosomal compartments as well as the tonoplast (Kim et al., 2007).

4.1.2 The Kinase effectors- CBL interacting protein kinases (CIPKs)

In contrast to calmodulins, CBLs specifically target a defined group of protein kinases known as CBL-interacting protein kinases (Halfter et al., 2000; Shi et al., 1999). CIPKs are the members of serine-threonine kinase family and like CBLs, they are also specific to the plant system only. Structurally, they are similar to the SNF1 (sucrose non-fermenting) kinase from yeast and AMPK (AMP-activated protein kinase) from the animal system (Albrecht et al., 2001; Luan et al., 2002). CIPKs have also been assigned to SnRK3, one of the three subgroups of plant SNF-like kinases (Hrabak et al., 2003). Functionally CIPKs are clearly distinct from SNF kinases and exhibit different modes of function and regulation (Kolukisaoglu et al., 2004). Recent studies have identified the presence of a large number of CIPKs from different plants species; e.g. 26 from Arabidopsis, 31 from rice, 27 from poplar, 43 from maize, 21 from grape and 32 from sorghum (Yu et al., 2014). CIPK proteins mainly comprise of a conserved N-terminal SNF1-type catalytic kinase domain and a C-terminal regulatory domain, which is absent in other SNF1-related kinases (Kolukisaoglu et al., 2004). The CIPK catalytic domain is identified by the presence of typical activation loop located between the N-terminal and C-terminal conserved amino acid motifs DFG and APA, respectively. There are three highly conserved amino acid residues, namely serine, threonine and tyrosine that are present within the activation loop. This loop is a target of phosphorylation by other protein kinases, however; mutational analyses have revealed that substitution of any these three conserved amino acids to aspartate residue results in a constitutively active form of the enzyme, the activity of which is no longer CBL-dependent (Gong et al., 2002b; Guo et al., 2001). The mechanism of phosphorylation-dependent activation of CIPKs suggests that there might be other, as yet unknown, CIPK-phosphorylating kinases providing the means of regulatory cross talk with other signaling pathways in plant cell (Kolukisaoglu et al., 2004). Within the C-terminal regulatory domain, one evolutionarily conserved asparagine–alanine–phenylalanine (NAF) domain has been identified as required and sufficient for mediating the interaction with CBL proteins (Albrecht et al., 2001).This domain is hydrophobic in nature and possibly involved in hydrophobic interaction between CBLs and CIPKs. There are reports, which suggest that

NAF domain itself is not sufficient to generate the observed interaction specificity, and other regions surrounding the interaction domain, or more distantly located are likely to be responsible for a high degree of interaction preferences between CBLs and CIPKs (Albrecht et al., 2001; Kim et al., 2000). It has been observed that deletion of the NAF domain resulted in constitutive, CBL-independent kinase activity; however, further deletion of the junction region between the NAF motif and the catalytic domain abolished the activation of the target protein (Guo et al., 2001). Adjacent to the NAF motif, another conserved domain has been identified and named as protein phosphatase interaction (PPI) motif (Ohta et al., 2003). It has been found to be involved in CIPKs interaction with protein phosphatases 2C, ABI1 and ABI2. To some extent, the sequence variations in PPI motifs determine the CIPK interaction with the protein phosphatase, ABA-insensitive 1 (ABI1) or ABA-insensitive 2 (ABI2). Currently, it has been suggested that CIPK kinases and a 2C-type protein phosphatase physically interact with the downstream target proteins, and modify their phosphorylation and dephosphorylation activities (Lee et al., 2007).

4.1.3 The CBL-CIPK complexes and functions

The CBL/CIPK signal system is involved in many kinds of signaling pathways (Zhu et al., 2013). Their general structures of signaling mechanism are shown in **Figure 4.1.** The Expression patterns and sub-cellular localization of both CBLs and CIPKs have been induced by many factors such as phytohormones, biotic and abiotic stresses (high salinity, drought, and high pH). SOS (salt overly sensitive) is one of the CBL–CIPK signaling pathways contributing to salt tolerance in the plants (Zhu et al., 1998). This pathway includes CBL4, CIPK24 and Na^+/K^+ antiporter during the tolerance activity. It is the first Ca^{2+} dependent CBL–CIPK signaling pathway discovered in plants in response to abiotic stress.

The CBLs/CIPKS have shown different expression patterns and are involved in various cellular mechanisms. *AtCBL1* is induced by drought, cold, wounding and also responds to glucose and gibberellin (GA) signals during seed germination and development (Kudla et al., 1999; Li et al., 2013). *AtCBL2* and *AtCBL3* expression is induced by light (Nozawa et al., 2001). *AtCBL4* responds to salinity stress, *AtCBL9* is involved in K^+ and NO_3^- uptake and *AtCBL10* regulates K^+ homeostasis (Yu et al., 2014). Similarly, CIPK genes are differentially expressed. For example, expression of *AtCIPK9* has been detected in nearly all tissues except roots and is induced mainly by ABA and slightly by drought and NaCl (Gong

et al., 2002a). In contrast to this *AtCIPK20* expression has been restricted only to leaves and is not induced by stress (Gong et al., 2002c).

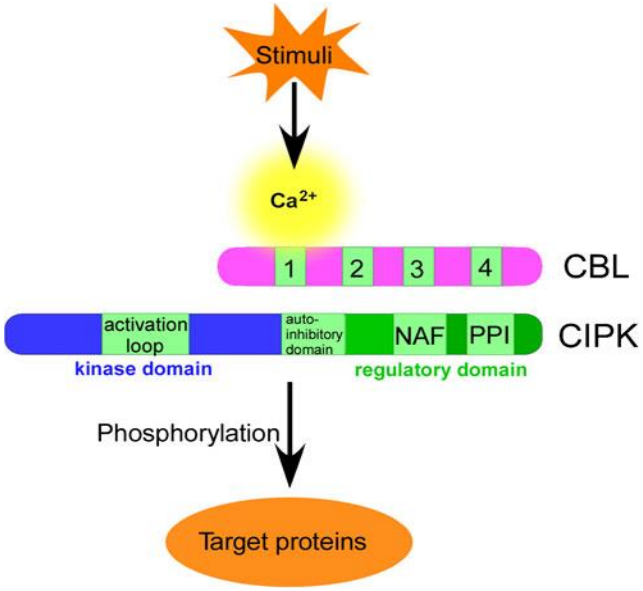

Figure 4.1 General structure of the Ca^{2+} sensor protein calcineurin B-like (CBL) and CBL-interacting protein kinases (CIPK). The CBL EF-hand domains are numbered 1–4. The asparagine–alanine–phenylalanine domain and protein–phosphatase interaction domain are labeled as NAF box and PPI box, respectively (Zhu et al., 1998).

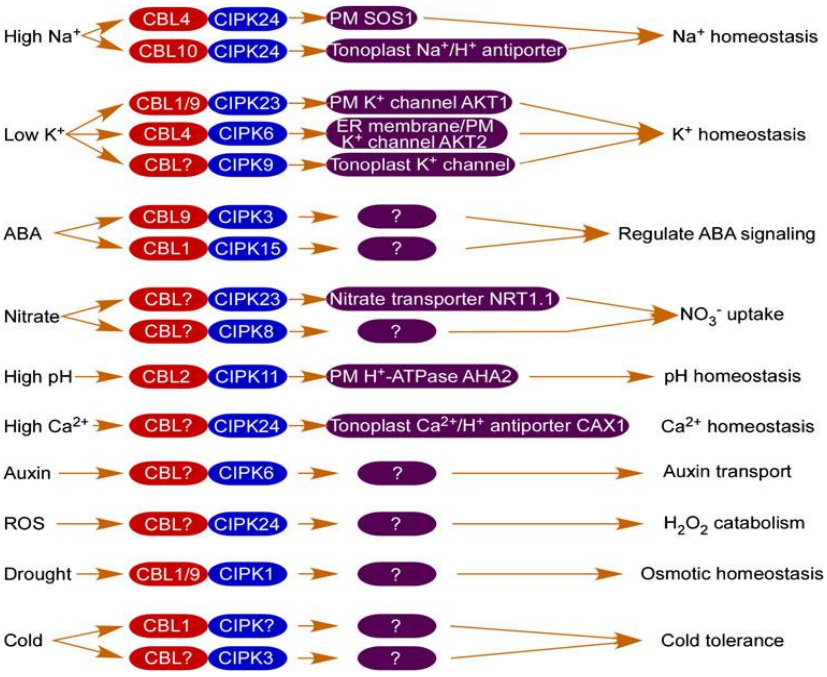

Figure 4.2 Stress signaling through the CBL-CIPK signaling system in plants (Zhu et al., 2013).

Like CBL4-CIPK24 system, others are also reported to be involved in different mechanisms. For example, The AtCBL1–AtCIPK1 complex is involved in ABA-dependent stress responses, while the AtCBL9–At-CIPK1 complex plays roles in ABA-independent stress responses (D'Angelo et al., 2006). In contrast, AtCBL9–AtCIPK3 plays roles in negatively regulating the ABA response during seed germination (Pandey et al., 2008). Another AtCBL2–AtCIPK11 system regulates the H^+-ATPase, AHA2 in the Arabidopsis plasma membrane (Fuglsang et al., 2007). Interestingly, depending on the conditions, one CBL can interact with more than one CIPK and vice-versa **(Figure 4.2)**; hence each CBL or CIPK can be involved in different mechanisms at different time periods.

In comparison to others, very little is known about the CIPKs from *Arachis* species. In the present study, using the available partial cDNA sequence of AdDR-5, a full length cDNA was amplified and cloned from the wild peanut, *Arachis diogoi* and named as *AdCIPK.* This 341bp partial sequence of AdDR-5 (NCBI Accession No. EF371923) was identified in *Arachis diogoi* during *Phaeoisariopsis personata* infection (Kumar and Kirti, 2011). We analyzed the *AdCIPK* expression in *Arachis diogoi* during various treatments and its subcellular localization by using GFP as fusion protein. Tobacco transgenic plants were raised and checked for the levels of tolerance against various stress treatments. Further, the transcript levels of various stress related genes were compared in WT and transgenic plants during the stress conditions.

4.2 Materials and Methods

4.2.1 Plant treatments

Wild peanut *(Arachis diogoi)* and tobacco (*Nicotiana tabacum* var Samsun) plants were maintained in the green house. Different hormone treatments to *A. diogoi* were done as described in section **3.2.1**. For stress treatments, 200 mM NaCl, 300 mM Sorbitol and 10% PEG were used. Samples were collected at regular interval and stored at -80^{o} C.

4.2.2 5'/ 3' RACE, isolation of full length cDNA and genomic sequence of *AdCIPK*

Rapid amplification of cDNA ends (RACE) was performed to derive full length *AdCIPK* 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. Primers used in this study were provided in **Table 4.1**.

4.2.3 Analysis of cDNA and protein sequence

DNA and protein sequences were analyzed using online tools as described in the earlier section **3.2.2**. Signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/).

4.2.4 qRT-PCR analysis

The total RNA (2 µg) was reverse transcribed to first strand cDNA by using SMART™ MMLV Reverse Transcriptase (Clontech, Becton Dickinson, USA). The total 20 µl of PCR mixture contained 2 µl of diluted cDNA, 10 µl of 2× SYBR Green PCR Master Mix (TAKARA BIO INC, Shiga, Japan) and 0.5 µl of specific primers (1.0 pM final concentration). The PCR samples were appraised in three replicates including three non-templates as the negative control. Real-time PCR analysis was carried out after pre-incubation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 1 min using Realplex PCR machine (Eppendorf, Germany). For *Arachis diogoi*, treated leaf samples and for tobacco, whole seedlings were used during real time analysis. Alcohol dehydrogenase class III (*adh3*) and Ubiqutin (*Ubq*) genes were used as internal control for *Arachis diogoi* and tobacco samples respectively. The Cycle threshold (C_T) values were obtained from the exponential phase of PCR amplification. To analyze the results, comparative C_T method was used (Livak and Schmittgen, 2001). Relative fold in expression

was estimated by using $2^{-\Delta\Delta C_T}$ formula where $\Delta\Delta CT = (C_{T\ target} - C_{T\ control})$ treated sample – $(C_{T\ target} - C_{T\ control})$ control sample. Primers used in this study were provided in **Table 4.2**.

4.2.5 AdCIPK subcellular localization analysis

The *AdCIPK* ORF was reamplified by using ORF-F1 and ORF-R1 primers with *Nco*I and *Spe*I restriction sites respectively. The amplified product was digested and cloned in binary vector pCAMBIA 1302, digested with same set of enzymes. Further, the confirmed recombinant vector was mobilized in *Agrobacterium* strain EHA105 using freeze thaw method. Similarly empty 1302 vector was also mobilized in same *Agrobacterium* strain.

4.2.6 Agroinfiltration and microscopy

Agroinfiltration and microscopy were done as described in section **3.2.6**. *Nicotiana benthamiana* leaves were used in this study.

4.2.7 Construct preparation, *Agrobacterium* mediated Tobacco transformation and molecular analysis of transgenic plants

The *AdCIPK* ORF was reamplified with ORF-F2 and ORF-R2 primers harboring *Apa*I and *Kpn*I restriction sites respectively and cloned in pTZ57R/T vector. Digested fragments were cloned in pRT100 vector at corresponding sites. The *AdCIPK* expression cassettes harboring CaMV35S promoter and polyadenylation signal from pRT100 vector were further digested with *Hind*III enzyme and cloned in pCAMBIA2300 vector at the same site in multiple cloning sites region. Confirmed vectors were mobilized into *Agrobacterium* strain EHA105. Tobacco (*Nicotiana tabacum* cv. Samsun) transformation was done as described in section **2.13**. Further the transformants were selected on 125mgL^{-1} kanamycin and T_0 putative transgenic were analyzed by polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) for *AdCIPK*. Nine different plants were checked for PCR and RT-PCR. Of them, plant 1 and 6 were taken as high expression and plant 2 as low expression line for further analysis. T_1 and T_2 seeds were raised via self-pollination and T_2 seeds were used in functional characterization for stress analysis.

4.2.8 Evaluation of fungal resistance in transgenic plants

Fully expanded leaves from high expression plants (line 1 and 6) along with non-transgenic controls were used for detached leaf anti-fungal bioassay in T_2 generation.

Resistance was checked against the phytopathogenic fungus *Phytophthora parasitica*. Actively growing fungus along with potato dextrose agar block (0.5 cm^2), were kept on the adaxial surface of the leaves after abrasion. Lesions were observed regularly, photographed after 5 days post-inoculation (dpi) and the percentage disease leaf areas (DLA) were calculated.

4.2.9 Seed germination assay

Mature WT and transgenic seeds were surface sterilized with 4% hypo solution and washed properly with water. From each T_2 generation of transgenic lines (1, 2 and 6), around 150 seeds of were taken and transferred to two separate sets of Half MS (MSH) medium without organic solvents, supplemented with 200 mM NaCl and 300 mM Sorbitol each. Germination was observed regularly and graphs were plotted after 7 days in both treatments. Control plates without any stress agent were also maintained simultaneously.

4.2.10 Seedling assay

Surface sterilized seeds transgenic lines were grown on MSH medium containing 125 mgL^{-1} kanamycin for 10 days. Simultaneously WT seeds were grown on MSH medium without kanamycin. Seedlings were maintained in a culture room at 27 ± 1°C with a photoperiod of 16 h light and 8 h dark. The seedlings from each of the transgenic plants (H1, H2 and Low) along with the WT were used in different stress treatments. For salt treatment, 200 and 300 mM of NaCl and for osmotic treatment 200 and 300 mM of sorbitol were used in MSH medium.

4.2.11 Leaf disc assay

Equal size leaf discs were excised from the healthy and fully expanded tobacco leaves of 7-week-old WT and transgenic plants from the glasshouse using a cork borer. Leaf discs were floated on 15 ml of the respective concentrations. For salt treatment, 100, 200 and 300 mM concentrations of NaCl were used whereas for osmotic stress 300, 400 and 500 mM concentration of sorbitol were used. The treatments were carried out in continuous white light at 27 ± 1°C until variations were seen among the lines.

4.2.12 Total chlorophyll and TBARS measurement

Total chlorophyll content and lipid peroxidation were measured as described earlier section **3.2.12** by using Arnon, and Heath and Packer method respectively. During salt stress to seedling stage, both chlorophyll and thiobarbituric acid reactive substances (TBARS) were measured after six days of treatment. However for leaf disc assay, they were measured after two days of treatment. In case of osmotic stress treatment to leaf discs, both chlorophyll and TBARS were measured after four days of treatment.

4.2.13 Catalase and H_2O_2 measurement

Ten days old WT and transgenic seedlings were transferred to different concentrations of NaCl and Sorbitol media. Both catalase and H_2O_2 were measured after 6 d of NaCl treatment whereas for sorbitol, they were measured after 10 d of treatment. Catalase activity was measured spectrophotometrically by following the oxidation of H_2O_2 at 240 nm according to the method described by Patterson et al. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 20 mM H_2O_2 and enzyme extract equivalent to 10 µg protein in a final volume of 1 ml. Δε for H_2O_2 at 240 nm was 43.6 mM^{-1} cm^{-1}.

For H_2O_2 measurement, seedlings samples (250 mg) with and without NaCl treatment were homogenized in 5 ml cold acetone. The extract and washings were centrifuged at 1,250 g^{-1} and the chlorophylls were adsorbed by activated carbon. The supernatant (200 µl) was added to 1 ml reaction buffer (0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 1.25 mM xylenol orange, and 1 mM sorbitol) at room temperature for 1 h. The H_2O_2 levels were quantified by noting the absorbance at 560 nm and were calculated as described by Xue et al. with three individual repetitions of the experiment.

4.2.14 ROS detection

By using the epidermal peels from the abaxial surface of fully expanded leaves, ROS formation was detected under confocal microscopy (Murata et al., 2001). Firstly, epidermal peels from WT and high expression lines 1 and 6 were treated with H_2DCFDA as described by Konopka-Postupolska et al. and then washed to remove excess dye. NaCl stress induced ROS formation was detected in the guard cells by incubating the epidermal peels in 100

mM NaCl for 30 min. Simultaneously, a control experiment was also performed in parallel with a mock treatment of buffer to WT and transgenic plants. The fluorescence (kex-488 nm and kem-530 nm) was pictured by using a Leica Laser Scanning Confocal Microscope (Leica, TCS-SP2 with AOBS, Heidelberg, GmbH, Germany) and the experiment was repeated thrice with at least n = 100 for each plant.

4.3 Results

4.3.1 Isolation of full length cDNA and genomic sequence of *AdCIPK*

A partial cDNA clone of 341bp designated as AdDR-5 encoding a putative CBL-interacting protein kinase gene was isolated in a previous study (Kumar and Kirti, 2011). Using 5'/3' RACE-PCR approach, 1254 and 768 bp products were obtained respectively **(Figure 4.3 A, B)**. Full length cDNA sequence was deduced based on the overlapping sequence of the obtained RACE products with existing partial cDNA sequence. The full length cDNA was amplified and confirmed after sequencing. It showed homology with CBL-interacting protein kinase and was hence, designated as *AdCIPK*. The cDNA was 2,031 bp long including the 1,386 bp ORF, 408 bp 5' and 237 bp 3' UTRs **(Figure 4.4)**. The ORF was potentially encoding a 461 amino acid polypeptide. The encoded protein had a predicted molecular mass of ~52 kDa with the isoelectric point value of 8.75. SignalP result showed the absence of any signal peptide in the deduced amino acid sequence. The amplification of corresponding genomic sequence revealed that *AdCIPK* gene did not harbor any introns.

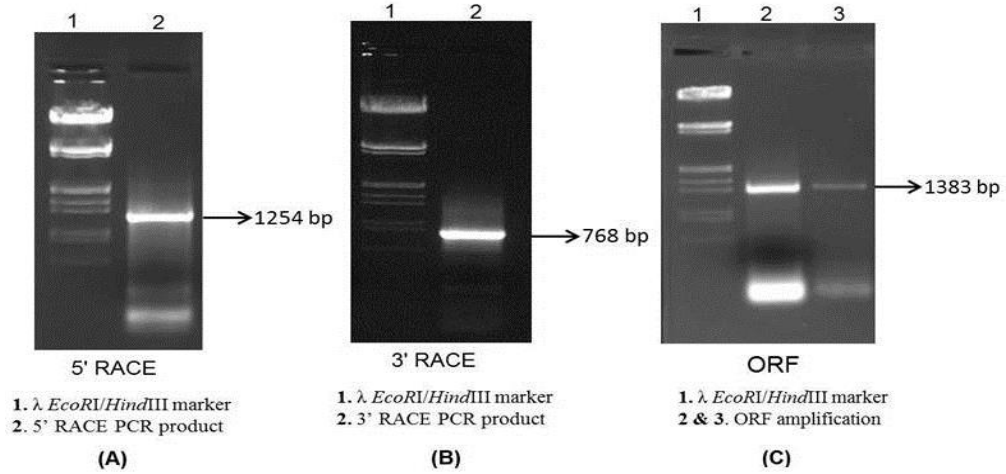

Figure 4.3 Amplification of cDNA ends using 5' **(A)** and 3' **(B)** RACE-PCR. Full length *AdCIPK* ORF amplification **(C)**.

AGCCCAATCCCAATCCCAATCCCAATCTCAATATATTCCCCCTCACTCCATTAATAAATTCCATCACAACACAAACTTCTT
CAACTTTCACTCAACCTAACCCAAAAACGGAAAAACCATACCCTTATTTTTTCCCTTCTTTTCTTCTTTCTCTCTTGCACG
TACATACACACCGGAGTTCCAATCTGGTGTATCACCTATGCACCATGAAGATCTTCAACCCTTCTGAATCCTTTCTCCGAA
ACGTTGTGTGTCCCTCGGACACTATATTCCTCAAAGGAATGAACCTCTCCAATCTTCACCACTCCACATTCTCCCTCCTCT
CTTGGCCATACAATTAGCCTTCCCAACCACTAATCTTAACTAACTTTCTTTCTTTTCCTTTTCTCTCTCTGGAATCTTCCT
CCCATGGATCTGAAAAACGAGATGGAGATCAAGTTGTCGAACAGCGCTAGGACGGTGATATTCAACAAGTACGAGATGGGA
CGCGTGTTAGGTCAGGGGAATTTTGCCAAGGTATACTACAGCAGGAACCTAGCGACGAACGAAAGTGTGGCGATTAAGGTG
ATAAAGAAGGATAAGCTGAGGAAGGAGAGGCTGATAGAGCAGATTAAGAGAGAGGTTTCGGTTATGAGGCTGGTGAGGCAT
CCGAACATAGTGGAGCTGAAGGAAGTCATGGCCACGAAGGGGAAGATATTCTTGGTTATGGAGTATGTTAAGGGAGGCGAA
TTGTTCAAGAAAGTCGAGAAAGGGAAGCTCAAGGAAGATGTAGCTAGAAAGTATTTTCAGCAGTTGATCTCGGCCGTTGAT
TTCTGCCATAGCCGCGGCGTCACTCACAGAGATCTCAAGCCGGAGAATCTGCTGCTTGATGAGAATGAAGAGCTTAAGGTC
TCCGATTTTGGCCTCTCTGCTTTGCCGGAACAACGCCGTGCAGATGGTATGCTGATAACACCGTGTGGAACACCTGCATAT
GTGGCACCAGAAGTGTTGAAGAAGAAAGGGTATGATGGATCCAAAGCAGATATATGGTCTTGTGGAGTGATTCTTTATGCT
CTGGTTTGTGGCTATCTACCCTTCCAAGGCGAAAACGTGATGAGAATCTACAGAAAAGCCTTCAAGGCTCAGTATGAATTC
CCTGATTGGGTTCCGGAAGGAGCAAAGAACTTGATCTCAAACCTACTTGTAGCTGATCCTGAAAAGAGGTTCTCAATTGCA
GATATCATGAAGGATCCTTGGTTCCAAGTCGGCTTCATGCGTCCGATTGCATTCTCCTTTAAGGAGTCTGCCATTGGGGAC
AATGTGGATGACTTCGATGAGGATGATAGTGATAGTAATAACAATCAAGAGTTTAACAAGGATGATTTGAAGCAGAGTAGT
TCTGCTAAGCCTGCGCGGCCGTTTTACAATGCTTTCGAGATAATTTCCTCGCTGTCGCATGGTTTTGATCTGAGGAGTTTG
TTTGAGACAAGGAAGAGGTCACCTTCAGTGTTTATATCAAAGCTTTCGGCTCAAGCAGTGGTGGGGAAGCTAGAGGCTATG
GCGAAGAAATTGAATTTCAGAGTGGCCGGGAAGAAGAAGAAGGAGTTTGTTGTGAGGATGGAAGGGGAGAGGGAAGGGAGG
AAAGGGAGGCTGGCCATGACGGTGGAAGTGTTCGAGGTGGCGCCGGAGGTGGCCGTGGTGGAGTTCTCGAAGTCTTCTGGG
GATACCTTGGAGTATATCAAATTCTGTGAGGAAGAAGTTAGGCCTTCATTGAATGACATTGTTTGGAGTTGGCAGGGAGAT
GGTAACAACTAATAATACTAGTCATGCCATTGGGATTATTTTGCCTATGTTGTTCTTCCTTTTTACTGTTTGATGCAGCTA
TATAATAATATAGTTTGATTTGCATAATAAGTAAAAGAAAAGGAAGATGCTAGGAAAGAAATAATGCTGTATTTTGATTTT
AGTTACAGGATTTCATTTCATTTGTGTAAAAAATTTGAGTTTAGTTTCTGATGATGTTTGAAGTTAATACTGAAAAAAAAA
AAAAA

Figure 4.4 Nucleotide sequence showing 2,031 bp long *AdCIPK* c-DNA sequence with 5' and 3' UTR sequences in red color.

4.3.2 Sequence analysis

BlastP analysis result showed the sequence similarity with serine-threonine kinases, protein kinases and CIPK family of proteins **(Figure 4.5 A)**. Based on sequence similarity, various conserved domains were drawn in AdCIPK protein **(Figure 4.5 B)**. The Kinase domain was present between 1 to 277 positions of amino acids, harboring a 29 amino acid long activation loop. Regulatory domain was 134 amino acids long and present between 327 to 461 amino acid positions. Highly conserved NAF domain (327-352) and less conserved PPI domain (355-386) were also present in regulatory domain. Between kinase and regulatory domain, a stretch of 48 amino acids was present as junction domain (278-326) responsible for the joining of both domains. The AdCIPK protein exhibited a maximum of 78% sequence similarity with GmCIPK25 from *Glycine max*. From Arabidopsis, the best CIPKs matches were AtCIPK5 with 62%, AtCIPK25 with 61% and AtCIPK16 with 56% of sequence similarity. Further the alignment of deduced amino acid sequence of AdCIPK with

CIPKs from other plants revealed the presence of conserved N-terminal activation loop and C-terminal NAF domain **(Figure 4.6)**. Like other CIPKs, the activation loop of AdCIPK was also harboring three conserved amino acid Serine (S), Threonine (T) and Tyrosine (Y) as targeting sites for phosphorylation by other protein kinases. Phylogenetic analysis showed the presence of two subgroups as intron-rich and intron-less, in phylogenetic tree **(Figure 4.7)**. AdCIPK was present in intron-less subgroup along with GmCIPK25, AtCIPK5, AtCIPK25 and AtCIPK16 as the evolutionarily closet ones.

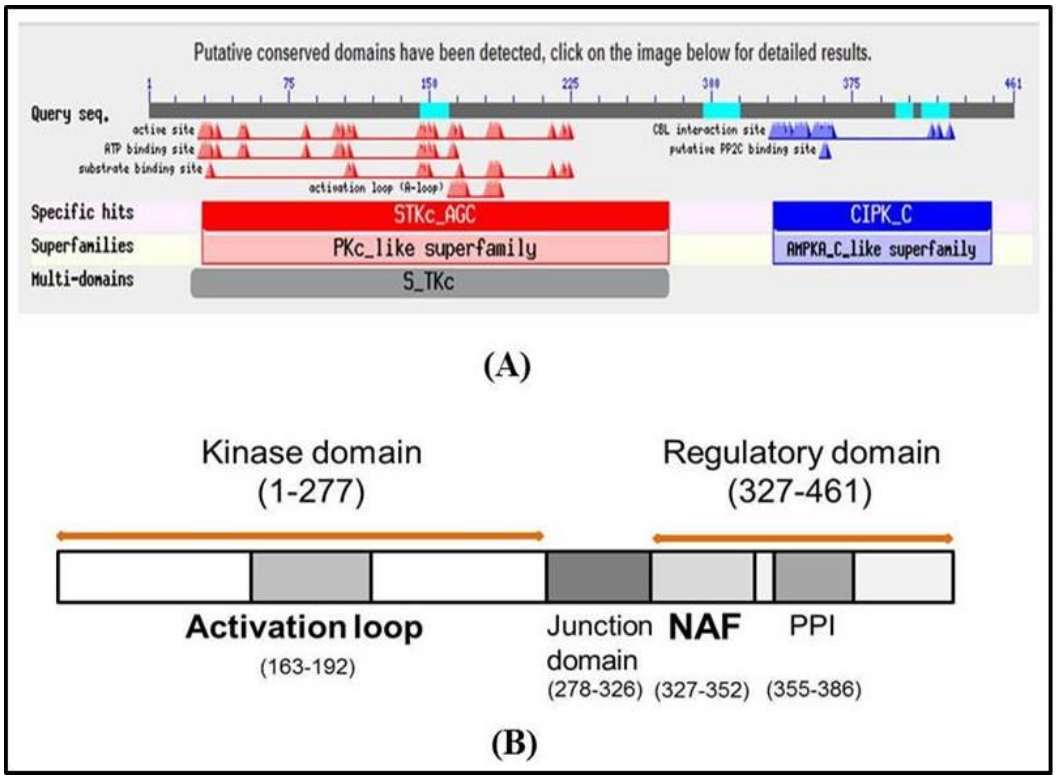

Figure 4.5 Blastp analysis of AdCIPK **(A)** and positions of conserved regions in protein sequence **(B).**

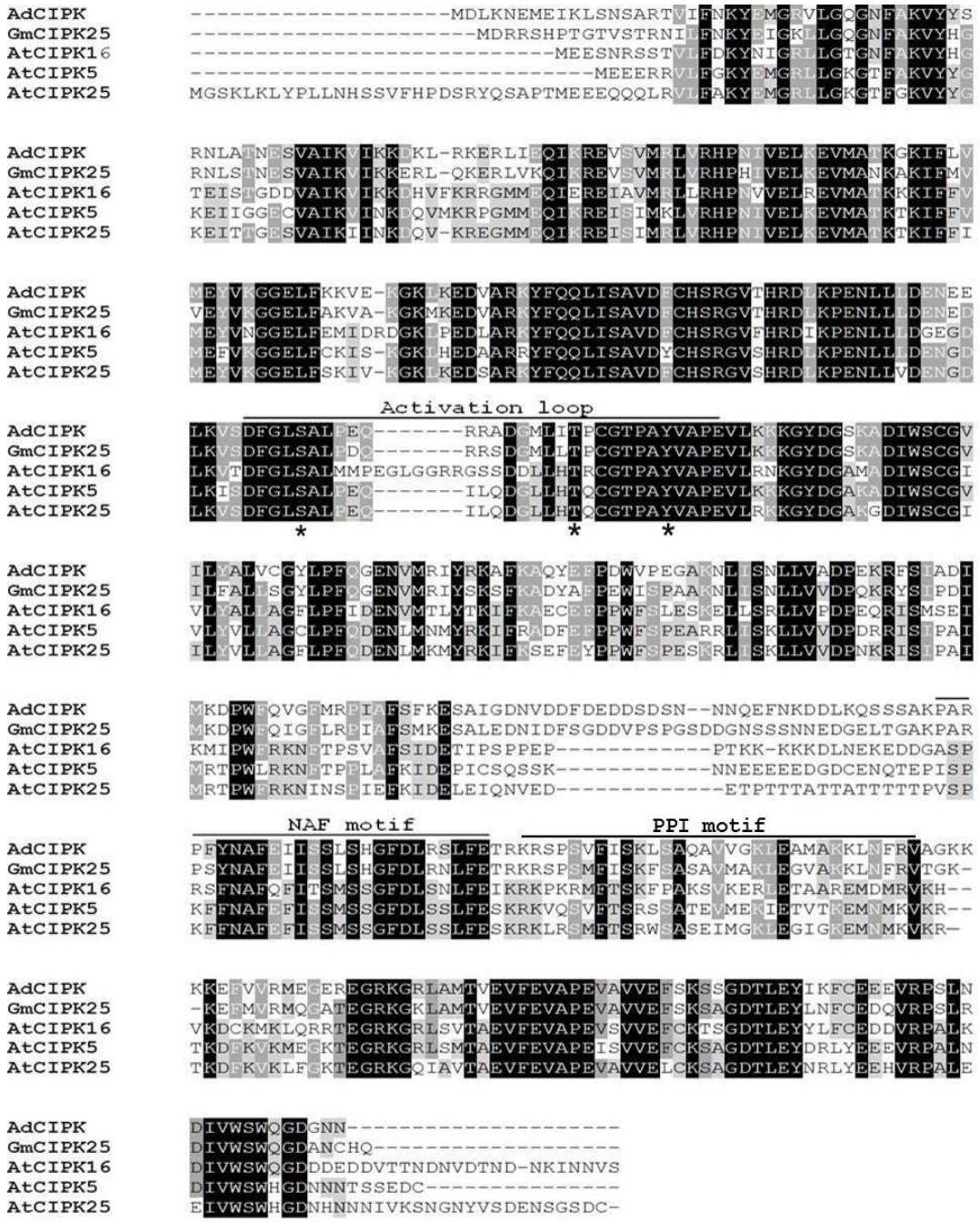

Figure 4.6 Alignment of deduced amino acid sequences of AdCIPK with closely related CIPKs from other plant species. Gm: *Glycine max*; At: *Arabidopsis thaliana*.

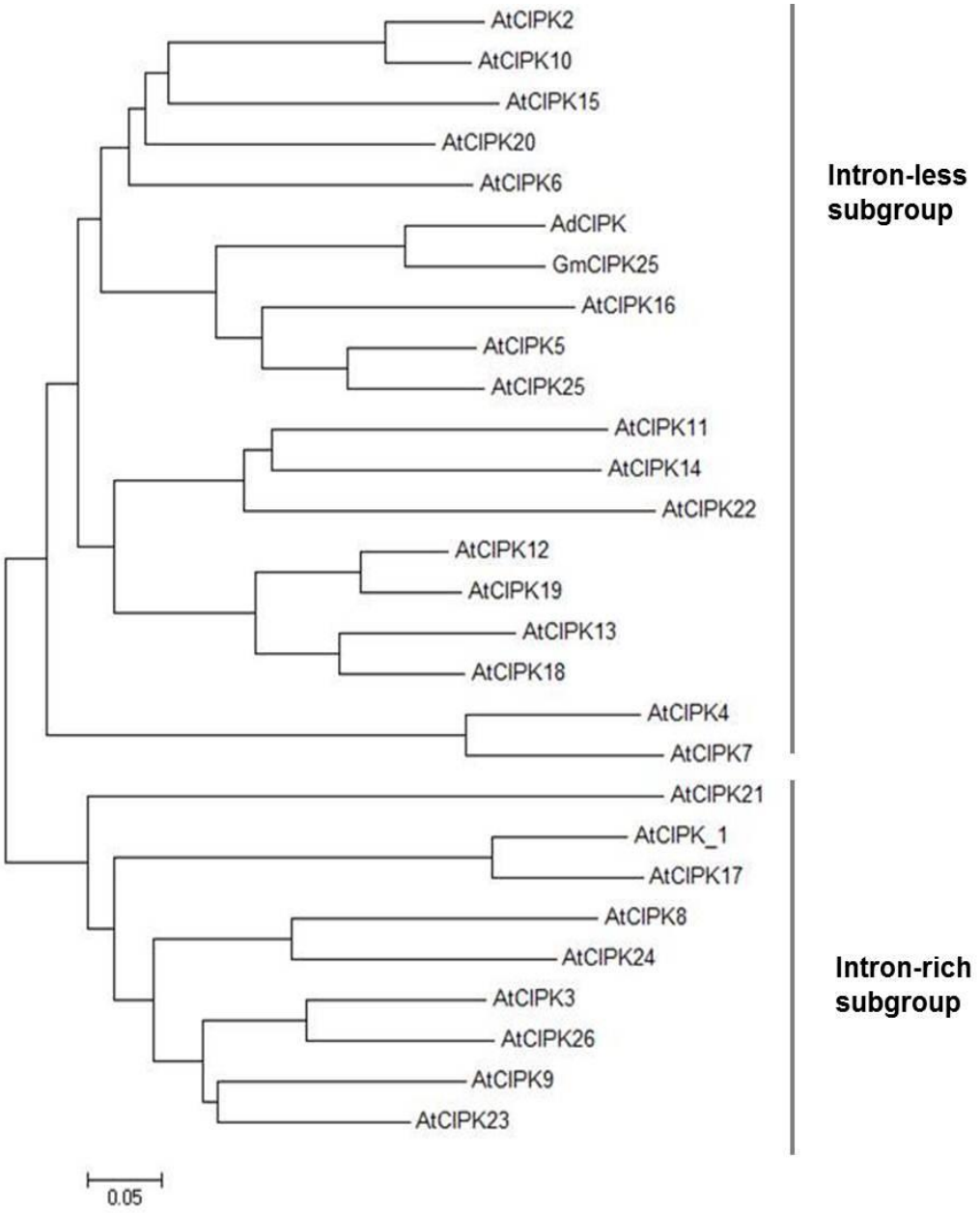

Figure 4.7 Phylogenetic tree of AdCIPK and related protein kinases: GmCIPK of *Glycine max*; AtCIPK 1–26 of *A. thaliana.*

4.3.3 *AdCIPK* expression analysis

CIPKs are involved in the signaling of various stress conditions. Hence the expression profiles of *AdCIPK* under different hormones and stress conditions were detected by qRT-PCR. The data demonstrated that during SA treatment the *AdCIPK* expression was induced early and reached maximum after 12 h **(Figure 4.8 A)** whereas continuous downregulation was observed in case of MJ treatment **(Figure 4.8 B)**. ABA caused strong upregulation of *AdCIPK,* which increased to around 9 fold after 6 h of treatment and got down regulated after 12 h. Again, a rebounding was observed after 24 h **(Figure 4.8 C)**. Slight upregulation was observed after 3 h of ethylene treatment **(Figure 4.8 D)**. *AdCIPK* also responded to abiotic stress conditions like NaCl, PEG and sorbitol. For all three conditions, maximum *AdCIPK* expression was observed after 6 h of treatment **(Figure 4.8 E, F, G)**. However, upregulation was observed after 24 h of treatment in case of NaCl.

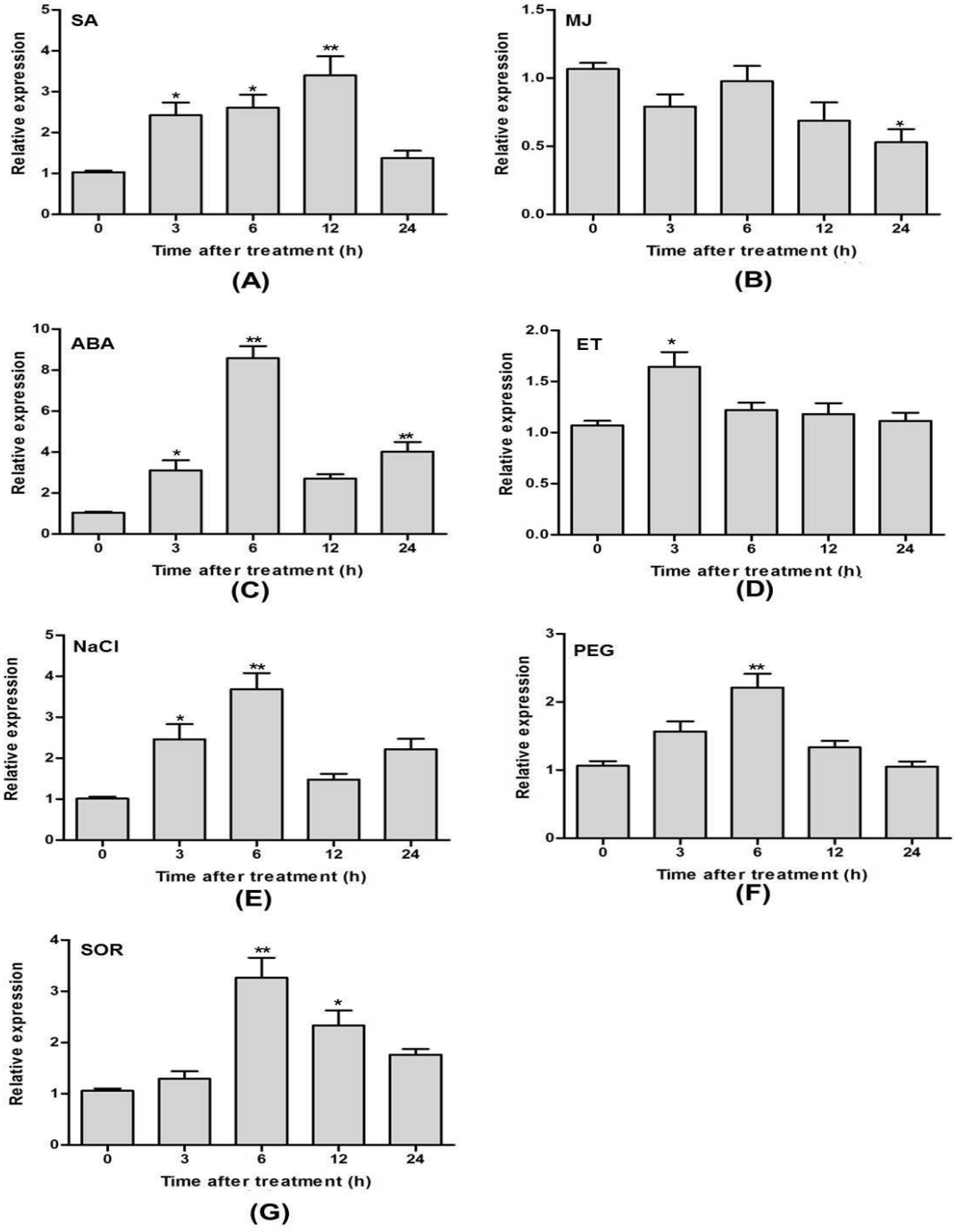

Figure 4.8 Expression analysis of *AdCIPK* in mature leaf of *Arachis diogoi* under different treatments by qRT-PCR. **(A)** 500 µM salicylic acid treatment; **(B)** 100 µM methyl jasmonate treatment; **(C)** 100 µM, abscisic acid; **(D)** 250 µM ethephon treatment; **(E)** 200 mM NaCl treatment; **(F)** 10% polyethylene glycol treatment; **(G)** 300 mM sorbitol treatment. Data represent means ±SE of three replicates. **P* <0.05; ***P* <0.01.

4.3.4 AdCIPK localization

Localization studies help in understanding the distribution of a particular protein inside the cell and could give some idea on its possible role in biological functions. Hence, AdCIPK subcellular localization was studied by constructing a C-terminal translational fusion of GFP with AdCIPK and used in transient expression studies in tobacco leaves through agroinfiltration method. The free GFP expressing from the control empty pCAMBIA1302 vector showed the presence GFP protein throughout the cells including the nucleus **(Figure 4.9 A, B, C)**. However, the recombinant CIPK::GFP protein was found to be localized mainly in plasma membrane with some expression in cytoplasm as well **(Figure 4.9 D, E, F)**.

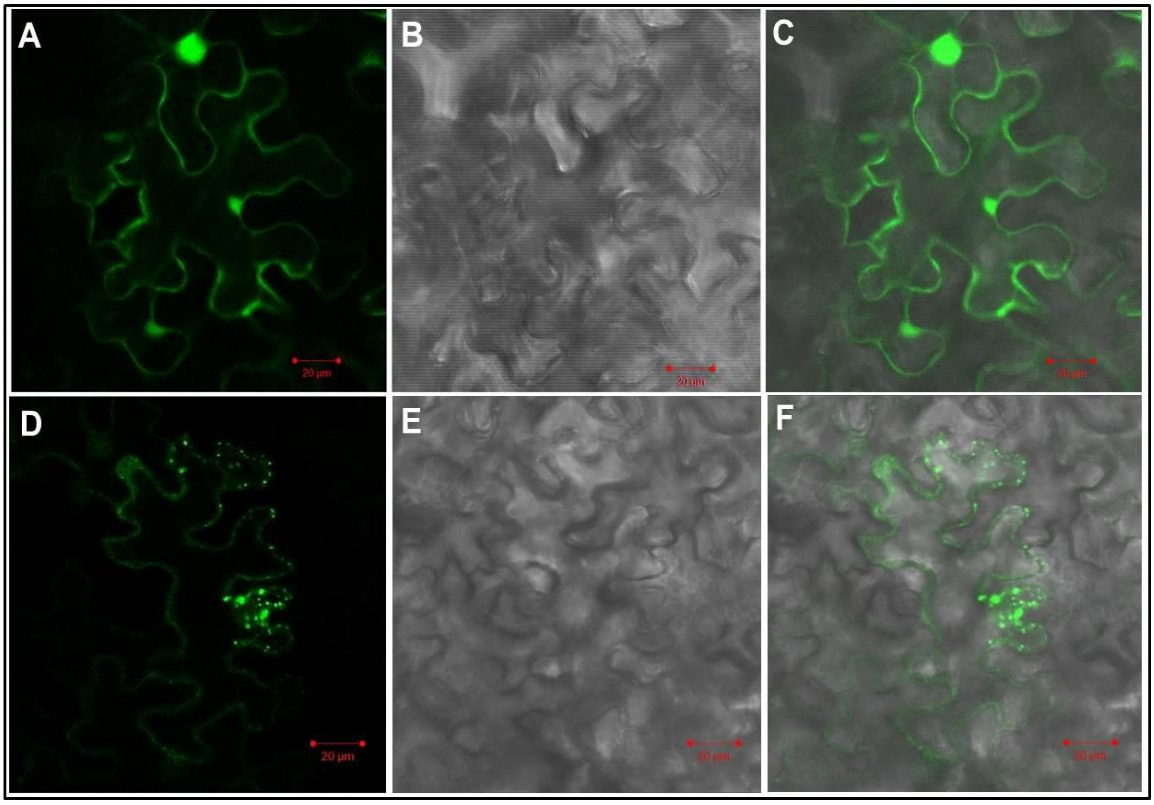

Figure 4.9 Subcellular localization of AdCIPK. The vectors, 35S::GFP (1302-*GFP,* positive control) **(A–C)** and 35S::AdCIPK-GFP (1302-*AdCIPK-GFP*) **(D–F)**, were agroinfiltrated into tobacco leaf cells and visualized under a confocal microscope.

4.3.5 Construct preparation, genomic DNA PCR and RT-PCR analysis in putative transgenic plants

Amplified ORF was cloned in PTZ57R/T vector and confirmed with PCR and restriction digestion with corresponding *Apa*I and *Kpn*I restriction enzymes **(Figure 4.10 A)**. The ORF was cloned in plant expression cassette of pRT100 vector. Subsequently the expression cassette was released with digestion with *Hind*III enzyme and cloned in binary vector pCAMBIA2300 vector. The cassette in pCAMBIA2300 vector was confirmed with restriction digestion **(Figure 4.10 B). Figure 4.10 C** shows the *AdCIPK* expression cassette orientation between left and right border of binary vector along with *npt*II expression cassette.

Genomic DNA was isolated from nine different kanamycin positive plants. PCR was performed for marker gene, *nptII* and *AdCIPK*. All nine plants gave expected amplification of 700 bp of *nptII* and 1383 bp of *AdCIPK*, respectively **(Figure 4.11 A, B)**. To identify the primary transgenic plants with high and low level expression of the target gene, *AdCIPK*, semi-quantitative RT-PCR was performed for all nine different lines **(Figure 4.12 A)**. Actin amplification used as the internal control. This analysis showed that the putative transgenic lines 1 and 6 were with highest and transgenic line 2 was with lowest expression level, respectively**.** The primary transgenic lines 1, 2 and 6 were further used for various analyses in T_2 generation after reconfirmation using PCR and RT-PCR **(Figure 4.12 B).** During our study both high expression lines 1 and 6 were designated as H1 and H2 whereas low expression line 2 designated as Low.

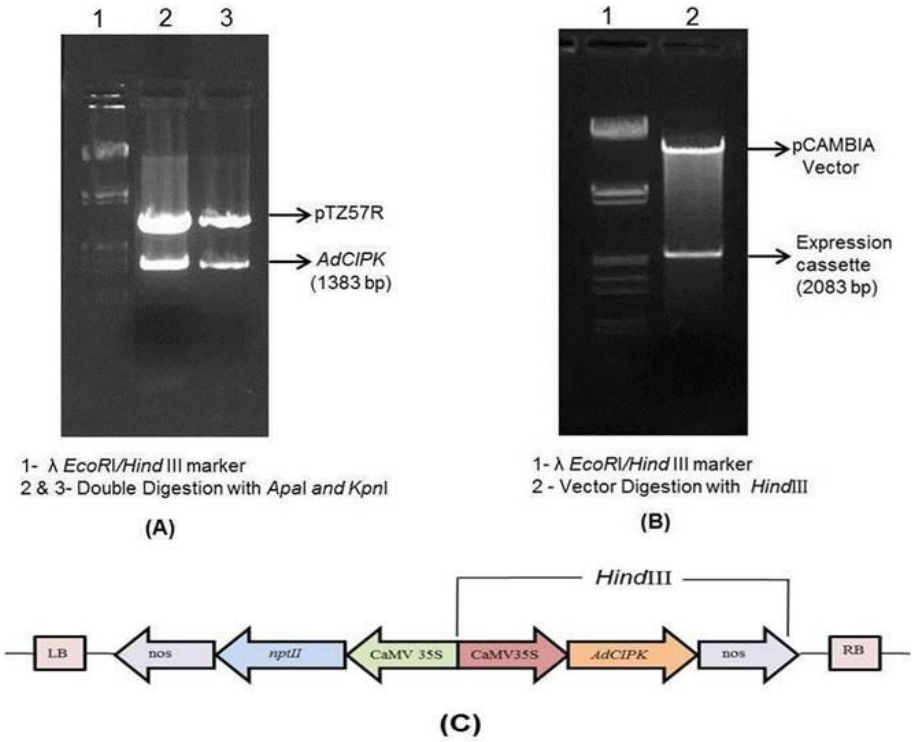

Figure 4.10 Restriction digestion confirmation of cloned *AdCIPK* ORF from pTZ57R/T vector **(A)** and expression cassettes from pCAMBIA2300 vector **(B)**. The orientation of *AdCIPK* and *npt*II cassettes is shown between left and right border of binary vector pCAMBIA2300 **(C).**

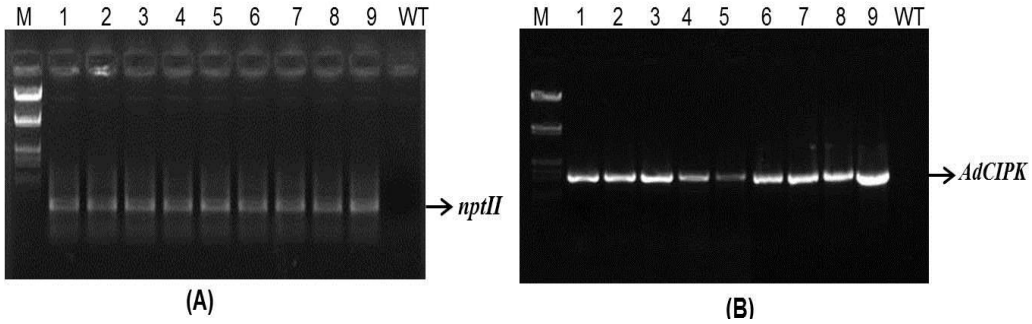

Figure 4.11 Gel pictures showing 739 bp amplified PCR product of *npt*II **(A)** and 1386 bp of *AdCIPK* ORF **(B)** from genomic DNA of nine different putative T_0 transgenic lines and WT plants. Lane M represents λ *Eco*RI/*Hind*III DNA ladder.

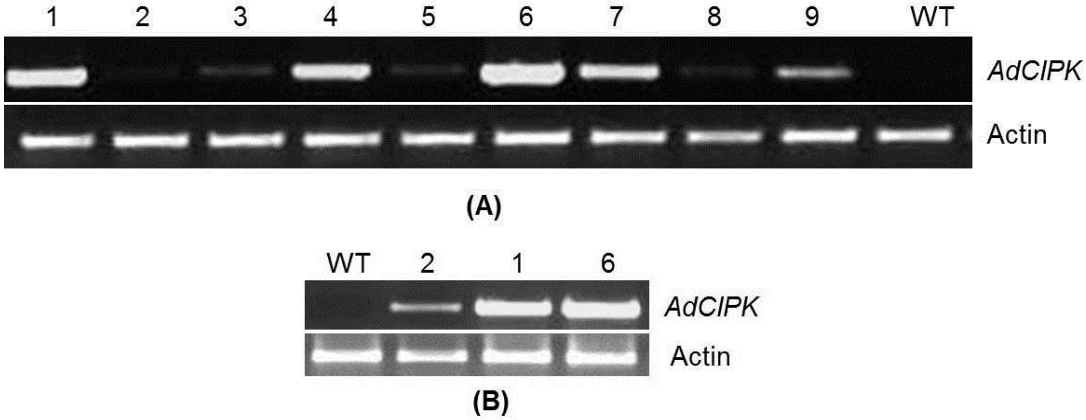

Figure 4.12 Semi-quantitative RT-PCR analysis of *AdCIPK* gene in putative T_0 **(A)** and T_2 **(B)** transgenic plants. Lane 1 and 6 are high expression lines and lane 2 represents the low expression line. Actin served as control to demonstrate equal loading

4.3.6 Antifungal analysis

Leaves of transgenic T_2 plants were collected and used in detached leaf anti-fungal bioassay using the phytopathogenic fungus *Phytophthora parasitica*. Infection symptoms started appearing after 2 days post inoculation (dpi) and observed regularly till 5 dpi **(Figure 4.13)**. After 5 dpi, necrosis covered almost 80% (22.4 ± 1.7 cm^2) of WT, 76 % (21.7 ± 2.2 cm^2) of H1 line and 72% (19.8 ± 2.5 cm^2) of H2 line of the surface of the leaves. This result showed that the level of *AdCIPK* expression in transgenic tobacco had no significant effect on resistance against the test fungus.

Figure 4.13 Resistance to fungal infection was checked in mature leaves of WT and high expression line plants against *Phytophthora parasitica* var. nicotianae. No significant differences were observed.

4.3.7 Seed germination analysis

Seeds from WT and transgenic plants were transferred to MSH media containing 200mM NaCl **(Figure 4.14 B)** and 300 mM sorbitol **(Figure 4.14 C)** along with the control plate **(Figure 4.14 A)**. After 7 d, almost 98 to 100 % seeds germinated on the control plate from all the lines. On NaCl plate, the germination percentage was around 20% for WT, 50% for Low expression line and 70% for both the high expression lines H1 and H2 **(Figure 4.14 D)**. These differences were significantly higher than WT. Similarly, 40-45% of WT, around 70% of Low and almost 90-95% seeds of both the high expression line H1 and H2 germinated on 300 mM sorbitol plate after 7 d. These results showed the higher level of tolerance in transgenic lines against abiotic conditions like salt and osmotic stress, during the seed germination stage.

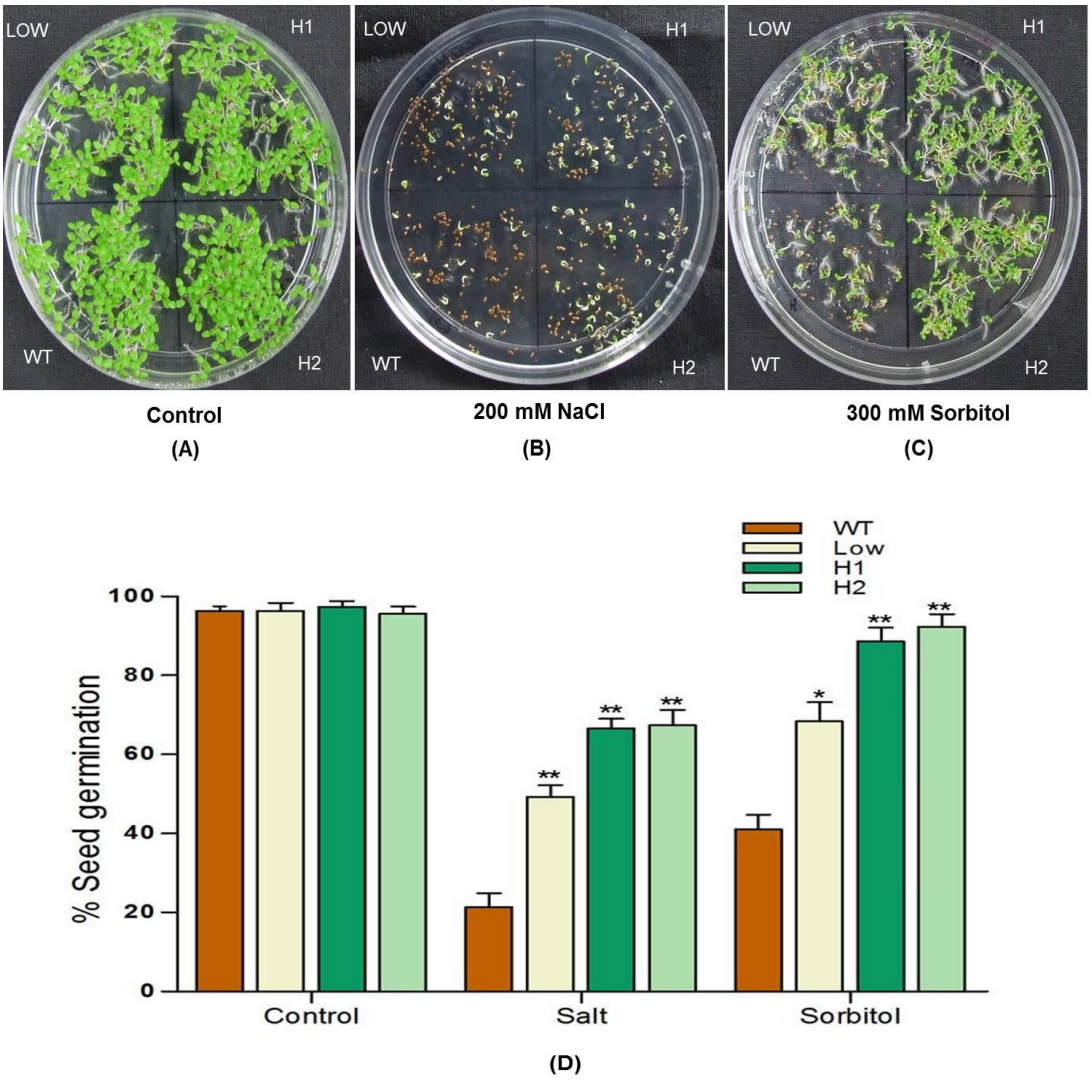

Figure 4.14 Germination rate assay under salt and osmotic stress. The germination rate (about 150 seeds) was detected on MSH medium supplemented 200 mM NaCl **(B)** and 300 mM sorbitol **(C)** for 7 days. Control plate, without any stress treatment, was maintained for the same period of time **(A)**. Graphical representation of percentage seed germination after 7 d of control as well as salt and osmotic stress conditions **(D)**. Three independent experiments were performed. *P <0.05, **P <0.01.

4.3.8 Salt stress tolerance

The 10 d old seedlings of the WT and transgenic lines 1, 2 and 6 germinated on NaCl-free medium were exposed to different levels of NaCl (200 and 300 mM) in MSH medium without organic nutrients. After the 10 d, WT seedling from 200 mM NaCl medium showed higher level of chlorosis and retarted growth compared to transgenic lines. Some chlorosis was also observed in Low expression line seedlings, whereas both the high expression lines

seedlings grew normally **(Figure 4.15 A)**. After 6 d, the WT and low expression line seedlings from 300 mM NaCl medium were severely affected **(Figure 4.15 B)**. However, the high expression lines showed a better growth, but with some degree of chlorosis. Further, the estimation of various physiological parameter revealed that the transgenic seedling retained higher chlorophyll content and catalase activity with lower H_2O_2 values compared to WT on 200 mM salt medium. On 300 mM medium, only high expression lines retained significantly higher chlorophyll and catalase value with low H_2O_2 content **(Figure 4.16 A, B, C)**.

The ability of seedlings to recover from stress condition was checked by transferring them from salt to salt free MSH medium. The WT and transgenic seedlings from both 200 and 300 mM medium were transferred after 6 d of treatment and observed for 15 d. Control plate was also maintained simultaneously **(Figure 4.15 C, D, E)**. Both the high expression line seedlings from 200 mM medium recovered completely with true leaf and root formation. However, among low expression line seedlings, around 50% were able to recover with slower growth rate whereas WT were completely bleached out after 15 d of recovery period. From 300 mM medium, almost all the seedlings from WT and low expression line were bleached out after the recovery period, whereas around 50% seedlings from high expression lines were able to recover with true leaf formation.

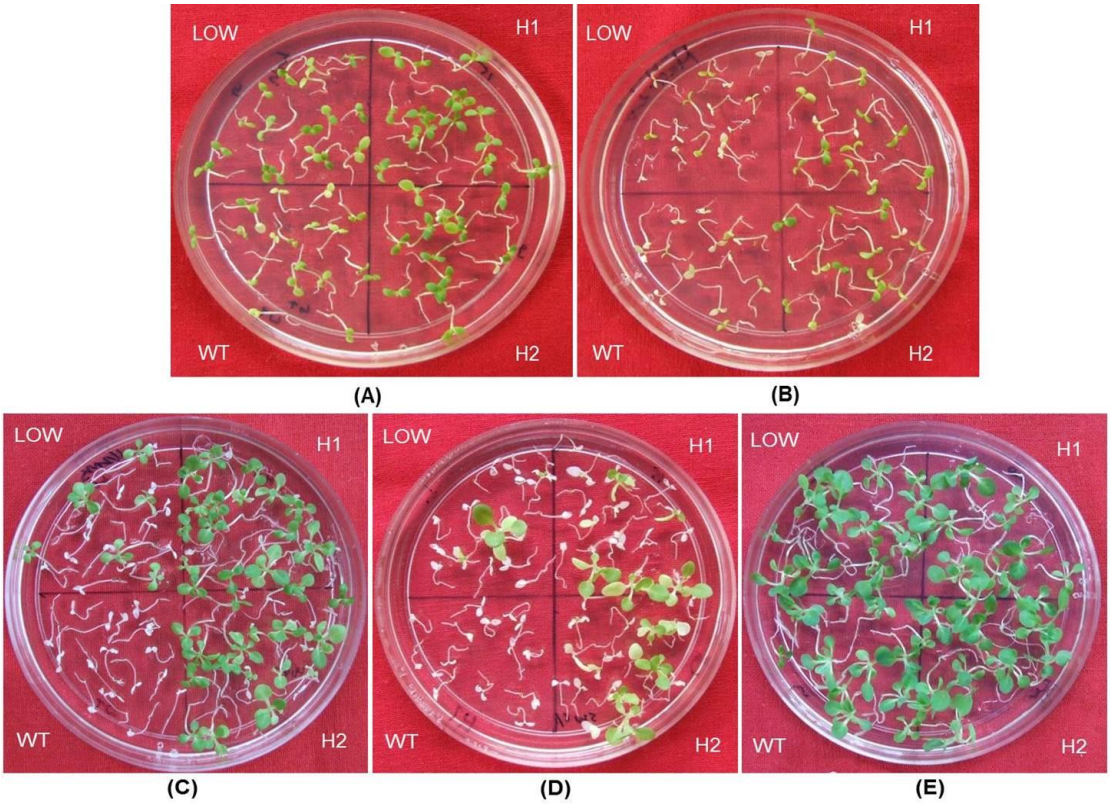

Figure 4.15 Effect of salt stress on the growth of WT and transgenic tobacco seedlings on MSH supplemented with 200 mM (for 10 d) **(A)** and 300 mM (for 6 d) **(B)** NaCl. Three independent experiments were performed with similar results. Seedlings on NaCl-free medium; recovery after treatment with 200 mM NaCl **(C)** and 300 mM NaCl **(D)**. Unstressed seedlings were regularly subcultured along with the NaCl-treated seedlings **(E)**.

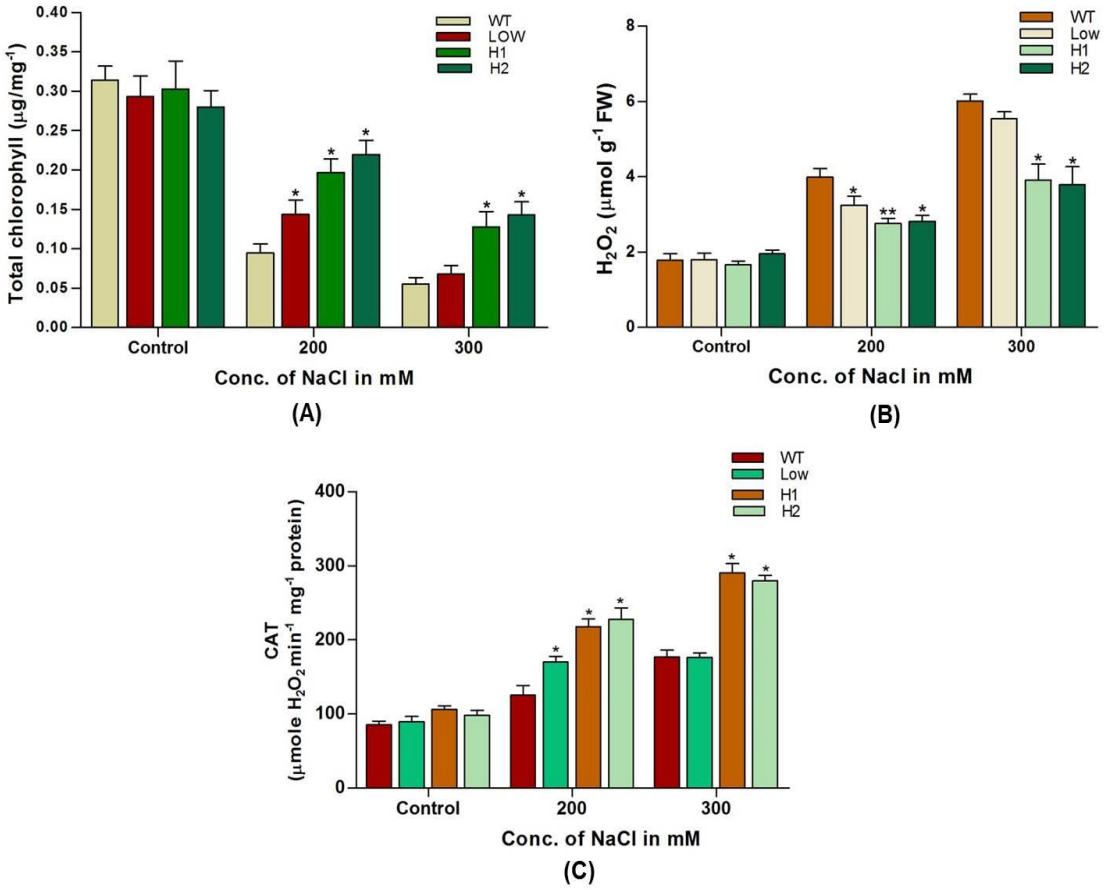

Figure 4.16 Analysis of physiological indices in the transgenic and control plants under salt stress. 10 d old different lines were exposed to salt treatment (200 and 300 mM NaCl) for 6 days. To assess the chlorophyll content, tobacco leaves were then sampled **(A)**. The seedlings were sampled to detect H_2O_2 content **(B)** and CAT activity **(C)**. Data represent means ±SE of three replicates. Three independent experiments were performed. *P <0.05, **P <0.01.

4.3.9 Osmotic stress tolerance

To check osmotic stress response at seedling stage, 10 d old WT and transgenic seedlings were transferred to medium containing 200 and 300 mM sorbitol and observed for 15 d. The transgenics grew normally in 200 and 300 mM sorbitol, but the WT plants showed growth retardation **(Figure 4.17 A, B)**. The root length of the WT was suppressed at both concentrations **(Figure 4.17 D)**. In contrast to this, all the transgenics exhibited significantly higher root length compared to the WT seedlings on 200 mM medium **(Figure 4.17 E)**. On 300 mM medium, in low expression line seedlings growth was also suppressed

with a non-significant difference in root length with WT, whereas both the high expression lines revealed better growth with significantly higher root length. Similar growth of the transgenic and the WT seedlings without sorbitol treatment was shown in **(Figure 4.17 C)**. Further on catalase and H_2O_2 analysis revealed that transgenic seedling retained higher catalase activity with lower H_2O_2 content on stress media **(Figure 4.18 A, B)**.

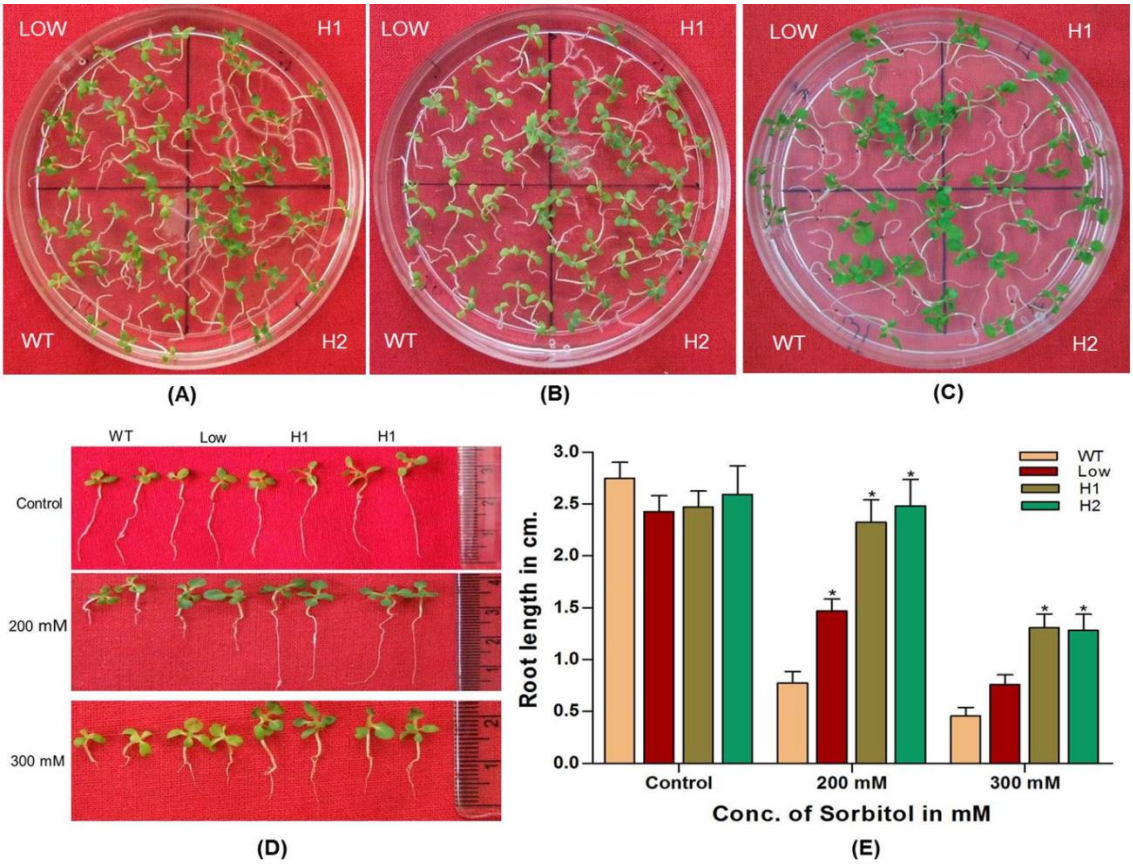

Figure 4.17 Transgenic and WT seedlings were transferred to 200 mM **(A)** and 300 mM **(B)** sorbitol medium. Seedlings not subjected to stress **(C)**. Seedlings morphology after 15 d of osmotic stress and control conditions **(D)**. Graphical representation of root lengths in sorbitol-stressed seedlings after 15 d treatment **(E)**. Three independent experiments were performed. *P <0.05.

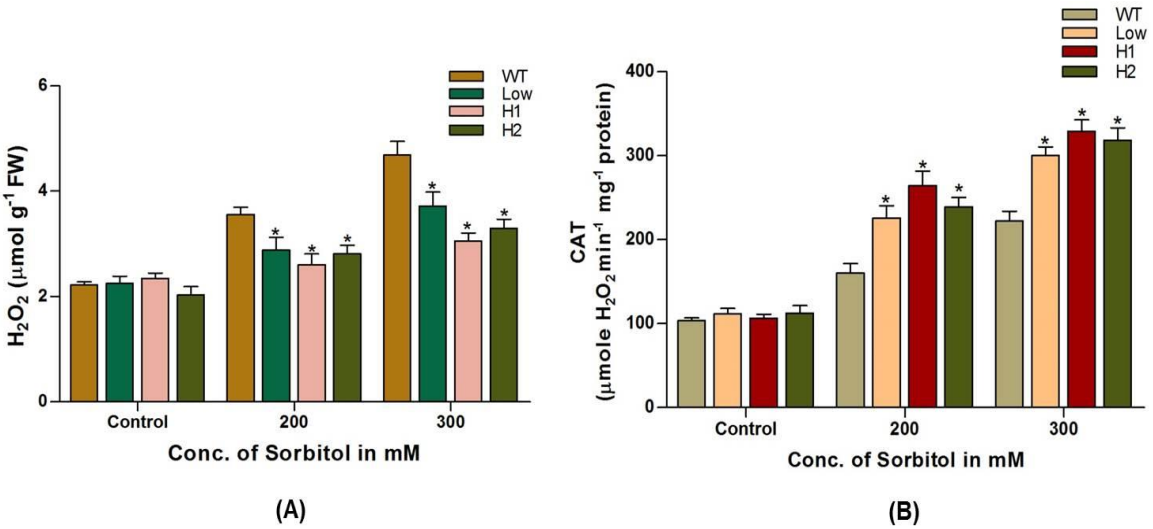

Figure 4.18 After 10 d of stress treatment, WT and transgenic seedlings from 200 and 300 mM sorbitol medium were sampled to detect H_2O_2 content **(A)** and catalase activity **(B)**. Data represent means ±SE of three replicates. Three independent experiments were performed. *P <0.05.

4.3.10 Leaf Disc assay for salt and osmotic stress

To assess the stress tolerance at more mature stage, leaf disc assay was performed against different concentrations of NaCl and sorbitol. Leaf discs from plants grown for 7-wks in the greenhouse were used for both the treatments. NaCl tolerance in these transgenic plants was observed for 3 d **(Figure 4.19 A)**. Chlorophyll estimation revealed the dose dependent loss of total chlorophyll in the WT compared with the transgenic plants, which were able to retain significantly higher chlorophyll content **(Figure 4.20 A)**. However, a non-significant difference was observed between WT and Low line plants on the 300 mM medium. Similarly, the levels of TBARS increased in the WT compared to the transgenic lines **(Figure 4.20 B)**. In case of sorbitol treatment, chlorosis started appearing after 3 d, which became more prominent after 5 d in WT with increasing concentration of sorbitol, whereas the leaf discs of transgenic plants showed little chlorosis **(Figure 4.19 B)**. Leaf discs from distilled water treatment remained green in both WT and transgenic plants. Further, the chlorophyll content and lipid peroxidation levels (TBARS) measurement of these leaf discs after 96 h treatment confirmed the observed phenotypic differences **(Figure 4.20 C, D)**.

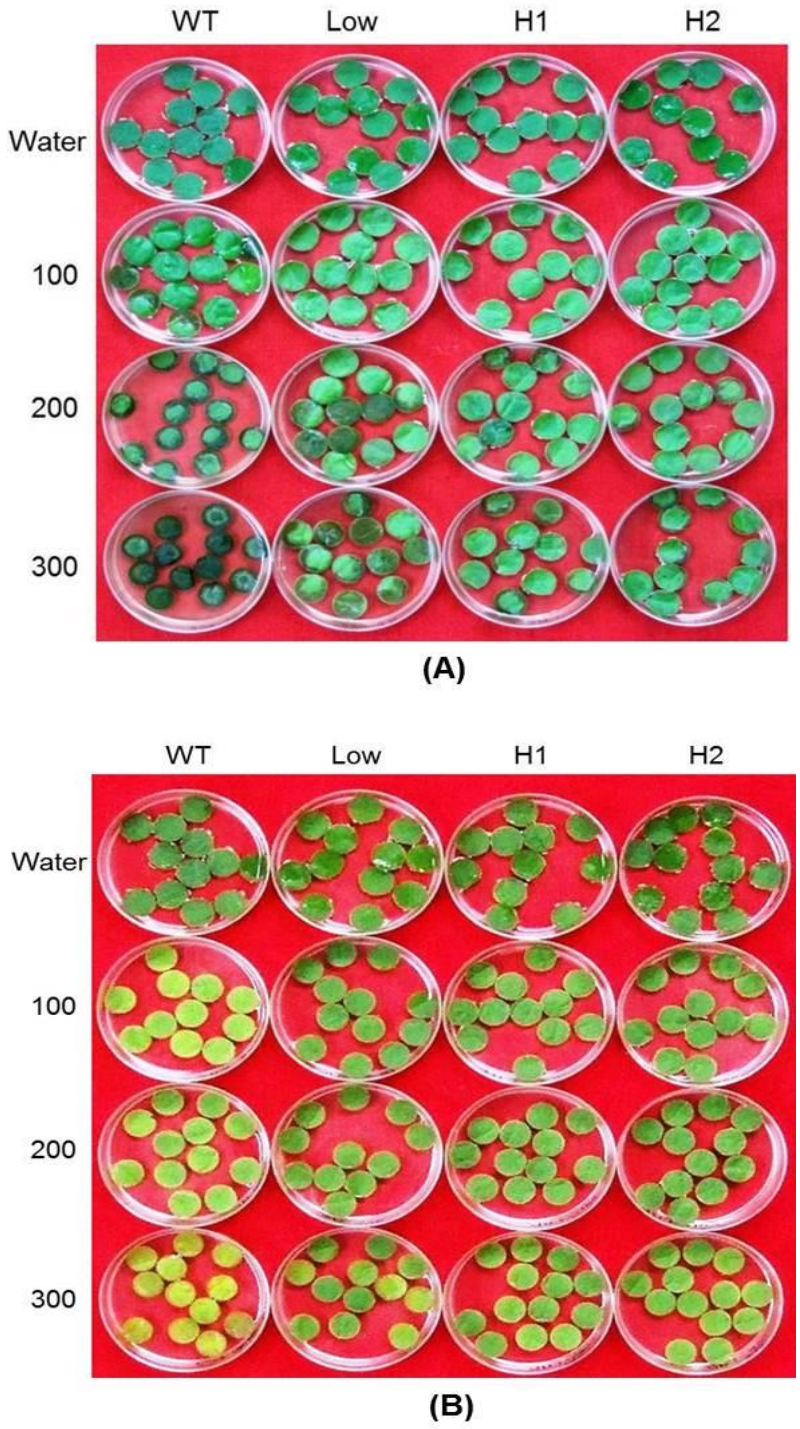

Figure 4.19 Stress tolerance exhibited by the leaf discs of WT and transgenic lines at various concentrations of salt (100, 200 and 300 mM) **(A)** and sorbitol (300, 400 and 500 mM) **(B)** treatments.

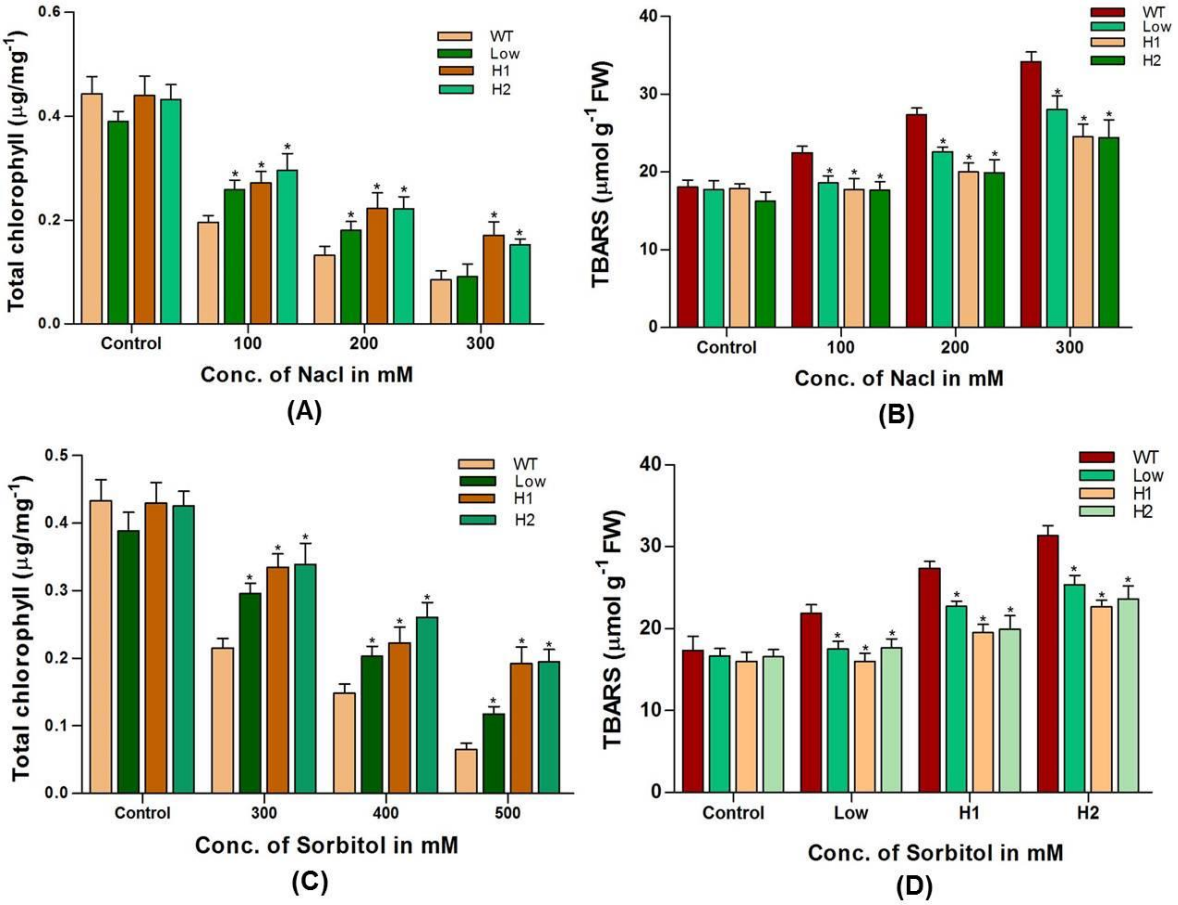

Figure 4.20 Total chlorophyll and TBARS were measured in WT and transgenic leaf discs after 2 d of NaCl treatment (**A** and **B**) and after 4 d of sorbitol treatment (**C** and **D**). Experiments were repeated three times and means ±SE were plotted. **P* <0.05; ***P* <0.01.

4.3.11 ROS analysis

Both the high expression lines H1 and H2 along with the WT were used for the ROS detection. In untreated control samples (without NaCl); the observed fluorescence due to the formation of H_2O_2 in stomatal guard cells was more or less similar in WT and transgenic plants **(Figure 4.21 D, E, F)**. Increased fluorescence was observed in WT and the transgenic plants guard cells with NaCl (100 mM) treatment **(Figure 4.21 A, B, C)**. However, the fluorescence displayed by the transgenic plants was less compared to the WT plants, which implied that there was reduced ROS formation in transgenic guard cells.

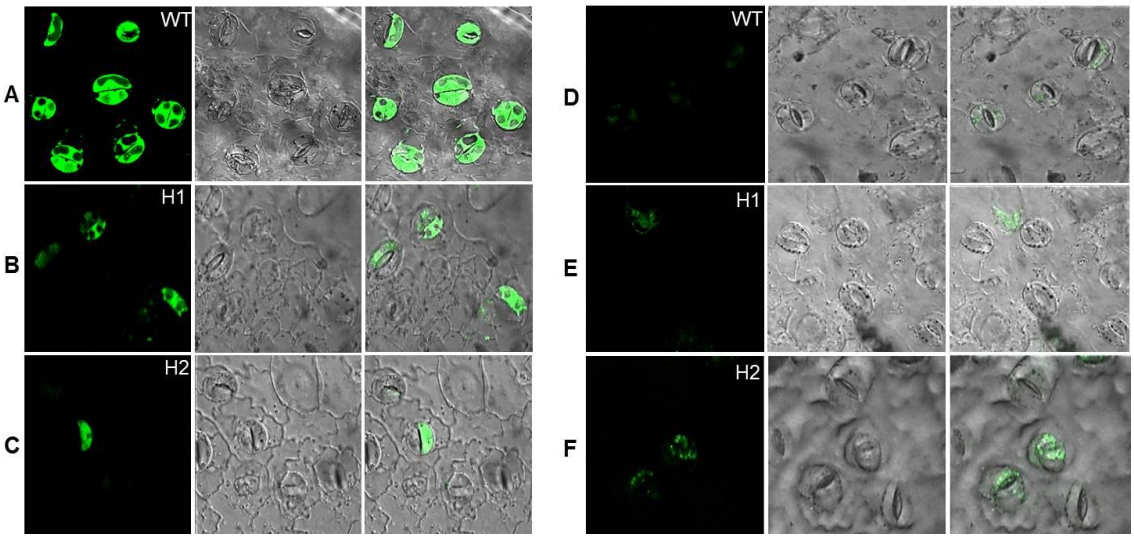

Figure 4.21 ROS detoxification by WT and high expression transgenic lines show fluorescence by staining with H_2DCFDA after 100 mM NaCl treatment in the epidermal guard cells as revealed by confocal microscopy **(A, B, C)**. Bright field images of WT and transgenic plants were also displayed. The basal florescence in control conditions (without NaCl) is also represented by confocal image **(D E, F)**.

4.3.12 Transcript level analysis of stress related genes

To understand further the effect of *AdCIPK* overexpression in salt and osmotic stress tolerance, the expression of different stress-related genes was detected in WT and the transgenic lines (Low and H1) with or without stress treatment. The transcript level of six genes (*NtCAT*, *NtERD10C*, *NtERD10D*, *NtNCED1, NtSus1* and *NtSOS1*) were analyzed, which are reported to be involved in response to abiotic stress. Two-week-old WT and transgenic plants grown in Petri dishes were exposed to salt stress treatment (200 mM NaCl) and sorbitol treatment (300 mM) for 3 d followed by qRT-PCR analysis. The results revealed that all stress-responsive genes analyzed were significantly induced in the high expression line in comparison to the control plants when exposed to salt and sorbitol treatment **(Figure 4.22)**. These results suggested that *AdCIPK* overexpression in tobacco could increase the level of expression of stress-related genes under salt and osmotic stress conditions.

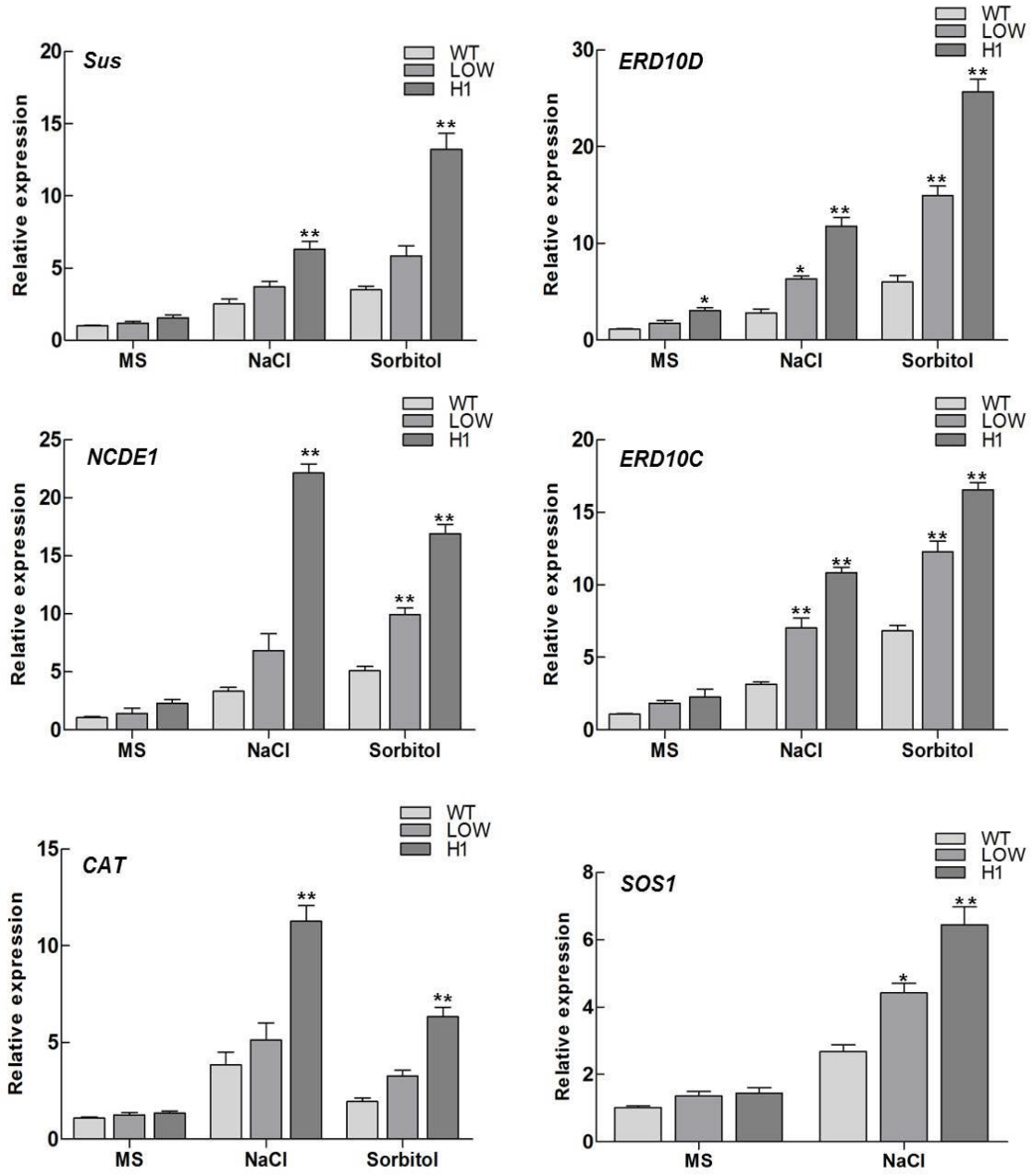

Figure 4.22 Transcript level analysis of stress-related genes in the control and transgenic plants under salt and osmotic stresses. Seedlings of two week old different lines were exposed to salt treatment (200 mM NaCl) and osmotic treatment (300 mM sorbiotol) for 3 d. Then the gene expression was measured by qRT-PCR. Data represent means ±SE of three replicates. Three replicated experiments were done with similar results. **P* <0.05; ***P* <0.01.

4.4 Discussion

In the present study we have identified, cloned and studied the possible role of a CIPK gene from *Arachis diogoi*. The 361 bp partial sequence of gene was identified during fungal infection (Kumar and Kirti, 2011). Based on the available partial sequence, a full length gene was RACE amplified and cloned from *Arachis diogoi*. For our study, it was named as *AdCIPK*. Sequence analysis of AdCIPK protein showed that the gene did not harbor any signal peptide sequence. Further, the amino acid sequence analysis revealed the presence of highly conserved domains like activation loop, NAF domain and less conserved PPI motif in the protein sequence. These conserved sequences are the characteristic feature of CIPK family proteins and important for their function in plants (Albrecht et al., 2001; Kolukisaoglu et al., 2004; Ohta et al., 2003). Examination of phylogenetic tree showed that *AdCIPK* was closely related to *AtCIPK5*, *AtCIPK25*, and *AtCIPK16* of *Arabidopsis thaliana*. Out of these 3, only *AtCIPK16* is well characterized and has been reported to be involved in enhanced salinity tolerance in transgenic barley plants (Roy et al., 2013). However, *AtCIPK5* was found to be induced during developmental processes and *AtCIPK25* during fungal infection (Huibers et al., 2009; Schmid et al., 2005). The transcript levels of *AdCIPK* in *Arachis diogoi* were checked during different hormones and abiotic stress treatments at different time intervals. The resulting analysis showed that except MJ, the transcript levels were differentially upregulated at some point of time during the SA, ABA and ET hormones treatment. Similarly higher levels of *AdCIPK* induction were also observed during salt, sorbitol and PEG treatments. The phytohormones used in the treatments, mimic both biotic and abiotic stress conditions; these results predicted the possible involvement of *AdCIPK* in both biotic and abiotic stress mechanism.

CIPKs appear not to harbor any recognizable localization signal (Kolukisaoglu et al., 2004). Their localization could be dependent on their respective interaction partners, which regulate the activity and localization of the interacting CIPKs at different sites inside the cell (Batistic and Kudla, 2004). For example, CBL3 is involved in dynamic translocation of CIPK5 from cytoplasm to tonoplast (Schlücking et al., 2013). Similarly AtCIPK1 was found to be localized to the plasma membrane, and to some extent also the nucleus and the cytosol, which was further recruited to the plasma membrane after interacting with AtCBL1 and AtCBL9 (D'Angelo et al., 2006). In case of AdCIPK, the protein was mainly found

to be localized to the plasma membrane and to some extent to the cytoplasm. Since no information is available about its interacting partners, it can be presumed that depending on the interacting partners and given conditions, the AdCIPK protein could be further localized at different sites within the cell.

To study further the role of AdCIPK in stress tolerance mechanisms, transgenic tobacco plants were raised. In comparison to abiotic stress, less is known about the role of CIPKs in biotic stress tolerance mechanisms. Recent report about rice *CIPK14/15* suggested that the genes were playing crucial role in rice cultured cells during MAMP-induced defense signaling pathways (Kurusu et al., 2010). Similarly, a set of *CIPK6/CBL10* from tomato was involved in plant immunity by generating ROS during ETI in the interaction of *Pseudomonas syringae* and *Nicotiana benthamiana* (de la Torre et al., 2013). The partial sequence of *AdCIPK* gene was identified induced during fungal infection (Kumar and Kirti, 2011), hence the levels of tolerance were checked in WT and tobacco transgenic lines against a phytopathogenic fungus *Phytophthora nicotaianae*. No significant differences were observed in damaged leaf area between them, showing that the ectopic expression *AdCIPK* gene did not enhance the level of fungal tolerance in the transgenic plant. It is likely that *AdCIPK*, which has been identified during the interaction with a hemibiotroph, would not be suitable in manipulating resistance to necrotrophic pathogen like *P. nicotianae*. The *AdCIPK* induction during various hormones and stress treatments could be the result of a cross talk among different stress mechanisms. However, further investigation of *AdCIPK* could draw a better picture about its importance during fungal infection.

Most of the reported CIPKs were found to be involved in abiotic stress mechanism (Kim et al., 2007; Quintero et al., 2002; Weinl and Kudla, 2009). The transcript levels of *AdCIPK* were also induced during abiotic stress conditions like NaCl, sorbitol and PEG. Hence, the tolerance levels were checked in WT and transgenic plants against salt and osmotic stresses by using NaCl and sorbitol. On both 200 mM NaCl and 300 mM sorbitol containing media, significantly higher percentage of transgenic seeds germination were observed compare to WT, showing higher level of tolerance to both salt and osmotic stress during the germination stage. Seed germination in stress is an important constituent of the stress tolerance mechanism as it is the first step towards tolerance.

Tolerance levels in transgenic lines were investigated further at seedling and mature stage. Various reports about different CIPKs suggest their involvement in salt tolerance mechanism. For example, *AtCIPK16* was involved in enhancing salt tolerance in transgenic barley plants (Roy et al., 2013). Overexpression of a CIPK gene from rice, i.e. *OsCIPK15*, enhanced salt tolerance in rice plants (Xiang et al., 2007). Similarly, ectopic expression of a wheat *TaCIPK14* gene conferred salinity tolerance in transgenic tobacco plants (Deng et al., 2013). Two different concentrations of salt were used for seedlings assay of *AdCIPK* transgenics. Transgenic lines were able to perform better than WT on both 200 and 300 mM NaCl medium. However, the 300 mM concentration was probably too high even for the transgenic plants. It was further confirmed on recovery medium where only 40-50% seedlings of high expression lines could recover from 300 mM salt stress, whereas most of them were able to recover from 200 mM concentration.

Various physiological parameters were checked to confirm the enhanced levels of tolerance in transgenic plants. Salinity stress, in general, can generate excessive reactive oxygen species (ROS), which are toxic to cell and can result in membrane destruction and cell death (Gouiaa et al., 2012). Increased ROS accumulation, due to high Na^+ resulted in damage to the activities of various enzymes and cellular membranes with higher Na^+ levels under salt stress (Zhu, 2001). However, K^+ is an essential co-factor for many enzymes (Blaha et al., 2000) and higher K^+/Na^+ ratio may help in maintaining the cytosolic ROS level (Mahajan et al., 2008). Higher CAT activity could reduce stress induced ROS level and protect cells from damage. Previous work in Arabidopsis revealed the direct interaction between AtCIPK24, and CAT2 and CAT3, thereby linking CIPK and H_2O_2 pathways. The *AtCIPK24* knock-out mutant accumulated more H_2O_2 during salt stress (Verslues et al., 2007). In case of AdCIPK, the higher chlorophyll content, higher CAT activity with generation of lower levels of H_2O_2 content in transgenic plants showed that the AdCIPK overexpression can enhance the salt stress tolerance by maintaining the cellular homeostasis. Similarly during the osmotic stress conditions also, transgenic seedlings showed better growth with higher CAT activity and lower H_2O_2 content.

To check the response of AdCIPK at more mature stage of the plants, WT and transgenic leaf discs were used against different concentrations of NaCl and sorbitol treatments. Total chlorophyll content and TBARS values were checked in all the treated

leaf discs. During both the treatments, transgenic lines were able to retain higher chlorophyll content with low TBARS value compared to WT plants. Analysis of TBARS is used to evaluate ROS-mediated membrane damage (Iturbe-Ormaetxe et al., 1998). Since the transgenic plants showed higher CAT, lower H_2O_2 and TBARS values during various stress treatments, the AdCIPK overexpression appeared to be involved in stress induced ROS detoxification. Hence, to confirm it further during salt stress, ROS levels were checked by using H_2DCFDA in stomatal cells. The results clearly showed higher level of fluorescence in WT plants compared to high expression transgenic lines, suggesting low ROS production due to AdCIPK overexpression. Similar results were observed in root cells of Arabidopsis *SOS2* (*AtCIPK24*) mutant where higher ROS was observed in mutant plant compared to WT during salt stress (Zhu et al., 2007). These results clearly suggest that AdCIPK overexpression resulted in the reduced damage to the membrane components of the cell and cell organelles in transgenic plants and made them tolerate different levels of NaCl and sorbitol at various stages.

To understand the function of AdCIPK in regulation of gene expression under salt and osmotic stresses, the transcript level of several stress-related genes was measured. The results showed that the expression of the genes, *NtERD10C*, *NtERD10D*, *NtCAT*, *NtNCED1*, *NtSus1* and *NtSOS1* was significantly higher in *AdCIPK* overexpressing high expression line plants than WT under salt stress condition. Previous reports revealed that these genes contributed in plant stress tolerance mechanism. For example, *NtERD10C and NtERD10D* encode LEA proteins, which are involved in protecting and stabilizing macromolecules and cellular structures during the stress adaptive responses in plant (Amara et al., 2012). *CAT* encodes catalase, which is directly involved in ROS detoxification and important for stress defense mechanism (Polidoros et al., 2001). *NtNECD1* plays important role in ABA biosynthesis regulation (Huang et al., 2010; Qin and Zeevaart, 1999). *Sus1* encodes a sucrose synthase enzyme, which responds to the cellular osmotic changes during stress conditions (Dejardin et al., 1999). A plasma membrane Na^+/H^+ antiporter, responsive to Na^+ exportation, is encoded by *SOS1* gene. Salt tolerance in transgenic Arabidopsis plants were improved by overexpressing *AtSOS1* (Shi et al., 2002). Similar results were also observed in osmotic stress tolerance. ROS detoxification and

maintenance of cellular homeostasis along with membrane integrity appeared to be the underlying mechanism of stress tolerance exhibited by *AdCIPK* transgenic plants.

4.5 Conclusion

In conclusion, a wild peanut CIPK gene, *AdCIPK*, was amplified and cloned. The partial sequence of gene was initially identified during fungal infection which was extended by RACE amplification. Transcript level analysis showed that the gene got upregulated against various hormone and abiotic stress conditions, identifying *AdCIPK* as a stress responsive gene. Overexpression of *AdCIPK* in tobacco plants enhanced salt and osmotic stress tolerance at various stages by regulating the expression of stress related genes and enhancing the antioxidant system to reduce ROS accumulation and membrane damage. Not much information is available about peanut CBL systems, hence future work will emphasize on identifying the direct targets of AdCIPK, which could help in understanding the precise function of *AdCIPK* during both biotic and abiotic stress conditions.

Table 4.1 Sequences of the oligonucleotides used in the study (see text for details).

Name of the primers	Primer sequences (5′-3′)
SMARTer II A oligo	AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG
5′ CDS	5′-$(T)_{25}$ V N-3′ (N=A, C, G or T; V= A, G or C)
3′ CDS	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ V N (N=A, C, G or T; V=A, G or C)
Universal Primer A Mix	Long 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ Short 5'-CTAATACGACTCACTATAGGGC-3'
Nested Primer	AAGCAGTGGTATCAACGCAGAGT
5′GSP-1	AGAACTACTCTGCTTCAA
5′GSP-2	CATCCTCATCGAAGTCATCCA
5′GSP-3	AAAGGAGAATGCAATCGGACG
3′ GSP1	TGGATGACTTCGATGAGGATG
ORF-F1	GCCATGGATCTGAAAAACGAGATGGA
ORF-R1	CCACTAGTGTTGTTACCATCTCCCT
ORF-F2	CCGGGCCCATGGATCTGAAAAACGAGATGGA
ORF-R2	CCGGTACCTTAGTTGTTACCATCTCCCTGC

Table 4.2 Sequences of the oligonucleotides used in qRT-PCR (see text for details). Nt: *Nicotiana tabacum*; Ad: *Arachis diogoi*.

Oligo name	Forward (5′-3′)	Reverse (5′-3′)
NtCAT	GGCCGCTACAACTCTCTCTTT	ACAGGACCTCTTGCACCAAC
NtERD10C	AAAGCCAACTCATGCCCAAG	AGAGCTGCTACTTGATCGATGG
NtERD10D	GCACGAGGGAAGAAGAGAAGG	TGGAGGCGCCACTTCCTC
NtNCED1	TGTCTGAAATGATCCGGGGC	AGTTTCCGGCTCTTCCCAAG
NtSUS1	CACGGATATTTCGCCCAGGA	GCAGCAGCCGAGTAGCAATA
NtSOS1	CAAATGTTATCCCCCGAAAGC	CGGAGAACCTGAGGAAATGTGA
NtUbq	GAGTCAACCCGTCACCTTGT	ACATCTTTGAGACCTCAGTAGACA
Adadh3	GACGCTTGGCGAGATCAACA	AACCGGACAACCACCACATG
Adcipk	CCATGACGGTGGAAGTGTTC	TACCATCTCCCTGCCAACTC

Chapter 5:

Cytochrome P450 Monooxygenase (*AdCYP97A3*)

5.1 Background

In both animals and plants, cytochrome P450 monooxygenases are described as heme protein-dependent mixed-function oxidase systems that utilize NADH and/or NADPH to reductively cleave atmospheric dioxygen to produce a functionalized organic substrate and a water molecule. In many cases, the products represent hydroxylated derivatives of the substrate at one of its carbon moieties, and in other cases, P450s mediate aromatic dealkylations, isomerizations, dimerizations, dehydrations, hydroxylations, epoxidations, carbon-carbon cleavages, nitrogen and sulfur oxidations, decarboxylations, ehalogenations, and deaminations (Schuler and Werck-Reichhart, 2003). Due to the regio-specific roles in the hydroxylations of various compounds and the large number of secondary compounds synthesized in plants, the P450 genes in plants have duplicated and diverged to a large number when compared with the P450 genes in vertebrates, insects, nematodes and yeasts (Schuler and Werck-Reichhart, 2003). The P450s were initially named according to the level of amino acid sequence similarity with other P450s. The same family P450s generally has > 40% amino acid sequence identity, those of the same subfamily have > 55% identity, and those that are thought to be allelic variants have >97% identity (Paquette et al., 2000). Currently, the grouping of sequences on multiple alignments also directs the naming of P450 genes. The CYP prefix is used to designate a P450, followed by a number for the family, a letter for the subfamily, and a number for the specific gene (Nelson et al., 1996).

In plants, cytochrome P450 monooxygenases are involved in synthesis and metabolism of many physiologically important compounds like steroids, lignins, terpenes, fatty acids, phenylpropanoids, alkaloids and phytoalexins (Chou and Kutchan, 1998; Durst and O'Keefe, 1995; Persans et al., 2001). They are the examples of these primary and secondary compounds that act as plant defense agents against a range of diverse pathogenic microbes and insect pests (Chou and Kutchan, 1998; Durst and O'Keefe, 1995; Persans et al., 2001). In the leaf tissue of Arabidopsis, cytochrome P450 enzymes are also involved in several biosynthesis pathways and function as part of the highly sophisticated network of plant defense reactions (Schuhegger et al., 2006). These defense responses include the hypersensitive response (Glazebrook, 2005) and inhibition of growth of specific pathogens (Kliebenstein et al., 2005). Microarray analysis of different cytochrome p450

genes has revealed that their expression are strictly regulated in response to phytohormones (salicylic acid, abscisic acid, jasmonic acid and ethylene), pathogens (fungal pathogens *Alternaria alternata* and *A. brassicicola*), heavy metal toxicity, drought, high salinity, UV damage, mechanical injury, and low temperatures (Narusaka et al., 2004). These observations suggest the possible involvement of cytochrome P450 genes in plant defense responses to biotic and abiotic stresses.

Carotenoids are generally a class of tetraterpenes formed by the condensation of eight isoprene units and prevalent in photosynthetic autotrophic plants and algae. Plant carotenoids are synthesized and accumulated in plastids and are involved in a variety of roles, including photosystem assembly and stabilization, energy transmission, excess energy dissipation and auxiliary light harvesting (Lv et al., 2012). Generally, carotenoids consist of carotenes and oxygenated forms of carotenes, i.e. xanthophylls. The interconversion of violaxanthin, antheraxanthin and zeaxanthin is commonly referred to as the 'violaxanthin cycle' or 'xanthophyll cycle' (Young et al., 1997). These xanthophyll cycle pigments exhibit important functions in protecting plants under environmental stress. Aside from antheraxanthin and zeaxanthin, carotene-derived xanthophylls such as lutein, which are structural components of the light-harvesting complex, contribute to the dissipation of excess absorbed light energy and protect plants from photooxidative damage (Niyogi et al., 1997). Lutein is a dihydroxy *β*, *ε*-carotene and the most abundant carotenoid in plant photosynthetic system. A minimum of four carotenoid hydroxylase genes were found to be involved in xanthophyll biosynthesis in Arabidopsis (Kim and DellaPenna, 2006; Pogson et al., 1996; Sun et al., 1996; Tian and DellaPenna, 2001). Two are nonheme *β*- ring hydroxylases and remaining two are P450-type; a *ε*- ring hydroxylase and *β*- ring hydroxylase. The synthesis of lutein requires hydroxylation of C-3 of both the *β*- and *ε*- rings by the action of *β*- ring and *ε*-ring hydroxylases **(Figure 5.1)**.

By using AFLP technique, a 591 bp partial sequence (Acession no. GQ922056) was identified from *Arachis diogoi* challenged with the conidia of *Phaeoisariopsis personata* (unpublished). Blastp analysis of partial sequence showed the similarity with different carotenoid hydroxylases, a Cytochrome P450 class of proteins. Since the carotenoid hydroxylases are well known for their importance in various mechanisms, in the present study, the remaining 5’ end c-DNA sequence of putative cytochrome p450 gene (Acession

no. GQ922056) was amplified from *A. diogoi*. In our analyses, it was named as *AdCYP97A3.* Transcript levels of *AdCYP97A3* were checked during different hormones treatment. The promoter of this gene was isolated and potential cis regulatory elements in the sequences were also characterized using bioinformatics tools.

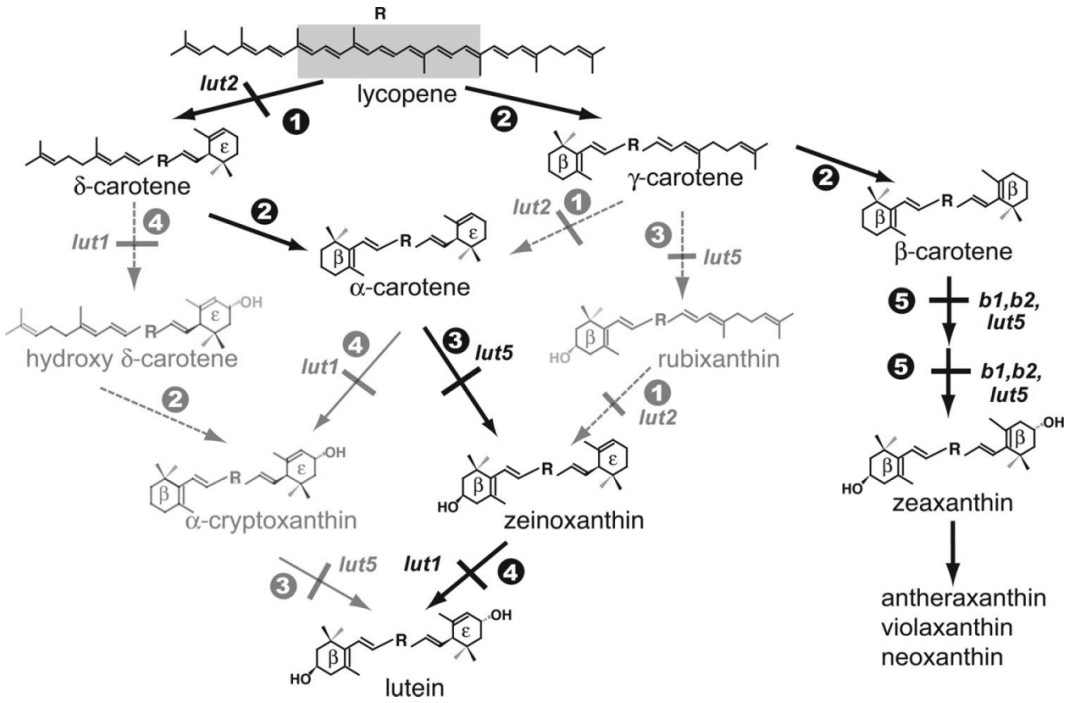

Figure 5.1 Pathway showing all possible routes to xanthophyll synthesis in *Arabidopsis thaliana*. Enzymatic reactions are indicated by numbers: ❶, *ε*-cyclization; ❷, *β* -cyclization; ❸, *β*- ring hydroxylation of *β, ε*- and, *β,ψ* -carotenoids; ❹, *ε*-ring hydroxylation; ❺, *β*-ring hydroxylation of *β*, *β*-carotenoids. Enzymatic reactions blocked by mutation of the indicated loci are shown: *lut1* (*ε*-ring hydroxylase), *lut2* (*ε*-cyclase), *lut5*, *b1*, and *b2* (three *β*-ring hydroxylases). Thick gray arrows indicate a reaction sequence that is supported by mutant phenotypes and/or enzyme activity assays in *Escherichia coli*, whereas dashed gray arrows are not. Thick black arrows, compounds, and mutant loci indicate major biosynthetic routes. (Kim and DellaPenna, 2006).

5.2 Material and methods

5.2.1 Plant treatments

For different treatments, detached leaves of *A. diogoi* were utilized. Different hormone treatments (salicylic acid, methyl jasmonate, abscisic acid, and ethephon) to *A. diogoi* were done as described in section **3.2.1**. Samples were collected at regular intervals, quick-frozen in liquid nitrogen, and stored at -80^{o} C till further use.

5.2.2 5' RACE and ORF amplification

The 3' end stop codon sequence was already present in the available partial sequence of *AdCYP97A3*, which was identified during AFLP study. A 5' RACE reaction was performed to amplify remaining 5' end sequence, by using SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's instructions and the method as described in earlier section **4.2.2**. All the RACE-PCR reactions were performed using hot-start DNA polymerase provided along with the kit. All PCR amplification products were cloned in to pTZ57R/T vector and sequenced for sequence confirmation. Primers used in this study were provided in **Table 5.1**.

5.2.3 Sequence analysis

DNA and protein sequences were analyzed using online tools as described in the earlier section **3.2.2**.

5.2.4 Isolation of upstream promoter region

Upstream promoter region of *AdCYP97A3* was cloned using GenomeWalker™ Universal Kit (Clontech, USA) following manufacturer's instructions. In brief, genomic DNA of *A. diogoi* was digested with different blunt cutters like *EcoR*V, *Dra*I, *Pvu*II and *Stu*I. Genome walker adaptors were ligated to the DNA fragments. Primary PCR was performed using adaptor primer 1 (AP1) and gene specific primer 1 (P-GSP1). Further diluted primary PCR products were amplified using gene specific primer 2 (P-GSP2) and adaptor primer-2 (AP2). The amplified products were run on 1.5 % agarose gel, eluted, cloned into pTZ57R/T and sequenced. P-GSP1 and P-GSP2 primers were designed based on the *AdCYP97A3* cDNA sequence. Used primers details are provided in **Table 5.1**. The upstream promoter region

isolated was analyzed for potential cis-regulatory elements using PLACE database (www.dna.affrc.go.jp/htdocs/PLACE/).

5.2.5 Construct preparation, *Agrobacterium* mediated Tobacco transformation and molecular analysis of transgenic plants

The *AdCYP97A3* was reamplified with ORF-F1 and ORF-R1 primers harboring *Xho*I and *Kpn*I restriction sites respectively, digested and cloned in pTZ57R/T vector. Digested fragments were cloned in pRT100 vector at corresponding sites. The *AdCYP97A3* expression cassettes harboring CaMV35S promoter and polyadenylation signal from pRT100 vector were further digested with *Pst*I enzyme and cloned in the binary vector, pCAMBIA2300. Confirmed vectors were mobilized to *Agrobacterium* strain EHA105. Tobacco (*Nicotiana tabacum* cv. Samsun) transformation was done as described in earlier section **2.13**. Further transformants selection and T_0 putative transgenic plant analysis were performed as described in earlier section **4.2.6**. Eight different lines were checked for PCR and RT-PCR. T_1 and T_2 seeds were raised via self-pollination.

5.2.6 Evaluation of fungal resistance in transgenic plants

Fungal resistance in WT and transgenic plants against the phytopathogenic fungus *Phytophthora parasitica* was checked in fully expanded leaves as described in earlier section **4.2.7**.

5.3 Results

5.3.1 *AdCYP97A3* ORF amplification and sequence analysis

The 3' end stop codon was already present in the *AdCYP97A3* partial sequence, which identified during AFLP study (Kumar and Kirti, unpublished). To get remaining 5' end sequence of gene, RACE-PCR was performed and around 1400 bp long DNA fragment was amplified **(Figure 5.1)**. The product was cloned and sequenced. Based on 5' and 3' end sequences, ORF-F and ORF-R primers were designed and the *AdCYP97A3* was amplified **(Figure 5.2)**. It was potentially encoding a 642 amino acid polypeptide. The encoded protein had a predicted molecular mass of ~72 kDa. with 6.36 isoelectric point. No signal peptide was detected in protein sequence. The BlastP analysis revealed that AdCYP97A3 protein shared the sequence similarity with Lutein deficient 5 (Lut 5) proteins from

different plants. It showed the closet sequence similarity with leguminous protein from *Cicer arietinum* (82%) and *Glycine max* (81%) and non-leguminous protein from *Arabidopsis thaliana* (80%) and *Solanum lycopersicum* (79%) **(Figure 5.3)**. The Lut 5 from *Arabidopsis thaliana* and *Solanum lycopersicum* are well characterized ones.

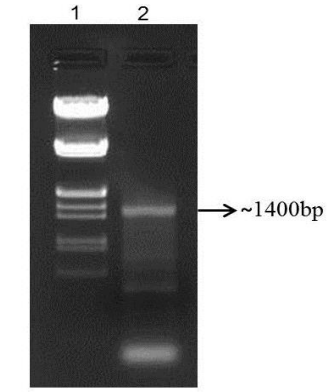

Figure 5.1 Amplification of 5' cDNA end using RACE-PCR.

acatgggggagtgttctccattaaccacttacttatctctttccaagtcctaacactaacccaaactgttacctttc
ttttttattctatttgtgaagattattattggtttggtgctttgttatgttacccATGGCTTCCCATGTTGCTTTTCCTC
ATGCTTCTCCTCTTTCTCTCTCTATTCCCAACAAACGATTCAGTTATAAACAGCTTCACATAAATGGAATCAAATCCATCACTT
CTTTCTCTTCATCCTCTTATTATTCTTCTTCTTGTTGGTGCTTCCGTTCACTTTCTACACAAAGAGGGTATTGTTTACCTGTGA
TTGCATGTTCCTCTTCCAATGGAAGGGGCCCTAATAGTTCTAAGGACGATGATGGTGTCAAGAGTGTTGAGAAGCTTCTTGAGG
AGAAAAGAAGAGCTGAATTGTCTGCTAGAACTGCCTCAGGTGAATTCACAGTGAAGCAGCAATCTGGTTTAAGTTCTATGTTGA
AGAAGAGCTTGTTAAAGGTGGGGGTGCCCAAAGAGGTTGTGGGGTTTTTGCTCGGTTGGGCGGCAGGGGAAGGTGATTACCCCG
AAATTCCTGAGGCAAAAGGATCTGTCAAGGCTATTAGAAGTGAGGCATTCTTCATTCCATTGTATGAACTTTACCTCACATACG
GTGGAATTTTCAGGTTGACTTTTGGACCAAAGTCCTTTTTGATTGTATCTGATCCGTCTATAGCAAAACACATCTTGAGAGACA
ATTCAAAGGCTTATTCGAAGGGTATCTTGGCTGAGATCCTAGAGTTTGTAATGGGGACAGGGCTCATCCCGGCTGATGGGGAAG
TGTGGCGAGTTCGACGACGAGCTATAGTCCCAGCATTGCATCTGAAGTACGTAGTGGCGATGATTAGCCTTTTTGGACAAGCCA
CAGGTAGGCTCTGCAAGAAGCTAGATGATGCTGCATCTGATGGGGAAGATGTTGAGATGGAATCACTTTTCTCTCGATTGACGT
TGGACATCATTGGAAAGGCAGTATTCAATTATGATTTCGACAGTTTAACAAATGATACCGGTATAGTTGAGGCTGTATATACTG
TACTGAGAGAAGCAGAAGATCGAAGCGTTTCTCCGATTCCAGTATGGGAGATCCCAATATGGAAAGACATATCACCGCGTCAAA
GGAAGGTTTCTGCTGCTCTCAAATTGATCAATGATACACTTAATGAGCTGGTAGCAATATGCAAGAGAATGGTGGACGAAGAAG
AGTTACAGTTTCACGAGGAGTACATGAATGAGCAAGATCCAAGTATTCTGCATTTCTTGTTGGCATCAGGAGATGATGTGTCGA
GTAAGCAACTCCGCGATGATTTAATGACAATGCTCATTGCTGGACATGAAACTTCAGCTGCTGTTTTAACTTGGACCTTCCATC
TTCTATCAAAGGAGCCTAGCGTCGTGTCGAAGCTCCAAGAAGAGGTTGACTCTGTACTAGGGGATCGATTTCCAACTATCGAAG
ACATGAAGAAACTCCGATATACAACCCGAGTGATCAATGAGTCAATGAGGCTTTACCCTCAACCACCAGTGTTGATCCGTCGCT
CTCTTGAGAATGATATGCTCGGCGAGTATACTATAAAAAGAGGTGAAGATATCTTTATATCTGTCTGGAACCTGCATCGTAGTC
CAAACCTATGGGATGATGCTGATAAGTTTCAACCGGAAAGATGGCCAGTAGATGGACCTAACCCCAATGAGACAAATCAAAACT
TCAAGTATCTTCCATTTGGGGGAGGACCGCGGAAATGTGTTGGTGATTTGTTTGCTTCATATGAGACCATAGTAGCACTTGCAA
TGCTTGTTAGACGGTTCAACTTTCAAGTGGCAGTTGGAGCCCCACCAGTTGAGATGACTACTGGAGCAACAATTCATACAACAC
AAGGGTTGAAGATGACAGTTACACGCAGAATAAAACCTCCAATTGTGCCCTCGTTACAAATGTCAACCATGGAAATTGATCCAT
CCATGAGGAAAGATGACACAAGTCAGAAAGGCGAAGTTTATCACGCTCAGTCCTAA

Figure 5.2 Showing 1929bp *AdCYP97A3* ORF (in upper case) with 133bp 5' UTR (in lower case).

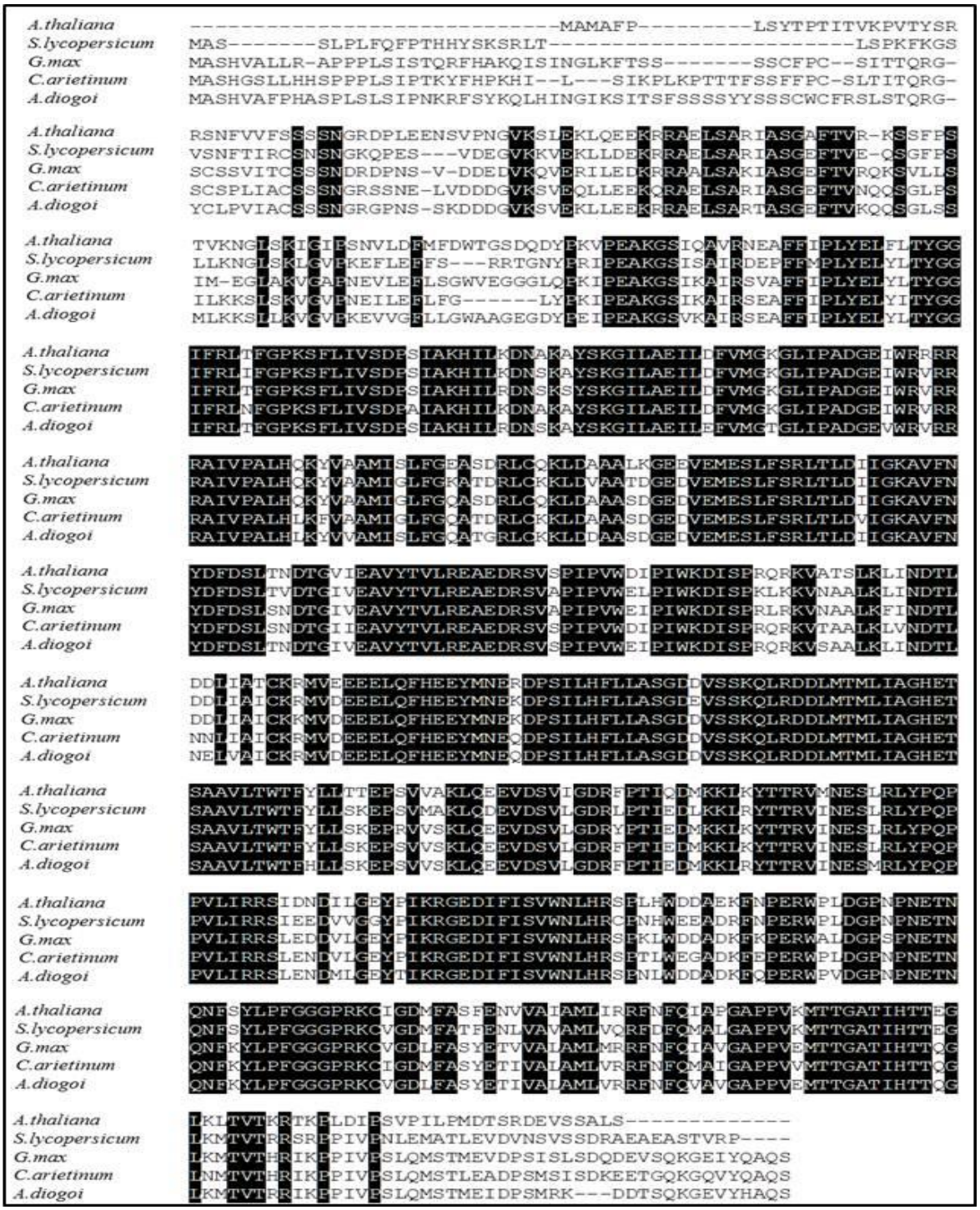

Figure 5.3 Alignment of deduced amino acid sequences of AdCYP97A3 with closely related proteins from other plant species.

5.3.2 *AdCYP97A3* expression in response to phytohormones

To understand the possible role of various phytohormones involved in plant defense in the regulation of *AdCYP97A3* expression, a semi-quantitative RT-PCR was performed. ET did not have any effect on the *AdCYP97A3* expression levels, whereas the treatment with MJ slightly increased the *AdCYP97A3* transcript levels after 12 h of treatment followed by a decline to basal levels. In response to SA, increased *AdCYP97A3* transcript levels were observed after 3 and 6 h followed by decline after 12 h and again increased after 24 h of treatment. In case of ABA, comparatively higher *AdCYP97A3* transcript levels were observed throughout the treatment. However the maximum inductions were observed after 6 and 12 h of treatment **(Fig. 5.4)**. These observations indicated that the gene expression is modulated by SA and ABA significantly.

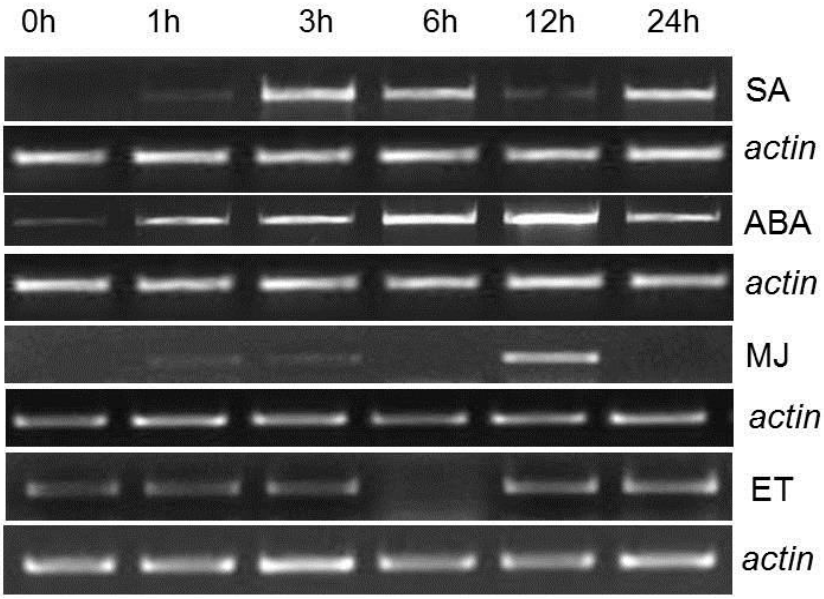

Figure 5.4 Transcript levels of *AdCYP97A3* in *A. diogoi* were analyzed using semi-quantitative RT-PCR, during various hormones treatments and at different time period (0-24 h).

5.3.3 Isolation and *in silico* analysis of 5'upstream promoter region of *AdCYP97A3*

Four different libraries were constructed by using *A. diogoi* genomic DNA. Primary and secondary PCRs were performed for each library, following the kit protocol. The longest fragment, with a size of around 1.1 kb, was amplified from *Stu*I, library which was further cloned and sequenced **(Figure 5.5)**. Based on the sequence of the 5' RACE products, the putative transcription start site was identified and designated as +1 site

(Figure 5.6). The transcription start site is present 133 bp upstream of translation initiation codon ATG. The sequence of *AdCYP97A3* promoter region was analyzed for potential cis-acting regulatory elements using the PLACE database software (www.dna.affrc.go.jp/htdocs/PLACE/) (Higo et al., 1999). A putative TATA box was found at –26 bp position upstream to the transcription start site. *In silico* analysis showed that various light and stress related cis-acting elements were present in promoter sequence **(Table 5.2)**.

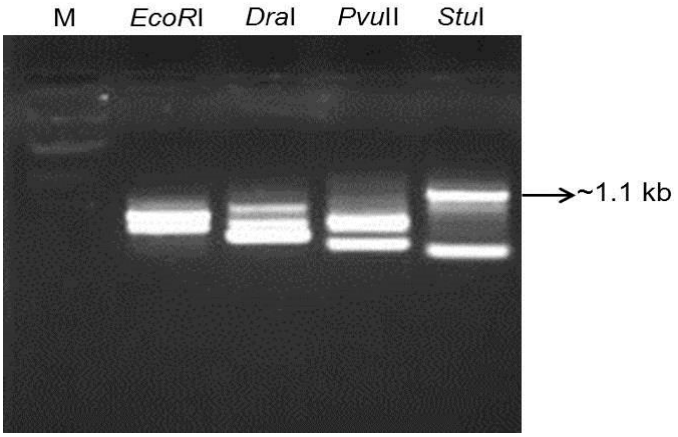

Figure 5.5 PCR amplification of 5' upstream promoter region of *AdCYP97A3* from different genomic DNA libraries. Lane M represents λ *Eco*RI/*Hind*III DNA ladder.

ACGGCCCTTGGGGCCGCAAGCATCCTAGTTTACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTCCTGTATTCAGATAA
CTCGAATTTACATGATTGGCTCTACAGAGGTGCAAGGAGTGGAGATGCTTTTCTTTTCTTTTCGACCATCTGGTGGATTTGG
AGAAGCAGGAATCATGACTTATTTAATATAGATGATTCATGGAGTGCTAGTAAAGTGGTGAGTTTGATTCGTAGTTCAGTAA
GGGAATTTCACACTATTTTTGCTATGCATCAATCTCTGTCTCCTCCTTCACTTTGTTTGCATTGGGTTCTACCTCCAGTTCA
TTCTGTTAAATTGAATTGTGATGCTAGTTGGTTTGCTCCTTCTGGCTATACTGGTTTTGGTTGTATTATTCGCAATCCTGAT
GGATGTTGGTTGAAAGGTTGCACTGGAAAAGTCGAAGTGTGCAGTGTTCTTTTTTGTTGAATTGTATGCAATTTGGAGAGGT
TTACTTCTTGCTTGGGAGAGTGGATTTTGTGAGGTTATTTGTGAAACAGACTGTTTAGAAGCTCTTTTCTTGGTAAACCAAA
GAATGCTTAGTAAGGATATTCCGGAATGGGATTTGGCAAAGCATATTCAGGAGGTTATGAATTGAAATTAGAGAGTCTCTAT
TCTTTTAATTCAGAGGACTGCAAATAGTGTTGCAGATTGTATGGCTAAAGCAGCTGCTTCTGTCGCGGACATTCACTCGAAT
TGGGGCCAACCATGGAGTGAGTTTCAACATCTAATAGCTTTAGATATGACCCTAGCCAATTAACTTGGTTTTGTCTCTTTTT
TTTCTTTCTTTTTTATTTAGTCACAAAAAAAAAAAAAAAACAAACGTGGCCTACTATGACACGAACATATTTAACAATGGAA
GCTAGAGAAAACGGTATCTTGTAACCAAGTTGCACATGTTTAGCATATGATGGTTATAAATATTCTTATGTACAATCCGACA
CACAACATGGGGGAGTGTTCTCCATTAACCACTTACTTATCTCTTTCCAAGTCCTAACACTAACCCAAACTGTTACCTTTTC
TTTTTTATTCTATTTGTGAAGATTATTATTGGTTTGGTGCTTTGTTATGTTACCCATGGCTTCCCATGTTGCTTTTCCTC

Figure 5.6 5' upstream promoter region of *AdCYP97A3*. Transcription start site was designated as (+1). TATA box was underlined.

5.3.4 Molecular analysis of putative transgenic plants and antifungal analysis

Eight different kanamycin resistance T_0 putative transgenic plants were analyzed by PCR and semi-quantitative RT-PCR. All plants showed *npt*II amplification **(Figure 5.7 A)**. However except plant one, remaining other seven showed *AdCYP97A3* amplification **(Figure 5.7 B)**. Semi-quantitative RT-PCR analysis revealed lines 2, 5 and 6 as high expression plants **(Figure 5.7 C).** T_2 generation of the high expression plants along with the WT were selected for the evaluation of fungal resistance, by using *Phytophthora parasitica*. No visible differences were observed between WT and transgenic leaves during repeated analysis (Data not shown), showing that *AdCYP97A3* overexpression did not alter the fungal resistance transgenic tobacco plants.

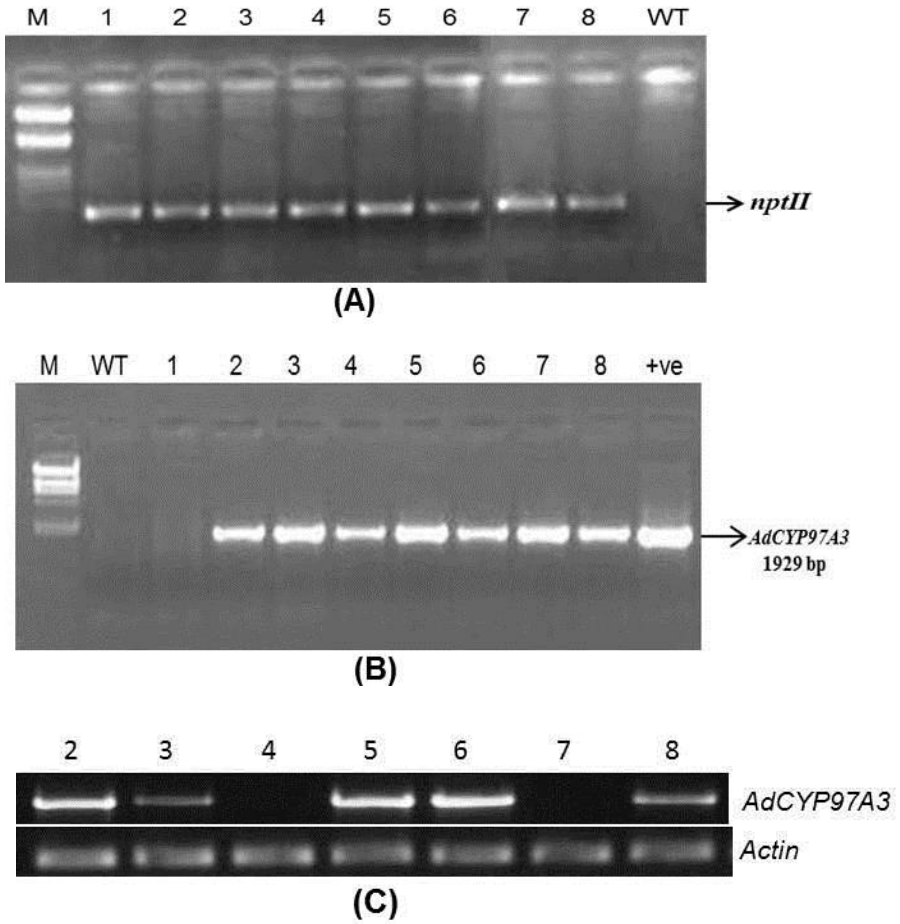

Figure 5.7 Gel pictures showing amplified PCR product of *npt*II (739 bp) **(A)** and *AdCYP97A3* (1929 bp) gene **(B)** from genomic DNA of eight different putative T_0 transgenic lines and WT plants. Semi-quantitative RT-PCR analysis of *AdCYP97A3* gene in putative T_0 transgenic plants **(C)**. Lane M represents λ *Eco*RI/*Hind*III DNA ladder.

5.4 Discussion

The carotenoids are essential components of the photosynthetic membranes in all cyanobacteria, algae and plants and serve an extraordinary variety of functions in plants (Cunningham Jr and Gantt, 1998). As described earlier, a minimum of four carotenoid hydroxylase genes were found to be involved in xanthophyll biosynthesis, the oxygenated forms of carotenes, in Arabidopsis. (Kim and DellaPenna, 2006; Pogson et al., 1996; Sun et al., 1996; Tian and DellaPenna, 2001). Several reports suggested that overexpression of *β*-carotene hydroxylase enhances abiotic stress tolerance in plants. For example, In *Arabidopsis thaliana*, overexpression of a bacterial *β*-carotene hydroxylase gene *chyB* caused a two fold increase in the size of the xanthophyll cycle pool and also made plants more tolerant towards the conditions like high light and high temperature (Davison et al., 2002). Similarly, the constitutive expression of a *β*-carotene hydroxylase gene, *DSM2* conferred drought and oxidative stress tolerance in transgenic rice plants by increasing xanthophyll and ABA synthesis (Du et al., 2010). The correlation between carotenoids and biotic stress mechanism is less studied. However, there are few reports postulating that carotenoids can play some role in plant-pathogen interaction. For example, it was found that the content of xanthophyll-cycle carotenoids significantly increased in *Phytophtora citricola* infected leaves of *Fagus sylvatica* (Fleischmann et al., 2004). Similarly, accumulation of carotenoids in parsley leaf was observed around the Septoria blight lesions (Baranski et al., 2005). Insect feeding on wheat leaves also stimulated the carotenoid accumulation (Ni et al., 2002).

During the AFLP study of *Phaeoisariopsis personata* infected *Arachis diogoi* leaves, a 591 bp long partial sequence of putative cytochrome p450 monooxygenases gene was found to be upregulated (Kumar and Kirt, unpublished). There are the reports suggesting the importance of cytochrome p450 monooxygenases family proteins in various plant mechanisms like different compound synthesis and stress responses (Chou and Kutchan, 1998; Durst and O'Keefe, 1995; Glazebrook, 2005; Kliebenstein et al., 2005). Hence, the full length c-DNA of putative cytochrome p450 monooxygenases gene was amplified using RACE-PCR, cloned and sequenced. Further the BlastP analysis of deduced amino acid was performed which showed closet sequence similarity with Lutein deficient 5 (Lut 5) proteins from different plants. Lut 5 is a cytochrome p450 monooxygenases family protein and in

Arabidopsis it is synthesized by *AtCYP97A3*, a *β*- ring hydroxylase gene (Kim and DellaPenna, 2006). Hence, during the study we named our gene as *AdCYP97A3*. To check the response of *AdCYP97A3* during different phytohormones treatment, the transcript levels were analyzed at different time period. The result analysis showed that the *AdCYP97A3* responded strongly during SA and ABA treatment. It is well known that SA regulates biotic stress responses and ABA abiotic stress responses; hence, exogenous application of both SA and ABA can mimic the biotic and abiotic stress conditions in plants. The induction *AdCYP97A3* gene during SA and ABA treatments along with initial observation made during AFLP study (unpublished), suggested the possible involvement of *AdCYP97A3* gene during both biotic and abiotic conditions. Other cytochrome P450 genes have also been reported being involved in plant responses to both biotic and abiotic stresses (Narusaka et al., 2004).

To understand much better about the role of *AdCYP97A3* gene in *A. diogoi*, the 5' upstream promoter sequence was amplified. *In silico* analysis showed that along with the light responsive, several biotic and abiotic stress related cis-acting elements were present in the promoter sequence. For example, E-BOX element is involved in light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. The MYB1 and MYC elements are involved in abiotic stress conditions like cold and dehydration. Two W-BOX elements and several WRKY71 elements were found in the identified promoter region. W-BOX is involved in wound responses whereas WRKY71 elements are involved in defense related gene activation. Ultimately, the analysis of other elements in promoter region predicted the possibility of *AdCYP97A3* gene involvement in various mechanisms in plants.

The results of transcript level analysis during various hormone treatments and promoter sequence study together suggested that *AdCYP97A3* could be involved in stress tolerance mechanism. Hence, transgenic tobacco plants overexpressing *AdCYP97A3* were raised to study its role during fungal infection. The partial sequence of *AdCYP97A3* was initially identified during fungal infection (Kumar and Kirti, unpublished), however, the transgenic plants did not show any sign of enhanced resistance suggesting that *AdCYP97A3* might not probably be involved in enhancing the fungal resistance in plants. To our best knowledge, there is no report available about the involvement of any *AdCYP97A3* homologs in biotic stress tolerance mechanisms. Hence, further in depth study about

AdCYP97A3 role during fungal infection could reveal some new aspects of this protein family.

5.5 Conclusion

In conclusion, a wild peanut cytochrome P450 monooxygenase gene, *AdCYP97A3*, was amplified and cloned. Transcript level analysis revealed that the gene got upregulated clearly during SA and ABA treatment. Promoter analysis showed the presence of various cis-acting elements related to different biotic and abiotic stress conditions. However, the transgenic tobacco plants overexpressing *AdCYP97A3* did not show any sign of enhanced resistance against the fungal infection suggesting the further study is required to reveal the precise function of *AdCYP97A3* during biotic stress condition.

Table 5.1 Sequences of the oligonucleotides used in the study (see text for details).

Name of the primers	Primer sequences (5'-3')
5' GSP1	GTGGTTGAGGGTAAAG
5' GSP2	CTTCATGTCTTCGATAGTTGG
ORF-F	ATGGCTTCCCATGTTGCTTTTC
ORF-R	TTAGGACTGAGCGTGATAAACTT
ORF-F1	CCCTCGAGATGGCTTCCCATGTTGCTTTTC
ORF-R1	CCGGTACCTTAGGACTGAGCGTGATAAACTT
P-GSP1	GGATGAAGAGAAAGAAGTGATGGATTTGAT
P-GSP2	AAGAGGAGAAGCATGAGGAAAAGCA
Actin-F	TGGCATCACACTTTCTACAA
Actin-R	CAACGGAATCTCTCAGCTCC

Table 5.2 *AdCYP97A3* promoter analysis using PLACE website.

Site Name	Position	Strand	Sequence	Function
-10 promoter element	654, 963	+	TATTCT	Involved in the expression of the plastid gene *psbD*, activated by blue, white or UV-A light
ABRE-related sequence	863, 41	+	MACGYGB	Ca^{+2}-responsive
ACGTAT-ERD1	864	+	ACGT	Required for etiolation-induced expression of *erd1* (early responsive to dehydration)
Amylase box	330	-	TAACARA	Alpha-amylase gene activity
ANAERO1 CONSENSUS	858 298	+ -	AAACAAA	Involved in the fermentative pathway
ARR1AT	690, 157, 514, 603, 95, 197, 229	+	NGATT	Cytokinin-regulated transcription factor, ARR1 binding site
BIHD	877	-	TGTCA	In disease resistance responses
BOXII	864	+	ACGTGGC	In light responsiveness
CACTFTPPCA1	257, 295, 431, 730, 377, 495,872,31	+	YACT	Elements for mesophyll-specific gene expression
CATATGGMSAUR	946	+	CATATG	Involved in auxin responsiveness
CBFHV	49	+	RYCGAC	Dehydration-responsive element (DRE) binding site
CGCG BOX	42	+	VCGCGB	Involved in multiple signaling pathway
E BOX	149,707,935,946	+	CANNTG	light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes
GT1 CONSENSUS	435, 564, 819	+, + -	GRWAAW	SA-inducible gene expression
I BOX	78	+	GATAA	Light-regulated transcription
MYB1	567,924 357,380,804,953	+ -	WAACCA	Dehydration response
MYC CONSENSUS	149,707,935,946	+	CANNTG	Dehydration, cold and ABA signaling
NODCON	554, 813	+	CTCTT	Nodule specific
P1BS	588, 615	+	GNATATNC	Binding sequence found in phosphate starvation responsive genes
RE alpha	568,925 356,385,416,803	+ -	AACCAA	Required for phytochrome regulation
SEBF CONSS	282 809	+ +	YTGTCWC	Found in promoter of pathogenesis-related gene (*PR-10a*)
W BOX	179 785	+ +	TGACY	Involved in activation of *ERF3* gene by wounding
WRKY71 OS	785, 179, 877 840	+ -	TGAC	Binding site of WRKY71, a transcriptional repressor also cause activation of defense-related genes

Chapter 6:

Summary of Work

The present study is focused on the characterization of three pathogen induced genes from *Arachis diogoi*, a wild relative of economically important crop *Arachis hypogea*. The partial sequences of these genes were identified from *A. diogoi* during its interaction with late leaf spot (LLS) causing fungus *Phaeoisariopsis personata*. Two different techniques, DDRT-PCR and AFLP were used for the identification of these genes during previous studies. The genes were named based on the sequence similarity with their homologs from Soybean and Medicago. A Thaumatin-like protein gene was the first to study and named as *AdTLP*. The partial sequence was identified during DDRT-PCR study. The AdTLP protein showed excellent *in vitro* inhibiting activity against various fungal pathogens. The calculated IC_{50} values for *F. oxysporum, F. solani* and *B. cinerea* were much lower than other reported legume TLP proteins. In general, the antifungal mode of action of TLP proteins was assumed to be due to the *β*-1, 3 Glucanase activity. However, the AdTLP protein did not show any such activity during our study. The protein modelling study predicted a strong electro-negative cleft in AdTLP protein, due to the acidic amino acid residues like D124, D129, E103, D222 and E111, which are essential for the antifungal activity of protein. Transgenic tobacco plants overexpressing AdTLP protein also showed strong antifungal activity against *Rhizoctonia solani*. The transgenic plants also exhibited tolerance to salt and oxidative stress with better recovery on recovery medium. Interestingly, the localization study showed that the AdTLP protein is mainly localized extracellular. However, protein expression in other subcellular structures, possibly ER regions, was also observed. Apart from that the transcript levels of various defence related genes were also analyzed and result showed higher transcript level of *PR1a*, *PI-I* and *PI-II* gene in transgenic plants compared to WT. *PI-I* and *PI-II* genes encode protease inhibitors that play important role in insect defense mechanism. Hence, it can be assumed that AdTLP protein might play some role in plant defense during insect attack.

Another partial sequence of a CBL-interacting protein kinase gene was also identified during DDRT-PCR study of *A. diogoi*. It was named as *AdCIPK.* Despite being induced during fungal infection, the gene also showed activity during abiotic stress conditions like salt and osmotic stress. Phytohormones treatments like SA and ABA also caused *AdCIPK* induction in *A. diogoi* at different time periods. Subcellular localization study revealed that the protein is mainly localized in cell membrane with some presence in

cytoplasm. Transgenic tobacco plants overexpressing AdCIPK were generated to study its *in vivo* role in stress tolerance mechanism. Since the gene was initially identified during fungal infection, the levels of fungal resistance were checked in WT and transgenic plants. A phytopathogenic fungus, *Phytophthora nicotaianae* was used for this analysis. However, no resistance level differences were observed between WT and transgenic leaves suggesting that AdCIPK protein did not enhance resistance against the test fungus in transgenic plants. Since most of the CIPKs from other plants are reported in abiotic stresses and *AdCIPK* transcript levels also showed significant expression during given abiotic conditions, the salt and osmotic level of tolerance were checked in WT and transgenic plants. At various growth stages, significant levels of tolerance were observed in transgenic plants against both salt and osmotic stresses. Further, the real time PCR analysis of defense related genes and ROS analysis revealed that *AdCIPK* overexpression maintains the integrity by enhancing the defence related gene expression and antioxidant system of cell. However, further study is required to understand the *AdCIPK* role in stress mechanisms.

A cytochrome P450 monooxygenase gene family protein was the last one to be studied in present work. P450s are involved in various plant mechanisms like different compound synthesis and stress responses. The partial sequence was identified during AFLP study of *A. diogoi*. The full length was amplified, cloned and sequenced and named as *AdCYP97A3*. The sequence showed 80% of similarity with a *β*- ring hydroxylase protein of *Arabidopsis thaliana*. It is involved in Lutein biosynthesis in plants. Lutein is a dihydroxy *β*, *ε*-carotene and a component of photosystem light-harvesting complex which is also involved in plant protection from photooxidative damages. The transcript levels of *AdCYP97A3* were checked during different hormone treatments in *A. diogoi* and at different time intervals. Clear upregulation was observed during SA and ABA treatment. To understand further its role in stress conditions, 5' upstream promoter region was isolated. Sequence analysis revealed the presence of various light and both biotic and abiotic stress related cis-acting elements in promoter sequence. Further the *AdCYP97A3* transgenic tobacco plants were generated and levels of fungal resistance were checked in WT and transgenic plants against the phytopathogenic fungus *Phytophthora nicotaianae*. No differences were observed in resistance levels showing that *AdCYP97A3* overexpression did

not alter the fungal resistance in transgenic tobacco plants. This study revealed that further study is needed about *AdCYP97A3* to understand its role during stress conditions, particularly during fungal infection.

All together, on the basis of present thesis work, it can be concluded that *AdTLP* and *AdCIPK* are good genes for the deployment in crop plants towards their improvement against various stress conditions.

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