Molecular cloning and functional characterization of biotic stress response genes of *Arachis* spp.

Dissertation submitted to the University of Hyderabad for the award of the degree of Doctor of Philosophy in Plant Sciences

By

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Certificate

This is to certify that I, **K. Raja Rajesh Kumar**, have carried out the research work embodied in the present thesis entitled "**Molecular cloning and functional characterization of biotic stress response genes of *Arachis* spp.**" and submitted for the degree of **Doctor of Philosophy** was accomplished for the full period prescribed under Ph.D ordinances of the University, under the supervision of Prof. P. B. Kirti, in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, and I declare to the best of my knowledge that no part of this thesis was earlier submitted in part or in full, for the award of any research degree or diploma to any University.

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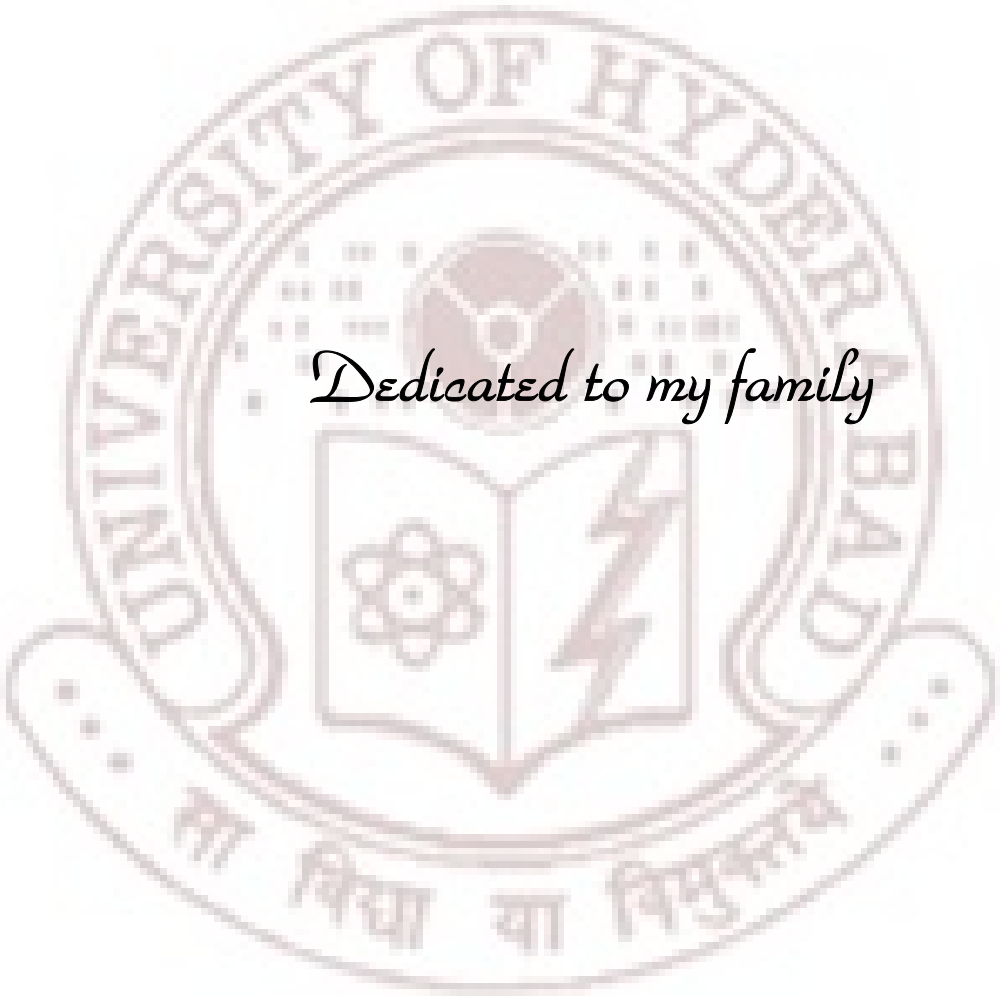

Dedicated to my family

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Abbreviations

µg	Microgram
µl	Microliter
µM	MicroMolar
ABA	Abscisic acid
ACP	Annealing Control Primer
ATP	Adenosine triphosphate
BAP	Benzyl amino purine
bp	Base pairs
BSA	Bovine serum albumin
CAMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
cm	Centimeter
CTAB	Cetyl trimethylammonium bromide
$\chi 2$	Chisquare -test
d	Day
DEPC	Diethyl pyrocarbonate
DEGs	Differentially Expressed Genes
DNA	Deoxy ribonucleic acid
DNTPs	Deoxy nucleotide triphosphates
EDTA	Ethylene diamine tetraacetic acid
G	Gram
GFP	Green Fluorescent Protein
h	Hours
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobases
kDa	Kilodalton
LB	Luria Bertani
M	Molar
MES	2-(N-Morpholino)-ethane sulfonic acid
min	Minutes
MeJa	Methyl jasmonate
ml	Milliliter
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
ng	Nanogram
GSP	Gene Specific Primer
O.D	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pI	Iso electric point
RACE	Rapid amplification of cDNA ends
RNA	Ribo nucleic acid

Rpm	Revolutions per minute
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
SNP	Sodium nitroprusside
TE	Tris.EDTA
Tris	Tris (hydroxymethyl) aminomethane
U	Units
UTR	Untranslated regions
WT	Wild type

Contents

Chapter 1

General Introduction

Plants and Stress

Plants are the primary producers in most terrestrial ecosystems and form the basis of the food web in these ecosystems. Virtually, all human nutrition depends on land plants directly or indirectly. Plants, being sessile, have to experience several stresses due to the changes occurring in the environment. Various environmental stresses have a potentially adverse affect on plant growth rate, chance of survival and reproductive success. Most valued for its general connotations, plant stress is often defined as any factor that decreases plant growth and reproduction below the potential of the genotype (Osmond et al. 1987). Stresses can be abiotic (nonliving) or biotic (living) including weather (rain, heat and temperature), soil conditions (water, pH and nutrients), insect populations, and disease incidence and in case of crop plants, management practices (cultivar, irrigation, fertilization and rotation) also play a role. As crop growth and crop yields are affected by abiotic and biotic factors, there is a need for greater understanding of the molecular and physiological mechanisms underlying plant response to various stresses. This will greatly enhance the chances of improving the plant performance against different stresses using biotechnological approaches.

1.1 Abiotic stress

Abiotic stresses include drought, flooding, salinity, heat and cold stresses. Plants acquire resistance to environmental stresses by reprogramming thier metabolism and gene expression, gaining a new equilibrium between growth, development and survival **(**Mazzucotelli et al. 2008). The perception of abiotic stresses and signal transduction to switch on adaptive responses are critical steps in determining the survival and reproduction of plants exposed to adverse environments (Chinnusamy et al. 2004). There are multiple stress perception and signaling pathways, some of which are specific, but others may cross-talk at various steps. Salt and drought stresses forms the major part of abiotic stress. Salt stress afflicts plant agriculture in many parts of the world, particularly irrigated land (Epstein et al. 1980). Compared to salt stress, the problem of drought is even more pervasive and economically damaging (Boyer 1982). Plant adaptation to

environmental stresses is controlled by cascades of molecular networks. These activate stress responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes (Wang et al. 2003). Genes involved in signaling cascades and transcriptional control, such as mitogen-activated protein (MAP) (Shou et al. 2004) and salt overly sensitive (SOS) (Qiu et al. 2002) kinases, phospholipases (Thiery et al. 2004) and transcription factors e.g. heat shock factor (HSF) and the C-repeat-binding factor /dehydration- responsive element binding protein (CBF/DREB) and ABA-responsive element binding factor/ABA-responsive element (ABF/ABRE) families (Zhang et al. 2004) have been extensively studied (Yamaguchi-Shinozaki and Shinozaki. 2006). Figure 1.1 depicts the abiotic stress signaling starting from perception to gene expression, finally resulting in tolerance or resistance.

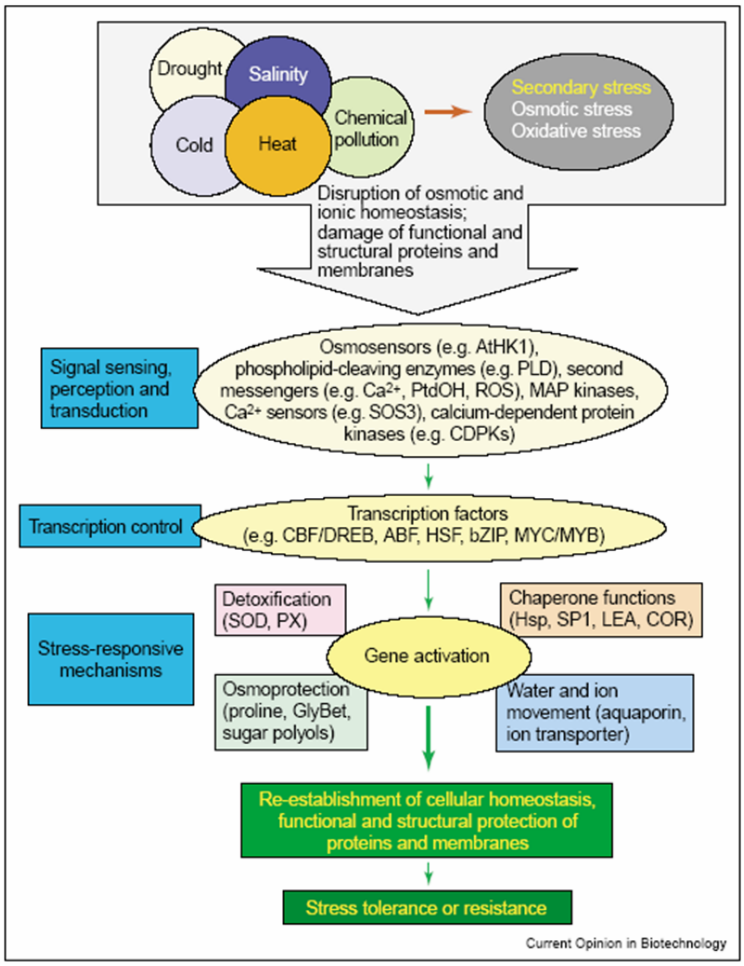

Figure 1.1 Complexity of plant response to abiotic stress. (Source: Wang et al. 2003)

1.1.1 Engineering abiotic stress tolerance

As several abiotic stresses severely affect the crop yield and productivity, engineering abiotic stress tolerance in plants has huge economical importance. There are several successful approaches to achieving tolerance through genetic engineering (Wang et al. 2003; Bhatnagar et al. 2008), although there are problems associated with them (Flowers 2004). Several candidate genes were analyzed and utilized for conferring abiotic stress tolerance in plants. Like constitutive expression of the tobacco mitogen-activated protein kinase kinase kinase/ Nicotinia protein kinase 1 (MAPKKK/NPK1) in maize leads to cold, heat, and salinity tolerance in the transgenic plants (Shou et al. 2004). Heat-shock proteins (Hsps) and molecular chaperones, as well as late embryogenesis abundant (LEA) protein families, are involved in plant abiotic stress tolerance (Wang et al. 2003, Wang et al. 2004a). Overexpression of HSP101 of *Arabidopsis* in rice plants resulted in a significant improvement of growth performance during recovery from heat stress (Katiyar-Agarwal et al. 2003). Overexpression of LEA proteins was correlated in several cases with desiccation tolerance, although the actual function of these proteins is still unknown (Villalobos et al. 2004). Severe osmotic stress causes detrimental changes in cellular components. A wide range of metabolites that can prevent these detrimental changes have been identified, including amino acids (e.g. proline), quaternary and other amines (e.g. glycine-betaine and polyamines) and a variety of sugars and sugar alcohols (e.g. mannitol and trehalose) (Vinocur and Altman 2005). Genetic engineering for increased biosynthesis of several specific polyamines resulted, in several cases, in stress tolerant plants (Capell and Christou 2004). Transcriptional activators that upregulate stress-responsive genes have been utilized to produce drought-tolerant transgenic plants. In addition to the enhancement of drought tolerance, overexpression of the DREB1/CBF3 (dehydration-responsive element binding protein/CRT binding factor) transcription factor in *Arabidopsis* controlled many stress inducible target genes (Fowler and Thomashow 2002; Maruyama et al. 2004) and increased tolerance to freezing and high salt exposure (Kasuga et al. 1999).

Since the application of functional genomics approaches, the identification of candidate genes related to abiotic stress response has increased, thus fueling this approach for genetic engineering. In future, the combination of transcriptomic, proteomic

or metabolomic analyses will also be useful for gene discovery in the engineering of abiotic stress tolerance.

1.2 Biotic Stress

As a rich source of sugars and amino acids, plants attract a variety of intruders, from viruses, bacteria and fungi to insects. Some of the pests and pathogens have a devastating impact on crop yields. To protect themselves, plants have in their armory, passive defense mechanisms such as strengthened cell walls and antimicrobial compounds, as well as active healing responses. Only a few microbes can breach these basal defenses, and are then fought by the plant's innate immune system. Plants have evolved sophisticated mechanisms to perceive such attacks, and translate the perception into an adaptive response.

Plant pathogens can be broadly divided into those that kill the host and feed on the contents (necrotrophs) and those that require a living host to complete their life cycle (biotrophs). However, many others behave as both biotrophs and necrotrophs, depending on the conditions in which they find themselves or the stages of their life cycles. Such pathogens are called hemi-biotrophs (Oliver et al. 2004). Hemibiotrophy has been defined by Perfect and Green (2001) as an 'initial period of biotrophy' followed by "necrotrophic hyphae", making it a subsidiary definition. Microbial necrotrophy is often accompanied by production of toxins. Viruses are quintessential biotrophs, although infection can lead eventually to host cell death. Bacteria and fungi can adopt either lifestyle. Many insects cause damage by chewing, induce a wound response that includes the production of protease inhibitors and other anti-feedants such as alkaloids. Additionally, wound responses include release of volatiles, which attract insects that feed on, or deposit eggs into, the larvae of the herbivorous insect. By contrast, sap-feeding insects and nematodes can adopt more intimate and sophisticated modes of biotrophic parasitism, imposing developmental responses on the plant cells, leading to the appearance of galls, root knots or cysts (Dangl and Jones 2001).

1.2.1 Plant-microbe interactions

It is increasingly evident that a plant-pathogen interaction may be compared to an open warfare, whose major weapons are proteins synthesized by both organisms. The outcome of each battle results in establishment of resistance or pathogenesis (Ferreira et al. 2007).

1.2.1.1 Plant defense responses

The molecular mechanisms underlying activation of plant defense responses are exceedingly complex. Pathogen attack leads to the activation of a large number of defense effector mechanisms and often begin with gene-for-gene recognition of the pathogen. *R* gene–mediated resistance is also associated with the activation of a salicylic acid (SA)-dependent signaling pathway that includes the production of antimicrobial metabolites and proteins and physical reinforcement of cell walls through production of callose and lignin (Glazebrook 2005).

1.2.1.2 R gene mediated defense responses

Plants have evolved the ability to recognize and respond to particular pathogen molecules, leading to rapid activation of defense responses. The innate immune response is well described genetically by what is known as the gene-for-gene model (Flor 1971), because it requires a pathogen protein encoded by an 'avirulence' (*Avr*) gene to be recognized by a plant protein encoded by a resistance (*R*) gene. This activates an array of defense mechanisms, including the hypersensitive response, in which a few plant cells at the site of infection die, thereby limiting the spread of disease. Pathogens that are recognized in this way and that therefore fail to cause disease are called avirulent pathogens, the host is called resistant, and the interaction is called incompatible. In the absence of gene-for-gene recognition, due to absence of the avirulence gene in the pathogen and/or of the *R* gene in the host, the pathogen is virulent, the host is susceptible, and the interaction is compatible. For example the Rpg1-b gene from soybean (*Glycine max*) confers resistance to *Pseudomonas syringae* pv glycinea (causing bacterial blight) carrying the avrB gene in a classic gene for gene specific manner (Ashfield et al. 2004).

R gene products may not directly bind to avirulence gene products, but rather detect alterations in host proteins that are caused by the pathogen gene products (Van der Biezen and Jones 1998a). This idea is known as the guard model (de Wit 2002; Marathe and Dinesh kumar 2003). Initiation of RPS2 mediated disease resistance against *Pseudomonas syringae* in *Arabidopsis* through elimination of RIN4 by AvrRpt2 is a typical example of Guard hypothesis (Axtell and Staskawicz 2003). Specific *R*-mediated innate immunity is superimposed on to one or more basal defense pathways. Basal defenses inhibit pathogen spread after successful infection and the onset of disease (Dangl and Jones 2001). Genetic overlap between specific and basal resistance responses suggests that one function of *R*-mediated signaling is to more rapidly and effectively activate defense mechanisms that are shared by both pathways (Yang et al. 1997; Mcdowell and Dangl 2000). *R* gene–mediated resistance and plant innate immunity were well explained in reviews by Nimchuk et al. (2003); Jones and Dangl (2006) respectively.

1.2.1.3 Systemic Acquired Resistance (SAR) and Induced systemic resistance (ISR)

The term 'cross protection' was initially used to describe enhanced tolerance to disease in previously infected perennials (Chester 1933). Today, cross protection comes in two guises, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Grant and Lamb 2006).

Upon recognition of a pathogen, plants often activate the hypersensitive response, resulting in rapid cell death of infected tissue to kill the pathogen and prevent it from spreading further. In addition to the locally effective hypersensitive response, pathogen recognition also triggers various inducible systemic defenses. In plant parts distant from the site of primary infection, systemic responses establish an enhanced defensive capacity against subsequent infection. This biologically induced resistance in systemic tissue is known as systemic acquired resistance (SAR) and has been shown to be effective in many plant species (Fig 1.2). The attained state of resistance is long-lasting and effective against a broad spectrum of pathogens, including pathogenic bacteria, fungi and viruses. SAR requires both local and systemic salicylic acid (SA) accumulation and the induction of a subset of the pathogenesis-related (PR) genes, but SA itself is not the mobile signal (Vernooij et al. 1994). In plant defense research, *PR* genes serve as powerful molecular

markers for the onset of SAR (Ryals et al. 1996). Although SA can be synthesized from phenylalanine, the predominant pathway for de novo SA biosynthesis during pathogen infection is through chorismate via isochorismate synthase (ICS).SA, synthesized from chorismate by means of ICS, is required for local and systemic acquired resistance responses (Wildermuth etal 2001)**.** NON-EXPRESSOR OF PATHOGENESISRELATED1 (NPR1, also known as NON-IMMUNITY 1 [NIM1]) is a central positive regulator of SAR signaling (Dong 2004). NPR1 protein contains an ankyrin repeat and a BTB/ POZ domain (Cao et al. 1997), and functions downstream of SA. NPR1 regulate PR gene expression through interaction with TGA transcription factors.

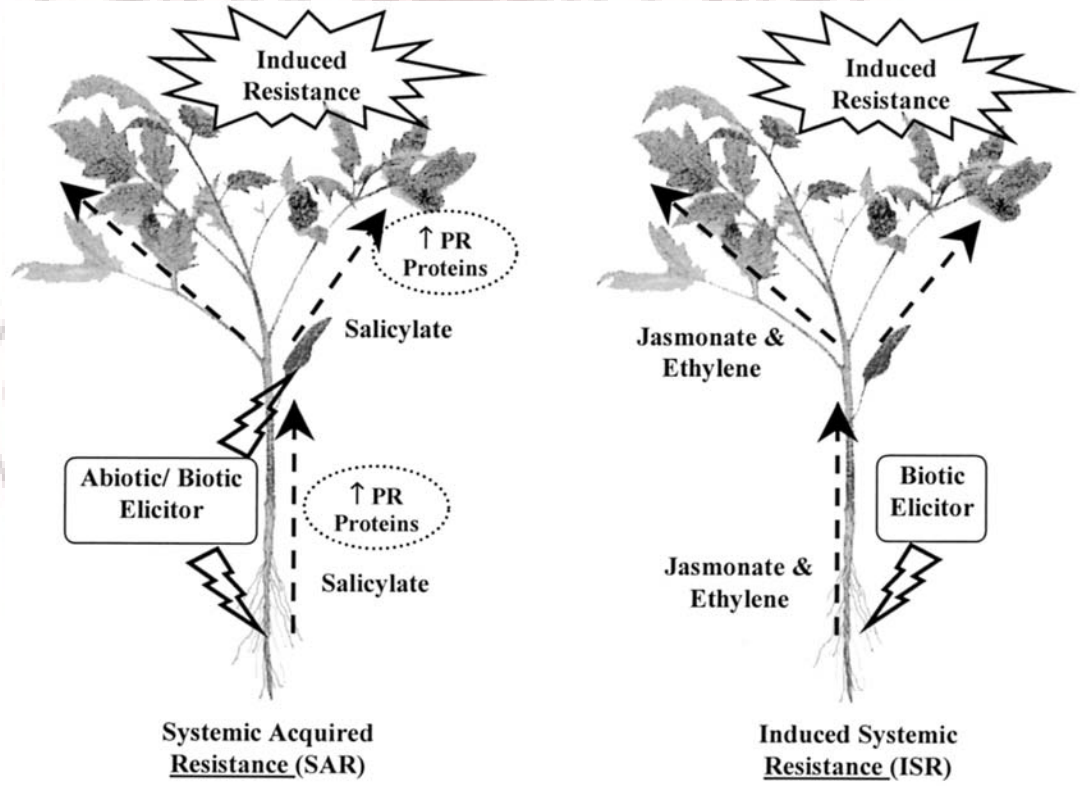

Fig 1.2 Induced resistance in plants (See text for details).

Source: Vallad and Goodman 2004.

Colonization of plant roots with selected strains of non-pathogenic, growth-promoting rhizosphere bacteria can also provoke broad-spectrum disease resistance in plants. This type of induced resistance is called induced systemic resistance (ISR) (Van Loon et al. 1998). ISR is less 'broad spectrum' in nature (Bostock 2005). In contrast to SAR, the ISR response does not require SA and generally requires jasmonic acid (JA) and ethylene (ET) (Fig 1.2) (Pieterse et al. 1998). Jasmonate synthesis occurs through the octadecanoid pathway and begins with the release of linolenic acid from the chloroplast membrane. Attack by necrotrophic pathogens, as well as herbivorous insects, elicits the production of a large chemically diverse set of oxygenated fatty acids (oxylipins) that can be potent regulators of defense signaling. JA and precursors of JA that are synthesized during JA biosynthesis have an important function as signaling molecules in various processes, including plant defense (Farmer et al. 2003). JA-dependent signaling proceeds through increased JA synthesis in response to pathogen attack and consequent increase in expression of defense effector genes such as *PDF1.2*. Some JA regulated genes are also regulated by ET. In the case of *PDF1.2*, induced expression requires both JA and ET. In contrast, ET is not required for expression of the JA inducible gene *VSP1* (Norman-Setterblad et al. 2000).

1.2.1.4 Signal Interplay between different Induced Defenses

Defense responses dependent on SA are often effective against biotrophic pathogens, whereas defenses dependent on JA are mostly effective against necrotrophic pathogens and insects. To achieve an effective state of resistance after recognition of the invader, plants are thought to fine-tune different defense signaling pathways by means of synergistic and antagonistic interactions (Beckers and Spoel 2005). A synergistic effect was reported between SA-dependent SAR and rhizobacteria-mediated induced systemic resistance (ISR). ISR is activated by root-colonizing, non-pathogenic fluorescent *Pseudomonas* spp. and provides broad spectrum resistance to pathogen attack (Pieterse et al. 2002). ISR signal transduction is dependent on an intact JA/ethylene response and, interestingly, requires the function of the regulatory protein NPR1 (Pieterse et al. 1998). Thus, SAR and ISR signaling pathways are distinct in their requirement for SA and JA/ethylene, yet unite in their downstream requirement of NPR1. Besides signal synergy

between SA- and JA-dependent defense responses, cases of antagonism between these two signaling molecules have also been reported. The JA-resisted tobacco hornworm *Manduca sexta* inflicted more damage on SAR induced tobacco plants compared to control plants (Preston et al. 1999). Moreover, tobacco plants silenced for the expression of the phenylpropanoid biosynthesis gene *PAL (phenyl ammonia-lyase)* exhibit reduced SAR against TMV, but exhibited enhanced resistance to insect infestation. Conversely, plants overexpressing *PAL* were more resistant to TMV, whereas resistance to insect attack was lost (Felton et al. 1999). Although cross-talk between the SA and JA signal may be regulated differently depending on the plant species, at least in *Arabidopsis* regulatory protein NPR1 plays a crucial role (Spoel et al. 2003).

1.2.2 Plant- herbivore interaction

Terrestrial plants are food source for an estimated one million or more insect species from diverse taxonomic groups (Howe and Jander 2008). Plant-herbivore interactions are played out on spatial scales that include the cellular responses, well-studied in plant-pathogen interactions, as well as responses that function at whole-plant and community levels. The plant's wound response plays a central role but is frequently altered by insect-specific elicitors, giving plants the potential to optimize their defenses (Kessler and Baldwin 2002). Insects use various strategies to obtain nutrients from all above and below ground plant parts. Around two-third of all known herbivorous insect species are leaf eating beetle (Coleoptera) or caterpillars (Lepidoptera) that cause damage with mouth parts evolved for chewing and tearing (Schoonhoven et al. 1998). Defense against microbes can be highly effective on small spatial scales like the hypersensitive response (HR), in which cells immediately surrounding the infection site rapidly die and fill with antimicrobial compounds to prevent the spread of the pathogen (Lam et al. 2001). Even HR can be effective against sedentary herbivores like aphids feeding on particular tissue like phloem (Miles 1999), But HR can not be effective against most free-living herbivores, which avoid an HR by simply moving to another feeding site, there by expanding the spatial scale of the plant-herbivore interaction to include not only whole-plant responses but also the community in which the plant lives (Fig 1.3).

1.2.2.1 Plant responses to Herbivores

As sessile organisms, plants rely heavily on chemical defenses to thwart the insect attack. Plant responses to herbivores are broadly categorized as direct and indirect defenses and tolerance (Kessler and Baldwin 2002). Compounds that exert repellent, antinutritive, or toxic effects on herbivores are commonly referred to as direct defenses. Proteinase inhibitors (PI) (antidigestive proteins) are inducible by wounding and herbivory and influence herbivore performance by inhibiting insect digestive enzymes (Koiwa et al. 1997a). Physical barriers such as leaf toughness and trichomes that increase plant fitness in the presence of herbivores come under direct defenses. Toxic compounds (e.g., alkaloids, terpenoids, phenolics) that poison generalist herbivores also constitute a part of direct defense. Indirect defenses are plant traits that attract predators and parasitoids of herbivores. Volatile organic compounds (VOCs) released by herbivore-attacked plants attract natural enemies of the herbivore (Kessler and Baldwin 2002). A plant genotype is termed tolerant if it can sustain tissue loss with little or no decrease in fitness relative to that in the undamaged state (Stowe et al. 2000). The mechanisms underlying in varying tolerance exhibited by different genotypes is not clearly understood.

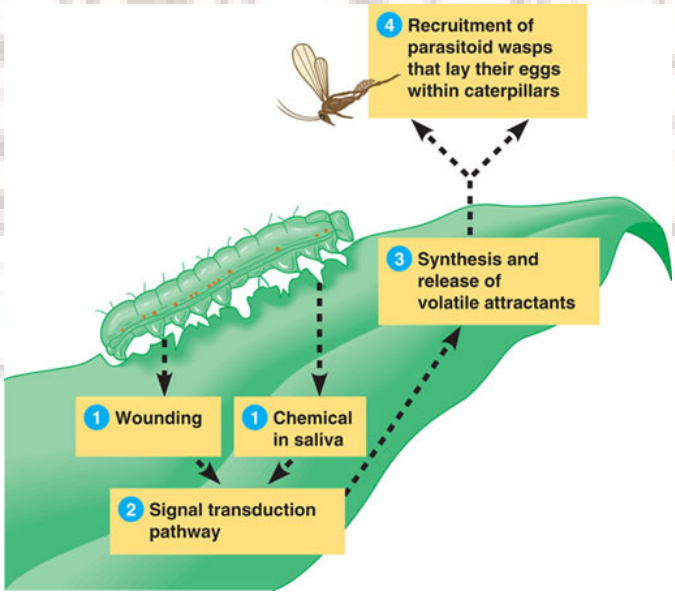

Fig 1.3 Plant responses to Herbivores

(Source: www.bio.miami.edu/dana/226/226F07_20print.html)

1.2.2.2 Role of jasmonates

The plant hormone jasmonic acid (JA) and related signaling compounds are collectively referred to as jasmonates and are ubiquitous signals for tissue injury and for the subsequent activation of defense responses to many, if not most, insect herbivores. In higher plants, JA is synthesized via the octadecanoid pathway. Wounding by chewing insects or mechanical damage results in rapid accumulation of JA at the site of wounding. Jasmonate mutants are compromised in resistance to a wide range of arthropod herbivores (Howe 2004). DNA microarray studies show that the jasmonate pathway has a dominant role in regulating global changes in gene expression in response to both mechanical wounding and herbivory (Reymond et al. 2000; Reymond et al. 2004; Ralph et al. 2006a). Jasmonates are also involved in the regulation of trichome-based defenses (Li et al. 2004; Boughton et al. 2005), priming of direct and indirect defenses (Engelberth et al. 2004: Ton et al. 2007), pathogen resistance (Glazebrook 2005), and systemic transmission of defense signals (Schilmiller and Howe 2005).

Severe mechanical tissue damage, abiotic stress, and developmental cues activate jasmonate synthesis and many associated defenses indicate that insect-derived factors are not strictly required for these responses. Rather, it would appear that insect-derived elicitors such as fatty acid-amino acid conjugates FACs, which stimulate JA synthesis when applied to artificial wounds, reinforce or amplify the jasmonate pathway (Kessler and Baldwin 2002). A strict requirement for JA synthesis in anti-insect defense was demonstrated by the use of mutants that are impaired in the β-oxidation stage of the octadecanoid pathway (Li et al. 2005). Major step towards identifying the signaling intermediates of JA responsive gene expression came from the identification of *COI* gene (Devoto and Turner 2005) and JAZ family of transcriptional repressors (Chini et al. 2007). JA signaling was reviewed extensively recently by Balbi and Devoto (2008). Induced expression of many anti-insect proteins is tightly regulated by the jasmonate signaling pathway. Insect feeding triggers the expression of plant defensive proteins that exert direct effects on the attacker. PIs, which impair various mechanistic classes of digestive proteases in the insect midgut, have been thoroughly studied for their role in the active defense response (Ryan 1990).

Other enzymes which disrupt the insect digestive physiology are members of the cysteine protease family of enzymes, plant lectins and chitinases which have different targets in insect gut (Konno et al. 2004; Lawrence and Novak 2006; Peumans and Vandamme 1995). Arginase and threonine deaminases (TD) degrade the essential amino acids arginine and threonine, respectively, in the lepidopteran midgut (Chen et al. 2005). Apart from theses defense proteins, plants exhibit other constitutive and inducible accumulation of several toxic secondary metabolites. Many terpenoids were known to form a part of the plant defense (Aharoni et al. 2005; Keeling and Bohlmann 2006). The alkaloids, widely distributed secondary metabolites that are best known for their metabolic effects in mammals (e.g., caffeine, nicotine, morphine, strychnine, and cocaine), likely evolved as a defense against insect herbivory (Howe and Jander 2008). Volatile organic compounds (VOCs) released from plants upon herbivore attack not only mediatie interactions with herbivores and their predators (Fig 1.3), and the damage induced volatiles can provide a signal that allows neighboring plants to prepare for imminent herbivory. This process is called priming.

1.2.3 Engineering plant resistance to disease and insect pests

Parasites and pathogens of plants are a significant and growing threat to crop production worldwide (Anderson et al. 2004). The goal of producing crops with increased and durable resistance to a spectrum of diseases is, therefore a major focus in plant research. Genetic engineering has the potential to solve these problems by inserting carefully selected and possibly multiple genes as transgenes (Campbell et al. 2002) and the search is therefore on for genes that confer durable broad-spectrum resistance that is also safe for all other organisms. Increasing knowledge of plant defense has led to more sophisticated transgenic approaches to enhancing resistance. The number of candidate genes put forward by transcriptomics, proteomics and protein interaction studies provides us with a large spectrum of genes that can be used.

A classic tactic for producing plants with increased disease resistance involves the manipulation of R genes and it is a strategy common to both transgenic approaches and classical breeding programs. The idea is to introduce an R gene and thereby confer on the plant the ability to recognize the pathogen and mount an effective defense. There have

been some notable reports on the successful deployment of these genes. The Bs2 gene confers durable resistance to bacterial spot disease in pepper (Tai et al. 1999). This disease is economically important in tomato and transformation of tomato plants with the pepper Bs2 gene led to resistance to bacterial spot disease (Tai et al. 1999). A promising approach to engineering disease resistance is to express a pathogen component in the plant that the plant can recognize. Recognition of these elicitor molecules then leads to the activation of a full defense response that is sufficient to inhibit the pathogen. Keller et al. (1999) expressed the elicitor cryptogein as a transgene in tobacco under the control of the pathogen-inducible hsr203J promoter. Under non-induced conditions, the transgene remained silent however after infection by the virulent oomycete, *Phytophthora parasitica* var. *nicotianae* localized necrosis similar to a hypersensitive response was observed.

Manipulating the expression of 'masterswitch' genes (McDowell and Woffenden 2003), such as kinases and transcription factors, which regulate quite a few target genes that could boost signaling through large portions of the pathogen induced signaling network and thereby lead to an increase in disease resistance. The disadvantage with this approach is that manipulation of some master switch genes could be detrimental to plant development (Gurr and Rushton 2005). The WRKY transcription factors have also been shown to be important in quantitative resistance to pathogens such as *Phytophthora infestans* (Trognitz et al. 2002). Another source of potential master-switch genes are protein kinases. MAP kinase (MAPK) signaling cascades are integral parts of many defence-signalling pathways, transient overexpression of MKK4a, MKK5a or constitutively active MEKK1 resulted in enhanced resistance to virulent *P. syringae* and *Botrytis cinerea* (Asai et al. 2002). In addition to kinases and transcription factors, other signaling molecules such as NPR1, NDR1, EDS1, PAD4, SGT1, COI1 and JAR1 that might represent important nodes in the signaling networks are candidates for this approach. For example in *Arabidopsis*, overexpression of NPR1 led to enhanced resistance to diverse pathogens (Cao et al. 1998) and crucially, this was achieved without a substantial yield penalty. The reason for this appeared to be that the NPR1-overexpressing plants did not constitutively turn on their defenses but rather appeared to be primed to respond to pathogen attack There have been numerous reports of transgenic

plants with increased disease resistance as a result of the overexpression of PR genes (Van Loon et al. 2006).

Care should be taken when using signaling component like MAPK and regulatory molecules like NPR1, as they might have several other roles in plants, which may adversely affect other important traits in the plants. For example, rice OsMAPK5 positively regulates drought tolerance and negatively regulates disease resistance (Xiong and Yang 2003). Recent work suggests that AtNPR1 negatively regulates viral resistance and abiotic stress tolerance in transgenic rice (Quilis et al. 2008). Apart from this, plant physiological environment also greatly influences the performance of different genetically modified (GM) plants. For example overexpression of rice *NPR1* makes the rice plants susceptible to herbivores (Yuan et al. 2007), whereas the *AtNPR1* overexpression did not cause any susceptibility in tobacco (Meur et al. 2008).

Genetic modification of plants is a powerful technology, with the potential to transform agriculture. Insect resistance, conferred via expression of a variety of *Bacillus thuringiensis* (Bt) delta-endotoxins, is the second most successfully used trait, after herbicide resistance, in commercial GM crops (James 2003). The primary threat to the long-term efficacy of Bt toxins is the evolution of resistance by pests and this can be countered by the modified Bt toxins, which may be useful against pests resistant to standard Bt toxins (Soberón et al. 2007). Proteinase inhibitors can be detrimental to the growth and development of many insects (Heath et al. 1997). Protease inhibitors (PIs) and lectins, whose ranges of insecticidal activity are generally broader than those of Bt toxins, are also being used in many experimental crops. Biotin-binding proteins (Kramer et al. 2000), chitinases (Wang et al. 1996), spider venom peptides (Penaforte et al. 2000), and plant hormones (Smigocki and Neal 1998) are some recent improvements along with caffeine production in tobacco as a potential pest repellant (Uefuji et al. 2005).

1.3 Priming

In everyday language, 'to prime' means 'to prepare' or make ready. In plant defense, priming is a physiological process by which a plant prepares to more quickly or aggressively respond to future biotic or abiotic stress (Frost et al. 2008). In addition to the direct activation of some antimicrobial defense reactions, systemic resistance responses in plants are frequently associated with a primed state, in which plants are able to 'recall' previous infection, root colonization or chemical treatment (Fig 1.4). As a consequence of this, primed plants respond more rapidly and/or effectively upon reexposure to biotic or abiotic stress (Conrath et al. 2002, Prime-A-Plant Group 2006). BABA (b-aminobutyric acid), a non-protein amino acid, induced priming for augmented stress responses is associated with enhanced resistance to a multiplicity of microbial pathogens, nematodes, insects, and abiotic challenges such as drought and salt stress (Prime-A-Plant Group 2006). In response to wounding or herbivore attack, plants often release extrafloral nectar or volatile organic compounds (VOCs), which may serve to attract parasitic or predatory natural enemies of the herbivores (Paré and Tumlinson 1999). Others have a role in enhancing the disease resistance in the wounded or herbivore-attacked plants themselves (Heil and Silva Bueno 2007) or neighbouring, unharmed plants (Heil and Kost 2006). VOCs from clipped sagebrush (*Artemesia tridentata*) prime nearby tobacco plants for accelerated production of trypsin proteinase inhibitors concomitant with reduced total herbivore damage and a higher mortality rate of young *Manduca sexta* caterpillars (Kessler et al. 2006). Thus, plants can use chemical signals in their environment to assess the risk of herbivory and use this information to adjust their overall defense strategies.

1.3.1 Costs and benefits of priming

The activation of direct defense reactions by external application of high doses of SA, jasmonic acid or by the action of resistance (R)-genes was shown to reduce plant fitness traits such as growth and fruit or seed set under pathogen-free conditions (Cipollini 2002; Tian et al. 2003; Heidel et al. 2004). These observations were also reported in the field when Heidel et al. (2004) demonstrated that *Arabidopsis* mutants, blocked in SA inducible defense, as well as mutants showing constitutive expression of these defenses,

were affected in growth and seed set. The trade-off dilemma between disease resistance and costs of defense activation could probably be overcome by priming. During a comparative study by Van Hulten et al. (2006), the costs and benefits of priming in *Arabidopsis* were determined and compared to those of the direct induction of defenses. Application of low doses of the non-protein amino acid β-aminobutyric acid (BABA) induced a primed state, caused only minor reductions in growth with no obvious effect on seed production. In contrast, direct induction of defense responses by high doses of either BABA or BTH strongly reduced both these fitness traits. Intriguingly, when under attack by pathogens, primed plants displayed even higher fitness than non-primed ones. Thus, in environments of pathogen challenge, the costs of fitness of the primed state seem to be outweighed by its benefits (Van Hulten et al. 2006).

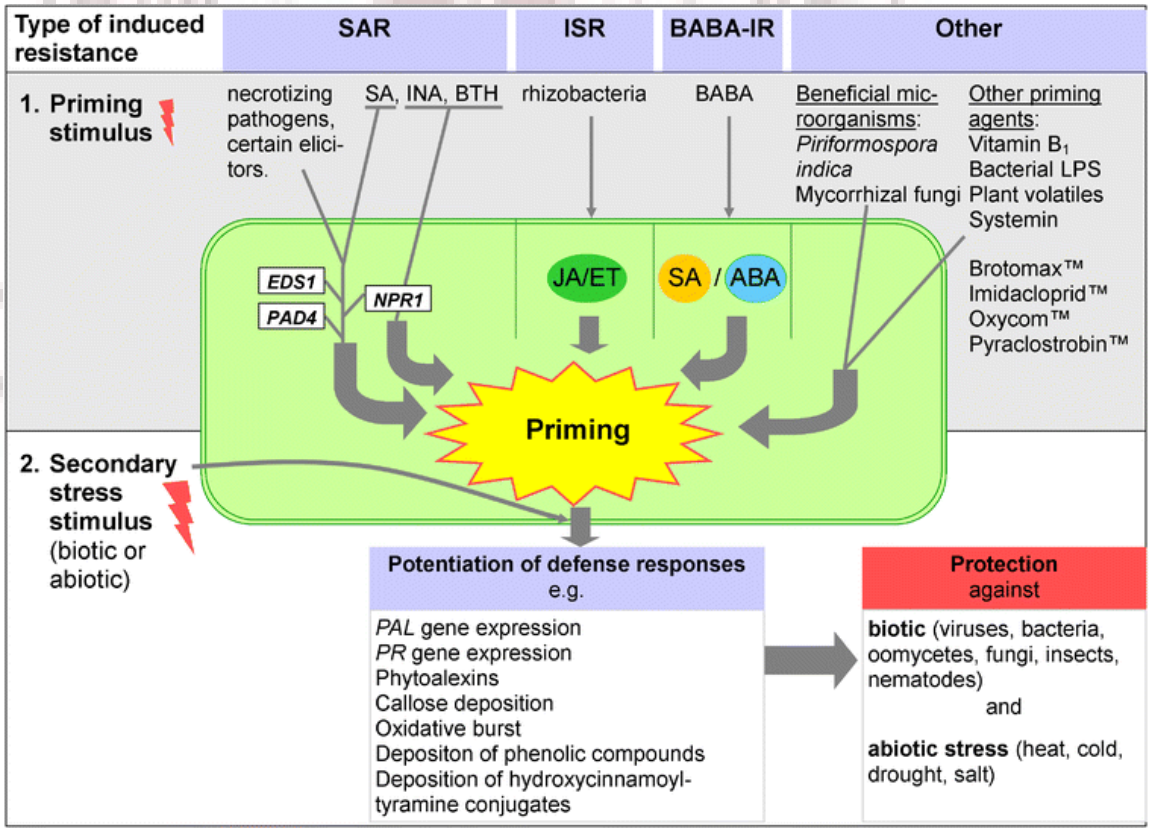

Fig 1.4 Events associated with priming in plants (Source: Goellner and Conrath 2008)

Chapter 2

General Materials and Methods

2.1 Plant materials

Arachis hypogaea cv. JL-24 seeds were obtained from local seed stations. *Nicotiana tabacum* cv. Xanthi seeds were obtained from Central Tobacco Research Institute (CTRI), Rajhamundry, Andhra Pradesh. *Arachis diogoi* (accession number: ICG 4983) seeds were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad.

2.2 Insect larvae and fungal pathogens

Spodoptera litura larvae were kindly provided by Dr. H. C. Sharma, ICRISAT, Hyderabad. *Phaseoisariopsis personata* condia were collected from infected peanut leaves with typical Late Leaf Spot (LLS) symptoms using a cyclone spore collector (Pande and Narayana Rao 2001). *Phytophthora parasitica* var. *nicotianae* was obtained from CTRI, Rajhamundry.

2.3 Chemicals

All the chemicals used in the present study were procured from Sigma-Aldrich, USA; Amersham Biosciences, UK; Promega Life Science, USA; Fermentas, Germany, Himedia chemicals, India and Qualigens fine chemicals, India.

2.4 Plasmid DNA vectors

- pTZ57R (MBI Fermentas, Germany) used for cloning all PCR products.
- pRT100 (Töpfer et al. 1987) is a plant expression vector for cloning complete ORFs, to obtain ORF flanked by 35S promoter and poly-adenylation signal. Kindly provided by Dr. D. Pental of the Delhi University.
- pRD400 (Datla et al. 1992) is a binary vector for plant transformation. This vector contains *npt*II gene conferring resistance to kanamycin as plant selectable marker.
- pEGAD vector (Cutler et al. 2000) is used to obtain N-terminal translation fusion with GFP.

- pET 32a (Novagen, USA) a bacterial T7 polymerase expression vector is used for expressing the recombinant protein as a fusion to Thioredoxin-Histidine (TRX-HIS) tag.

2.5 Bacterial strains

The bacterial strain *Escherichia coli* (DH5α) was used in the maintenance of the plasmid clones and *E. coli* BL-21 (DE3) pLysS was used for the expression of Trx-His tagged fusion recombinant protein. The *Agrobacterium tumefaciens* strain EHA105 was used for both transient and stable transformation of tobacco.

2.6 Restriction enzymes, modifying enzymes and Markers

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

2.7 Growth conditions

The different strains of *E. coli* were incubated and cultured either in liquid LB medium (Himedia, India) with continuous shaking at 200 rpm or in the solid LA medium at 37 ºC. Agrobacterium strains were incubated in liquid LB medium with continuous shaking at 200 rpm or in the solid LA medium at 28 ºC. Plants under *in vitro* culture were maintained in culture room at 28±1°C and a photoperiod of 16:8 (Light: Dark). Plants growing in green house were maintained at 30±1°C under natural light.

2.8 Extraction and purification of plant genomic DNA (Murray and Thompson, 1980)

Two to three grams healthy leaf material was homogenized to a fine powder using liquid N_2. About 10 ml of freshly prepared lysis buffer (100 mM Tris-Cl, pH 8.0, 20 mM EDTA, pH 8.0, 2% CTAB, 0.2% β-mercaptoethanol, 1.4 M NaCl) was taken in oakridge tube, prewarmed to 65^0C and the homogenized powder was added to the buffer. The homogenate was mixed thoroughly with the buffer and incubated in a water bath at 65^0C for ~1 h. After lysis, tubes were allowed to come to room temperature. Equal volume of chloroform: isoamyl alcohol (24:1) was added and the two phases were mixed thoroughly

by gently inverting several times. Following this phases were separated by centrifugation at 14,000 rpm for 20 min at room temperature. The clear aqueous phase in the top layer was collected in a fresh tube with cut tips and an equal volume of isopropanol was added. After gently inverting for several times, the DNA which appeared as threads, was spooled out with a bent thin glass rod and transferred to a 1.5 ml eppendorf tube. DNA was washed with 1 ml of 70% ethanol. The tubes were centrifuged at 12,000 rpm for 5 min at room temperature. Ethanol was decanted by inverting the tube carefully; the pellet was briefly dried and dissolved in 100-200µl TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA pH8.0).

For purification, DNA was treated with RNase (1mg/ml) for 2 h at 37^0C. The residual protein contamination was removed by extracting once with phenol: chloroform: isoamyl alcohol (25: 24:1) and twice with chloroform: isoamyl alcohol (24:1). Each time the organic phase was mixed thoroughly with the aqueous phase by inverting the tubes several times, centrifuging at 12,000 rpm for 15 minutes at room temperature and collecting carefully the upper clear aqueous phase in a fresh tube. Finally, the DNA was precipitated by adding $1/10^{th}$ volume of 3M sodium acetate, (pH5.2) and two volumes of absolute ethanol or one volume of isopropanol followed by centrifugation at 12,000 rpm for 15 minutes at 4^0C. The pellet was washed with 70% ethanol, briefly dried and dissolved in 50-100µl of TE. Genomic DNA samples were stored at 4^0C for immediate use and at -20^0C for long-term use.

2.9 Extraction of total RNA

About 100mg of leaf material was ground with mortar and pestle to a fine power using liquid nitrogen. Total RNA was extracted using TRI reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. Isolated RNA was dissolved in DEPC-treated water.

2.10 Quantification of DNA and RNA

The quality and concentration of RNA and DNA samples were examined by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis. The concentration of DNA and RNA was determined using spectrophotometry by measuring

the absorbance at 260 nm and 280 nm. A value of OD_{260} =1 corresponds to 50 µg/ml for DNA, while OD_{260} =1 corresponds to 40 µg/ml for RNA. For pure DNA, the value of OD_{260}/OD_{280} must be between 1.8 and 2.0. A value below 1.8 means the contamination of DNA with proteins and phenolic compounds. For the purity of RNA the value of OD_{260}/OD_{280} must be between 1.9 and 2.1.

2.11 First strand cDNA synthesis

The synthesis of first strand cDNA was carried out using MMLV- Reverse transcriptase (Sigma –Aldrich, USA) or (Promega Corporation, USA) following manufacturer's instructions with total RNA as template using Oligo-dT or gene specific primers.

2.12 Polymerase Chain Reaction (PCR)

Consumables for all the PCR reactions were obtained from Invirogen, USA. PCR reactions were performed on Eppendorf Personal Thermal cycler, Germany or Biorad thermal cycler, USA. Depending on the template and primer combinations, PCR conditions were optimized empirically in each case.

2.13 Restriction endonuclease treatments

General molecular biology methods were adopted from Sambrook et al. (1989). DNA digestion was carried out in a reaction volume of 50 µl with an appropriate reaction buffer (10 X) and 5U of restriction enzyme was used per 1 µg of DNA to be digested, while for double digestions the restriction digestions were carried out sequentially.

2.14 Purification of DNA fragments from the agarose gel

After the restriction digestion, or PCR amplification of plasmid DNA constructs, DNA bands or plasmid inserts were identified using standard molecular weight marker (Lambda *Hind* III + *Eco*RI or Lambda *Hind* III digest). After the extraction, purification of DNA from the agarose gel pieces was done with Eppendorf Perfectprep gel cleanup kit (Eppendorf, Germany) according to the manufacturer's instructions.

2.15 Dephosphorylation

The single digested DNA fragments were dephosphorylated at their 5'-ends with Calf intestine alkaline phosphatase (Fermentas, Germany) in order to avoid self ligation of cohesive / blunt-end termini of plasmid DNA during DNA recombination. The reaction was carried out in a total volume of 50 µl comprising 5 µl dephosphorylation buffer (10X), 1 µl (1.0 unit) of Calf intestine alkaline phosphatase and appropriate µg of plasmid DNA. The mixture was made up to 50 µl with sterile double distilled water and incubated at 37°C for 30 min, followed by heat inactivation at 85°C for 15 min.

2.16 Ligation

For plasmid DNA constructs, different DNA inserts were ligated in various independent experiments using T4 DNA ligase (Fermentas, Germany). The ligation reaction mixture was made in a total volume of 20 µl comprising 2 µl ligation buffer (10X), appropriate volumes (in µl) each of linear digested plasmid DNA and insert DNA, and finally T4 DNA ligase (1-2 units for cohesive ends and 5 units for blunt ends). The reaction mixture was incubated for 16 h at 16°C for cohesive ends and at 22°C for blunt ends respectively.

2.17 Preparation of *E. coli* competent cells and transformation

A single colony of *E. coli* DH5α or BL-21 (DE3) pLysS cells were inoculated into 5 ml of LB broth and incubated overnight with constant shaking at 37°C. One ml of the overnight culture was inoculated in 50 ml of fresh LB medium with vigorous shaking until the OD_{600} reaches 0.5. The cells were cooled on ice for 30 min and pelleted by centrifugation at 4000 rpm for 5 min at 4°C. The pellet was suspended in 40 ml of ice cold 100mM $CaCl_2$ and incubated on ice for 20 min and further centrifuged as above. The pellet was finally resuspended in 3 ml ice-cold 100 mM $CaCl_2$, a final concentration of 15% (v/v) sterile glycerol, mixed and stored at -70°C in aliquots of 0.2 ml of competent cells. Plasmid DNA or the ligation mixture was added to competent cells, carefully mixed and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 90 sec, and left on ice for 2 min. LB medium (0.8 ml) was added to the treated cells and further incubated by shaking at 200 rpm at 37°C. Aliquots (100-200 µl) of the

transformed cells were spread on LA plates with appropriate antibiotics and incubated at 37°C overnight.

2.18 *Agrobacterium* competent cell preparation and transformation

Agrobacterium tumefaciens (EHA105) competent cells were prepared as described for *E. coli* except that cells were grown at 28°C. Transformation of Agrobacterial competent cells was performed using freeze thaw method (Holsters et al. 1978), which involved immediate freezing in liquid nitrogen after adding plasmid DNA, followed by incubating in a 37°C water bath for 5 min. To this, 1 ml of LB medium was added and incubated at 28°C for 2-4 h with shaking. The cells were pelleted at 4000 rpm for 5 min and plated on LB agar medium with rifampicin and the corresponding selectable marker of the plasmid DNA used for transformation.

2.19 Isolation of plasmid DNA (mini-prep) by alkaline lysis method (Birnboim and Doly, 1979)

For plasmid mini-prep, a single colony of the transformed *E. coli* was inoculated in 5 ml of LB medium containing appropriate antibiotics and allowed to grow overnight with shaking at 200 rpm at 37°C. The bacteria were harvested by centrifugation at 12,000 rpm at room temperature for 1 min. The pellet was resuspended in 100 µl ice-cold solution-I (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg RNase A) and vortexed thoroughly. The cells were lysed by adding 200 µl freshly prepared solution-II (200 mM NaOH, 1% (w/v) SDS) and mixed by gentle inversion. To this lysate, 150 µl of pre-chilled solution-III (3.0 M potassium acetate, pH 4.8) was added, mixed well and incubated on ice for 5 min. The supernatant was collected by centrifugation for 15 min at 12,000 rpm at 4°C. To the supernatant, an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added, mixed gently and centrifuged as above. The DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.7 volumes of isopropanol. Finally the DNA was pelleted by centrifugation at 12,000 rpm for 15 min at room temperature and the pellet was washed in 70% (v/v) ethanol, vacuum-dried and resuspended in 50-100 µ l of TE buffer and stored at -20°C.

2.20 Southern blot analysis

Total genomic DNA (15-20 µg) was digested with the appropriate restriction enzymes as per the requirement, in their respective 1X buffers, incubating overnight at 37^0C in a water-bath. The reaction was stopped by adding of 1µl of 0.5 M EDTA, pH 8.0. Samples were stored in -20^0C until use. For resolving the digested DNA, 0.8% agarose gel was prepared by melting 0.8 g agarose in 100 ml of 1 X TAE buffer (diluted from 50 X TAE: 2M Tris, 1M Acetate, 100mM EDTA, pH 8.1) and pouring onto a casting tray with a comb. After the gel polymerized, digested samples were loaded in the wells after adding 6x DNA Loading Dye (0.15% bromo phenol blue, 0.15% xylene cyanol, 5 mM EDTA, 40% sucrose) to a final concentration of 1X. The gels were run by submerging in 1x TAE buffer in the gel running tank at 30V (5v/cm) for 12 h till the dye front of bromophenol blue reaches 12cm from the well. As a reference for size marker, Lambda DNA digested with *Hin*dIII/*Eco*RI was separated along with the samples. After run was over, the gel was soaked in water in a tray containing ethidium bromide for staining the marker bands and for visualization of the separation of the digested DNA. The ethidium bromide stained gels were documented to mark the position of the λ-*Hind*III/*Eco*RI double digest marker.

The gel was then treated with the depurination solution (0.2 N HCl) for 15 min followed by 30 min each in the denaturation solution (1.5 M NaCl, 0.5 M NaOH) and the neutralization solution (1 M Tris-HCl, pH 7.4, 1.5 M NaCl) respectively. The DNA was transferred onto Hybond-N+ nylon membrane (Amersham Biosciences, UK) overnight by the capillary method with 20X SSC (1.5 M NaCl, 0.15 M Sodium citrate, pH 7.0) as a transfer buffer. The DNA transferred on the membrane was UV-cross linked, prehybridized at 65°C for 3-4 h in phosphate buffer (0.5 M phosphate buffer, pH 7.2, 7% (w/v) SDS, 10 mM EDTA and 1% BSA) and hybridized for 16 h with $^{\alpha\text{-}32}$P dATP radiolabelled DNA using Prime-a-gene labeling system of Promega, USA. After hybridization, the membranes were washed twice with 2 X SSC, 0.1% SDS at 65°C for 10 min followed by 1 X SSC, 0.1% SDS and 0.1 X SSC and 0.1% SDS for 5 min each respectively. The membranes were exposed at -70°C and autoradiographed.

2.21 Northern blot analysis

Total RNA (20 µg) was separated by electrophoresis at 70 V for 2-3 h through 1.2% (w/v) formaldehyde agarose gel in formaldehyde gel buffer. The gel was washed in DEPC water for 30 min. Transfer of RNA onto Hybond-N+ nylon membrane, hybridization and washings were carried out at 65°C as described in the protocol of southern blot analysis. All glassware, plasticware, buffer solutions used for RNA work were treated with 0.1% (v/v) DEPC.

1.2% formaldehyde gel (100 ml):

To 1.2 g of agarose, 10 ml 10X formaldehyde gel buffer, RNase-free water to 100 ml were added and agarose was melted. This was then cooled to 65°C and 1.8 ml of 37% (12.3 M) formaldehyde and 1µl of ethidium bromide (10 mg/ml) was added, mixed thoroughly and poured onto gel support.

10 X formaldehyde gel buffer:

200 mM MOPS (free acid)
50 mM Sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

1 X formaldehyde running buffer:

100 ml 10x formaldehyde gel buffer
20 ml 37% formaldehyde (12.3 M)
880 ml RNase-free water

RNA sample preparation:

The RNA samples were prepared by adding 1 volume of 5 X RNA loading buffer per 4 volumes of RNA sample (for example: 10 µl of 5X RNA loading buffer and 40 µl of RNA). This was incubated for 3-5 min at 65°C, chilled on ice and loaded onto gel.

5X RNA loading buffer:

Saturated bromophenol blue -16 µ l

0.5 M EDTA, pH 8.0 - 80 µl

37% formaldehyde (12.3 M) -720 µl

100% glycerol - 2ml

Formamide - 3084 µl

10x formaldehyde gel buffer - 4ml

RNase-free water to 10 ml

2.22 Preapration of $^{\alpha-32}$P dATP labeled DNA probes for hybridization

DNA was radiolabled using Primer-a-Gene® Labeling System (Promega Corporation, USA) according to the manufacturer's instructions. In brief, 25ng of the denatured template DNA, 10 µl labeling buffer (5 X), 2 µl of a mixture of unlabeled dNTPs (dGTP, dCTP and dTTP), 2 µl of nuclease-free BSA, 5 µl of 50 µ Ci $^{\alpha-32}$P dATP and 5 units of DNA polymerase-I (Klenow fragment) were added and finally the volume of the reaction mixture was made up to 50 µl. This mixture was then incubated at room temperature for 1 h followed by heating at 95-100°C for 5 min and chilling on ice and adding EDTA to 20 mM. This was used directly in a hybridization solution.

2.23 Custom primer synthesis

All the primers used in this study were custom synthesized from Bioneer Corporation, Korea, MWG Biotech, India and Sigma-Aldrich, India.

2.24 Custom sequencing

All the recombinant clones were sequenced at Macrogen, Korea or MWG Biotech, India.

Chapter 3

Molecular cloning and functional characterization of *AhMPK3*, a mitogen-activated protein kinase gene of Peanut (*Arachis hypogaea* L.)

3.1 Introduction and Background

3.1.1 Peanut

The genus *Arachis* is native to South America, probably from a region including Central Brazil and Paraguay (Gregory and Krapovickas 1980). There are 69 species described in the genus, assembled into nine sections, according to the morphology, geographic distribution and crossability (Krapovickas and Gregory 1994). Some of these species have been used for forage in South America, but the most important species is the cultivated peanut, *Arachis hypogaea* L. This crop is widely grown in more than 80 countries in the Americas, Asia and Africa (Singh and Singh 1992). The origin of the genus *Arachis*, its taxonomy, cytogenetics and genomes relationships, the botanical classification, and reproductive development have been extensively covered in a review article by Holbrook and Stalker (2003).

Cultivated peanut (*Arachis hypogaea* L) is one of the world's most important oilseed crop, along side soybean, cottonseed, rapeseed, and sunflower. It is also a rich source of edible oil and vegetable protein and an important crop for both human and animal food. Peanut is the second most important grain legume crop worldwide after soybean, with a production of 33 million tons in 2003/04 (FAO 2003). Soybean and peanut provide more than 35% of the world's processed vegetable oil. Peanut is produced throughout the tropics and warmer regions of the subtropics, but is particularly important in Africa, Asia and in the United States (FAO 2003). The cultivated peanut (*Arachis hypogaea* L.) is an allotetraploid, with two genomes, classified as AA and BB, according to cytogenetic characters. Similar genomes to those of *A. hypogaea* are found in the wild diploid species of section *Arachis*, which is one of the nine *Arachis* sections.

Peanut genomics

With the completion of the *Arabidopsis,* rice and poplar whole genome sequencing projects, a vast amount of valuable data has been generated to facilitate cross-species genome comparison in the plant kingdom. The peanut genome size is significantly larger (2,800 Mb) than the currently sequenced plants (Temsch and Greilhuber 2000), such as

Arabidopsis (128 Mb), rice (420 Mb), and *Medicago* (500 Mb) (Guo et al. 2008). It is practically unrealistic to completely sequence the whole peanut genome in the near future. The EST projects of peanut have started couple of years back only and by 2007 dbEST release (032307), the number of *Arachis* ESTs deposited in genbank database were a meager 19,790 (Guo et al. 2008), in sharp contrast to the large number of ESTs in the database of the top five plant species including *Arabidopsis* (1,276,131), rice (1,211,154), maize (1,161,193), wheat (855,272) and barley (437,728). This clearly shows that peanut genomics is at a very early stage and the identification and isolation of genes of peanut and their characterization will greatly help in increasing its genomic resources and understanding of the plant development and response to various environmental cues.

3.1.2 Plant MAPKs

For living cells to respond to the external stimuli or environment and adapt to them, it is necessary to transmit the signal from out side the cell to inside in a coordinated manner, and finally to the nucleus, where the required gene expression takes place. Living in inevitably changing environmental conditions, plants should respond to them in the most befitting manner in order to survive against the odds. For this purpose they have evolved a variety of signal transduction mechanisms, which transduce the perceived external signal to the inner cellular components for appropriate response to combat such pressures. At the molecular level, the perception of extracellular stimuli and the subsequent activation of defense responses require a complex interplay of signaling cascades, in which reversible protein phosphorylation plays a central role (Yang et al. 1997). Activation and de-activation of enzymes through phosphorylation / de-phosphorylation by kinases and phosphatases allows for fast and specific signal transduction. One particular signal transduction mechanism, the MAP kinase cascade, plays an important role in many different eukaryotic organisms, from yeast, through *Dictyostelium*, *Drosophila* and *Caenorhabditis* to mammals, and also plants. Mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine protein kinases highly conserved among eukaryotes, which mediate intracellular phosphorylation events

linking receptor activation to the control of cell proliferation, chemotaxis, differentiation and stress response (Schaeffer and Weber 1999).

Mitogen-activated protein kinase (MAPK) cascade is a conserved transduction mechanism involving three functionally related components (Widmann et al. 1999). The upstream MAPKKKs (MAP kinase kinase kinases), which phosphorylate and activate the downstream MAPKKs (MAP kinase kinases), which in turn phophorylate and activate the MAPKs (MAP kinases). Phosphorylation targets of activated MAP kinases include both nuclear and cytosolic proteins (Morris 2001).The mammalian MAPKs have been classified into three subgroups based on the phylogeny and function (Kultz 1998). The first subgroup is referred to as extracellular signal-regulated kinases, which are involved in differentiation and cell cycle regulation. The MAPKs in this subgroup are characterized by the specific dual phosphorylation motif TEY (Seger and Krebs 1995). The other two subgroups are the stress-activated protein kinase/Jun N-terminal kinase subfamily, in which TPY is the phosphorylation motif, and the p38/HOG1 subfamily, which uses TGY as the phosphorylation site (reviewed by Canman and Kastan 1996; Kyriakis and Avruch 1996).

Numerous protein kinases, with close sequence similarity to mammalian MAPKs, have been identified in plants (reviewed by Stone and Walker 1995; Hirt 1997; Mizoguchi et al. 1997; Tena et al. 2001; Zhang and Klessig 2001; Agarwal et al. 2003). Most plant MAPKs are associated with the subgroup of extracellular signal-regulated kinases based on phylogeny (Kultz 1998). Several plant MAPKs has been identified, which are activated in response to pathogens (Ligterink et al. 1997; Zhang and Klessig 1997, 1998b; He et al. 1999), cold (Jonak et al. 1996), salinity (Mikolajczyk et al. 2000), drought (Jonak et al. 1996), and wounding (Seo et al. 1995, 1999; Bögre et al. 1997; Zhang and Klessig 1998a; He et al. 1999). Plant MAPKs can also be activated by fungal elicitors (Suzuki and Shinshi 1995), hormones like salicylic acid (Zhang and Klessig 1997), and abscisic acid (Knetsch et al. 1996; Burnett et al. 2000), jasomonates and ethylene (Schweighofer and Meskiene 2008). Apart from this, several MAPKKs (Morris et al. 1997; Hackett et al. 1998; Hardin and Wolniak 1998; Ichimura et al. 1998a; Kiegerl et al. 2000; Yang et al. 2001; Xu et al. 2008) and MAPKKKs (Ichimura et al. 1998b;

Kovtun et al. 2000; Frye et al. 2001; Zhang et al. 2007) have been cloned and characterized.

Mitogen-activated protein kinase (MAPK) pathways in plants have been implicated in signal transduction for a wide variety of stress responses (Jonak et al. 2002; Colcombet et al. 2008; Menges et al. 2008). In the monocot model plant rice, several MAPKs were characterized to be involved in both biotic and abiotic stress responses (Agrawal et al. 2003; Cheong et al. 2003; Reyna et al. 2006; Roshila and Yang 2007; Lee et al 2008). For most of the rice MAPKs only expression data are available with few exceptions of functional characterization (Roshila and Yang 2007) like OsBWMK1 whose ectopic expression in tobacco resulted in constitutive PR gene expression and enhanced resistance to fungal (*Phythophthora parasitica*) and bacterial (*Pseudomonas syringae*) infection (Cheong et al. 2003). RNAi (RNA interference) of OsMAPK5 was shown to induce constitutive PR gene expression and enhanced resistance to fungal (*Magnaporthe grisea*) and bacterial (*Burkholderia glumae*) infection (Xiong and Yang 2003). *OsMAPK5* was found to positively regulate drought, salt, and cold tolerance and negatively modulate *PR* gene expression and broad-spectrum disease resistance. And recently, OsMPK6 was shown to negatively regulate rice disease resistance to bacterial pathogens (Yuan et al. 2007).

Based on the completed *Arabidopsis* genome sequence, 20 MAPKs, 10 MAPKKs and 60 MAPKKKs were identified; they were divided into four groups (A-D). MAPKs belonging to groups A, B and C all possess a TEY motif in their activation loop, while members of group D harbor a TDY motif (MAPK group 2002). Group A MAPKs have been most frequently found to be involved in environmental and hormonal responses. AtMPK3 and AtMPK6 and their apparent orthologs in other species are present in group A and are activated by many environmental stresses (MAPK group 2002). Group B MAPKs have been less well studied, but appear to be involved in both environmental stress responses and cell division. Extensively studied MAPK of group B is AtMPK4, which was shown to be activated in response to abiotic stresses, bacterial elicitor flagellin and harpin (Teige et al. 2004; Suarez-Rodriguez et al. 2007). Loss of function mutants concluded that AtMPK4 functions as a negative regulator of defense responses. Recently Qiu et al. (2008) demonstrated the *in vivo* interactions of AtMPK4 responsible for its

negative regulatory role in defense. Information on the Group C MPKs is limited, although microarray analysis detected circadian-rhythm-regulated expression of *MPK7* (Schaffer et al. 2001). Recent reports suggest that group C MAPKs also play a role in stress responses (Maisa et al. 2008; Zong et al. 2008). Group D MPKs, which include eight members of the *Arabidopsis* MPKs, are notable for the TDY motif in their T-loop and their extended C-terminal region relative to Groups A, B and C. Group D MPKs also lack the C-terminal CD domain, which is consistently found in members of the other MPK groups. Group D is not studied extensively and members were found to be involved in biotic stress responses like, rice *BWMK1* and alfalfa *TDY1* are induced by blast fungus and wounding (He et al. 1999; Schoenbeck et al. 1999; Reyna et al. 2006) respectively.

AtMPK3, *AtMPK6* and their apparent orthologs in other species are present in group A and are found to be activated by many environmental stresses and shown to be involved in non-host resistance (Zhang and Klessig 1998a; Zhang et al. 2000), gene for gene signal transduction (Zhang and Klessig 1998b; Romeis et al. 1999), hypersensitive response (Liu et al. 2003; Stulemeijer et al. 2007), wounding (Seo et al. 1995), response to elicitors (Zhang et al. 2000; Daxberger et al. 2007), and several abiotic stresses (Jonak et al. 1996**;** 2004; Samuel et al. 2000; Ahlfors et al. 2004). Recent studies showed their involvement in phytoalexin biosynthesis (Ren et al. 2008), response to herbivores (Wu et al. 2007), key regulators of stomatal development and patterning (Wang et al. 2007), anther (Hord et al. 2008) and ovule (Wang et al. 2008) development.

AtMPK3 orthologs, *NtWIPK* (*Nicotiana tabaccum*), *LeMPK3* (*Lycopersicon esculentum*) and Ms*MMK4* (Medicago *sativa*) were very well studied and were found to be induced in response to various biotic and abiotic elicitors (Mizoguchi et al. 1996; Jonak et al. 1996; 2004; Bogre et al. 1997; Zhang et al. 2000; Holley et al. 2003; Mayrose et al. 2004; Wan et al. 2004;). In tobacco, mechanical wounding induced rapid transcript accumulation and activation of WIPK (wound-induced protein kinase, Seo et al. 1995). Transgenic plants overexpressing *NtWIPK* showed constitutive *PI-II* transcript accumulation, WIPK activity, and higher jasmonic acid (JA) levels compared to wild type (Seo et al. 1999). JA quantification in *NtWIPK* silenced plants demonstrated that *NtWIPK* is involved in the production of wound induced JA (Seo et al. 2007). Several orthologs of *AtMPK3* were shown to play a crucial role in plant defense responses like

over-expression of *MK1*, which encodes the *Capsicum* ortholog of *NtWIPK*, display elevated JA levels and resistance to blast fungus in transgenic rice plants (Lee et al. 2004). Plants overexpressing *TIPK* (*Trichoderma*-Induced MAPK), an *AtMPK3/NtWIPK* ortholog from cucumber were more resistant to pathogenic bacterial attack than control plants (Shoresh et al. 2006). And the suppression of *NtWIPK* or its orthologs led to increased susceptibility against pathogens as well (Sharma et al. 2003; Shoresh et al. 2006). For example *AtMPK3* mutant plants exhibit reduced camalexin accumulation after *B. cinerea* infection in *Arabidopsis* (Ren et al. 2008). Although the involvement of *AtMPK3* and its orthologs was well established in wound and systemin signaling responses (Seo et al. 1995; Holley et al. 2003), their role in plant response to herbivore attack was not well explored till recently. Kandoth et al. (2007) and Wu et al. (2007) demonstrated that *LeMPK3* and *NaWIPK* are involved in regulating the defense response against herbivore attack in *L. esculentum* and *N. attenuata* respectively. Although several *AtMPK3/NtWIPK* orthologs from various plants were overexpressed and shown to confer resistance to microbial pathogens, there were no reports on the performance of plants against herbivore attack.

3.1.3 Objectives of the present work:

Though peanut is one of the widely cultivated oilseed crops with economical and nutritional importance, extensive genomic information is not available in public databases pertaining to it. The availability of genetic information would enhance the understanding of mechanisms involved in plant development and stress responses (Guo et al. 2008). This has been accomplished to some extent by the completion of some of the peanut EST projects (Luo et al. 2005a; Guo et al. 2008). Considering the lack of extensive genomic information of peanut and the significant role of MAPKs in regulating plant defense and stress responses, the following objectives were framed.

1. Isolation of partial cDNA corresponding to MAPKs of peanut (*Arachis hypogaea* L.) using degenerate primers.
2. Obtaining corresponding full length cDNA using RACE.
3. Sequence and phylogenetic analysis of isolated gene.
4. Expression analysis in response to various cues.
5. Functional characterization of peanut MAPK by heterologous expression in tobacco.

3.2 Materials and Methods

3.2.1 Plant material

Detached leaves of peanut (*Arachis hypogaea* cv. JL-24) from 2-3 week old plants grown in the greenhouse were used in all experiments. Tobacco (*Nicotiana tabacum* cv. Xanthi) seeds were surface sterilized with 4% sodium hypochlorite for 10 min, washed 4-5 times with sterile distilled water and allowed to germinate on Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Individual germinated seedlings were transferred to culture bottles with MS medium and maintained aseptically.

3.2.2 Treatment with chemicals and abiotic stresses

Compound leaves (quadrifoliate) detached from peanut plants were kept in a tray with a moist filter paper saturated with sterile distilled water and covered with a polythene bag to maintain humidity and left overnight to stabilize the wound signal. For various chemical treatments, leaves were floated in the corresponding solution. The treatments given were 500 μM salicylic acid (SA), 100 μM methyl jasmonate (MeJA), 100 μM abscisic acid (ABA), 25 mM hydrogen peroxide (H_2O_2), 200 mM mannitol, 100 mM sodium chloride (NaCl), 100 μM sodium nitroprusside (SNP) and the treatment with water served as control. Wounding was performed by damaging the leaf lamina with a sharp blade and a pointed forceps and cold treatment was given by shifting the leaves to a cold chamber (4^{o}C). Samples were collected at regular intervals, quickly frozen in liquid nitrogen, and stored at -80^{o}C until use. Mannitol, NaCl and H_2O_2 are obtained from Himedia, India. Rest of the chemicals used for treatments were purchased from Sigma-Aldrich, USA.

3.2.3 DNA and RNA isolation

Leaves of peanut and tobacco were frozen in liquid nitrogen and ground into a fine powder. Total Genomic DNA was then extracted by the cetyltrimethylammonium bromide (CTAB) procedure (Murray and Thompson, 1980). RNA was isolated from

samples harvested at various intervals using TRI reagent (Sigma-Aldrich, USA) following the manufacturers instructions. The quality and concentration of RNA and DNA samples were examined by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis.

3.2.4 RT-PCR and amplification of Partial cDNA and cloning of PCR products

Reverse transcription reaction was performed using 4 µg of total RNA, 500 ng of Oligo-dT primer and 2 µl of 10mM dNTP, 2 µl of 10X RT buffer and 1 µl (200 units) of reverse transcriptase enzyme (Sigma Aldrich, USA) in a 20 µl reaction at 37^{o}C for 60 min followed by 15 min at 75^{0} C for heat inactivation of the enzyme. One-tenth volume of the RT reaction mixture was used as a template for PCR using degenerate primers, IntF as the sense primer and IntR as the antisense primer (Table 3.1). PCR was performed in a volume of 25 µl containing 200µM dNTP mix, 2mM $Mgcl_2$, 0.8 pmol each of forward and reverse primer, 1X PCR buffer and 1.25 units of *Taq* polymerase (Invitrogen, USA). A gradient PCR was performed with varying annealing temperatures with cycling parameters of 94^{o}C for 3min for initial denaturation followed by 33 cycles of 94^{o}C for 1 min (denaturation), 51.5^{o}C /53^{o}C /54.4^{o}C /56^{o}C for 1 min (annealing) and 72^{o}C for 1min (extension), a final extension at 72^{o}C was performed for 10 min in an Eppendorf Thermal Cycler.

3.2.5 Cloning of PCR products

All the PCR amplified products were electrophoresed, gel eluted (Gel cleanup kit, Eppendorf, Germany) and ligated into cloning vector pTZ57R/T (Insta clone T/A cloning kit, Fermentas, Germany). The ligation reactions were transformed into *Escherichia coli* (DH5α) host cells and the bacterial colonies carrying the inserts were identified by blue/white selection and subsequently confirmed with colony PCR.

3.2.6 DNA sequencing and Sequence analysis

For all the clones, both DNA strands were completely sequenced on an automated DNA sequencer commercially. The sequence similarity search was performed using BLASTN and BLASTP at NCBI website (www.ncbi.nlm.nih.gov). Nucleotide translations were

performed using (DNA/RNA to protein) Translate tool at ExPASy. (www.expasy.ch). Sequence alignments were done using CLUSTALW multiple sequence alignment tool at European Bioinformatics Institute (www.ebi.ac.uk). Phylogenetic analysis was performed using CLC Free Workbench (http://www.clcbio.com)**.** Reverse complementation and other sequence formatting were done using BCM search launcher (www.searchlauncher.bcm.tmc.edu). The promoter sequences were analyzed using the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/).

3.2.7 Isolation of full length cDNA of *AhMPK3*

To obtain the full length sequence of *AhMPK3*, 5' and 3' Rapid amplification of cDNA ends (RACE) reactions were performed using 5'/3' RACE kit (Roche applied sciences, Germany) following the manufacturer's instructions with minor modifications. Based on the available partial cDNA sequence, primers were designed for 5' and 3' RACE. The list of gene specific primers and their sequences were given in Table 3.1. For 5' RACE, the first strand cDNA was synthesized in a 20 µl reaction containing cDNA synthesis buffer, dNTP mix, 6 µg of total RNA, 12.5 µM gene specific primer, and Transcriptor reverse transcriptase at 55^{o}C for 60 minutes, followed by 85^{o}C for 5 minutes to heat inactivate the enzyme. Instead of using a gene specific primer, a degenerate primer WyrR (Table 3.1) designed against conserved (WYRAPE) amino acids of subdomain VIII of MAPKs was used in reverse transcription. To add a homopolymeric tail, the purified cDNA was incubated with dATP in the presence of terminal transferase at 37^{o}C for 30 minutes, followed by heat inactivation of enzyme at 70^{o}C for 10 min. The dA-tailed cDNA was used as a template to amplify the 5' region using oligo dT-anchor primer (Table 3.1) and gene specific primer Ah443R in a 50µl reaction with 1.5mM $MgCl_2$, 200uM dNTP (Invitrogen), 1X PCR buffer, and 2.5 units of *Taq* DNA polymerase (Invitrogen). The cycling conditions were 94^{o}C for 3 min, followed by 34 cycles of 94^{o}C for 45 sec, 55^{o}C for 45 sec, 72^{o}C for 1 min and a final extension of 10 min at 72^{o}C. The PCR product obtained was diluted and used as template in nested PCR using Ah270R and PCR anchor primer (Table 3.1) following similar cycling conditions. For 3' RACE, the cDNA synthesis reaction conditions were the same as in 5' RACE, except that an OligodT-Anchor primer was used, instead of Gene specific or degenerate primer. One twentieth of

the cDNA was used in a PCR reaction of 50 µl, amplified with PCR anchor primer and gene specific primer AH3P1F. PCR conditions were 94^0C for 3 min followed by 35 cycles of 94^oC for 30n sec, 54^oC for 30 sec and 72^oC for 1 min 30 sec with a final extension of 10 min at 72^oC. The PCR product was diluted and used in the nested PCR using nested primer Ah3P2F in combination with PCR anchor primer. The full length cDNA of AhMPK3 was deduced by aligning 5' and 3' RACE product sequences with partial cDNA. The full length cDNA, including the 5' and 3' UTR was amplified using gene specific primers $AhMK_3$1F and $AhMK_3$1497R.The PCR reaction was performed in a 50µl volume containing 2.0mM $MgCl_2$, 200µM dNTP (Invitrogen), 1X PCR buffer, and 2.5 units of *Taq* DNA polymerase (Invitrogen). The cycling conditions were 94^oC for 3 min, followed by 34 cycles of 94^oC for 45 sec, 55^oC for45 sec, and 72^oC for 2 min.

3.2.8 Isolation of the *AhMPK3* genomic clone

Two gene specific primers $AhMK_3$1F and $AhMK_3$1497R were used to PCR amplify the *AhMPK3* gene using *Arachis hypogaea* genomic DNA as template. PCR was carried out using 100ng of genomic DNA in a 50 µl reaction volume containing 2.0 mM $MgCl_2$, 200 µM dNTP (Invitrogen), 1X PCR buffer, and 2.5 units of *Taq* DNA polymerase (Invitrogen). The PCR conditions were 94^oC for 3 min, followed by 34 cycles of 94^oC for 45 sec, 55^oC for45 sec, 72^oC for 3 min. and a final extension of 15 min at 72^oC

3.2.9 Genomic Southern blot analysis

Peanut genomic DNA (20 µg) was digested with *Bcl*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I (Fermentas, Germany) respectively, fractionated on 0.8% agarose gel and visualized by ethidium bromide staining. The DNA from the gel was transferred onto a Hybond N+ membrane (Amersham Pharmacia, UK) through capillary transfer and the blot was UV-cross-linked. A 281 bp fragment of the 3′ UTR was amplified using primers $AhMK_3$1216F and $AhMK_3$1497R and labeled with [$^{\alpha\text{-}32}$P] dATP using Primer-a-Gene® Labeling System (Promega, USA) according to the manufacturer's Instruction. The membrane was pre-hybridized for 3-4 h at 65 °C and hybridized for 16–18 h at 65 °C using [$^{\alpha\text{-}32}$P] labeled probe. Following hybridization, the membrane was washed with 2 X SSC, 0.1% SDS and 1 X SSC, 0.1% SDS for 10 min and 0.1X SSC and 0.1% SDS for 5

min each respectively at 65 °C and then exposed to an X-ray film (Kodak, Japan) using two intensifying screens at −80 °C.

3.2.10 Semi-quantitative RT-PCR.

Reverse transcription was performed as described earlier in Material and Methods except that instead of 4 μg, only 2 μg of RNA was used. The amount of cDNA and number of cycles for linear increase of PCR products was determined (data not shown). Conditions, which consistently gave product in linear range, were used for all experiments. The expression of *AhMPK3* in peanut was studied using specific primers for amplifying the entire coding region, ORF-F and ORF-R (Table 3.1). Gene specific primers were employed for expression analysis of the defense related genes in transgenic and wild type (WT) tobacco plants (Table 3.3). The house-keeping gene actin amplified using primers Act-F and Act-R served as the internal control. The amplified products were analyzed on 1.2% agarose gel and visualized by staining with ethidium bromide.

3.2.11 Localization of AhMPK3

AhMPK3 cDNA was amplified from reverse-transcribed RNA using Primers ORF-F2 and ORF-R (Table 3.1) engineered with *Sma*I and *Bam*HI restriction sites respectively. The resulting fragment was cloned into pEGAD vector (Cutler et al. 2000) digested with appropriate restriction enzymes to make an in-frame fusion with GFP to obtain pEGAD: AhMPK3. The pEGAD control vector and pEGAD: AhMPK3 constructs were mobilized into *A. tumefaciens* strain EHA105 by freeze thaw method (Holsters et al. 1978). The resulting strains were utilized in transient transformation of tobacco leaves by agroinfiltration as described by Yang et al. (2000). In brief, agrobacterial strains harboring the corresponding clones were grown overnight at 28^{o}C in the presence of appropriate antibiotics, pelleted at 3000g for 5 min and diluted to an OD_{600} of 1.0 in 10 mM MES pH 5.6, 10 mM MgCl2, 150 μM acetosyringone and infiltrated into the leaves using a needle less syringe. After 48 h, GFP was visualized with a laser scanning confocal microscope (Leica). 10mM H_2O_2 was infiltrated into the leaves 60min before observation to study the dynamic localization of AhMPK3 in response to oxidative stress. Water was used for mock infiltration.

3.2.12 Development of Transgenic tobacco plants

The complete open reading frame of *AhMPK3* was amplified using primers ORF-F and ORF-R (Table 3.1) cloned into $_{P}$TZ57R vecor, and confirmed by sequenceing. *Nco*I and *Bam*HI restriction sites were incorporated in the primers at 5' and 3' ends to facilitate cloning into plant expression vector pRT100 by digesting it with the same set of enzymes such that the coding region will be flanked by 35S promoter and Poly A signal in the sense orientation. The entire cassettes with *AhMPK3* coding region flanked by 35S promoter and Poly A signal was released from pRT100 by digesting with *HindIII* and cloned into binary vector pRD400 digested with *HindIII.* The recombinant binary vector was mobilized into *Agrobacterium tumefaciens* EHA105 using freeze thaw method. Transgenic tobacco plants were generated by standard leaf disc transformation method (Horsch et al. 1985). Leaf discs (0.5 cm in diameter) were cut out from leaves of tobacco plants grown aseptically and the leaf discs were agroinfected by soaking them in agrobacterium suspension for 4-5 min. *Agrobacterium*-infected leaf discs were cultivated on MS medium with 2 mgl^{-1} BAP and 0.1 mgl^{-1} NAA at 28°C for 2 d, and then transferred to the MS medium containing 2 mgl^{-1} BAP and 0.1 mgl^{-1} NAA supplemented with 150 mgl^{-1} kanamycin and 250 mgl^{-1} cefataxime. Control uninfected leaf discs were kept directly on selection medium with kanamycin. The explants were subcultured to fresh selection medium every 15 days. Shoots obtained were rooted on MS medium containing100 mgl^{-1} kanamycin and 250 mgl^{-1} cefataxime. The rooted shoots were transferred to soil, acclimatized at 28°C and shifted to greenhouse.

3.2.13 Molecular analysis of transgenics

DNA was extracted from 5-6 week old T_0 transgenics, and around 100ng of DNA was used for PCR amplifications. Putative transgenics were confirmed by amplifying the genomic DNA with 35SF (Table 3.1) as the sense primer designed against the CaMV35S promoter region and AhMPK3 ORF-R as the antisense primer. Southern Analysis for transgenic plants was performed as described earlier in Materials and Methods, except that the genomic DNA was digested with *Eco*RI and hybridization was done using [$^{\alpha-32}$P] dATP labeled *nptII* fragment obtained by the amplification of neomycin

phosphotransferase gene with nptII F and nptII R primers. For Northern analysis of transgenic plants, 20 µg of total RNA was fractionated on a 1.2% agarose-formaldehyde gel. Equal loading and RNA integrity was checked by ethidium bromide staining, and fractionated RNA was transferred by capillary action overnight to a Hybond-N+ nylon membrane (Amersham Pharmacia, UK) using 20X SSC. The RNA on the membrane was fixed by UV cross-linking. Probe labeling, hybridization, and detection were the same as in the procedure described for southern blot hybridization. [$^{\alpha-32}$P] dATP labeled *AhMPK3* ORF was used as probe in Northern hybridization.

3.2.14 Herbivore bioassay

Bioassay was performed according to detached leaf method described by Sharma et al. (2005) with minor modifications. In brief, leaves of two month old WT and transgenic plants were cut at their petiole with a sharp blade and immediately planted into 3% agar-agar in a petri dish. Bioassays were conducted with first, second and third instar larvae of the generalist herbivore, *Spodoptera litura* with five larvae per leaf and five replications for each sample. The bioassay plates were maintained in a culture room at 28°C ± 1 and a photoperiod of 16:8 (Light/ Dark). The experiments were terminated when > 80% of the leaf area was consumed in WT plants, generally 5days for first instar, 3 days for second instar, and 2 days for third instar larvae respectively. The area of leaf damage and mass of larvae were recorded after each experiment and mean of five replications was plotted. The data were analyzed by ANOVA and student's *t*-test.

3.3 Results

3.3.1 Isolation of full length cDNA of *AhMPK3*

In an attempt to clone mitogen-activated protein kinases from *Arachis hypogaea*, degenerate primers were designed from two conserved regions of MAP kinases. The forward primer IntF corresponded to the ATP binding motif (GAYG I/VVC) in subdomain I of protein kinases (Hanks et al. 1988) and the reverse primer IntR corresponded to the region including TEY motif (MTEYVVT) present between subdomain VII and VIII. Using RT-PCR, a single fragment of 465 bp was obtained, which might be the product of amplification of several different MAP kinases, because the primers were designed against highly conserved regions. The PCR product was cloned and several clones were sequenced and identical clones were grouped together. Among them two clones were identified as diverse, but closely related and considered as different clones (Fig 3.1). Sequence similarity search using BLANTN and BLASTP showed a high similarity to the existing MAPKs from several plant species. One of the two clones i.e MPK2 (Fig.3.1) was further extended using 5' and 3' RACE to obtain the full length cDNA.

By utilizing the sequence information of the partial cDNA fragment, gene specific primers were designed. The same RNA used for amplification of the partial clones was used as a template for reverse transcription using oligo dT- Anchor primer (for 3′ RACE) and degenerate gene specific primer WyrR (for 5′ RACE) followed by the amplification of corresponding 5′ and 3′ cDNA ends with designed nested gene specific primers. By using a degenerate primer for reverse transcription, several members of the gene family would get reverse transcribed. Hence, the same cDNA could be used as template for isolating 5′ regions of different genes of the same family using nested gene specific primers in combination with PCR anchor primer. In 5′ RACE, a 750 bp product was obtained with the gene specific primer Ah443R in combination with PCR anchor primer. The product was further confirmed by amplification with the nested primer Ah270R. In 3′ RACE, a 1100 bp product was obtained with the primer Ah3P1F, which was confirmed with the nested primer Ah3P2F. All the PCR products obtained were cloned and

sequenced. The sequences were aligned to obtain the overlapping regions and the full length cDNA of *AhMPK3* was deduced. Based on the sequences of RACE products, two gene specific primers $AhMK_31F$ and $AhMK_31497R$ were designed and the full length cDNA was amplified and sequenced, which was identical to the deduced cDNA. *AhMPK3* cDNA was submitted to NCBI Genbank database under the accession number DQ068453.

MPK1

IntF

5′**GGCGCTTATGGCATCGTTTG**TTCCGCTCTAAATTCGGAGACGGATGAGCATGTTGCCATTAAGAAGATT
GCCAATGCATTTGACAACAAGATTGACGCCAAGAGGACCCTCCGTGAAATCAAGCTGCTTCGTCACATGGA
TCATGAAAACGTGGTTGCAATAAGGGATATAGTGCCACCACCTCAGAGGGAGGCATTCAATGATGTTTACA
TTGCCTATGAGTTGATGGACACTGATCTTCACCAAATCATTCGCTCGAATCAAGCATTGTCAGAGGAGCAC
TGTCAGTACTTTCTGTATCAAATCCTCCGTGGTTTGAAGTACATACATTCAGCAAATGTTTTGCATAGGGA
CTTGAAACCTAGCAACCTTCTCCTGAATGCCAACTGCGACTTAAAAATTTGTGATTTTGGACTGGCCCGTG
TCACCTCTGAGACTAATTTCAT**GACTGAATATGTTGTTACA** 3′

IntR

MPK2

IntF

5′**GGAGCTTACGGAATCGTTTG**CTCGGTGTTGAATACTGAGACTAATGAGCTGGTTGCTGTTAAGAAGATA
GCTAACGCGTTCGATAATCACATGGATGCGAAGCGCACACTCCGTGAGTTTAAGCTCCTGAGGCATCTGGA
TCATGAAAACGTGATTGGCTTAAGAGATGTTATTCCTCCACCCCTTCGTAGAGAGTTTAATGATGTCTATA
TTGCAACGGAGCTCATGGATACTGATCTTCATCACATTATTCGCTCCAATCAGGGCCTGTCGGAGGAACAC
TGCCAGTACTTCTTGTATCAGATTCTTCGTGGGCTGAAGTACATACATTCTGCAAACATAATTCATAGAGA
TTTGAAACCAAGCAATCTGTTGCTGAATGCAAATTGTGACTTGAAGATTATTGATTTTGGTCTTGCGCGGC
CAACTTTAGAAAATGATTTCAT**GACAGAGTATGTTGTCACA** 3′

IntR

Figure 3.1 Sequences of the two partial clones of MAPK (MPK1 and MPK2) isolated from *A. hypogaea* using degenerate primers. IntF and IntR are degenerate primers used and the letters in bold indicates the primer annealing region.

```
1     cac taa cct tcc ttc gtc cct ctc aac ggt ttc tag aga gag aga   45

46    gag aga aac taa aag tct ccg ttt aga gag aga aag tag aaa aca   90

91    ccg ATG GCC GGC GTT AAT CCA AAC GGT GCC GCG GAT TTT CCG GCG   135
          M   A   G   V   N   P   N   G   A   A   D   F   P   A    14

136   GTT CCG ACT CAC GGT GGA CAG TTC ATT CAG TAC AAC ATC TTC GGT   180
15     V   P   T   H   G   G   Q   F   I   Q   Y   N   I   F   G    29

181   AAC CTC TTT GAG GTC ACC GCT AAG TAC CGT CCT CCG ATC ATG CCT   225
30     N   L   F   E   V   T   A   K   Y   R   P   P   I   M   P    44

226   ATC GGT CGT GGA GCT TAC GGA ATC GTT TGC TCG GTG TTG AAT ACT   270
45     I   G   R   G   A   Y   G   I   V   C   S   V   L   N   T    59

271   GAG ACT AAT GAG CTG GTT GCT GTT AAG AAG ATA GCT AAC GCG TTC   315
60     E   T   N   E   L   V   A   V   K   K   I   A   N   A   F    74

316   GAT AAT CAC ATG GAT GCG AAG CGC ACA CTC CGT GAG TTT AAG CTC   360
75     D   N   H   M   D   A   K   R   T   L   R   E   F   K   L    89

361   CTG AGG CAT CTG GAT CAT GAA AAC GTG ATT GGC TTA AGA GAT GTT   405
90     L   R   H   L   D   H   E   N   V   I   G   L   R   D   V   104

406   ATT CCT CCA CCC CTT CGT AGA GAG TTT AAT GAT GTC TAT ATT GCA   450
105    I   P   P   P   L   R   R   E   F   N   D   V   Y   I   A   119

451   ACG GAG CTC ATG GAT ACT GAT CTT CAT CAC ATT ATT CGC TCC AAT   495
120    T   E   L   M   D   T   D   L   H   H   I   I   R   S   N   134

496   CAG GGC CTG TCG GAG GAA CAC TGC CAG TAC TTC TTG TAT CAG ATT   540
135    Q   G   L   S   E   E   H   C   Q   Y   F   L   Y   Q   I   149

541   CTT CGT GGG CTG AAG TAC ATA CAT TCT GCA AAC ATA ATT CAT AGA   585
150    L   R   G   L   K   Y   I   H   S   A   N   I   I   H   R   164

586   GAT TTG AAA CCA AGC AAT CTG TTG CTG AAT GCA AAT TGT GAC TTG   630
165    D   L   K   P   S   N   L   L   L   N   A   N   C   D   L   179

631   AAG ATT ATT GAT TTT GGT CTT GCG CGG CCA ACT TTA GAA AAT GAT   675
180    K   I   I   D   F   G   L   A   R   P   T   L   E   N   D   194

676   TTC ATG ACA GAG TAT GTT GTC ACA AGG TGG TAC AGG GCT CCT GAA   720
195    F   M   T   E   Y   V   V   T   R   W   Y   R   A   P   E   209

721   CTG CTG TTG AAC TCG TCT GAT TAC ACT TCT GCA ATT GAT GTT TGG   765
210    L   L   L   N   S   S   D   Y   T   S   A   I   D   V   W   224

766   TCT GTT GGT TGC ATC TTT ATG GAA CTC ATG AAT AAA AAG CCT CTC   810
225    S   V   G   C   I   F   M   E   L   M   N   K   K   P   L   239

811   CTC CCA GGG AAG GAT CAC GTG CAT CAG ATG CGC CTA TTG ACA GAG   855
240    L   P   G   K   D   H   V   H   Q   M   R   L   L   T   E   254

856   CTT CTT GGC ACT CCA ACT GAG GCA GAC CTT GGG TTA GTG AAA AGT   900
255    L   L   G   T   P   T   E   A   D   L   G   L   V   K   S   269

901   GAG GAT GCG AGA AGA TAC ATC CGA CAA CTT CCT CAA TAT GCT CGC   945
270    E   D   A   R   R   Y   I   R   Q   L   P   Q   Y   A   R   284

946   CAA CCT TTA GCT AGG ATC TTC CCC CAT GTT CAT CCC TTG GCC ATT   990
285    Q   P   L   A   R   I   F   P   H   V   H   P   L   A   I   299
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991   GAT CTT GTT GAT AAA ATG TTG ACA ATT GAT CCA ACT AAA AGA ATT   1035
300    D   L   V   D   K   M   L   T   I   D   P   T   K   R   I    314

1036  ACA GAT GAA GAA GCA CTG GCC CAT CCA TAT CTT GAA AAG CTG CAT   1080
315    T   D   E   E   A   L   A   H   P   Y   L   E   K   L   H    329

1081  GAT ATA GCC GAC GAA CCT GTC TGC ATG GAA CCA TTC TCA TTT GAC   1125
330    D   I   A   D   E   P   V   C   M   E   P   F   S   F   D    344

1126  TTT GAG CAA CAG CAG TTG GAT GAA GAA CAA ATA AAA GAG ATG ATC   1170
345    F   E   Q   Q   Q   L   D   E   E   Q   I   K   E   M   I    359

1171  TAC AGA GAG GCA TTG GCA CTC AAT CCT GAG TAT GCT TAA agt aaa   1215
360    Y   R   E   A   L   A   L   N   P   E   Y   A   *

1216  atg aca tga gca att tga agt tta gaa ggt aat aat aaa tta tta   1260

1261  aaa aaa aat aat gtg taa tca att ctt tta ttg caa agt tca aca   1305

1306  cag tgg gaa gtt gtt aca ggc agg aaa gaa cca agt tag aac gag   1350

1351  gat tga atc cta ttg ctg atc aaa cct tcc cgg gta aat cct gtt   1395

1396  caa tat gtg aga tta gtt agg ttc aat tta atg ctt tta aaa caa   1440

1441  atg agt gcc tta gtg ttg taa agc acc ata taa tga tta tta ctc   1485

1486  ccc aga att caa taa aaa aaa aaa aaa   1512
```

Figure 3.2 Nucleotide and deduced amino acid sequences of *AhMPK3*. The nucleotides and amino acids are numbered. Asterisk (*) indicate the stop codon. The conserved TEY motif is underlined. The UTR regions are represented by lower case letters.

3.3.2 Nucleotide and protein sequence analysis.

The full length *AhMPK3* cDNA is 1514 bp long including the ORF, 5′, 3′ untranslated regions and the poly-A tail (Fig 3.2). Sequence analysis revealed an open reading frame of 1113 bp potentially encoding a 371 amino acid polypeptide. The reading frame shown was the only possible reading frame in the cDNA and had both the translational initiation codon ATG at nucleotide 94 and translational stop codon TAA at nucleotide 1207. A 93 bp of 5′ untranslated region and a 292 bp 3′ untranslated region followed by poly-A tail were present flanking the open reading frame. A potential polyadenylation signal (AATAAA) was found in the 3' UTR at1249bp. Threonine and Tyrosine amino acids of TEY motif were present at 197 and 199 positions respectively. The 371 amino acids encoded protein had a predicted molecular mass of 42615.98 Da and a calculated isoelectric point (pI) of 5.52 (Compute pI/MW tool, ExPASy).

3.3.3 Multiple sequence alignment and phylogenetic analysis

Sequence alignment of the predicted amino acid residues of AhMPK3 with closely related MAP kinases indicated that it contained all the eleven conserved subdomains of protein kinases described previously (Hanks et al. 1988) and possessed a dual phosphorylation activation motif (TEY) located between subdomains VII and VIII (Fig. 3.3). The phylogenetic analysis showed that AhMPK3 belongs to the A1 subgroup of MAPK family (Fig. 3.4) (MAPK group 2002). The AhMPK3 protein exhibited 94% sequence identity to GmMPK1 from *Glycine max*, 91% to MsMMK4 from *Medicago sativa*, 85% to CsTIPK and PtMPK3-1 of *Cucumis sativus* and *Populus trichocarpa* respectively. Most well characterized A1 subgroup members of MAPK family, AtMPK3 (*Arabidopsis thaliana*), NtWIPK (*Nicotiana tabacum*) and LeMPK3 (*Lycopersicon esculentum*) shared 81% similarity with AhMPK3 protein.

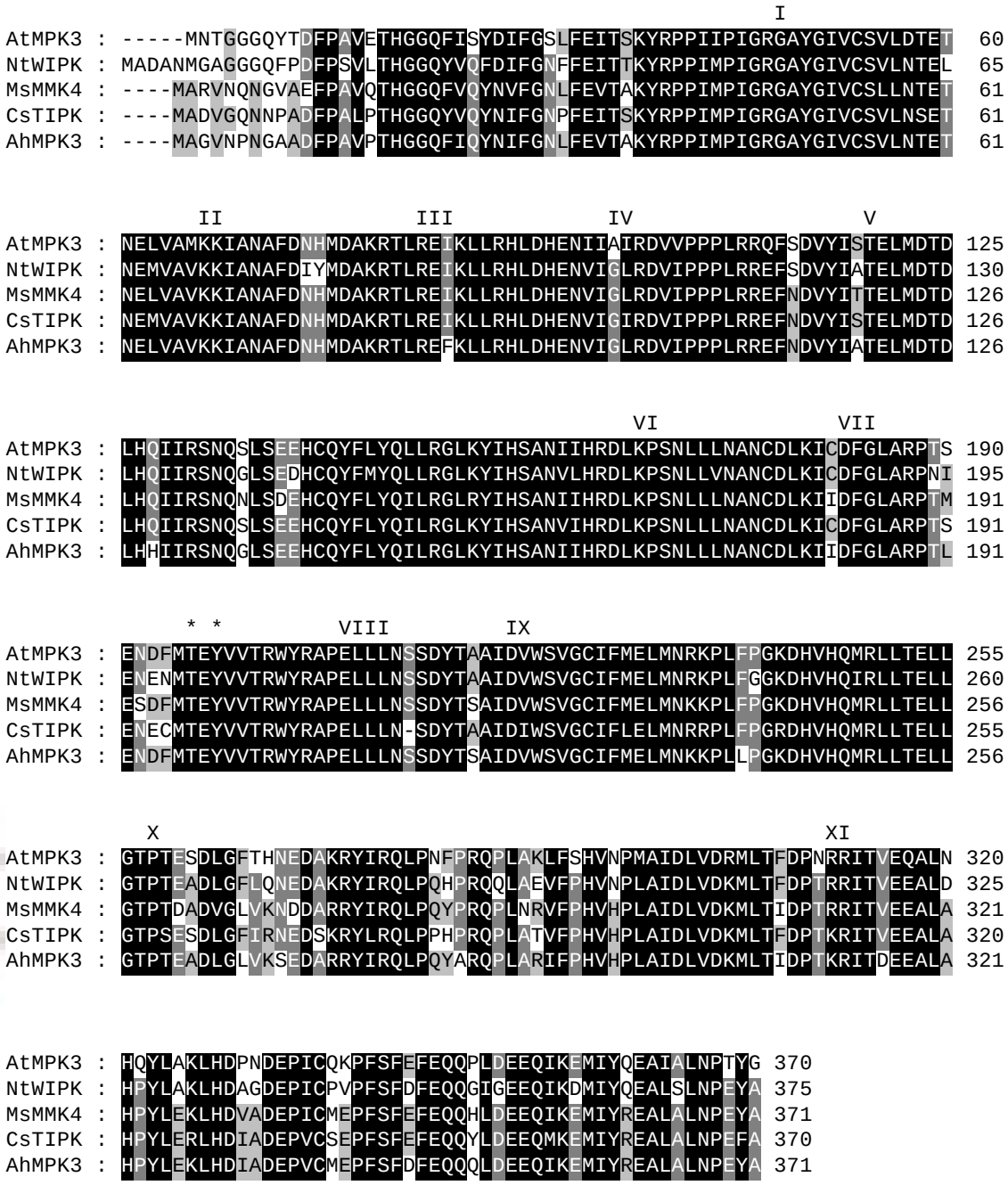

Figure 3.3 Alignment of deduced amino acid sequences of AhMPK3 with closely related MAPKs from other plant species. The eleven subdomains of protein kinases are marked with roman numerals. Threonine (T) and Tyrosine(Y) residues whose phosphorylation is required for MAPK activation are indicated by Asterisk. At: *Arabidopsis thaliana*, Nt: *Nicotiana tabacum*, Ms: *Medicago sativa*, Cs: *Cucumis sativus*.

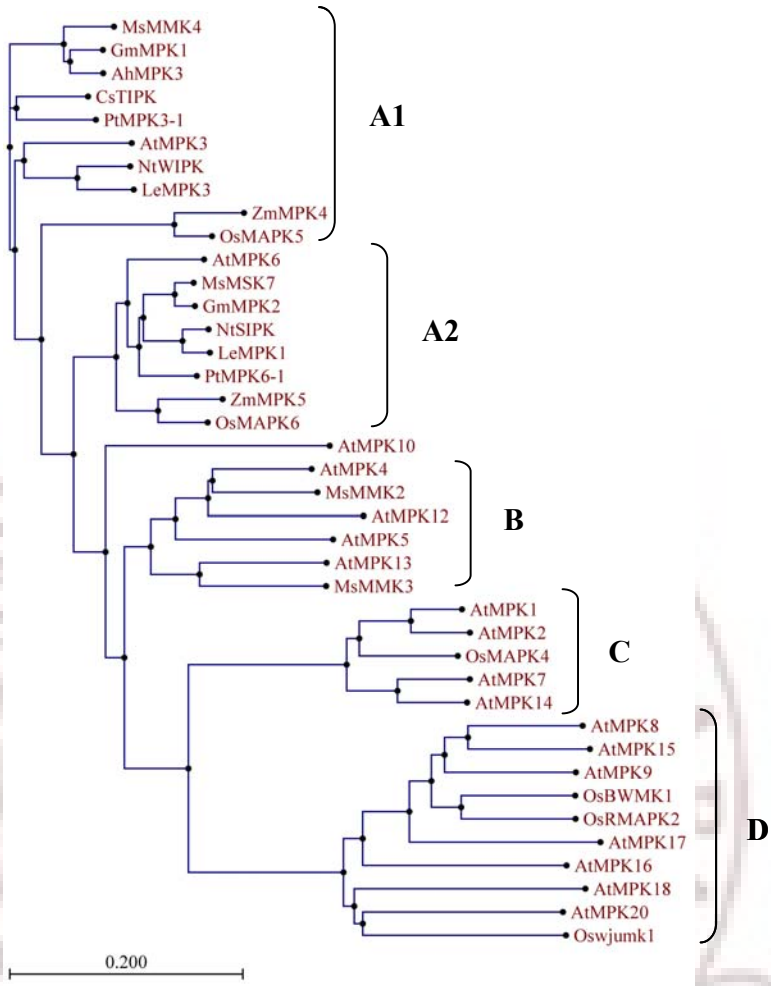

Figure 3.4 The phylogenetic relationship of AhMPK3 with other MAPK family members from different plant species. A phylogenetic tree based on the genetic distance of the protein sequences was constructed using ClustalW program and CLC-free workbench 3.1. The MAPK members used for construction of the tree are listed in the GenBank database under the following accession numbers: *AhMPK3*(DQ068453); *AtMPK1*(NM_100895); *AtMPK2*(NM_202320); *AtMPK3*(NM_114433); *AtMPK4*(NM_116367); *AtMPK5*(AK176361); *AtMPK6*(NM_129941); *AtMPK7*(NM_127374); *AtMPK8*(NM_179354); *AtMPK9*(NM_112686); *AtMPK10*(NM_115841); *AtMPK12*(NM_130170); *AtMPK13*(NM_001035913); *AtMPK14*(NM_119808); *AtMPK15*(NM_106026); *AtMPK16*(NM_121906); *AtMPK17*(NM_126206); *AtMPK18*(NM_104229); *AtMPK20*(NM_129849); *CsTIPK*(DQ118734); *GmMPK1*(AF104247); *GmMPK2*(AF329506);

LeMPK1(AY261512); *LeMPK3*(AY261514); *MsMMK2*(X82268); *MsMMK3*(AJ224336); *MsMMK4*(X82270); *MsMSK7*(X66469); *NtWIPK*(D61377); *NtSIPK*(U94192); *OsBWMK1*(AF177392); *OsMAPK5*(AF479883); *OsMAPK6*(AJ535841); *Oswjumk1*(AJ512643); *OsRMAPK2*(AF194416); *OsMAPK4*(AJ251330); *PtMPK3-1*(estExt_fgenesh4_pm.C_LG_IX0462); *PtMPK6-1*(estExt_fgenesh4_pm.C_LG_VII0025); *ZmMPK4*(AB016801); *ZmMPK5*(AB016802); Ah: *Arachis hypogaea*, At: *Arabidopsis thaliana*, Cs: *Cucumis sativus*, Gm: *Glycine max,* Le: *Lycopersicon esculentum,* Ms: *Medicago sativa*, Nt: *Nicotiana tabacum*, Os: *Oryza sativa,* Pt: *Populus trichocarpa,* Zm: *Zea mays.*

3.3.4 Genomic and structural organization of *AhMPK3* gene

The copy number of *AhMPK3* gene was analyzed by Southern blot analysis. *Arachis hypogaea* genomic DNA was digested with restriction enzymes *Bcl*I, *Eco*RI, *Eco*RV, *Hind*III , *Xba*I and subjected to hybridization using a 281 bp 3' UTR region of *AhMPK3* as a probe. This 281 bp region did not harbor restriction sites of any of the above enzymes used. Two distinct bands were detected in samples digested with *Eco*RI, *Eco*RV and *Hind*III (Fig. 3.5). This can be explained by the fact that peanut (*Arachis hypogaea*) is an amphidiploid, which carries two sets of diploid chromosomes. Hence, one band corresponds to the *AhMPK3* gene and the second band in the southern blot presumably belonged to its ortholog in the second genome of peanut. The sample digested with *Bcl*I showed three distinct bands, which could be explained by the possibility of *Bcl*I site in the *AhMPK3* othrolog in the second genome of peanut. The single band detected in *Xba*I digested sample could be due to the near equal size of two bands which co-migrated giving the appearance of a single band. The simple hybridization pattern suggested that gene encoding *AhMPK3* might exist as a single copy in peanut genome (Fig. 3.5).

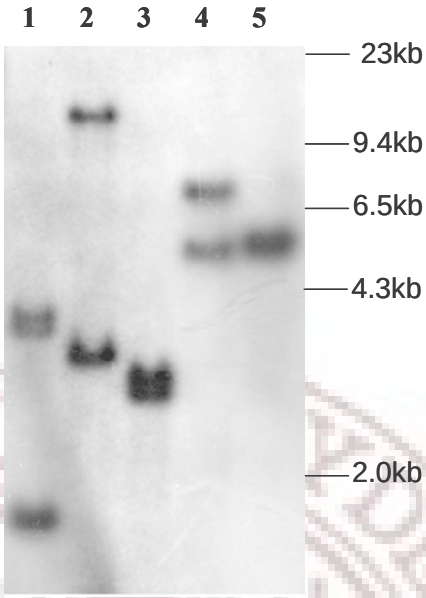

Figure 3.5 Genomic organization of *AhMPK3*. 1- *Bcl*I, 2- *Eco*RI, 3- *Eco*RV, 4-.*Hin*dIII, 5- *Xba*I. *Arachis hypogaea* genomic DNA was digested with the indicated restriction enzymes, fractionated on 0.8% agarose gel, blotted on to a nylon membrane and hybridized with *AhMPK3*- 3'UTR fragment as a probe.

The genomic clone of*AhMPK3* was amplified using gene specific primers designed against 5′ and 3′ termini of full length *AhMPK3* transcript using peanut genomic DNA as template. A 3036 bp fragment was obtained which was cloned and sequenced. The genomic structure of the *AhMPK3* gene was established by the alignment with the corresponding cDNA, which revealed that coding region of *AhMPK3* contained six exons and five introns (Fig. 3.6). The size of introns varied from 104bp (III intron) to 810bp (II intron). All the 5′ and 3′ splice junctions follow the typical, canonical consensus di-nucleotide sequence GT-AG (Table 3.2). All the introns are A + T-rich; in particular they present an elevated T content (Table 3.2), which is a peculiar feature of many plant introns (Ko et al. 1998).

AhMPK3 gene structure was compared with its orthologs from *Arabidopsis* (*AtMPK3*) and poplar (*PtMPK3-1 &3-2*) (Fig. 3.6), where *PtMPK3-2* is presumed paralog of *PtMPK3-1* (Nicole et al. 2006). Comparative analysis of exon-intron junctions in all the three species indicate that the numbers of exons, and their sizes as well as the intron phases were extremely well conserved. Whereas the intron lengths were varied

among the species, poplar and peanut introns were much longer than the corresponding *Arabidopsis* introns (Fig. 3.6). *AhMPK3* genomic clone can be accessed from NCBI GenBank under the accession number EU182580.

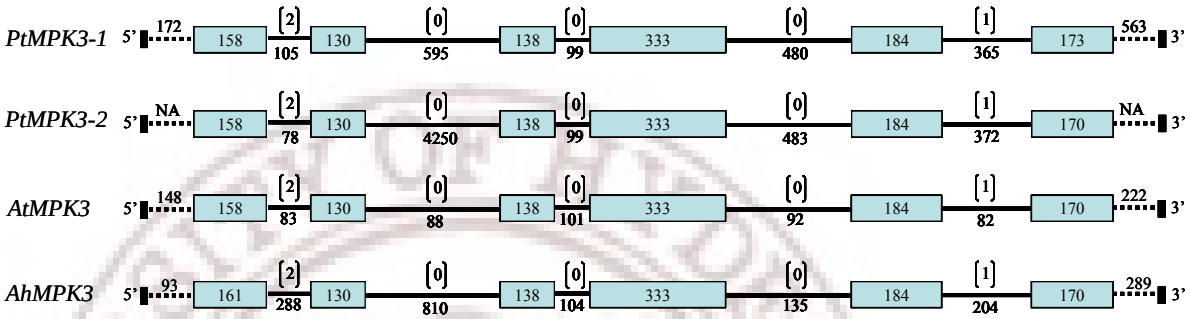

Figure 3.6 Graphical representation of *AhMPK3* gene structure and its comparison with its orthologs from poplar (*PtMPK3-1&3-2*) and *Arabidopsis* (*AtMPK3*). Exons are represented by closed boxes and introns by dark lines, the dotted lines represent the 5′ and 3′ UTRs respectively. The individual exons, introns and UTRs length were given in base pairs. Numbers between brackets correspond to the intron phase. Drawings are not exactly to scale. NA: Not available. *PtMPK3-1&3-2* genomic sequences were retrieved from DOE Joint Genome Institute database (http://genome.jgi.psf.org/Poptr1_1/Poptr1_1.home.html) and *AtMPK3* genomic sequence was obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org).

3.3.5 Expression analysis of *AhMPK3*

To determine the expression pattern of *AhMPK3* in response to various stress treatments, a semi-quantitative RT-PCR was carried out using RNA samples harvested from the materials frozen at various intervals (Fig. 3.7). The results showed that a basal level of *AhMPK3* is maintained in leaves, which got upregulated upon the incidence of stress. The difference observed in transcript levels at 0 h in various treatments could be due to the plant physiological differences or Circadian rhythms. In response to wounding, *AhMPK3* transcript expression reached to peak in 15 min, which gradually came down to the basal level by 6 h. Since both the pathogen and wound stress lead to H_2O_2 accumulation in plants, we studied the effect of H_2O_2 on the expression pattern of *AhMPK3*. With H_2O_2 application, *AhMPK3* got upregulated gradually up to 30min after treatment followed by a sudden decline at 60 min and gradual rebound by 12 h. In response to salicylic acid and methyl jasmonate, which are the signaling molecules for SAR and wound signaling respectively, the gene expression showed an upregulation during the later stages of the treatment. To examine the influence of nitric oxide (NO), which is an emerging essential component of plant defense, treatment with SNP which is a NO donor caused steady state increase in the *AhMPK3* transcript reaching a peak by 12 h. The analysis of *AhMPK3* transcript in response to mannitol, which causes osmotic stress, showed a gradual increase by 30 min, and declined to basal level by 24 h. ABA, which is the major signaling molecule for abiotic stress responses, induced *AhMPK3* transcript accumulation at 30min and a gradual decline before rebounding at 24 h after treatment. NaCl treatment had no significant impact on the expression pattern of *AhMPK3* (data not shown). Treatment with water served as control and it showed a slight increase in *AhMPK3* transcripts after 15 min. This suggested that the increase observed at 15 min in various chemical treatments was a combined effect of placing the leaves in an aqueous solution and its corresponding chemical.

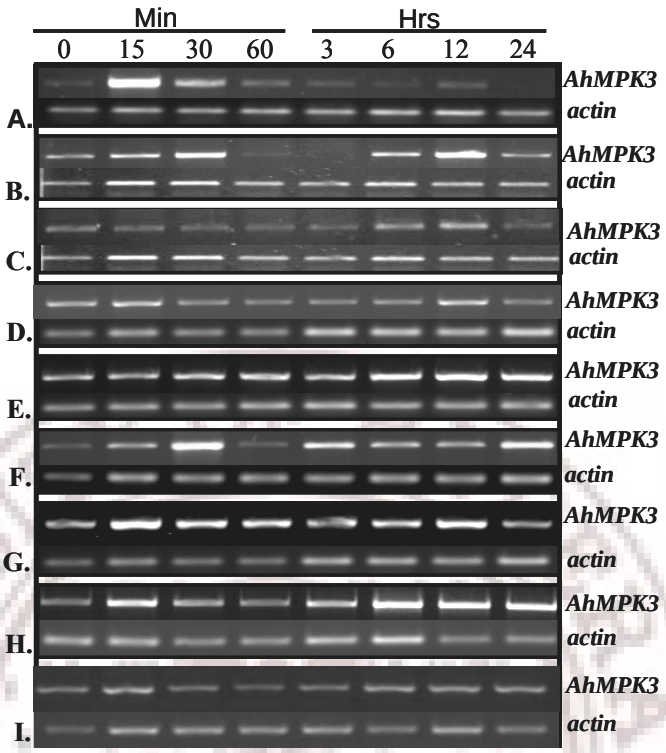

Figure 3.7 Expression analysis of *AhMPK3* in response to various treatments using semi-quantitative RT-PCR. A. wounding, B. hydrogen peroxide (H_2O_2), C. methyl jasmonate (MeJA), D. salicylic acid (SA), E. Sodium nitroprusside (SNP), F. abscisic acid (ABA), G. mannitol, H. cold and I. water (H_2O). The semi-quantitative RT-PCR reactions of *AhMPK3* are performed as described in materials and methods. cDNA synthesized from RNA samples collected at specific intervals of different treatments were amplified using gene specific primers *AhMPK3* (ORF-F and ORF-R). Actin, which served as an internal control, was amplified using Act F and Act R primers

3.3.6 Subcellular localization of AhMPK3

Studies in mammals and yeast have shown that stimulus-induced activation of MAPKs correlated with dynamic changes in their localization, whereby the proteins often translocated to, and accumulated in, the nucleus of the cell. This is often required due to the nuclear localization of key MAPK substrates, including transcription factors involved in the control of gene expression (Brunet et al. 1999). Localization of AhMPK3 was analyzed by constructing an N-terminal GFP fusion and transiently expressing in tobacco

leaves using agroinfiltration. As previous studies showed that AtMPK6/NtSIPK and AtMPK3/NtWIPK were activated by hydrogen peroxide and superoxide (Kovtun et al. 2000; Samuel et al. 2000; Moon et al. 2003), we studied the dynamic changes in the localization of AhMPK3 in response to H_2O_2. Under untreated conditions, AhMPK3 localized simultaneously in nucleus and cytoplasm. Upon treatment with H_2O_2 the staining intensity and frequency of nuclear staining further increased in a majority of cells observed, showing predominant nuclear localization (Fig. 3.8). Cells expressing GFP from control vector alone showed GFP in the entire cell and was unaffected by water or H_2O_2 treatments.

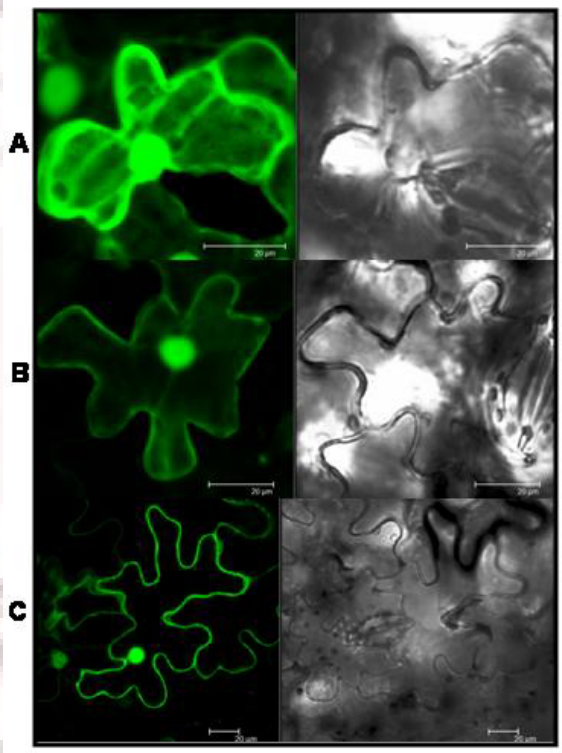

Figure 3.8 Subcellular localization of AhMPK3. Control vector pEGAD and pEGAD:AhMPK3 were transiently transformed to *Nicotiana tabacum* leaves through agroinfiltration. GFP was visualized in epidermal cells using Confocal laser scanning microscope 48 h post agroinfiltration. Water (H_2O) or 10mM hydrogen peroxide (H_2O_2) were infiltrated 1 h before GFP visualization. (Continued…)

A. pEGAD control vector shows expression of GFP throughout the cell with out any treatment or when treated with H_2O or H_2O_2.

B. pEGAD: AhMPK3 shows GFP localized to both cytoplasm and nucleus with out any treatment or upon treatment with H_2O

C. pEGAD: AhMPK3 shows predominant nuclear localization of GFP upon treatment with H_2O_2. (Bar 20μm)

3.3.7 Generation of transgenic plants and Herbivore resistance assay

Transgenic tobacco plants with *AhMPK3* under CaMV35S promoter were raised using *Agrobacterium* mediated leaf disc transformation (Horsch et al. 1985). T_0 transgenic plants were confirmed by genomic PCR (Fig 3.9A) and Southern hybridization (Fig 3.9B). Northern analysis confirmed the *AhMPK3* expression in T_0 transgenic plants (Figure 3.9C). Two single copy, high transgene expression lines (T-5, T-8) and a moderately expressing line (T-9) derived from the corresponding progeny of the primary transgenic plants by selfing were selected for further analysis in T_2 generation. T_2 seeds were germinated on half strength MS medium supplemented with 100mg/l kanamycin. The kanamycin tolerant plants were selected and transferred to soil in green house along with WT plants germinated on half strength MS medium.

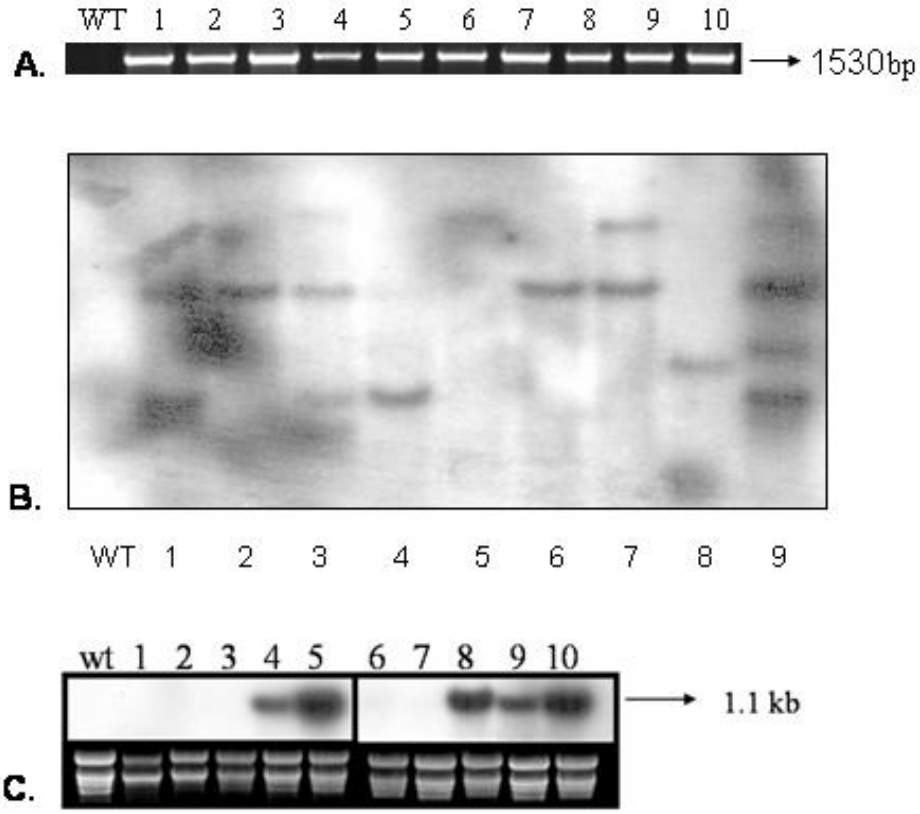

Figure 3.9 A. PCR confirmations of T_0 transgenic plants. Genomic DNA was isolated from wild type and T_0 transgenic plants and used as template. A fragment of 1530bp was amplified using promoter and gene specific primers (35SF and *AhMPK3* ORF-R). That indicated both the presence of the transgene and the correct promoter–transgene fusion/ orientation.

B. Southern analysis of T0 transgenic plants. Genomic DNA of wild type (WT) and T_0 transgenic plants (1-9) was digested with *Eco*RI and electrophoresed, blotted and hybridized with [$^{\alpha\text{-}32}$P] -labelled *npt*II gene as probe

C. Northern analysis of T_0 transgenic plants. Total RNA was prepared from wild type (wt) and T_0 transgenic plants (1-10). RNA samples (20μg) were separated by denaturing formaldehyde-agarose gel electrophoresis, blotted, and hybridized with [$^{\alpha\text{-}32}$P] -labelled *AhMPK3* probe. Ethidium bromide stained ribosomal RNA bands are shown as loading controls.

All the T_2 transgenic plants were first confirmed with genomic PCR (data not shown) followed by northern hybridization to analyze the expression of *AhMPK3* in T_2 transgenic plants (Fig.3.10). As we performed all the hybridization and washing steps at high stringency conditions, we did not observe any signal due to cross reactivity in wild type plants using AhMPK3 as probe. Herbivore resistance of transgenic plants against the common cut worm *Spodoptera litura* was examined by the level of leaf damage and gain of larval weight upon feeding on leaves of two month old WT and transgenic plants. All the transgenic plants showed a high level of resistance to the first instar larvae, a moderate resistance towards second instar and low resistance towards third instar larvae respectively (Fig. 3.11; Fig. 3.12A). Analysis of larval weights after feeding showed that the final biomass of larvae fed on WT plants was significantly higher compared to the larvae fed on high expression transgenic lines (Fig.3.12B).

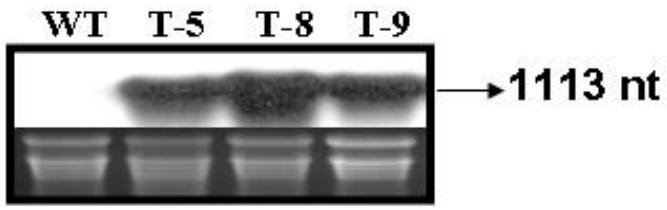

Figure 3.10 Northern analysis of T_2 transgenic plants for *AhMPK3* expression. Total RNA was prepared from WT and T_2 transgenic plants (T-5, T-8 and T-9). RNA samples (20 μg) were separated by denaturing formaldehyde-agarose gel electrophoresis, blotted, and hybridized with $^{\alpha\text{-}32}$P-labelled *AhMPK3* probe. Ethidium bromide stained ribosomal RNA bands are shown as loading controls.

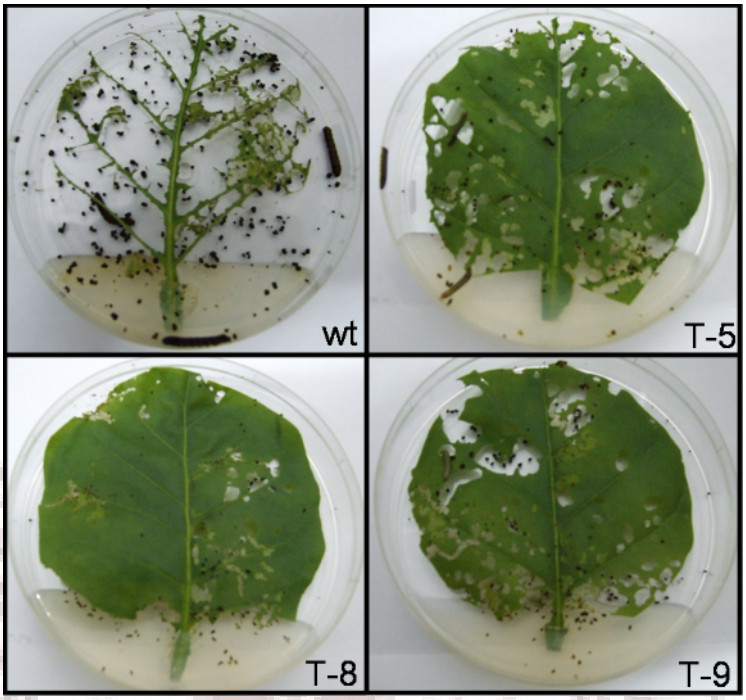

Figure 3.11 Representative pictures of leaf damage in wild type (WT) and transgenic plants (T-5, T-8 and T-9) after feeding by first instar larvae of *S. litura* for five days.

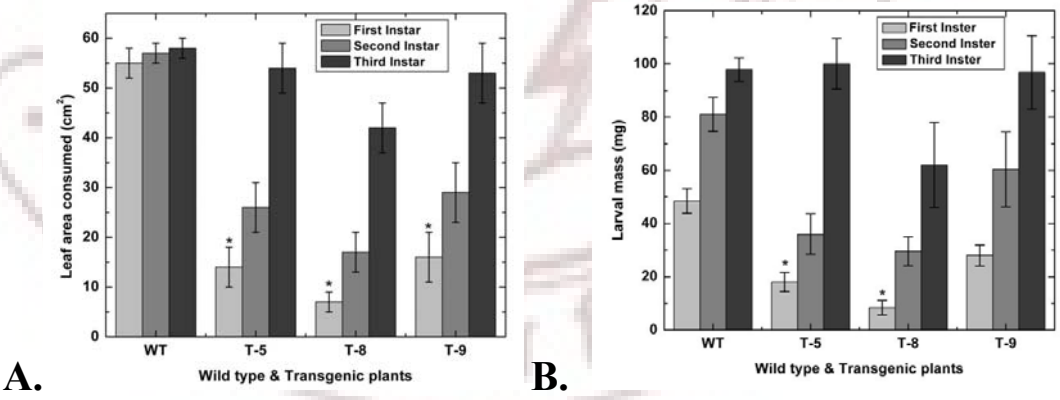

Figure 3.12 **A.** Leaf area consumed (cm^2) in WT and transgenic plants with first, second and third instars of *S. litura* larvae. Data are mean values ±SE and asterisks indicate significant difference between WT and transgenic plants. (* indicates $P<0.05$)

B. Mean mass (±SE) of individual *S. litura* larvae after feeding on Wild type (WT) and transgenic plants (T-5, T-8, T-9). Asterisks indicate significant difference between WT and transgenic plants. (* indicates $P<0.05$)

3.3.8 Transcript levels of defense response genes in transgenic plants.

Transgenic plants showing enhanced resistance to *Spodoptera litura* were analyzed for the levels of various defense related transcripts using semi-quantitative RT-PCR. Transgenic plants displayed constitutively higher levels of lipoxygenase1 (*LOX1*), pathogenesis related proteins *PR1a, PR1b, acidic ß-1,3-glucanase, acidic chitinase*, *protease inhibitor II* (*PI-II*) and *ornithine decarboxylase* (*ODC*) transcripts compared to WT plants (Fig. 3.13). Transcript levels of isochorismate synthase (*ICS*), lipoxygenase 3 (*LOX3*), and *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS3a*) which are the key enzymes in salicylic acid (SA), jasmonic acid (JA) and ethylene biosynthesis respectively, were unaffected. Apart from these, other wound or JA responsive gene transcripts like *protease inhibitor I* (*PI-I*), *allene oxide synthase* (*AOS*), *allene oxide cyclase* (*AOC*) were almost similar in both WT and transgenic plants (Fig. 3.13). Transcripts levels of basic PR5 (osmotin) and defensin, which are known to be regulated by ethylene and JA synergistically were also unaffected. *AhMPK3* transgenics also exhibited slightly higher transcript levels of tobacco native *MPK3* i.e *NtWIPK* (Fig. 3.13).

To study the effect of *AhMPK3* overexpression on wound induced defense responses, we analyzed the level of *PI-II* transcripts upon wounding in WT and transgenic plants. Upon wounding, transgenic plants accumulated *PI-II* rapidly to high level by one hour and maintained it through out the study (Fig. 3.14), whereas the WT plants exhibited a gradual increase of *PI-II* transcripts in a time dependent manner but not to the level exhibited by the transgenic plants. *PR1b*, which is a wound inducible pathogenesis related protein, also displayed a similar rapid induction in transgenic plants (Fig.3.14). *LOX3,* a key regulator of wound induced JA biosynthesis, was induced rapidly in both WT and transgenic plants reaching peak level by 1 h after wounding. In WT plants, *LOX3* transcripts reached the basal level in a time dependent manner, but the transgenic plants maintained higher *LOX3* transcript levels even 24 h after wounding. Neither the constitutive accumulation of defense related transcripts nor the rapid accumulation of *PI-II* transcripts upon wounding was observed in aged transgenic plants of five months or older (data not shown).

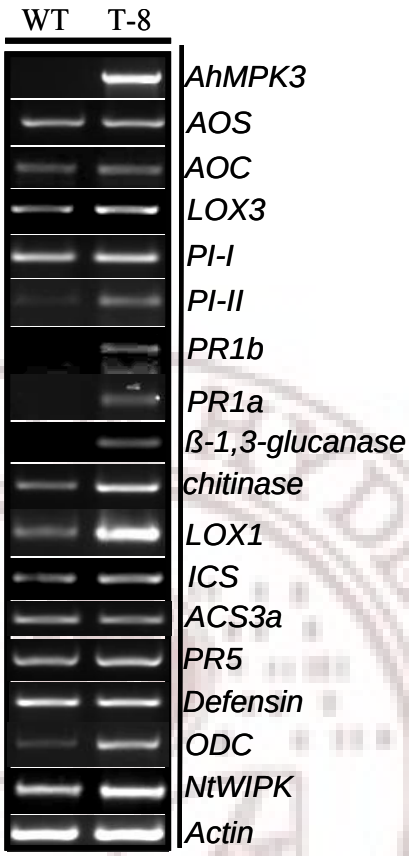

Figure 3.13 Transcript profile of defense responsive genes in WT and Transgenic plants (T-8). Data for line T-8 alone is provided here. The experiments were performed on all other lines with similar results. Semi-quantitative RT- PCR was performed using total RNA of WT and transgenic plants. AOS: allene oxide synthase, AOC: allene oxide cyclase, LOX: lipoxygenase, PI: Protease inhibitor, PR: pathogenesis related protein, ICS: isochorismate synthase, ODC: ornithine decarboxylase.

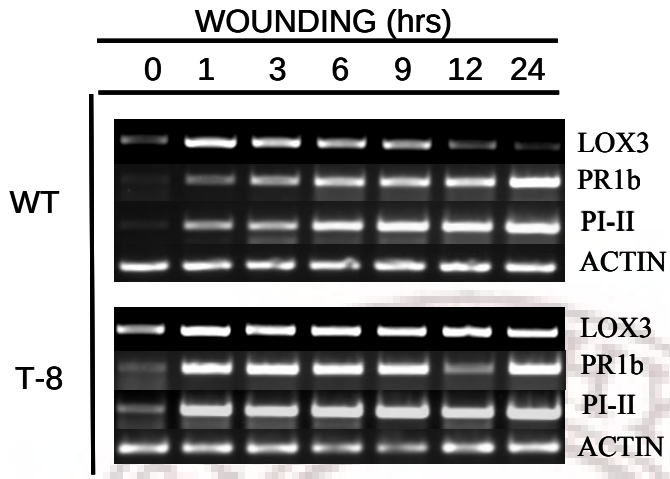

Figure 3.14 Time course analysis of wound induced expression of *LOX3, PR1b* and *PI-II* in WT and transgenic plants (T-8). Data for line T-8 alone is provided here. The experiments were performed on all other lines with similar results. Semi-quantitative RT-PCR was performed using total RNA extracted from samples collected at the indicated time intervals of WT and transgenic plants after wounding. LOX: lipoxygenase, PR: Pathogenesis related protein, PI: Protease inhibitor.

3.3.9 *In silico* analysis of promoter regions

A previous study by Yap et al. (2005) identified *NtWIF* (*N. tabacum* WIPK interacting-factor) as a downstream target of *NtWIPK* in tobacco plants. Based on overexpression and suppression of *NtWIF* in transgenic tobacco plants, Chung and Sano (2007) demonstrated that *NtWIF* regulates the wound-responsive genes containing the core sequence of auxin-responsive element (ARE). *AhMPK3*, being an ortholog of *NtWIPK*, probably might share common downstream targets of *NtWIPK*, when expressed in tobacoo plants. With increased transcript levels of *PR1b*, *acidic ß-1, 3-glucanase, acidic chitinase* in *AhMPK3* transgenics, it was tempting to speculate that *NtWIF* might be working downstream of *AhMPK3* in transgenic tobacco plants.

As a positive feedback regulation was reported in case of NtWIPK and NtWIF interaction (Chung and Sano 2007), we tried to ascertain the possible role of NtWIF in the current transgenic plants by analyzing the transcripts levels of *NtWIPK*.. The higher

transcript levels of *NtWIPK* further demonstrated the possible role of NtWIF in regulating gene expression in *AhMPK3* transgenic plants (Fig. 3.13). *Acidic ß-1,3-glucanase*, *acidic chitinase and NtWIPK* were already shown to harbor ARE motifs in their promoter regions (Chung and Sano 2007). Hence, a preliminary *in silico* analysis was carried out for other genes like *NtPI-II, NtPR1a, NtLOX1 and NtODC-1*, which showed differential regulation in *AhMPK3* transgenic plants and their upstream regions are available to identify if any of them harbor ARE motif (TGTCTC) in their promoter regions. An ARE motif was found in the *NtPI-II* gene (Z29537) promoter region at -1564 from transcription start site on the negative strand (Fig. 3.15). However, the upstream regions of *NtPR1a* (~1.5kb, X05959), *NtLOX1* (~2.2kb, EF397141) and *NtODC-1*(~2.0kb, AF233849) did not display any ARE motifs in the available upstream regions, although we can not completely rule out the presence of an ARE motif further upstream in these genes.

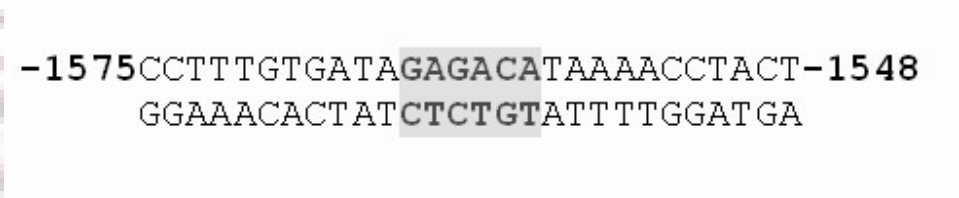

Figure 3.15 ARE motif in promoter of *NtPI-II*. Numbers indicate nucleotide positions upstream to transcription start site (TSS). ARE motif (TGTCTC) is shaded. The *NtPI-II* coding region only is available in NCBI database (Z29537). Upstream region was taken manually from Balandin et al. (1995).

3.4 Discussion

Being sessile, plants have to defend themselves against a wide range of unfavorable conditions for which they have developed elaborate and complex signaling networks to perceive the signal and respond. Mitogen-activated protein kinase (MAPK) cascade is one such signaling network, which is present in all eukaryotic organisms form yeast to mammals and also in plants. The cascade comprises three classes of hierarchically organized protein kinases, namely MAPKKKs, MAPKKs, and MAPKs, which rapidly amplify and transduce extracellular signals into various appropriate intracellular responses (Morris 2001).

A full length cDNA corresponding to a mitogen- activated protein kinase (MAPK) gene from peanut was cloned and based on its high homology with Arabidopsis *AtMPK3*, the present cDNA was designated as *AhMPK3*. AhMPK3 contains TEY motif in its activation loop and belongs to the A1 subgroup of MAPK family (MAPK group 2002). AhMPK3 protein shows very high homology with A1 subgroup members from other plants like GmMPK1 (*Glycine max),* MsMMK4 (*Medicago sativa*), AtMPK3 (*Arabidopsis thaliana*), NtWIPK (*Nicotiana tabacum*). Southern blot analysis revealed that *AhMPK3* might exist as a single copy gene in peanut genome and analyzing its genomic clone showed it contains six exons and five introns. Structural organization of AhMPK3 when compared with AtMPK3 (Arabidopsis, a herbaceous plant) and PtMPK3 (Poplar, a woody plant) revealed that the number of exons and introns, exon length and intron phases are well conserved, whereas the intron lengths and length of UTRs varied. This highlighted the conservation of these signaling molecules across various species and a strong negative selection for any alteration in protein sequence (Nicole et al. 2006).

Transcriptional regulation offers an important level of control in plants. Hence, analysis of transcriptional regulation of MAPK cascade components in a given plant species would provide an insight into possible biological functions of these components (Nicole et al. 2006). *AhMPK3* orthologs from other plant species were found to be transcriptionally regulated in response to wounding (Seo et al. 1995; Mysore et al. 2004), Systemin and UV light (Holley et al. 2003), cold and drought (Jonak et al. 1996). Like other counterparts of A1 subgroup of MAPK family, *AhMPK3* transcripts in peanut were

also induced in response to various cues. In response to wounding, H_2O_2, NO, mannitol, ABA and cold AhMPK3 exhibited distinct expression. SA and MeJA did not induce significant expression of *AhMPK3* at early stage but an upregulation at later stages of the treatments was observed. Previous reports of on *TIPK* (Shoresh et al. 2006) and *LeMPK3* (Mayrose et al. 2004) also suggested that there was no effect of JA on their expression levels.

Identification of subcellular localization of MAPKs would provide an insight into the potential functional roles they harbor in plants. It has long been known that the activation of MAPKs in yeasts and mammals involved their simultaneous transport to the nucleus (Cobb and Goldsmith 2000). The phosphorylated AtMPK3 translocated rapidly to the nucleus upon ozone (O_3) exposure (Ahlfors et al. 2004). Elicitation of parsley cell cultures with Pep-13 resulted in the translocation of PcMPK3a/b to the nucleus (Ligterink et al. 1997; Lee et al. 2004). NtWIPK was also shown to simultaneously locate in nucleus and cytoplasm (Yap et al. 2005). Like its homolog NtWIPK, AhMPK3 was also found to localize in both nucleus and cytoplasm. In our experimental system, we utilized the agroinfiltration for transient expression of GFP fusions in tobacco leaves and *Agrobacterium* itself is known to activate AtMPK3 (Djamei et al. 2007). Although we made observations 48 h after infiltration, we can not completely rule out the possibility of *Agrobacterium* induced activation and nuclear localization of some portion of AhMPK3 protein. However, AhMPK3 protein predominantly accumulated in the nucleus after H_2O_2 application, which clearly showed that H_2O_2 induced activation of AhMPK3 resulted in subsequent translocation to the nucleus. In a recent report, Qui et al. (2008) elegantly demonstrated that WRKY33 was sequestered with MPK4 and MKS1 in the nucleus under normal conditions. But, challenge with *Pseudomonas syringae* or flagellin lead to the activation of MPK4 and phosphorylation of MKS1 and subsequent release of WRKY33, which activates camalexin synthesis through regulation of PAD3. This provides a new mechanism by which plant MAPKs could also regulate the gene expression by releasing transcription factors in the nucleus upon activation. A study in yeast also suggests that MAPKs may physically associate with promoters and influence the transcription of certain genes (Pokholok et al. 2006). Hence, the nuclear localization of AhMPK3 might have significant implications in gene regulation.

Recent evidence demonstrates the involvement of *AtMPK3/NtWIPK* orthologs and *AtMPK6/NtSIPK* orthologs in regulating plant defense response against herbivores using VIGS (Wu et al. 2007; Kandoth et al. 2007). Co-silencing *LeMPK1*and *LeMPK2* orthologs of *AtMPK6/NtSIPK* compromised pro-systemin mediated resistance to *Manduca sexta* herbivory (Kandoth et al. 2007). However, no direct experimental data was available in case of plants overexpressing or silenced *AtMPK3/NtWIPK* or its orthologs in terms of the effect of their expression conferring resistance against chewing insects. Hence, the transgenic tobacco plants ectopically expressing *AhMPK3* were studied for their resistance against *Spodoptera litura*. *AhMPK3* transgenic plants showed enhanced resistance to the attack by the first instar larvae and moderate resistance against second instar larvae. Analyzing the defense response transcripts in transgenic and WT plants showed higher transcript levels of *LOX1, PR1a, PR1b, acidic ß-1, 3-glucanase, acidic chitinase and PI-II*. The transcript levels of *isochorismate synthase* (ICS) which is a key enzyme in salicylic acid (SA) biosynthesis (Wildermuth et al. 2001; Catinot et al. 2008) were similar in WT and transgenic plants indicating a possible SA-independent upregulation of PR genes in *AhMPK3* transgenics.

Except for *PI-II* and *PR1b*, whose transcripts were upregulated in transgenics, other genes, which are known to be involved in wound or JA responses like *AOS, AOC, LOX3 and PI-I* displayed no apparent differences in transcript levels. As *PI-II* are also regulated by ethylene as well (Balandin et al. 1995; Kim et al. 2003), we studied the transcript levels 1-aminocyclopropane-1-carboxylic acid synthase (*ACS3a*) involved in ethylene biosynthesis and other ethylene responsive genes like basic *PR5* (osmotin) and defensin in transgenic plants, which showed no apparent differences between WT and transgenic plants. The transcript levels of ornithine decarboxylase involved in biosynthesis of nicotine, which is herbivore or wound or JA inducible and ethylene suppressible (Shoji et al. 2000), were higher in transgenic plants compared to WT. This implied a less possible role of ethylene in controlling *PI-II* levels in transgenic plants.

A time course analysis of *PI-II* transcripts in response to wounding showed that in WT plants, the accumulation of *PI-II* transcripts occurred gradually and reached maximum level by 12 to 24 h, whereas the transgenic plants accumulated high levels of *PI-II* transcripts within 1h, which was maintained through out the time of study. A similar

expression pattern observed for *PR1b* suggested that other wound induced genes were also induced in a similar way. *LOX3* is a wound induced lipoxygenase and *LOX3*-mediated JA signaling accounts for a major part of induced resistance, when plants are damaged by insect herbivores (Rayapuram and Baldwin 2006). Antisense suppression of *LOX3* resulted in herbivore susceptibility indicating a crucial role in herbivore tolerance (Halitschke and Baldwin 2003). Sustained transcript levels of *LOX3* in transgenic plants after wounding suggested a better wound induced JA or JA responsive gene induction.

Transcript abundance at a given time is an important prerequisite to subsequent production of the corresponding protein required for proper execution of its function. The activation of NtWIPK an ortholog of AhMPK3 was delayed and it requires transcriptional activation and *de novo* synthesis of a WIPK protein (Zhang et al. 2000). It was postulated that a delayed or lack of activity of WIPK, when treated with phosphatase inhibitors, was likely because of the reduction in upstream kinase activity by the time WIPK accumulated to a significant level (Liu et al. 2003). Like other MAPKs, AhMPK3 also presumably might be activated by its upstream MAPK kinase, which in turn phosphorylates and activates effector proteins that directly or indirectly regulate a spectrum of responses. Hence, by overexpressing *AhMPK3*, the protein would be available readily to be activated by its upstream kinase upon appropriate signal. In such a case plants overexpressing *AhMPK3* would be primed to respond rapidly.

The activities of MAPKs in a cell are controlled by the opposing actions of MAPKKs, which phosphorylate and activate them and MAPK phosphatases, which dephosphorylate and inactivate them (Widmann et al. 1999). The observed constitutive upregulation of defense response genes in transgenic plants could be due to the basal level activity of upstream kinase or the level of corresponding phosphatase is not sufficient enough to inactivate the entire pool of protein in a transgenic plant with high expression levels. It is also possible that more than one MAPKK is involved in the activation of a particular MAPK under different conditions as in case of yeast and animal systems (Widmann et al. 1999; Davis 2000). The higher transcript levels of *PR1b*, *acidic ß-1, 3-glucanase, acidic chitinase* and *NtWIPK* in *AhMPK3* transgenics suggested a possible role of *NtWIF*. *In silico* analysis of promoter regions of other upregulated genes for possible *NtWIF* targets identified *NtPI-II* harboring ARE motif in its promoter region.

Whereas available upstream regions of *NtPR1a, NTLOX1 and NtODC-1* did not have ARE motifs. But surprisingly no protease inhibitor gene was reported by Chung and Sano (2007) in their analysis of *NtWIF* downstream targets. It is not known whether this particular gene was part of their analysis or not. Further experimental evaluation is required to confirm the *PI-II* regulation by *NtWIF*. This suggests that genes like *acidic ß-1, 3-glucanase, acidic chitinase*, *NtWIPK* and *PI-II* might be regulated by the NtWIF, whereas genes like *PR1a, LOX1 and ODC* might be under the control of one or more different transcription regulators in *AhMPK3* transgenic plants.

For example, AtVIP1 a bZIP type of transcription factor was demonstrated to be regulating *AtPR1a* expression upon activation of AtMPK3 in *Arabidopsis* (Djamei et al. 2007). And *NaWIPK* was found to regulate the transcript levels of *MPK4, NaSIPK*, *WRKY* and several *CDPKs* (Wu et al. 2007). Our results of higher *LOX*3 and *PI-II* transcript levels in *AhMPK3* transgenic plants upon wounding are in agreement with the VIGS functional analysis of *LeMPK3* and *NaWIPK*, in which silencing of *LeMPK3* in 35S:: *prosys* tomato plants resulted in significant reduction in *LoxD* transcripts (a homolog of tobacco *LOX3*) as well as reduced PI-II levels (Kandoth et al. 2007). The silencing of *NaWIPK* resulted in reduced Trypsin proteinase inhibitor (TPI) activity and reduced *LOX3* transcripts with wounding alone and in combination with the *Manduca sexta*'s oral secretions (OS) application in *N. attenuata*. This shows that *PI-II* levels are positively regulated by *AhMPK3* or its homologs.

In a recent review, Beckers and Conrath (2007) reported that in *Arabidopsis* priming by the chemical agent benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) is based on enhanced accumulation of mitogen-activated protein kinase 3 (AtMPK3) protein with out displaying MPK3 activity. However, upon exposure to biotic or abiotic stresses, MPK3 enzyme activity was induced to enhanced levels in primed plants, which was associated with boosted defense gene activation and stress resistance. The physiological state, in which plants are able to faster or better activate defense responses, or both, is called the primed state of the plant (Beckers and Conrath 2007). Due to the overexpression of *AhMPK3*, the transgenic plants presumably are in a primed state, which resulted in rapid induction of *PI-II* upon wounding. Lack of these enhanced levels of defense response transcripts observed in older *AhMPK3* transgenics could be

imputed to the unavailability of downstream regulatory molecules, or due to the absence of active physiological environment in aged plants. Constitutively higher level of various defense gene transcripts as well as rapid induction of protease inhibitor II (*PI-II*) transcripts upon wounding, which encodes the antidigestive protein and functions as a direct defense against herbivore, might have collectively resulted in the resistance of *AhMPK3* transgenic tobacco against *Spodoptera litura*. The observed transcript levels of various genes in WT and transgenics indicate that regulation is independent of hormones as all the subset of genes known to be regulated by specific hormone were not affected. Sustained levels of *LOX3* in wounded transgenic plants suggested the possible role of *AhMPK3* in regulating stress induced hormone levels. This indicates that AhMPK3 probably activates transcription factors with well-defined downstream targets.

Essentially, most of the studies on priming in response to wounding and or herbivore attack were associated with use or involvement of plant derived cues like volatile organic compounds(VOCs) that are emitted in response to herbivory (Frost et al. 2008). In the present investigation, we demonstrated the wound induced priming of defense responses in tobacco plants ectopically expressing *AhMPK3* of peanut. The regulatory molecules connecting *AhMPK3* and gene expression are being currently investigated. Our results substantiate the function of *AtMPK3/NtWIPK* orthologs in defense against herbivore attack in plants.

Table 3.1 Sequences of oligonucleotides used in the study. See text for details

Name of the Primer	**Primer Sequence (5'-3')**
IntF	GG(A/C)GC(A/T)TA(C/T)GG(A/C)(A/G)T(A/T/G/C)GT(A/T/G/C)TG
IntR	(A/T/G/C)GT(A/T/G/C)AC(A/T/G/C)AC(A/G)TA(C/T)TC(A/T/G/C)GTC
WyrR	(C/T)TC (A/T/G/C)GG(A/T/G/C)GC (A/T/G/C)C(G/T)(A/G)TA CCA
Ah443R	TGACGACATACTCGGTCATG
Ah270R	AGTTCTGGCAGTGTTCCTCCG
Ah3P1F	TCCACCCCTTCGTAGAGAGT
Ah3P2F	CGGAGGAACACTGCCAGAACT
AhMK$_3$1F	CACTAACCTTCCTTCGTCC
AhMK$_3$1216F	ATGACATGAGCAATTTGAAGT
AhMK$_3$1497R	GAATTCTGGGGAGTAATAATC
ORF-F	CATCCATGGCCGGCGTTAATCCAA *Nco*I
ORF-R	AGGATCCTTAAGCATACTCAGGATTGAGT *Bam*HI
ORF-F2	GCCCGGGATGGCCGGCGTTAATCCAA *Sma*I
PCR anchor primer	GACCACGCGTATCGATGTCGAC
Oligo d(T) anchor primer	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV
Act F	TGGCATCACACTTTCTACAA
Act R	CAACGGAATCTCTCAGCTCC
nptII F	GAGGCTATTCGGCTATGACTG
nptII R	ATCGGGAGCGGCGATACCGTA
35SF	ACGACACTCTCGTCTACTC

Table 3.2 Properties of *AhMPK3* gene introns. Upper and lower case letters indicate exons and intron regions, respectively. Letters in bold lower case indicate canonical di-nucletide 5'& 3' splice sites.

Intron No	Intron size (bp)	Splice junction sequences	A+T (%)	T (%)
I	288	CGTTTG**gt**aact .....ttga**ag**CTCGGT	68	49
II	810	GAAAAC**gt**gagt….aaac**ag**GTGATT	68	39
III	104	TGCCAG**gt**gaat…...ttgc**ag**TACTTC	62	45
IV	135	ACAGAG**gt**tagt…...ctgt**ag**CTTCTT	66	34
V	204	TTACAG**gt**aact……tggc**ag**TTGAG	64	37

Table 3.3 Sequences of gene-specific primers used in RT-PCR for amplification of defense related gene transcripts. See text for descriptions.

Oligo Name	Forward	Reverse
PI-I	ATGGTGAAGTT TGCTCACGT	AATCCCTTAGCCAACCTGG
PI-II	GTTAGTTTCGTCGCTCATCT	CTGCGTTACAACAGTTGATG
AOS	CTTGGTCTTCCGAAGGTTC	GACGTCGATATCCAACGTG
PR1a	CTTCTTGTCTCTACACTTCTC	GCAAGAGACAACATATCCTC
PR1b	TCTTAACCCTCACAATGCAG	AGGGTTGCTCCTCAAGATC
LOX1	CACTTCCTACTGATCTCATC	CTCATCGACATTCATCTGCA
LOX3	AATGACAGAGAACTCCAAGC	TAGAACGCTTCGACAATCTC
Glucanase	ATGGCTTTATGCATTAAAAATGGC	AGCATTGAAGACATTTGTTTCTGG
Chitinase	CTGAAGAATAGGAACGACGGTAG	ATACCTCCTGTAGTATCCAATTCG
Defensin	GAGGCGAGAACTTGTGAGTC	AAGCCGAAACCATTATTCATAAC
PR5	CTTGAGATCTTCTTTTGTTTTCTTC	ACTTCCAGGCATTTCCAAGGGAAA
ODC	CCCTTTGATTCCTTCCTCTCA	TAAATTACTACCAAAAACAACAAAATT
ICS	TGCATATCAGTTCTGTTTGCAAC	CCAGCATACATTCCTCGGTCA
AOC	CTCCTCAGCCTCTGCTGCTCTTAGA	ATATAAAGGACAATAATTATTTATC
ACS3a	ATAGTTATGAGTGGAGGAGC	CCGTGTCTTTTCCCTAGTCT

3.5 Summary

Mitogen-activated protein kinase cascade plays a very important role in plant signal transduction mechanism. A full length cDNA of 1514 bp length, corresponding to a mitogen- activated protein kinase gene was cloned from peanut (*Arachis hypogaea*). Based on its high homology with *Arabidopsis AtMPK3*, the cDNA was designated as *AhMPK3*. It carried an open reading frame of 1113 bp encoding a 371 amino acid polypeptide. AhMPK3 bears TEY motif in its activation loop and belongs to the A1 subgroup of MAPK family. Southern blot analysis revealed that *AhMPK3* might exist as a single copy gene in peanut genome and its structural organization revealed well-conserved nature of these signaling components across different species. *AhMPK3*, when transiently expressed in tobacco leaves was found to localize in both nucleus and cytoplasm. Transgenic tobacco plants ectopically expressing *AhMPK3* exhibited enhanced resistance to the first and second instar larvae of *Spodoptera litura* and constitutively higher transcripts levels of defense response genes like *PR1a, PR1b*, *LOX1*, *PI-II* etc. Apart from this when wounded, transgenic plants accumulated high levels of *PI-II* and *PR1b* transcripts rapidly compared to wild type indicating the occurrence of a priming phenomenon.

Chapter 4

Identification and isolation of genes induced early in response to *Phaseoisariopsis personata* infection in *Arachis diogoi*: A wild relative of peanut

4.1 Introduction and Background

4.1.1 Legumes

The Leguminosae (Fabaceae) represent one of the great flowering plant radiations and the diversity within this family (of an estimated 20 000 species) is difficult to comprehend. In the Latin American and African tropical rainforests, the majority of the trees that form the canopy overhead are legumes. However, the Leguminosae is not just a tropical woody family. There have also been notable radiations of herbaceous species in temperate regions. The legumes are highly diverse and can be divided into three subfamilies: Mimosoideae, Caesalpinioideae, and Papilionoideae (Doyle and Luckow, 2003). Of these, the Papilionoideae subfamily contains nearly all economically important crop legumes, including soybean (*Glycine max*), peanut (*Arachis hypogaea*), mungbean (*Vigna radiata*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), and alfalfa (*Medicago sativa*).With the notable exception of peanut, all these important crop legumes fall into two Papilionoid clades, namely, Galegoid and Phaseoloid, which are often referred to as cool season and tropical season legumes, respectively.

Significance

Legumes, broadly defined by their unusual flower structure, podded fruit, and the ability of 88% of the species examined to date to form nodules with rhizobia (De Faria et al. 1989), are second only to the Graminiae in their importance to humans (Graham and Vance 2003). Even in intensive animal and milk production, where grain crops are major feed sources, forage legumes are required to maintain animal health (Wattiaux and Howard, 2001). Nitrogen (N_2) is the primary nutrient limiting plant production in most natural ecosystems (Seastedt and Knapp 1993; Vitousek et al. 1997). Legumes, through their symbiotic abilities, can play an important role in colonizing disturbed ecosystems, including those that are fire prone (Arianoutsou and Thanos 1996).

4.1.2 Legume- microbe interactions

Diseases and pests are major constraints to legume production, especially in the tropics and subtropics. The ability of legumes to form symbiotic mutualistic relationships with certain bacteria in the Rhizobiales (collectively called rhizobia) and harness the ability of the bacteria to fix atmospheric N_2 into ammonia has a tremendous impact on natural and agricultural ecosystems. The interaction enables legumes to produce protein-rich seeds and foliage that are critical to many human and animal diets. Past research has illuminated many of the facets of plant-bacterium recognition, nodule formation, nitrogen fixation, and ammonia assimilation. The mechanisms that allow bacterial colonization without triggering plant defense responses is not well understood, raising an important question how do legumes recognize friend from foe?

Role of Phenylpropanoid pathway

All incompatible interactions were characterized by strong, rapid upregulation of genes encoding enzymes in the phenylpropanoid pathway, particularly for the synthesis of isoflavones and isoflavanones. Down regulating isoflavone synthase genes in soybean roots using an RNAi approach resulted in a 95% reduction in isoflavone accumulation and an enhanced susceptibility to the pathogens (Subramanian et al. 2005). Sustained up-regulation of genes involved in phenylpropanoid metabolism has been associated with R-gene-mediated resistance responses in *M. truncatula* responding to foliar pathogens (Torregrosa et al. 2004).

Flavonoid compounds, particularly isoflavonoids, are key components in defense responses of legumes to pathogens in which they likely have roles in restricting microbial growth and as antioxidants. However, many of the same compounds also play key roles in establishing an effective symbiotic mutualism with rhizobia. Flavonoid compounds attract rhizobia to host roots, stimulate growth, modify composition of bacterial cell wall components, induce expression of nod genes leading to production of Nod factors, induce expression of the Type Three Secretion System (TTSS), and induce expression of plant cell wall degrading enzymes (Cooper 2004). After initial nodule formation, the host inhibits additional rhizobial infections to limit nodule number. Recent research using an alfalfa split root system provides evidence for systemic suppression of nod gene-inducing

flavonoid compounds after initial nodulation as a means of inhibiting new infections (Catford et al. 2006). Inhibiting production of salicylic acid, which plays a key role in inducing the HR and in systemic acquired resistance, increases nodulation in *Lotus japonicus* and *M. truncatula* (Stacey et al. 2006), Suggesting that SA-induced defenses are also involved in controlling nodulation.

The emerging picture from recent research indicates that legumes utilize similar mechanisms to recognize pathogens and symbiotic microbes. Recent reports suggest that LysM domains and GRAS transcription factors are important for recognition of both friend and foe. This complexity makes the Legume-microbe interactions fascinating as well as challenging.

4.1.3 Constrains in Peanut production

Peanut is extensively grown in the semi-arid tropics (SAT) where many abiotic and biotic factors limit its productivity and seed quality. Rust (*Puccinia arachidis* [Speg.]), Early Leaf Spot (ELS: *Cercospora arachidicola* [Hori]), and Late Leaf Spot (LLS: *Phaseoisariopsis personata* [Berk. and MA Curtis]) are widely distributed foliar diseases of peanut. Other diseases include virual groundnut rosette disease (GRD), bacterial wilt and aflatoxin contamination of kernels by *Aspergillus flavus,* which is a serious problem for marketing of peanut kernels as well as cake. Late leaf spot (LLS) caused by *Phaeoisariopsis personata* (Berk. and M.A. Curtis) and rust caused by *Puccinia arachidis* (Speg.) are among the most serious diseases of peanut. LLS alone can cause yield losses up to 80 % (McDonald et al. 1985; Grichar et al. 1998) and will be devastating combined with rust. Biotic stresses often occur in combinations and their severity and extent of distribution vary with cropping systems, growing seasons, and regions. The estimates of the global annual yield losses caused by these peanut diseases are estimated to be US$ 467 mn (RUST), US$ 326 mn (ELS) and US$ 599 mn (LLS) (Dwivedi et al. 2003).

Although fungicide application is effective in controlling these diseases, problems associated with it like high cost, environmental pollution and chances of pathogen developing resistance do not make a good choice for future applications. Other environmental friendly approaches were tried against foliar diseases of peanut with less

success. Several elicitors of SAR and ISR also failed to reduce symptoms of late leaf spot of peanut and in some cases exacerbated symptoms (Zhang et al. 2001). It is conceivable that disease was unaffected because the pathogen was able to thwart host defenses or because the plant lacked effective defenses or lacked the capacity to initiate defenses against the pathogen (Vallad and Goodman 2004).

4.1.4 Peanut improvement: Role of wild relatives

The cultigen *A. hypogaea* probably arose from a single or few events of hybridization involving AA and BB genome species. The hybrid underwent spontaneous duplication of chromosomes to produce the allotetraploid *A. hypogaea* with genome type AABB (Simpson 2001). In common with many other polyploids, it seems that a severe genetic bottle-neck was imposed at the species origin, via hybridisation of two wild species and spontaneous chromosome duplication (Proite et al. 2007). This has led to lack of variability in some important traits, limited availability of allelic combinations and consequently restrictions in productivity. In addition, the very low level of polymorphism in cultivated peanut has hampered genetic and genomic characterization.

Wild relatives are an important source of genes for resistances to biotic and abiotic stresses that affect crop species. Wild *Arachis* species and exotic germplasm are usually agronomically inferior to modern cultivars (Dwivedi et al 2003). However, reports in rice and tomato suggest that wild germplasm may contain alleles capable of improving both yield and seed quality of elite cultivars (Xiao et al. 1996; Tanksley and McCouch 1997).

In contrast to cultivated peanut, most wild *Arachis* species are diploid with high genetic diversity (Galgaro et al 1997) and harbor resistance to pests and diseases that affect the cultivated peanut and are a potential source of genes to increase the resistance levels in peanut. Wild *Arachis* species harbor a range of genes conferring resistance to pests and diseases, oil and protein contents, and oleic (O)/linoleic (L) fatty acid ratios (Dwivedi et al 2003). Some genotypes show very high levels of resistance to rust, ELS, LLS, nematodes, GRD, PBNV, thrips, leaf miner, *Spodoptera*, and aphids (Dwivedi et al. 2003).

The difference in ploidy rendered peanut sexually isolated from its wild relatives, giving this species a very narrow genetic basis (Raina et al. 2001). Therefore, all land races of peanuts might have derived from a single plant (Kochert et al. 1991). Due to this sexual isolation, the introgression of genes from wild species is only possible through complex crosses or genetic transformation. To date, there is only one case of successful introgression of genes from wild species into *A. hypogaea* to produce commercial cultivars of peanut (Simpson 2001). Many of the wild *Arachis* species are not cross compatible with cultivated peanut leading to the interspecific progenies carrying a lot of linkage drag. Progress in ELS and LLS resistance breeding has been limited by the absence of high levels of resistance in cultivated peanut and the linkage of resistance with undesirable traits (Wynne et al.1991; Singh et al. 1997). In contrast, several wild *Arachis* species show a very high level of resistance to ELS and LLS. The success in transferring ELS and LLS resistance from wild *Arachis* species to cultivated peanut has been limited mainly because of cross compatibility barriers, the linkage of resistance with many undesirable characteristics, and the long periods of time required for developing stable tetraploid interspecific derivatives. Apart from pathogen resistance, many of the wild *Arachis* species possess a high degree of resistance to herbivores like leafminer and *Spodoptera*. However, these are not readily cross compatible with cultivated peanut (Dwivedi et al 2003). As transgenic technologies were well established for peanut now, genetic transformation would be the best option to incorporate genes for resistance into cultivated peanut, provided that genes conferring reasonable levels of resistance can be identified and isolated.

Hence, for improvement of the peanut crop, there is a need to both identify and isolate novel genes with potential agronomic interest in its wild relatives which can be utilized in genetic transformation, there by avoiding the deleterious linkage drag that often becomes a problem while transferring genes from wild species through conventional breeding techniques.

***Arachis diogoi*: A wild relative of peanut**

A. diogoi Hoehne (Syn. *A. chacoense*) is diploid wild relative of cultivated peanut *Arachis hypogaea* L. and belongs to section *Arachis*. Several studies on wild *Arachis* species confirmed that *A. diogoi* is highly resistant to several fungal and viral pathogens (Table 4.1) (Abdou et al. 1974; Subrahmanyam et al. 1985a; 1985b; 2001; Rao et al. 2003).

Table 4.1 Resistance of *A. diogoi* against various pathogens and insect pests.
(Source: 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	+			+								+	+	

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.

4.1.5 Objectives of the present work:

Based on the Significance of *Arachis* wild species with their potential to harbor genes for resistance against late leaf spot disease, the following objectives were framed.

1. Identification of transcripts which are upregulated early in response to *Phaseoisariopsis personata* infection in *A. diogoi* using GeneFishing DEG premix kit from Seegene.
2. Cloning of the upregulated partial cDNAs.
3. Sequence analysis and annotation of the isolated clones.
4. Time course expression analysis of selected genes in response to *Phaseoisariopsis personata* infection.

4.2 Materials and Methods

4.2.1 Plant Material

The *Arachis diogoi* seeds were germinated *in vitro*, transferred to the greenhouse and the seedlings were allowed to establish. To avoid any environmental variations between control and treated samples like soil water content or insect/ aphid damage, which may also induce a subset of genes, healthy growing twigs were cut with a razor blade and the cut stems were immediately kept under water. The twigs were washed thoroughly with sterile double distilled water and kept in trays with moist filer paper with cuttings wrapped in moist tissue paper towel. Care was taken so that paper towels remained wet all the time. The trays were covered with plastic covers to maintain high humidity. The twigs were maintained at 28 ºC with 16 hrs of light and 8 hrs of dark photoperiod. Twigs were acclimatized for 10-15 days so that the twigs would get stabilized by the formation of adventitious roots at the cut stem regions. Pathogen inoculation was performed after acclimatization.

4.2.2 Pathogen inoculation

Conidia of *P. personata* were harvested from *Arachis hypogaea* leaves with typical disease symptoms using a cyclone spore collector. The conidia were suspended in sterile double distilled water to obtain a concentration of 10^5 conidia per milliliter**.** Tween 20 was added to a final concentration of 0.02 % which will serve as a surfactant. The spore suspension was applied on leaves with a paint brush homogenously, and the treatment was carried in duplicate. Leaves treated with 0.02 % tween 20 served as control. Both the control and treated twigs were covered with polythene bags and maintained at $26^0 \pm 1^oC$ with a photoperiod of 16 h light supplied by cool white fluorescent tubes with a light intensity of about 1500 lux. Leaves were collected from both control and treated leaves at regular intervals and immediately frozen in liquid nitrogen and stored at -80^oC until usage. During all the inoculation experiments, susceptible *Arachis hypogaea* leaves were also inoculated simultaneously with the same spore suspension to check the pathogenecity of spores and the disease symptoms were scored after 20 days post inoculation.

4.2.3 RNA isolation

Total RNA was isolated and quantified from both control and treated leaves as described earlier in section: 3.2.3

4.2.4 Identification of DEGs (differentially expressed genes) in response to *P. personata* infection

Genes upregulated during the *A. diogoi* and *P. personata* interaction were identified using Genefishing DEG premix kit (Seegene, Korea) following manufacturers instructions. In brief it involves two steps, first step is reverse transcription where 3µg of total RNA from control and treated samples was reverse transcribed in the presence of 10µM dT-ACP1, 4 µl of 5 X RT buffer, 5 µl of 2 mM dNTP mix, 0.5 µl of RNase inhibitor (40 u/µl) and 200u of M-MLV reverse transcriptase (Promega, USA) in a 20µl reaction volume. The reaction was incubated at 42ºC for 90 min. The reaction was terminated by incubating at 94ºC for 2 min. The first strand cDNA synthesized was diluted with 80 µl of DNase-free water and used directly in second step i.e GeneFishing PCR. Around ~50 ng of first strand cDNA was amplified in a two step reaction in the presence of 0.5µM arbitrary ACP, 0.5 µM dT-ACP2 and 1 X SeeAmp ACP Mastermix in a total volume of 20µl. The first stage of PCR for second strand cDNA synthesis involved a single cycle of 94ºC for 5min, 50ºC for 3 min and 72ºC for 1 min. The cycling conditions for second stage of PCR were 94ºC for 40sec, 65ºC for 40sec and 72ºC for 40 sec (40 cycles) and a final incubation at 72ºC for 5 min. The PCR products were run on 2% agarose gel and visualized using ethidium bromide staining.

4.2.5 Cloning and sequencing DEGs

Fragments which were upregulated in treated samples were cloned and sequenced as described earlier in sections: 3.2.5 and 3.2.6

4.2.6 Sequence analysis and Annotation

Sequence analysis was performed by searching for homologous sequences in NCBI Genbank non-redundant and EST databases using basic local alignment search tools

(BLASTN and BLASTX) (Altschul et al. 1997). Annotation was based on the best match found in blastx alignment against protein databases at NCBI. Annotated clones were assigned with putative function based on their sequence homology with other genes in the database. For sequences for which sufficient coding sequence was not available, the putative coding gene was identified by blastx analysis of the highly homologous EST clones available in Genbank database.

4.2.7 Expression analysis of genes in response to *P. personata* infection

Semi-quntitative RT-PCR was performed as described earlier in section: 3.2.10. Using 1 µg of total RNA and gene specific primers, expression analysis of genes involved in phenylpropanoid pathway and lignification were studied in a time dependent manner in both control and treated leaves. Sequences of gene specific primers were given in Table. 4.2.

4.3 Results and Discussion

4.3.1 Identification and isolation of genes from *A. diogoi* upregulated early in response to *P. personata* infection.

Using Seegene's genefishing DEG premix kit, we isolated partial cDNAs of genes from *Arachis diogoi* upregulated during interaction with *P. personata*. The kit is based on Annealing Control Primer (ACP) technology (Hwang et al 2003) that utilizes high annealing temperatures, thereby reducing the false positives. Although there are several amplicons, which are downregulated we were interested in only genes which are upregulated upon pathogen inoculation. We have cloned 60 partial cDNAs, which were upregulated in reponse to *P. personata* infection (Table.4.1). The partial cDNA clones were designated as AdDR (clone number) cDNAs for *Arachis diogoi* Defense Responsive.

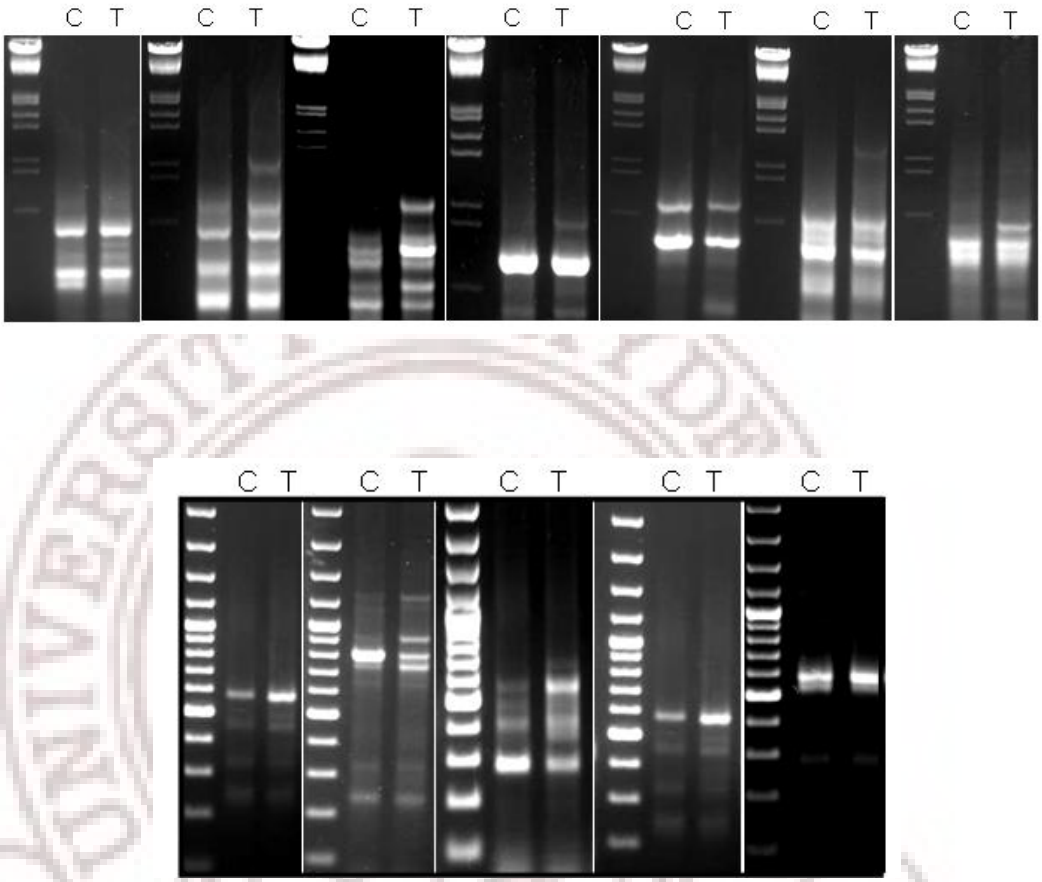

Figure 4.1 Representative gel pictures showing differential expression pattern between Control/mock treated (C) and *P. personata* treated (T) samples. Differential expression pattern was observed using Genefishing DEG kit, involving isolation of total RNA from control (C) and pathogen treated (T) leaves, cDNA synthesis followed by amplification of cDNA with different random arbitrary primers (See Material and Methods for details).

Table 4.1 Analysis of AdDR cDNA clones. AdDR: *Arachis diogoi* Defense Responsive; Asterisk (*) indicate clones whose annotation is based on the BlastX analysis of the highly homologous sequences in EST database.

Clone	**Length (~bp)**	**Accession No.**	**Annotation, Best BLASTX hit (Accession.No)[Organism]**	**E-value**
AdDR1	700	DQ861992	ADP-ribosylation factor, AAR29293 [*Medicago sativa*]	4e-85
AdDR2	1100	EF371920	Cinnamic acid 4-hydroxylase, Q96423 [*Glycyrrhiza echinata*]	1e-95
AdDR3	750	EF371921	HMG-1 like protein gene CAA41200 [*Glycine max*]	4e-36
AdDR4	750	EF371922	SecY protein; ABC transporter related ABE85367 [*Medicago truncatula*]	2e-89
AdDR5	800	EF371923	CBL- interacting protein kinase, BAD95894 [*Lotus japonicus*]	1e-29
AdDR6	800	EF371924	Hypothetical protein, unknown protein ABE80371 [*Medicago truncatula*]	2e-25
AdDR7	1100	EF371925	Zinc finger Protein, SCOF-1, AAB39638 [*Glycine max*]	2e-53
AdDR8	700	EF374057	unknown protein, NP_563935 [*Arabidopsis thaliana*]	2e-73
AdDR9	750	EF374058	Asparagine synthetase, AAL91002 [*Securigera parviflora*],	6e-101
AdDR10	650	EF374059	Putative Leucine Rich Repeat, ABE91519 [*Medicago truncatula*]	4e-09
AdDR11	370	EF374060	Thaumatin like protein, AAB38064 [*Prunus avium*]	1e-17
AdDR12	650	EF392852	Tonoplast intrinsic protein, CAB45653 [*Pisum sativum*]	7e-86
AdDR13	640	EU170616	Cyclophilin, ABL67655 [*Citrus cv. Shiranuhi*]	9e-49
AdDR14	450	EF392854	Hypotheticalprotein AI84653 [*Nicotiana abacum*]	6e-51
AdDR15	700	EF392855	S-adenosylmethionine synthase. ABB29942 [*Solanum tuberosum*]	2e-60
AdDR16	420	EF392856	Phenylalanine ammonia-lyase, BAA21643 [*Populus kitakamiensis*]	5e-21
AdDR17	1000	EF392857	1-deoxy-D-xylulose 5-phosphate synthase,ABF18929 [*Hevea brasiliensis*]	7e-53
AdDR18	850	EF392858	ATPase. AAO73433 [*Citrus limon*]	1e-73
AdDR19	550	EF392859	glutathione S-transferase GST AAG34804 [*Glycine max*]	6e-43

AdDR20	216	EF419306	Putative translation initiation protein AAD39892 [*Medicago truncatula*]	0.24
AdDR21	490	EF419307	No similarity found	
AdDR22	400	EF419308	No similarity found	
AdDR23	800	EF419309	No similarity found	
AdDR24	310	EF419310	No similarity found	
AdDR25	370	EF419311	No similarity found	
AdDR26*	622	EU310407	Putative membrane associated protein	
AdDR 27	379	EU310408	No significant similarity found	
AdDR 28	597	EU310409	ZTL (ZEITLUPE) ubiquitin-protein ligase, ABC25060.2 [*Ipomoea nil*]	5e-24
AdDR 29	734	EU310410	nucleic acid binding protein CAC69852.1 [*Nicotiana tabacum*]	7e-43
AdDR 30	394	EU310411	terminal flower 2 protein BAF75817.1 [*Malus x domestica*]	5e-11
AdDR 31*	521	EU310412	lysin motif-type receptor-like kinase	
AdDR 32	598	EU310413	pyridoxine biosynthesis protein AAZ67142.1 [*Glycine max*]	6e-21
AdDR 33	325	EU310414	proline dehydrogenase AAR86686.1 [*Glycine max*]	3e-14
AdDR 34	401	EU310415	papain-like cysteine proteinase AAF61441.1 [*Ipomoea batatas*]	2e-08
AdDR 35	413	EU310416	No significant similarity found.	
AdDR 36	735	EU331146	harpin binding protein 1 AAR26479.1 [*Glycine max*]	2e-72
AdDR 37	727	EU331147	high molecular weight heat shock proteinAAF34134.1[*Malus x domestica*]	1e-66
AdDR 38	691	EU331148	dirigent-related protein ABE73781.1 [*Tamarix androssowii*]	9e-19
AdDR 39	~800	EU331149	dirigent-like protein, ABD52120.1 [*Picea engelmannii x Picea glauca*]	7e-13
AdDR 40	563	EU331150	Cu-Zn superoxide dismutase ABF51006.1[*Arachis hypogaea*]	8e-42
AdDR 41*	330	EU331151	ubiquitin-associated (UBA)/TS-N domain-containing protein [*Arabidopsis*]	
AdDR 42	1250	EU331152	cinnamyl alcohol dehydrogenase CAK22318.4 [*Leucaena leucocephala*]	6e-56

AdDR 43	600	EU331153	chaperonin 60 alpha subunit AAC68501.1 [*Canavalia lineata*]	3e-43
AdDR 44	560	EU331154	putative 60S ribosomal protein L1 BAE71215.1 [*Trifolium pratense*]	3e-54
AdDR 45	439	EU331155	Rubisco activase ABF38996.1 [*Pachysandra terminalis*]	
AdDR 46	592	EU338519	unknown protein NP_180103.2 [*Arabidopsis thaliana*]	2e-41
AdDR 47	624	EU338520	Rubisco activase ABF38996.1 [*Pachysandra terminalis*]	2e-64
AdDR 48	524	EU338521	Chloroplast oxygen-evolving enhancer protein AAV74404.1[*Manihot esculenta*]	2e-43
AdDR 49	631	EU338522	3-ketoacyl-CoA thiolase CAA63598.1 [*Brassica napus*]	4e-49
AdDR 50	506	EU338523	mitochondrial acyl carrier protein 1 NP_181990.1 [*Arabidopsis thaliana*]	9e-29
AdDR 51	392	EU338524	lipid transfer protein I AAQ74627.1 [*Vigna radiata*]	5e-04
AdDR 52	381	EU338525	No significant similarity found	
AdDR 53*	369	EU338526	putative splicing factor	
AdDR 54*	280	EU338527	putative splicing factor	
AdDR 55*	394	EU338528	putative nifU-like protein	
AdDR 56	638	EU338529	chloroplast 30S ribosomal protein S13-1 CAA79013.1 [*Arabidopsis thaliana*]	4e-43
AdDR 57	555	EU338530	chloroplast 30S ribosomal protein S13-2 CAA79013.1[*Arabidopsis thaliana*]	4e-43
AdDR 58	700	EU338531	putative histone H2A AAM60967.1 [*Arabidopsis thaliana*]	1e-43
AdDR 59	527	EU338532	hypothetical protein BAD43819.1 [*Arabidopsis thaliana*]	1e-23
AdDR 60	512	FJ481981	putative cyclophilin ABL67655 [*Citrus cv. Shiranuhi*]	2e-25

Cultivated peanut (*Arachis hypogaea*) is susceptible to *P. personata*, which causes late leaf spot (LLS) disease. Except for a couple of fungicides, which have their own advantages and disadvantages, there is no efficient methodology to reduce the LLS on peanut. The best way of improving the peanut resistance against *P. personata* would be to develop resistant cultivars. Because of the ploidy levels of cultivated peanut which

lead to its sexual isolation from its wild relatives, cultivated genotypes exhibit a very narrow genetic basis.

Though LLS disease on peanut is of great economical importance as it alone can reduce the crop yields drastically, the interaction has not been studied at molecular level for a long time. There are only two reports on identification of genes involved in peanut (*A. hypogaea*) and *P. personata* interaction (Luo et al 2005b; Nobile et al 2008). In spite of the known fact that wild relatives of *Arachis* are resistance against several pathogens including *P. personata* (Rao et al 2003) for quite some time, most of the works using wild species of peanut were confined to breeding programmes. *A. diogoi,* which is a wild relative of peanut, was shown to be resistant against *P. personata* and several other pathogens. In plants, response to any particular stress whether it is abiotic or biotic, a subset of genes will be induced, some are early responsive and some are late responsive to cope up with that stress. Although both are required to defend it against various environmental cues the early responsive genes will hold the key in identification and amplification of different stress signals and induction of further downstream gene expression. Hence we are interested in identification of the early responsive genes during the infection process. This identification of genes which are upregulated in *Arachis diogoi*, during the early stages in response to *P. personata* infection will greatly help in understanding the resistance mechanisms involved as well as the isolation of full length genes that might work as good candidate genes from peanut transformation, once their role in pathogen resistance is established.

4.3.2 Role of isolated genes in plant defense

As the Genefishing DEG kit generates 5' partial clones, i.e they will have complete 3' UTR region and based on the arbitrary 10-mer complementary region on cDNA different sizes of partial coding sequences would be obtained. Partial coding sequences were used in blastx analysis to identify putative homologs. For sequences, where less coding sequences were available for blastx analysis, putative homologs were identified by blastx analysis of clones which were showing high homology in EST database. Majority of the cloned genes were well known for their role in plant defense or stress responses, apart from the genes involved in secondary metabolism and unknown/hypothetical or

sequences with no similarity found in public sequence databases were high in number (Table 4.1). Genes classified as receptors, cell signaling components, involved in primary metabolism and gene expression regulators were also identified. The following are some of the annotated clones and their significance in plant defense in concise.

AdDR1: ADP-ribosylation factor.

ADP-ribosylation factors (ARFs) make up a family of myristoylated small GTP-binding proteins (~21 kDa) that are similar in structure to members of the Ras–GTPase superfamily and heterotrimeric G-protein α-subunits. The best characterized function of ARF are the activation of phospholipase D and involved in vesicle trafficking. Plant ARFs were shown to be upregulated in responsed to fungal and elicitor treatment as well as heavy metal stress (Xiong et al. 2001; Kim et al. 2004; Minglin et al. 2005). Constitutively over-expressed rice ARF1 triggered spontaneous induction of lesion mimics, induced an array of pathogenosis-related (PR) genes, reduced susceptibility to a fungal pathogen, and caused accumulation of SA (Lee et al. 2003). A recent study proved that ADP-ribosyation factor induces cell death upon overexpression in *N. benthamiana* and involved in non-host resistance and R-gene mediated resistance (Coemans et al. 2008).

AdDR3: High mobility group (HMG) protein

The high mobility group (HMG) proteins are non-histone components of chromatin that regulate gene expression via interactions with chromatin and transactivating protein factors (Klosterman and Hadwiger 2002). Expression of pea defense gene DRR206 is inversely correlated with HMG-I/Y expression in pea tissue, suggesting a potential role for HMGI/Y in the regulation of plant defense genes (Klosterman and Hadwiger 2002). The HMG contains a DNA-binding domain and is part of a family of proteins that can enhance the binding of monocot TFs to transcriptional elements. HMGs in plants were also shown to be up regulated by treatment with elicitors (Verica et al. 2004).

AdDR4: ABC transporter protein

ATP-binding cassette (ABC) transporters, which belong to a protein superfamily found in all living organisms, mediate the translocation of a wide range of structurally unrelated molecules across biological membranes and use ATP hydrolysis as a source of energy. Several of these proteins have been identified by their ability to confer drug resistance, hence their designation as multidrug resistance (MDR) or pleiotropic drug resistance (PDR) proteins. Recent studies suggested that plant plasma membrane transporters are involved in the secretion of endogenous metabolites that play a role in defense against biotic stress. Transgenic plants of *Nicotiana plumbaginifolia* in which NpPDR1 expression was prevented by RNA interference showed increased sensitivity to sclareol and reduced resistance to *B. cinerea* (Jasin´ski et al. 2001; Stukkens et al. 2005). The pen3 (PENETRATION 3) mutant plants permitted both increased invasion into epidermal cells and initiation of hyphae by *B. g. hordei*, suggesting that PEN3 contributes to defenses at the cell wall and intracellularly. The phenotypes conferred by pen3 result from the loss of function of PLEIOTROPIC DRUG RESISTANCE8 (PDR8), a highly expressed putative ATP binding cassette transporter shows that involvement of ABC transporter proteins in plant defense responses (Stein et al. 2006).

AdDR5: CIPK: CBL-interacting protein kinase

Calcineurin B–like proteins (CBLs) are small Ca^{2+} binding proteins that do not have any enzymatic activity and that function by interacting with their target proteins. CBLs specifically target a family of protein kinases referred to as CIPKs (CBL-interacting protein kinases). CBLs and CPKs were found to be involved in ABA, salt, drought and wound responses (Batistic and Kudla 2004; Luan 2008). It has been speculated that ‘‘inter-phosphorylation’’ events between the CDPK and CIPK signaling systems could provide a mechanism for coordinating and/or enhancing calcium-induced signals in plant cells. Role of CIPKs in biotic stress signaling is not explored till now.

AdDR10: Leucine rich repeat protein

While over 40 R genes have been isolated from plants (Martin et al. 2003), only two have been isolated from a legume. Isolation of R genes from legumes has been slow due to lack of detailed genetic maps, appropriate mapping populations, and chromosome

walking tools. In addition, the polyploid nature genomes of many crop legumes makes obtaining mutants difficult, and high frequency transformation systems for legumes are limited. Five main classes of R genes have been defined according to the structural characteristics of their predicted protein products (Dangl and Jones 2001). The majority of functionally described R genes are the nucleotide-binding site (NBS)-leucine-rich repeat (LRR) type and an N-terminal putative leucine-zipper (LZ) or other coiled-coil (CC) sequence. Other well studied class is TIR-NBS-LRR proteins, which have a region with similarity to the N terminus of the Toll and Interleukin 1 receptor (IL-1R) proteins (Martin et al. 2003). To date, there have been several reports of induced R gene expression. Rice *Xa1* gene was induced on pathogen inoculation and wounding (Yoshimura et al. 1998); *Pib* gene was induced under altered environmental conditions (Wang et al. 1999); RPW8 from A. thaliana increased after infection of powdery mildew pathogens (Xiao et al. 2001). In soybean, the R gene candidates DD6 and KR1 were also induced during the HR (Seehaus et al. 1998; He et al. 2003).

AdDR11 and AdDR51: Thaumatin like protein and lipid transfer protein

Thaumatin like proteins are class-V pathogenesis related proteins with antimicrobial activity (Van Loon et al 2006). Lipid transfer proteins (LTPs) belong to PR-14 family and known for antifungal and anti microbial activity (Van Loon et al 2006).

AdDR13: Cyclophilin

Cyclophilins (Cyps) have an endogenous enzymatic peptidyl-prolyl cis–trans isomerase (PPIase or rotamase) activity that catalyzes the cis–trans isomerization of the amide bond between a proline residue and the preceding amino acid residue. They can aid protein folding and serve as molecular chaperones, which are involved in various stress responses (Marivet et al. 1995; Romano et al. 2004). The antifungal activity of cyclophilins was also reported for some isoforms of Cyps (Ye and Ng 2000; Ye and Ng 2001). The functional role of Cyclophilins in plant-microbe interactions is not clearly understood.

AdDR17: 1-deoxy-D-xylulose 5-phosphate synthase

Isopentenyl diphosphate (IPP), which is the central intermediate in the biosynthesis of isoprenoids, is produced by the mevalonate pathway and the recently discovered deoxyxylulose 5-phosphate (DXP) pathway. 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) catalyzes the condensation of glyceraldehyde-3-phosphate and ‘‘activated acetaldehyde’’ generated from pyruvate (Lange et al 2000). Recent study proves the requirement for DXPS2-dependent MEP pathway-based isoprenoid products to sustain mycorrhizal functionality at later stages of the symbiosis in *Medicago truncatula* (Floß et al. 2008). It will be interesting to study in legumes if terpenoids also play a role in both symbiotic and pathogenic interactions like isoflavanones.

AdDR19: glutathione S-transferase (GST)

GSTs are dimeric enzymes that catalyse the conjugation of electrophilic molecules to glutathione. In plants, these conjugates are sequestered in the vacuole, where they are further processed and detoxified. Stress-inducible GSTs also have glutathione peroxidase activity, thereby protecting cells from oxidative injury by detoxifying organic hydroperoxides of fatty and nucleic acids (Dixon et al. 2002). The proposed function of GST is the detoxification of organic peroxides to prevent continuing cell death caused by free radicals produced during the hypersensitive response in the incompatible interaction (Wagner et al. 2002).

AdDR20: putative translation initiation protein

Very recent study demonstrates that translation initiation protein is a key element of the signal transduction pathway resulting in plant programmed cell death (Hopkins et al. 2008).

AdDR31: lysin motif-type receptor-like kinase

The cascade of plant responses leading to nodule formation is triggered by the recognition of bacterially produced lipo-chitooligosaccharide Nod factors by a family of LysM receptor kinases (Samac and Graham 2007). LysM domains may also be involved in pathogen recognition. (Kaku et al. 2006; Knogge and Scheel 2006).

AdDR32: pyridoxine biosynthesis protein

Vitamin B_6 represents a highly important group of compounds ubiquitous in all living organisms. It has been demonstrated to alleviate oxidative stress and in its phosphorylated form participates as a cofactor in >100 biochemical reactions (Wagner et al. 2006). It was also shown to play essential role in plant salt tolerance (Shi et al. 2002).Vitamin B_6 is also a strong antioxidant with potential importance during the plant-pathogen defense response (Denslow 2005).

AdDR33: Proline dehydrogenase

This enzyme catalyzes the first step of the degradation of proline to glutamic acid. Proline is the most diversely used osmolyte that accumulates in osmotically stressed organisms, and in plants its concentration is controlled by the balance of biosynthesis and metabolism. Proline dehydrogenase (ProDH) was transiently induced both in tobacco and in *Arabidopsis* upon *R. fascians* infection (Simón-mateo et al. 2006). Proline dehydrogenase homologues in maize and barley are up-regulated by a compatible infection with the corresponding species-specific rust. Proline catabolism plays an as yet undefined role in the interaction between rust fungi and the host plant (Ayliffe et al. 2002).

AdDR34: Papain-like cysteine proteinase

Cysteine proteases have emerged as key enzymes in the regulation of animal PCD. In soybean cells, PCD-activating oxidative stress induced a set of cysteine proteases. Inhibition of the cysteine proteases by ectopic expression of cystatin, an endogenous cysteine protease inhibitor gene, inhibited induced cysteine protease activity and blocked PCD triggered either by an avirulent strain of *Pseudomonas syringae* pv *glycinea* or directly by oxidative stress (Solomon et al. 1999). *Rcr3* (papain-like cysteine endoprotease) is specifically required for the function of *Cf-2*, a *Lycopersicon pimpinellifolium* gene bred into cultivated tomato (*Lycopersicon esculentum*) for resistance to *Cladosporium fulvum* (Krüger et al. 2002). The highest levels of up-regulation of *StCathB* (encoding the cysteine protease cathepsin B) were observed early

in *R* gene-mediated resistance in potato upon infection with *Phytophthora infestans* (Avrova et al. 2004). Gilroy et al. (2007) demonstrated that the plant cysteine protease cathepsin B is required for the disease resistance hypersensitive response (HR). VIGS of cathepsin B prevented programmed cell death (PCD) and compromised disease resistance induced by two distinct non-host bacterial pathogens. An excellent review by Shindo and Van der hoorn (2008) gave a comprehensive coverage of the role played by papain-like cysteine protease in plant-pathogen interactions.

AdDR36: Harpin binding protein

Since its discovery as a plant protein interacting with pathogen typeIII effector molecule harpin, its functional role in plants is not clear yet. Harpin binding protein trancript was found to be up regulated up induction with lipopolysaccharides from *Burkholderia cepacia* in *Nicotiana* (Sanabria and Dubery 2006).

AdDR37: HSP 70

Kanzaki et al. (2003) demonstrated the involvement of Hsp90 and Hsp70 in defense signaling of plants. Challenge with *P. cichorii* revealed that HR and non-host disease resistance were severely compromised in NbHsp90c-1 and NbHsp70c-1 silenced *N. benthamiana* plants. During hypersensitive reaction, Hsp70 was found to be upregulated in coffee as an early response gene (Fernandaz et al. 2004).

AdDR 38 and AdDR39: Dirigent like proteins.

We isolated two cDNA clones, AdDR38 and AdDR 39, which shows high homology with dirigent like proteins. Dirigent proteins are involved in directing stereoselective phenolic radical coupling in the biosynthesis of lignans from two molecules of coniferyl alcohol in the phenyl propanoid pathway (Davin et al. 1997). Dirigent protein coding genes were found to be differentially regulated during mechanical wounding and weevil feeding in spurse (Ralph et al. 2006b). Dirigent-like genes were also highly induced in cotton (*Gossypium barbadense* and *G. hirsutum*) after infection by *Verticillium dahliae* (Zhu et al. 2007).

Other cloned genes like PAL, C4H and CAD are part of the phenylpropanoid pathway. Chaperonin 60 alpha subunit, which is hsp60 family protein is also showed to be upregulated during HR along with Hsp70. Similarly, genes coding for 60S ribosomal subunit and Rubisco activase were also shown to be differentially regulated during plant defense responses. There are several hypothetical proteins and unknown/ unclassified proteins, whose function is yet to be established in plants.

4.3.3 Expression analysis of selected genes in response to *P. personata* infection

The role 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. Time course expression analysis was performed for cloned genes involved in phenylpropanoid pathway and lignification process. Among the genes cloned, three genes were part of phenylpropanoid pathway i.e Phenylalanine ammonia lyase (PAL), Cinnamate 4-hydroxylase (C4H) and cinnamyl alcohol dehydrogenase (CAD).Two genes encoding dirigent like proteins were cloned which functions in liginification process. Semi-qunatitative RT-PCR was performed to observe the variations in expression pattern of these five genes in response to *P. personata* infection.

PAL got upregulated by 24 hours post inoculation (hpi) and reached to basal levels by 36 hpi and again rebound by 72 hpi. C4H has shown initial upregulation at 36 hpi followed by decline and reaching maximum expression by 60 hpi. CAD has shown upregulation only at 36 hpi and maintained steady state levels at all other time points observed. Among the two dirigent proteins named as DIR1 and DIR2, whose expression is analyzed, DIR2 maintained higher transcript levels at all time intervals compared to 0 hpi. On the other hand the DIR1 exhibited a very interesting expression pattern. Although the primers used for amplification of DIR1 were designed against ORF as well as 3' UTR regions, still the amplification resulted in appearance of two bands. In control or mock treatment these two bands were observed in all the time points, whereas in treated samples at 24 hpi and 60 hpi smaller fragment disappeared completely, at the same time the intensity of the larger fragment was increased (Fig 4.2). All the genes studied did not

show any prominent fluctuation in transcript levels in control or mock treated samples (Fig 4.2).

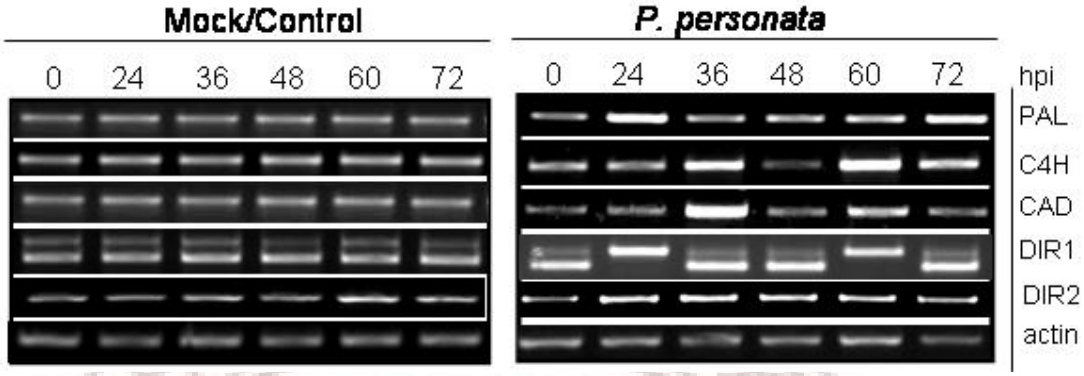

Figure 4.2 Transcript analysis in Control/Mock and *P. personata* treated leaves. Semi-quantitative RT-PCR was employed to study the expression analysis of PAL, C4H, CAD, DIR1, and DIR2 during a time course of 0 to 72 hours post inoculation (hpi). Actin served as the internal control. PAL: Phenylalanine ammonia lyase; C4H: Cinnamate 4-hydroxylase; CAD: Cinnamyl alcohol dehydrogenase; DIR: Dirigent like protein.

Many crop plants are susceptible to pathogens because of years of selective breeding leading to the removal of natural products found in their more resistant, wild counterparts (Stuiver and Custers 2001). This might be the case also in peanut, but with out much knowledge of secondary metabolites or their levels in peanut and its wild relatives and due to lack of genomic information, which will hinder the study of expression levels of various key genes involved in secondary metabolism, it can not be concluded.

An almost ubiquitous feature of plant responses to incompatible pathogens or to elicitors is the activation of phenylpropanoid metabolism in which phenylalanine ammonia-lyase (PAL) catalyses the first committed step of the core pathway of general phenylpropanoid metabolism (Goâmez-vaâsquez et al. 2004). Typically, the accumulation of PAL activity and mRNA is more rapid, higher and longer lasting in

incompatible plant-pathogen interactions (Cui et al. 1996). The higher transcript levels of PAL as early as 24 hpi and again at 72 hpi is in accordance with the incompatible interactions observed in other plant pathogen interactions. For example, upon inoculation with orange rust, two peaks of PAL activity were observed in resistant coffee plants 2 days and 5 days post inoculation (Silva et al. 2002). In contrast to this, only one peak was observed in susceptible coffee plants 5 days post inoculation (Silva et al. 2002). Cinnamate 4 hydroxylase is a cytochrome P450 monooxygenase (P450s) catalyzing hydroxylation of cinnamic acid to 4-coumaric acid in the second step of phenylpropanoid pathway. CAD catalyzes the last step in the production of the monolignols, the reduction of cinnamaldehydes into cinnamyl alcohols. Both C4H and CAD are known to be induced in response to pathogens and elicitors in several plant-microbe interaction studies (Betz et al. 2001; Zabala et al. 2006).

There could be several possibilities for differential regulation of various phenylpropanoid pathway genes. Certain phenylpropanoid intermediates are known to have feedforward (Loake et al. 1992) or feedback (Blount et al. 2000) regulatory properties acting at the level of enzyme activity or gene transcription (Mavandad et al., 1990; Loake et al. 1992). For example, cinnamic acid has been shown to inhibit PAL expression at the transcriptional level (Mavandad et al. 1990; Blount et al. 2000). Other possibility is that all these genes were coded by multigene families (Dixon et al 2002). For example, seven different PAL or PAL -like gene transcripts were found in *M. truncatula* EST libraries and CAD has ten or more members (Dixon et al. 2002). Some of the genes annotated as encoding a particular enzyme may in fact encode related enzymes with different functions (Dixon et al 2002).

Legumes, in particular, use phenylpropanoid compounds as both phytoalexins and signal molecules for the attraction of symbiotic microbes, and the independent regulation of such pathways would be necessary (Dixon et al 2002). Different members of a single gene family may have different induction patterns (upregulation or downregulation) in response to stress or development. Hence, there is a necessity for more gene-selective approaches to expression profiling than the often non-discriminatory RNA gel blot analyses (Dixon et al 2002). Keeping this in mind, we designed our primers directed against ORF and 3' UTR regions for RT-PCR analysis, which might reduce the chances

of amplification of other family members to some extent. Although we can not completely rule out the possibility of other family members being amplified.

Dirigent proteins are involved in directing stereoselective phenolic radical coupling in the biosynthesis of lignans from two molecules of coniferyl alcohol in phenyl propanoid pathway. Lignans have antifungal, antibacterial, and anti-insect activities (Davin and Lewis, 1992). Apart from their role in lignification, the other possible role of lignins in plant defense is yet to be determined. Dirigent proteins were shown to be induced in response to fungal infection (Rinaldi et al. 2007; Coram et al. 2008) and insect and mechanical wounding (Ralph et al 2006b; 2007). In case of DIR1, the present expression studies suggest a possible chance of alternative splicing taking place. Other possibility is that, there are two genes with similar ORF and 3' UTR regions complementary enough for primer annealing. In either case, it will be an interesting phenomenon, because of the disappearance of one band completely at one point of time with increased levels of the other corresponding band. Further work is required to confirm possibility of alternative splicing, which is well known in plant defense especially in case of R genes like N-gene (Dinesh-Kumar and Baker 2000) and RPS4 (Zhang and Gassmann 2007) of tobacco and *Arabidopsis* respectively.

It is surprising that in two previous studies of *A. hypogaea* and *P. personata* interaction at molecular level using micro array of EST libraries (Luo et al. 2005b) as well as Subtractive hybridization (Nobile et al. 2008) did not report the upregulation of genes like PAL, C4H, CAD and dirigent proteins. In contrast, we identified and cloned these genes using random arbitrary primers in wild *Arachis*, which practically covers less transcriptome compared to the above two techniques. It is not known whether these genes were not upregulated in *A. hypogaea* within the time periods studied or they were not identified by the above techniques.

The expression studies suggest that phenylpropanoid pathway play a very important role in defense responses of *A. diogoi* to *P. personata*. Further work aiming at the comparison of expression pattern of these genes in a susceptible species will advance the understanding of the key role they hold in defense against *P. personata* as well as other pathogens. The previous works on *P. personata* and *A. hypogaea* interaction showed only the higher transcript level of caffeic acid methyl transferase (Luo et al.

2005b; Nobile et al. 2008) and cinnaamoyl-CoA reductase (Nobile et al 2008) of phenylpropanoid pathway. Hence, it will be interesting to study the whole set of phenylpropanoid pathway genes, once more and more genomic resources for *Arachis* increases. Identification of regulatory molecules upstream to Phenylpropanoid pathway which are regulating the expression of different genes of pathway with very high specificity would also help in interpreting the control mechanisms plants utilize in regulating a family of genes with utmost precision.

Table 4.2 Sequence details of the oligoes used in expression analysis

Oligo Name	Forward	Reverse
PAL	GGAACCGGTATGCTCACC	GCATCATGTATTTCATTGGCA
C4H	GCGAAATCAACGAAGACAAC	AATGGGAACAAGCAGAGTTAC
CAD	GTTCACTACTGTGGACTCC	CGGGTTTATGCATAGCACC
DIR1	CTACACAATGGTGACACAAC	AGTCAAATGCAGTTGTATTAAC
DIR2	CTACACAATGGTGACACAAC	GCAGTTGTATTTACTTTATACG
Actin	TGGCATCACACTTTCTACAA	CAACGGAATCTCTCAGCTCC

4.4 Summary

Arachis diogoi, a diploid wild relative of peanut, exhibits high resistance to several pathogens and pests including resistance against *Phaseoisariopsis personata,* the causal agent of late leaf spot in peanut. Sixty partial cDNA clones were identified and cloned as transcripts upregulated in *A. diogoi* in response to *P. personata* infection during the early stage of interaction. The isolated partial cDNAs were annotated based on the best match found in blastx alignment against protein databases at NCBI. Annotated clones were assigned with putative function based on their sequence homology with other genes in the database. Majority of the cloned genes were well known for their role in plant defense (hypersensitive cell death) or stress responses, apart from that genes involved in secondary metabolism and unknown/hypothetical proteins or sequences with no similarity found in public sequence databases are more in number. Isolated partial cDNAs were annotated to genes classified as receptors like Leucine rich repeat protein, cell signaling components like CBL-interacting protein kinase, transcriptional regulators like High mobility group (HMG) protein, Antioxidants like glutathione S-transferase (GST), Defense related proteins like Thaumatin like protein, lipid transfer protein, Papain-like cysteine proteinase, and components of secondary metabolism like PAL, C4H, CAD and 1-deoxy-D-xylulose 5-phosphate synthase were present. Time course expression analysis of genes encoding proteins involved in phenylpropanoid pathway and lignification show differential regulation pattern for each gene. Early regulation of the genes involved in phenylpropanoid pathway and lignification suggest that they play a very important role in defense responses of *A. diogoi* against *P. personata.*

Chapter 5

Functional characterization of *Arachis diogoi* cyclophilin (*AdCyp*) in plant defense response

5.1 Introduction and Background

5.1.1 Cyclophilins

Cyclophilins (Enzyme Commission (EC) number 5.1.2.8) were originally identified as the cellular targets of immunosuppressant drugs. There are two ubiquitous protein families: the FK-506 binding proteins or FKBPs and cyclosporin-binding proteins or cyclophilins together called as immunophilins. Immunophilins are present in organisms ranging from bacteria to animals and plants. These proteins are characterized by their peptidyl–prolyl cis–trans isomerase (PPIase) enzymatic activity (Romano et al. 2004). While immunophilins are defined by their ability to bind immunosuppressant ligands, they share their PPIase activity with a third family of proteins known as the parvulins (Fischer and Aumüller 2003). Recent studies have revealed that many immunophilins possess a chaperone function independent of PPIase activity (Barik 2006).

PPIase: Role in Protein folding

Biological processes in the cell are extremely dynamic and complex events that are superbly choreographed, both spatially and temporally. In order to perform their function within the cell, newly synthesized proteins are rapidly and efficiently converted from their primary linear sequences into their well-defined functionally competent tertiary structures. Most proteins possess peptide bonds connected in the *trans* conformation in both the newly synthesized unfolded form and in the folded native structure; however, prolyl bonds occur in both *cis* and *trans* conformations there by providing an intrinsic backbone switch that is controlled by prolyl *cis-trans* isomerization (Lu et al. 2007). Peptidyl–prolyl *cis–trans* isomerases catalyze the rapid isomerization of prolyl bonds from the *cis* to the *trans* configuration. *Cis* -prolyl bonds are uncommon, being most likely due to unfavorable contacts between adjacent amino acid residues in this isomeric form, whereas the *trans* isomer usually takes preference (MacArthur and Thornton 1991; Reimer and Fischer 2002). The ability of prolyl isomerases to catalyze conformational rearrangements of folded proteins likely provides the basis for their participation in

different biological functions. Cyclophilins participate in the protein folding process not only as prolyl isomerases but also as chaperones (Barik 2006).

Cyclophilins: Mammals, yeast and plants

Cyclophilins have been found in mammals, plants, insects, fungi, and bacteria. They are structurally conserved throughout evolution and all have PPIase activity (Wang and Heitman 2005). There are 7 major cyclophilins in humans - hCypA, hCypB, hCypC, hCypD, hCypE, hCyp40 (40 kDa), hCypNK and a total of 16 unique proteins (Galat 2003). Drosophila has at least 9 cyclophilins (Galat 2003) and the model plant *Arabidopsis thaliana* has 29 putative cyclophilins (He et al. 2004), whereas 8 cyclophilins, Cpr1-Cpr8, have been found in *Saccharomyces cerevisiae* (Arevalo-Rodriguez et al. 2004). In mammals, CypA and Cyp40 are cytosolic whereas CypB and CypC have amino-terminal signal sequences that target them to the ER protein secretory pathway (Galat 2003). CypD has a signal sequence that directs it to the mitochondria (Andreeva et al. 1999). In Arabidopsis, nine of the cyclophilins are predicted to be cytosolic, five are predicted to be targeted to the secretory pathway and two to the mitochondria (Romano et al. 2004), although the precise localization of the majority of these isoforms awaits experimental corroboration. The yeast cyclophilin Cpr1 is a homolog of hCypA that shares 65% identity in amino-acid sequence and is present in the cytoplasm and also enriched in nuclei (Arevalo-Rodriguez et al. 2005). AtROC1 is the *Arabidopsis* ortholog of CypA and Cpr1.

Several protein-folding processes depend on the catalytic and/or chaperone-like activities of cyclophilins. For example, CypA promotes both the formation and the infectivity of virions of the human immunodeficiency virus (HIV)-1 (Luban et al. 1993; Sokolskaja et al. 2004). CypA is incorporated into HIV-1 virions, where it interacts with HIV-1 Gag, and the polyprotein precursor of virion structural proteins. Cyclophilins can also act as modulators of protein function. CypA has been associated with YY1, a zinc-finger suppressor of gene transcription (Yang et al. 1995), and Zpr1, an essential zinc-finger protein (Ansari et al. 2002). The CPR1, yeast ortholog of hCypA is involved in controlling meiosis in yeast (Arevalo-Rodriguez et al. 2004). Plant cytosolic cyclophilin transcripts were shown to be induced by various biotic and abiotic factors (Marivet et al.

1994; 1995; Birch et al. 1999). AtROC1 was found to interact with *Agrobacterium tumefaciens* VirD2 protein (Deng et al. 1998) and a type III effector molecule AvrRpt2 (Coaker et al. 2005). One of the cyclophilins in secretory pathway has been shown to be under the control of NPR1 in *Arabidopsis* (Wang et al. 2005).

Most of the reports on cyclophilin in plant-microbe interactions are confined to its transcript regulation, with no direct evidence in plant defense. Due to the functional redundancy, analysis of mutants (Loss of function) is not a feasible option. Gain of function through overexpression or ectopic expression can only help in understanding the role of cyclophilin in plant physiology and responses against various environmental cues.

5.1.2 With this background, we formulated the following objectives

1. 5' RACE to get the full length cDNA of the partial cyclophilin cDNA identified as an early response gene in *A. diogoi* upon infection with *P. personata.*
2. Sequence and phylogenetic analysis of the isolated full length cyclophilin.
3. Isolation and *in silico* analysis of upstream promoter region of cyclophilin (*AdCyp*).
4. Recombinant AdCyp protein expression and purification.
5. Generation and molecular analysis of *AdCyp* overexpressing tobacco transgenic plants.
6. Evaluation of disease resistance in AdCyp transgenic plants.

5.2 Materials and Methods

5.2.1 Plant materials and Pathogen inoculations

As described earlier in sections: 4.2.1 and 4.2.2

5.2.2 5' RACE to isolate full length cDNA and Isolation of the genomic clone

Performed as described earlier in sections: 3.2.7 and 3.2.8.

The primers utilized for RACE-PCR were cyclo 262R and cyclo 141R respectively. The primer sequences were given in Table 5.1. ORF-F and ORF-R primers (Table 5.1) used for amplification of genomic clone of *AdCyp* using genomic DNA of *A. diogoi* as template.

5.2.3 Sequence alignments and phylogenetic analysis

As described earlier in sections: 3.2.6

5.2.4 Isolation of upstream promoter region

Upsream promoter region of *AdCyp* was cloned using Universal Genome Walking kit (Clontech, USA) following manufacturers instructions. In brief, *A. diogoi* genomic DNA was digested with different blunt cutters like *Dra*I, *Eco*RV, *Stu*I and *Ssp*I. Genome walker adaptors were ligated to restriction digested DNA fragments. Primary PCR was performed using adaptor primer 1 (AP1) and gene specific primer 1 (GSP1). Diluted primary PCR products were amplified using Adaptor primer 2 (AP2) and gene specific primer 2 (GSP2). The amplified products were run on 1.5% agarose gel, eluted, cloned into pTZ57R and sequenced. GSP1 and GSP2 primers were designed based on the *AdCyp* cDNA sequence (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 *P. personata* induced expression of *AdCyp*

AdCyp expression analysis in response to *P. personata* was performed using semi-quantitative RT-PCR as described in sections: 4.2.3 and 4.2.7.

An internal control reaction was performed using AdCyp-511F designed against promoter region and AdCyp ORF-R designed against coding region, to confirm that the amplification of *AdCyp* was from cDNA and not from the genomic DNA.

5.2.6 Bacterial expression and purification of Trx-His tagged AdCyp and analysis of antifungal activity of purified recombinant protein

AdCyp ORF was cloned into *Nco*I and *Xho*I sites of pET 32a vector after digesting it with corresponding restriction enzymes to obtain thioredoxin–histidine tagged (Trx-His tagged) AdCyp. Sequencing of the construct confirmed that AdCyp ORF was inserted in frame with the vector. AdCyp**:**pET32a was introduced into *E. coli* BL21 (pLysS) and recombinant protein expression was induced by adding 0.4 mM IPTG to logarithmic phase bacteria grown in LB medium. The cells were harvested by centrifugation after 4 h, washed with buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl), and sonicated. The supernatant containing the recombinant His-tagged protein was collected by centrifugation for 15 min at 12,000 rpm. The recombinant fusion protein was then affinity-purified by Ni^{2+}–NTA resin and eluted with 0–500 mM imidazole gradient. The eluted protein was dialyzed with 10 mM phosphate buffer (pH 7.0). Antifungal activity of purified recombinant AdCyp protein was performed against *Rhizoctonia solani* and *Botrytis cinerea* by placing the filter paper discs containing different concentrations (100µg, 200µg and 300µg) of protein 0.5 cm away from the hyphal growth on potato dextrose agar.

5.2.7 Generation of transgenic tobacco plants overexpressing *AdCyp.*

Construction of binary vectors and generation of transgenic plants was performed as described earlier in section: 3.2.12

The *AdCyp* ORF-F and ORF-R primers were used to amplify the AdCyp ORF (Table 5.1). Restriction sites *Nco* I and *Xba* I were used for cloning *AdCyp* ORF into plant

expression vector pRT100. *Hin*dIII was used for further subcloning into pRD400 binary vector to obtain AdCyp: pRD400.

5.2.8 Southern blot analysis and Semi-quantitative RT-PCR

Performed as described earlier in sections: 3.2.9 and 3.2.10

Genomic organization of *A. diogoi* cyclophilin genes was analyzed with Southern hybridization using cyclophilin partial cDNA (AdDR-13), isolated using GeneFishing DEG premix kit described in section: 4.2.4 and Table 4.1, as a probe. Transgene copy number was analyzed using *nptII* as probe. Semi-qunatitative RT-PCR for analyzing *AdCyp* expression levels in transgenic plants was performed using AdCyp ORF-F and ORF-R primers. Actin amplified using primers described in Table 5.1 served as an internal control.

5.2.9 Bioassay of *AdCyp* transgenic plants against *Phytophthora parasitica* var. *nicotianae*

Detached leaf assay was performed to analyze the disease resistance of *AdCyp* transgenic plants against fungal pathogen *Phytophthora parasitica* var. *nicotianae.* The fungus was grown on potato dextrose agar (PDA) (Himedia, India) for 5-7 days at 24°C. Fully expanded leaves of two month old plants of wild type (WT) and transgenic plants (3, 16 and 21) were placed on moist filter papers for fungal inoculation. A 0.5cm diameter plug of fungal mycelium grown on PDA was placed on the middle of the adaxial side of leaf after damaging the leaf with a razor blade to promote fungal infection. The leaves were placed in growth room with 16h: 8h of light: dark photoperiod. Leaf damage and symptoms were recorded after seven days. All the experiments were performed in duplicate.

5.2.10 Analysis of defense related transcripts in *AdCyp* transgenic plants

Performed as described earlier in section: 3.2.10

Primer sequences were described earlier in table: 3.3

5.3 RESULTS

5.3.1 Isolation of *P. personata* induced cyclophilin partial cDNAs

Two clones corresponding to cyclophilin like proteins were identified in a screen for genes which were upregulated in response to *P. personata* infection in *Arachis diogoi* as described in section: 4.2.4. Two clones (AdDR-13 and AdDR-60) were isolated using different ACP primers and were varied in length based on the ACP primer annealing region. Pairwise sequence alignment showed that except for a couple of nucleotide differences and two stretches of nucleotide sequences in 3' UTR, the rest of the sequence was essentially similar (Fig 5.1). Translation of both the available partial sequences showed that both code for essentially similar amino acid polypeptide with a single amino acid variation (Fig 5.2), which could be possible due to nucleotide sequencing errors.

5.3.2 Genomic organization of AdDR-13 and AdDR-60 clones

To identify the genomic organization of AdDR-13 and AdDR-60 in *Arachis diogoi* genome, Southern analysis was performed. The hybridization pattern showed two prominent bands in all lanes along with a low intensity band (Fig 5.3). This simple hybridization pattern suggest that both AdDR-13 and AdDR-60 clones might be coded by two different genes and the third band detected could be a other closely related cyclophilin gene, as cyclophilins are represented by multigene families in plants (Chou and Gasser 1997).

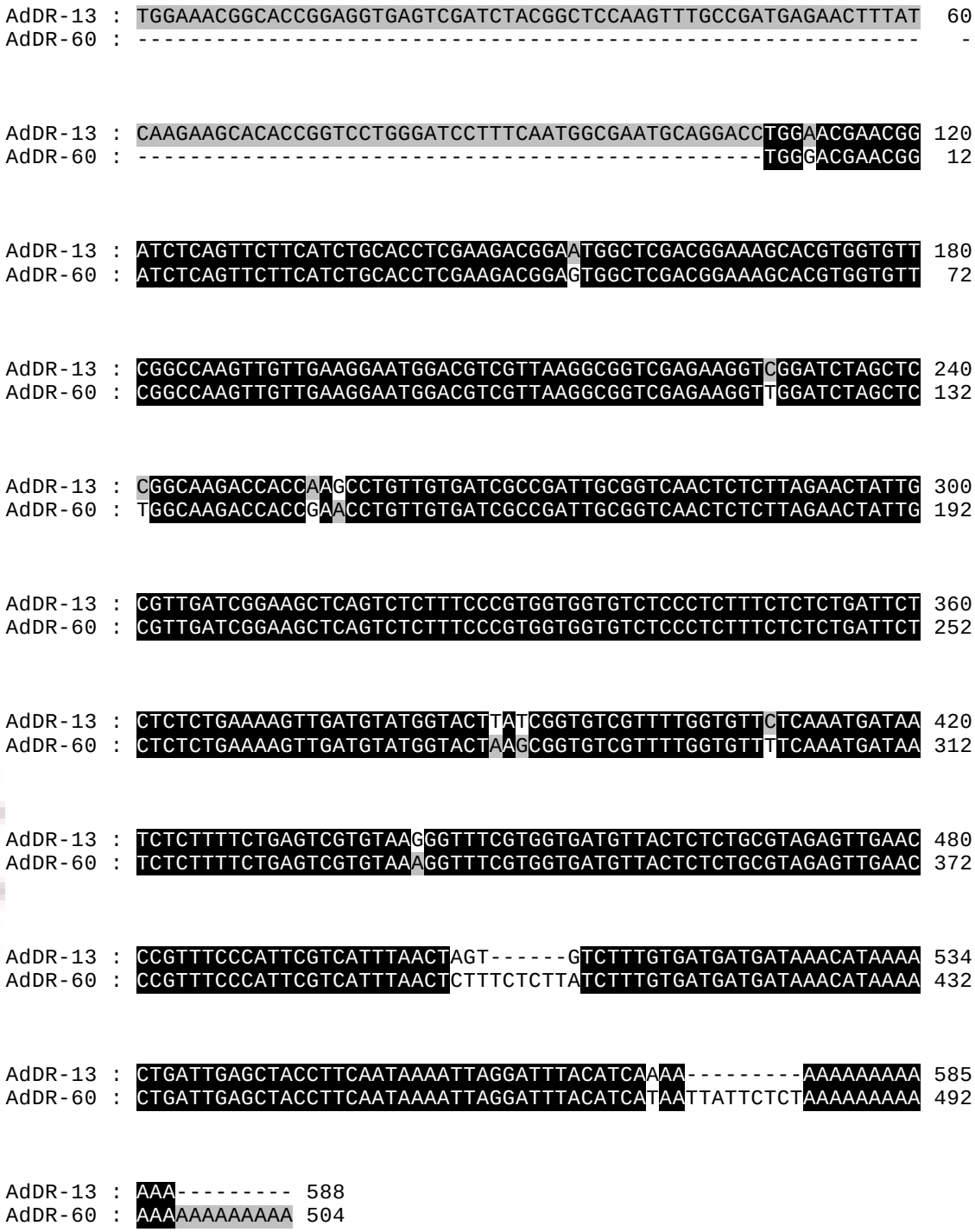

Figure 5.1 Pairwise sequence alignment of AdDR-13 and AdDR-60 partial cDNA nucleotide sequences.

```
AdDR-13 : GNGTGGESIYGSKFADENFIKKHTGPGILSMANAGPGTNGSQFFICTSKTEWLDGKHVVF  60
AdDR-60 : ------------------------------------GTNGSQFFICTSKTEWLDGKHVVF  24


AdDR-13 : GQVVEGMDVVKAVEKVGSSSGKTTKPVVIADCGQLS  96
AdDR-60 : GQVVEGMDVVKAVEKVGSSSGKTTEPVVIADCGQLS  60
```

Figure 5.2 Pairwise sequence alignment of deduced amino acid sequence of AdDR-13 and AdDR-60 partial cDNA clones

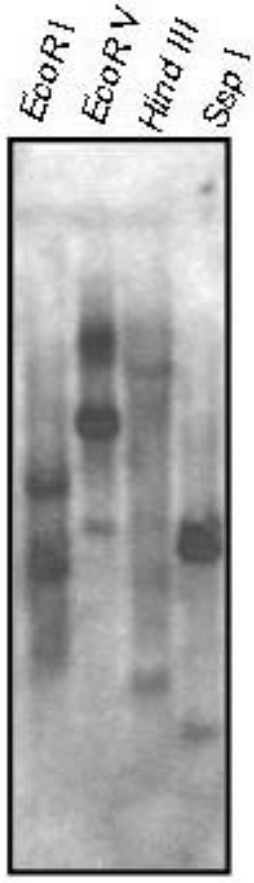

Figure 5.3 Genomic organization of AdDR-13 and AdDR-60 clones. *Arachis diogoi* genomic DNA was digested with the indicated restriction enzymes, fractionated on 0.8% agarose gel, blotted on to a nylon membrane and hybridized with AdDR-13 cDNA as a probe.

5.3.3 Isolation of full length cDNA, genomic clone and sequence analysis

One of the clones, AdDR-13 was further extended using 5' RACE to obtain the full length cDNA. The full length cDNA was designated as *AdCyp* for *Arachis diogoi* cyclophilin. The full length cDNA was 885 bp long potentially encoding a single polypeptide of 172 amino acids (Fig 5.4). A 69 bp 5' UTR region and a 297 bp 3' UTR region including the poly A tail were present flanking the open reading frame. The predicted polypeptide has a molecular weight of 18115.57 Da and theoretical *pI* of 8.68. Lack of any N-terminal signal sequence suggests a possible cytosolic localization for this protein. The genomic clone of *AdCyp* amplified using ORF-F and ORF-R primers using *A. diogoi* genomic DNA as template showed that *AdCyp* gene did not harbor any introns. The full length cDNA of AdCyp was submitted to the genbank under the accession number EU170616.

5.3.4 Multiple sequence alignment and phylogenetic analysis

Alignment of predicted amino acid sequence of *AdCyp* with close homologs from other organisms like *Arabidopsis thaliana* (AtROC1), *Saccharomyces cerevisiae* (ScCPR1) and *Homo sapiens* (HsCypA) shows that this protein is evolutionarily conserved. AdCyp and AtROC1 are characterized by a seven-amino acid insertion between amino acids 47 and 55 compared with HsCypA or ScCPR1 (Fig. 5.5). Phylogentic analysis of AdCyp with other Cyps shows that the closest homolog of AdCyp in *Arabidopsis thaliana* is AtROC1 with 86% similarity. Among human cyclophilin genes Cyclophilin A shows maximum similarity of 75% with AdCyp. Cyclophilin from *Lupinus luteus,* a legume and *S. tuberosam* shows 84% similarity with the AdCyp. Cyclophilins from monocots TaCyp and OsCyp of *Triticum aestivum* and *Oryza sativa* show 81% and 80% similarity respectively. PpCyp of *Physcomitrella patens* shows 76% similarity and AdCyp homolog from yeast ScCPR1 shows 69% similarity.

```
1     aaa acc cta aaa ttc tca tcc ttc ttc gtt gca ata cga atc gat   45

46    cga ttt ctt ttc tct aca gca aaa ATG GCT AAC CCT AAG GTT TAC   90
                                      M   A   N   P   K   V   Y    7

91    TTC GAC ATG TCC ATC GGA GGA CAA CCA GCC GGA AGA GTC GTC TTC   135
8      F   D   M   S   I   G   G   Q   P   A   G   R   V   V   F    22

136   GAG CTC TTC GCC GAC ACG GTT CCC CGC ACC GCC GAG AAT TTT CGG   180
23     E   L   F   A   D   T   V   P   R   T   A   E   N   F   R    37

181   GCC CTC TGC ACC GGT GAG AAA GGC GTC GGT CGC GGC GGC AAG CCT   225
38     A   L   C   T   G   E   K   G   V   G   R   G   G   K   P    52

226   CTC CAC TAC AAG GGA TCA TCC TTC CAC CGT GTG ATC CCT AAC TTC   270
53     L   H   Y   K   G   S   S   F   H   R   V   I   P   N   F    67

271   ATG TGT CAG GGA GGT GAC TTC ACC GCT GGA AAC GGC ACC GGA GGT   315
68     M   C   Q   G   G   D   F   T   A   G   N   G   T   G   G    82

316   GAG TCG ATC TAC GGC TCC AAG TTT GCC GAT GAG AAC TTT ATC AAG   360
83     E   S   I   Y   G   S   K   F   A   D   E   N   F   I   K    97

361   AAG CAC ACC GGT CCT GGG ATC CTT TCA ATG GCG AAT GCA GGA CCT   405
98     K   H   T   G   P   G   I   L   S   M   A   N   A   G   P   112

406   GGG ACG AAC GGA TCT CAG TTC TTC ATC TGC ACC TCG AAG ACG GAG   450
113    G   T   N   G   S   Q   F   F   I   C   T   S   K   T   E   127

451   TGG CTC GAC GGA AAG CAC GTG GTG TTC GGC CAA GTT GTT GAA GGA   495
128    W   L   D   G   K   H   V   V   F   G   Q   V   V   E   G   142

496   ATG GAC GTC GTT AAG GCG GTC GAG AAG GTT GGA TCT AGC TCC GGC   540
143    M   D   V   V   K   A   V   E   K   V   G   S   S   S   G   157

541   AAG ACC ACC AAA CCT GTT GTG ATC GCC GAT TGC GGT CAA CTC TCT   585
158    K   T   T   K   P   V   V   I   A   D   C   G   Q   L   S   172

586   TAG aac tat tgc gtt gat cgg aag ctc agt ctt ttt ccc gtg gtg   630
173    *

631   gtg tct ccc tct ttc tct ctg att ctc tct ctg aaa agt tga tgt   675

676   atg gta cta agc ggt gtc gtt ttg gtg ttt tca aat gat aat ctc   720

721   ttt tct gag tcg tgt aag ggt ttc gtg gtg atg tta ctc tct gcg   765

766   tag agt tga acc cgt ttc cca ttc gtc att taa cta gtg tct ttg   810

811   tga tga tga taa aca taa aac tga ttg agc tac ctt caa taa aat   855

856   tag gat tta cat caa aaa aaa aaa aaa aaa   885
```

Figure 5.4 Nucleotide and deduced amino acid sequences of *AdCyp*. The nucleotides and amino acids are numbered. Asterisk (*) indicates the stop codon.

Figure 5.5 Alignment of deduced amino acid sequences of AdCyp with closely related Cyps from other organisms. Ad: *Arachis diogoi* At: *Arabidopsis thaliana*, Hs: *Homo sapiens*, Sc: *Saccharomyces cerevisiae*

5.3.5 Isolation and *in silico* anlysis of 5' upstream promoter region of *AdCyp*

Using genome walking approach a 841 bp 5' upstream promoter region of *AdCyp* was obtained (Fig 5.6). Based on the sequence of the 5' RACE products the putative transcription start site was identified and designated as +1 (Fig 5.6). Transcription start site is present 69 bp upstream of translation initiation codon ATG. The sequence of *AdCyp* promoter region was analyzed for potential *cis*-acting regulatory elements using the PLACE database (www.dna.affrc.go.jp/htdocs/PLACE/) (Higo et al. 1999). A putative TATA box was found at –34 bp position upstream to transcription start site. *In silico* analysis showed that several biotic and abiotic stress related *cis*-acting elements were overrepresented (Fig 5.6). Two W-BOX elements and several WRKY71 elements were found in the identified promoter region. W-BOX elements were recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins. WRKY71 element is the core of TGAC-containing W-BOX. Several GT1GMSCAM4 elements were also present in the promoter region. Apart from these, there are ABRE elements, known to be presnt in the ABA regulated genes and MYB and MYC transcription factor binding sites as well.

Figure 5.6 5' upstream promoter region of *AdCyp*. Transcription start site was desiganated as (+1). Putative *cis*-regulatery elements were shaded and named. The arrows indicate the presence of *cis*-element on positive or negative strand.

5.3.6 P. personata induced expression of *AdCyp*

As AdCyp was isolated as an early response gene upon infection with *P. personata* from *Arachis diogoi* and expression analysis of AdCyp was performed in time dependent manner from 0 to 72 hours post inoculation (hpi). AdCyp transcript levels were increased by 36 hpi and maintained till 72 hpi (Fig 5.7). On the other hand in control or mock treatment there was no significant variation in transcript levels. As the *AdCyp* is an intron less gene, PCR amplification of the same cDNA samples with primers designed against *AdCyp* promoter and ORF was performed. Which did not gave any amplification confirming that the amplification of *AdCyp* observed was in fact from cDNA and not from genomic DNA contamination (Fig 5.7).

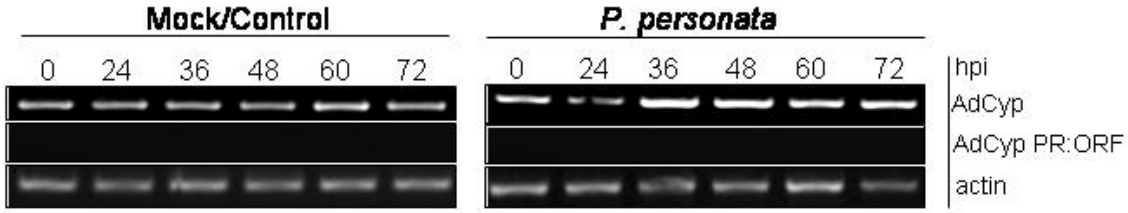

Figure 5.7 Time-course analysis of *AdCyp* expression in mock and *P. personata* treatments. Semi-quantitative RT-PCR was performed using total RNA extracted from samples collected at the indicated time intervals. AdCypPR:ORF represent the amplification of cDNA with primers designed against *AdCyp* promoter and ORF regions to observe the amplification of *AdCyp* from possible genomic DNA contamination.

5.3.7 Antifungal activity of AdCyp

AdCyp recombinant protein was obtained by expression in bacteria as Trx-His tagged fusion protein and after purification (Fig 5.8). As some of the previous reports suggested antifungal activity for plant cyclophlins, we analyzed the purified, recombinant protein, for antifungal activity using *Rhizoctonia solani* and *Botrytis cinerea*. There was no potent

antifungal activity was observed for the recombinant protein against the both the tested fungal pathogens.

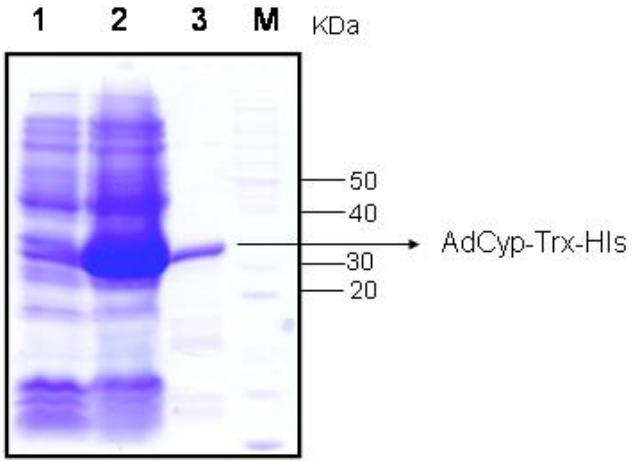

Figure 5.8 SDS gel showing the bacterial expression and purification of recombinant AdCyp protein. 1. Uninduced, 2. Induced with 0.4 mM IPTG, 3. Purified recombinant AdCyp protein, M. Protein molecular weight marker (kDa)

5.3.8 Generation of transgenic tobacco plants expressing *AdCyp* and their molecular analysis

Transgenic tobacco plants ectopically expressing *AdCyp* were generated by *Agrobacterium* mediated leaf disc transformation of tobacco (Horsch et al. 1985). Southern and semi-quantitative RT-PCR was performed for T_0 transgenics to know the copy number of the transgene as well as transgene expression levels in the transgenic plants. Two high expressing and one low expressing plants were selected for further analysis. All the three selected transgenics plants #3, #16 and #21 were harboring single copy of transgene (Fig 5.8A). Plants 3 and 21 were having high expression levels and plant 16 was having low expression levels of *AdCyp* (Fig 5.8B). T_0 plants were selfed for two generations to obtain T_2 seeds. T_2 Seeds from transgenic lines 3, 16 and 21 were germinated on 100mg/l kanamycin containing half strength MS medium, and resistant seedling were selected and transferred to soil and established in green house. Similarly,

wild type plants germinated on half strength MS medium with out antibiotics were also shifted to the green house.

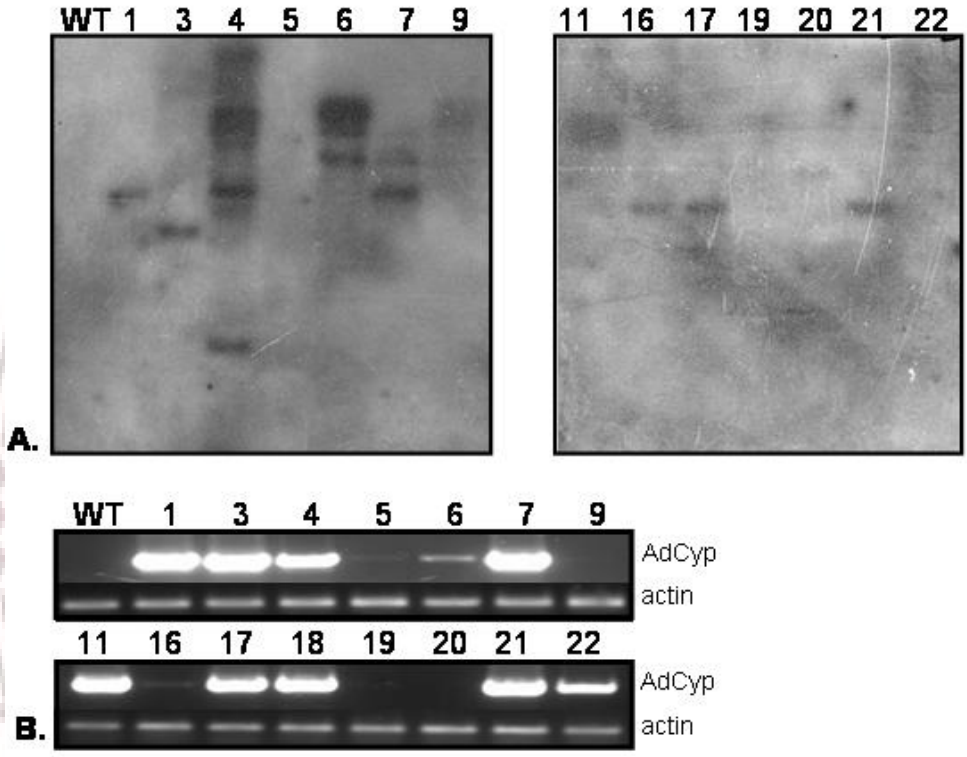

Figure 5.8 Molecular analyses of T_0 AdCyp transgenic tobacco plants.

A. Southern analysis of T_0 trangenic plants. Genomic DNA of wild type (WT) and T_0 transgenic plants were digested with *Eco* RI and electrophoresed, blotted and hybridized with [$^{\alpha\text{-}32}$P] -labelled *nptII* gene as probe

B. Semi-quantitative RT-PCR analysis of *AdCyp* expression in WT and transgenic plants. cDNA was synthesized from total RNA of WT and transgenic plants and amplified with *AdCyp* ORF-F and ORF-R primers. Actin served as internal control.

5.3.9 Disease resistance in *AdCyp* transgenic plants

Expression of AdCyp in T_2 trangenic plants was confirmed with RT-PCR (Fig 5.10). Leaves of WT and Transgenic lines 3, 16 and 21 were used to analyze the resistance of transgenic plants against black shank disease causing fungal pathogen *Phytophthora parasitica* var. *nicotianae.* Transgenic plants 3 and 21 with high *Adcyp* expression levels exhibited enhanced resistance against *P. parasitica* compared to WT plants (Fig 5.9). Whereas transgenic line 16, which is a low expressing line displayed delayed susceptibility against pathogen.

Figure 5.9 Resistance exhibited by *AdCyp* transgenic plants against *Phytophthora parasitica* var. *nicotiana.* Wild type (WT) and *AdCyp* transgenic plants (3, 21 and 16). Pictures were taken 7 days post inoculation (dpi).

5.3.10 Analysis of defense response gene transcripts levels in *AdCyp* transgenic plants

As *AdCyp* transgenic plants displayed enhanced resistance against *Phytophthora parasitica* var. *nicotianae*, transcript levels of various defense related genes were studied in transgenic plants using semi-qunatitative RT-PCR. *AdCyp* transgenic plants exhibited higher transcript levls of PR1a, acidic chitinase, osmotin, protease inhibitor I and

glucanase (Fig 5.10). Whereas PR1b, defensin, Lox1 and Lox3 transcript levels were similar in both WT and transgenic plants.

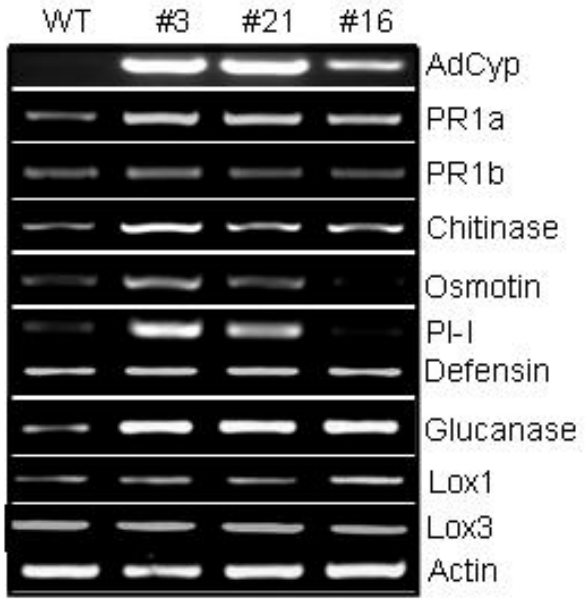

Figure 5.10 Transcript profile of defense response genes in WT and transgenic plants (3, 21 and 16). Semi-quantitative RT- PCR was performed using total RNA of WT and transgenic plants. PR: Pathogenesis Related, PI: Protease inhibitor, Lox: Lipoxygenase

5.4 Discussion

Cyclophilins are ubiquitous proteins with an intrinsic enzymatic activity of peptidyl-prolyl cis-trans isomerase and participate in the protein folding process not only as prolyl isomerases but also as chaperones (Ou et al. 2001). Two partial cDNAs encoding cyclophilin like proteins (AdDR-13 and AdDR-60 Refer to Section: 4.2.4) were isolated as early response genes from *A. diogoi* upon infection with *P. personata.* There are several studies in plant pathogen interaction, where cyclophilins were found to be induced (Birch et al. 1999; Bozkurt et al. 2007). Southern analysis suggested that both the partial cDNAs might be coded by different genes and there is atleast one more very closely related gene in the genome of *A. diogoi*. As both the clones were essentially coding the same polypeptide, one of the partial clones was extended using 5' RACE. An 885 bp full length cDNA was isolated with an open reading frame of 519 bp encoding a polypeptide of 172 amino acids with a predicted molecular weight of 18115.57 Da, and designated as *AdCyp* for *Arachis diogoi* cyclophilin. Predicted amino acid sequence contains the conserved tryptophan residue at position 128, which is necessary for cyclosporin A (CsA) binding (Liu et al. 1991).

Multiple sequence alignment shows that these proteins are conserved across the kingdoms. Analysis of genomic clone of *AdCyp* and also the available genomic information of homologs from other organisms like *AtROC1*, *LiCyp*, *ScCPR1* and *PpCyp* showed that they did not harbor any introns. Only exception is *HsCypA* which harbors four introns. *PpCyp* is a cyclophilin from *Physcomitrella patens,* a bryophyte for which whole genome sequence is available. All currently sequenced cytosolic Cyp genes of higher plants do not contain introns. In contrast, *ROC4* gene, encoding *Arabidopsis* chloroplast Cyp, contains six introns (Chou and Gasser 1997). Phylogenetic analysis shows that AtROC1 from *A. thaliana* is the closest homolog and showed a maximum similarity of 86% with AdCyp. A cyclophilin from legume LlCyp, which was shown to be induced during nodulation (Nuc et al. 2001) showed 84% similarity. AdCyp homologs from human and yeast, HsCypA and ScCPR1 showed 75% and 69% similarity respectively.

Analysis of *AdCyp* promoter exhibited several *cis*-regulatory elements known to be involved in biotic and abiotic stress responses suggesting that *AdCyp* might be

regulated in response to various stimuli. The presence of W-BOX, WRKY71 and GT1GMSCAM4 elements suggest a possible involvement in pathogen response. GT1GMSCAM4 plays a role in pathogen and salt-induced SCaM-4 gene expression (Park et al. 2004). However functional anlalysis of promoter using deletion analysis is required to confirm the role of different cis-regulatory elements in *AdCyp* expression in-planta.

Expression analysis of *AdCyp* exhibited an early upregulation at 36 hpi in response to *P. personata*, suggesting a possible role in plant defense response against pathogens. Early responding genes may have pivotal roles in downstream transcriptional regulation (Kirsch et al. 2001). Such early pathogen induced expression of cyclophilin genes were previously reported (Birch et al. 1999; Godoy et al. 2000; Kong et al. 2001; Campo et al. 2004; Dubey 2007). While there are number of reports suggesting a role for cyclophilins in plant–microbe interactions, yet there is no direct role assigned to them. Cyclophilin were identified as host eukaryotic factors utilized by microbes for pathogenesity (Kromina et al. 2008). For example, Protein VirD2 of *Agrobacterium tumefaciens* binds to Cyps of *A. thaliana* and inhibition of interaction of VirD2 and Cyps hampers the T-DNA transfer into the nucleus (Deng et al. 1998). Polypeptide Gag of human immunodeficiency type 1 virus (HIV-1) is found in mature virions and has high affinity to most of 15 tested human Cyp proteins (Braaten et al. 2001). Till date, there was no direct role of cyclophilins proved in plant defense except for its involvement in RPS2 mediated resistance in *A. thaliana* (Axtell and Staskawicz 2003, Coaker et al. 2005). AtROC1, which is the ortholog of AdCyp from *A. thaliana* was involved in activation of pathogen effector molecule, AvrRpt2 in host plant cells. The activated AvrRpt2 cleaves RIN4 (Coaker et al. 2005). Because RIN4 is a negative regulator of the R protein RPS2, elimination of RIN4 activates RPS2 and defense responses. AtROC1 can activate AvrRpt2 in rps2 mutant background also suggesting no direct role for AtROC1 in defense.

Although some of the previous reports showed antifungal activity for cyclophilins (Ye and Ng 2000; Ye and Ng 2001) we did not observe any potent antifungal activity of recombinant AdCyp expressed and purified from bacteria against *R. solani* and *B. cinerea*. This could be due to the lack of posttranslational modifications in bacteria or

lack of inherent antifungal activity. As our emphasis was on studying the regulatory role of *AdCyp* in plant defense, we went ahead with raising transgenic tobacco plants ectopically expressing *AdCyp* under the 35S promoter. Transgenic plants with high expression levels of *AdCyp* exhibited enhanced resistance against *Phytophthora parasitica* var. *nicotianae*. *AdCyp* transgenic plants showed higher transcript levels of various defense related genes. Analyzing different classes of PR gene transcripts showed that PR1a, osmotin, chitinase, glucanse and protease inhibitor type I were constitutively upregulated in transgenic plants. Atleast in case of animal systems the regulatory role of cyclophilins was showed at several levels. For example human *CypA*, one of the extensively studied cyclophilin and homolog of *AdCyp*, was shown to be involved in transmembrane signaling, mitochondrial function, RNA splicing, stress response, gene expression and regulation of kinase activity (Wang and Heitman 2005). For example CyPA plays a role in CXCR4-mediated nuclear export of hnRNP A2 (Pan et al. 2007), activation and nuclear translocation of ERK1/2(REFF). CypA is important for the activity of essential Zn finger proteins, YY1 and Zpr1 (Ansari et al. 2002).Very recent report demonstrated that the recombinant cyclophilin from yellow lupine was able to interact with nucleic acids (Nuc et al. 2008).

Hence *AdCyp* might possibly be involved in activation of regulatory molecules there by playing a role in plant defense responses. It needs to be studied whether the regulatory role of AdCyp is because of possible PPIase or chaperone or nucleic acid binding capacities. The transcript levels of different PR genes like acidic PR1, chitinase and glucanse suggest a possible role of NPR1 like transcription factors, but upregulation of PI-I, whose regulation in plants is not well established, indicates that more complex mechanism involved. Further work is required to elucidate the mechanism in detail. However our results for the first time prove the direct involvement of plant cyclophilins in plant resistance mechanism.

Table 5.1 Sequences of the oligoes/primers used in the present study

Name of the Primer	Primer Sequence (5'-3')
Cyclo 262R	CTT AAC GAC GTC CAT TCC TTC
Cyclo 141R	ATT GAA AGG ATC CCA GGA CC
ORF-F	GCCATGGCTAACCCTAAGGTTTAC
ORF-R	GTCTAGACTAAGAGAGTTGACCGCAATC
GSP1	GGAAGGATGATCCCTTGTAGTGGAGA
GSP2	ACATGTCGAAGTAAACCTTAGGGTTA
Cyclo -541 R	GGAATTCCCTGACATATACCAGAGTTTC
Actin F	TGGCATCACACTTTCTACAA
Actin R	CAACGGAATCTCTCAGCTCC

5.5 Summary

Two partial cDNAs encoding cyclophilin like proteins (AdDR-13 and AdDR-60) were isolated as early response genes from *A. diogoi* upon infection with *P. personata*. Southern analysis suggested that both the partial cDNAs might be coded by different genes and there is atleast one more very closely related gene in the genome of *A. diogoi*. As both the clones were essentially coding the same polypeptide, one of the partial clones was extended using 5' RACE to obtain the full length cDNA. The full length cDNA was designated as *AdCyp* for *Arachis diogoi* Cyclophilin. The full length cDNA of AdCyp was 885bp encoding a polypeptide of 172 amino acids with a predicted molecular weight of 18115.57 Da. AdCyp protein showed maximum homology with AtROC1 of *Arabidopsis* and HsCypA of humans and the cyclophilin protein appears to be conserved across the kingdoms. Analysis of *AdCyp* promoter exhibited several *cis*- regulatory elements known to be involved in biotic and abiotic stress responses. AdCyp transcript levels were found to be induced as early as 36 hours post inoculation (hpi) with *P. personata.* Transgenic tobacco plants ectopically expressing *AdCyp* exhibited enhanced resistance against *Phytophthora parasitica* var. *nicotianae.* Constitutively higher levels of PR1a, acidic chitinase, osmotin, protease inhibitor I and glucanase transcripts were present in *AdCyp* transgenic plants compared to wild type.

Chapter 6

Molecular cloning and partial characterization of *P. personata* induced genes of *A. diogoi* encoding Lipid transfer protein, Thaumatin-like protein and TIR-NB-LRR class Resistance protein

Three AdDR partial cDNA clones identified in previous study (Refer to section: 4.3.1) encoding lipid transfer protein, thaumatin-like protein, and a resistance protein were extended using RACE to obtain the corresponding full length cDNAs. All the three genes are at an early stage of characterization. Isolation of these full length cDNA sequences, their expression patterns up-on pathogen treatment, some of the interesting features of the proteins encoded by these genes and their significance in plant defense are discussed in this chapter in concise.

PR-Proteins

Plants possess both preformed and inducible mechanisms to resist the pathogen invasion. Pathogen has to overcome the preformed morphological barriers, secondary metabolites (phytoanticipins) and antimicrobial proteins to invade plants. The elicitors produced and released by the pathogen and their recognition by the plant induce further defense responses like reinforcement of cell walls, production of phytoalexins and synthesis of various defense- related proteins. Inducible defense-related proteins were first discovered in tobacco reacting hypersensitively to Tobacco Mosaic Virus (TMV) and later shown to occur in plant species from different families upon infection by various pathogens like viruses, bacteria, fungi, nematodes or insect attack (Van Loon et al. 2006). Most of these defense-related proteins correspond to Pathogenesis-Related (PR) proteins.

Originally, PR proteins were classified on the basis of their characteristics as plant proteins induced in pathological or related situations. However, this proposed definition caused confusion in the past as the term PR proteins was often used to designate all microbe-induced proteins, including enzymes like phenylalanine ammonia lyase (PAL) which are constitutively present, but also increase during most infections. Van Loon et al. (2006) recently introduced the term ‘‘inducible defense-related proteins’’ referring to the originally intended definition of PR proteins. The recognized PR-proteins currently comprise 17 families of induced proteins (Table. 6.1). The families are numbered in the order in which they were discovered. A type member, usually the first or most prominent

one, was chosen and families were defined further on the basis of their common biochemical and biological properties (Table 6.1).

Table 6.1 Recognized families of Pathogenesis-related proteins.

Source: http://www.bio.uu.nl/%7Efytopath/PR-families.htm

Families	Type member	Properties	Gene symbol
PR-1	Tobacco PR-1a	antifungal	*Ypr1*
PR-2	Tobacco PR-2	b-1,3-glucanase	*Ypr2*, [*Gns2* ('*Glb*')]
PR-3	Tobacco P, Q	chitinase type I,II, IV,V,VI,VII	*Ypr3, Chia*
PR-4	Tobacco 'R'	chitinase type I,II	*Ypr4, Chid*
PR-5	Tobacco S	thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	proteinase-inhibitor	*Ypr6, Pis* ('*Pin*')
PR-7	Tomato P_{69}	endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	chitinase type III	*Ypr8, Chib*
PR-9	Tobacco 'lignin-forming peroxidase'	peroxidase	*Ypr9, Prx*
PR-10	Parsley 'PR1'	'ribonuclease-like'	*Ypr10*
PR-11	Tobacco 'class V' chitinase	chitinase, type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	thionin	*Ypr13, Thi*
PR-14	Barley LTP4	lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxOa (germin)	oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	'oxalate oxidase-like'	*Yrp16*
PR-17	Tobacco PRp27	unknown	*Yrp17*

6.1 Lipid transfer protein (LTP)

The lipid transfer proteins (LTPs) were denominated due to their ability to facilitate the transfer of phospholipids between a donor and an acceptor membrane, in vitro (Kader 1996). The term “non specific lipid-transfer protein” (nsLTP) was also used in reference to the apparent lack of specificity for the various phospholipids (Kader 1996). Lipid transfer proteins (LTPs) are small, cationic, cysteine-rich peptides found in various plant species (Kader 1997).

LTPs comprise two families. The LTPs that form the first family, namely LTP1, have molecular masses of approximately 10 kDa and are basic, with isoeletric points (*pI*) between 9 and 10. These LTPs have 90–95 amino acid mature peptides, of which eight are conserved cysteines. These eight cysteines, bound to each other, form four disulfide bridges that help the stabilization of the peptide tertiary structure. The LTP2 family is formed of peptides that have molecular masses of approximately 7 kDa, possessing mature peptide of around 70 amino acids; their other characteristics, such as a high pI, lipid transfer activity and another pattern of four conserved disulfide bridges, are shared with the LTP1 family (Liu et al. 2002; Castro et al. 2003). Both families present a signal peptide at the amino terminal region, which in general varies between 21 and 27 amino acids, for the LTP1 family (Kader 1996), and from 27 to 35 amino acids, for the LTP2 family (Kalla et al. 1994). This signal peptide is required for targeting the LTPs to cell secretory pathway where they are exported to the apoplast. This extra-cellular location is not a general rule as LTPs have also been found in glyoxosomes, where their activity was linked to lipid catabolism (Tsuboi et al. 1992) and in protein storage vacuoles of seeds (Carvalho et al. 2001). LTPs were suggested to play a role in cutin synthesis, β-oxidation, somatic embryogenesis, alergenics, plant signaling and plant defense against phytopathogens, but the true physiological roles fulfilled by the LTPs have yet to be determined (Carvalho and Gomes 2007).

LTPs in plant defense

LTPs belong to PR-14 family of pathogenesis-related proteins. LTPs are known to be induced in response to pathogen attack (Jung et al. 2003; Carvalho and Gomes 2007) as

well as other abiotic factors like drought and cold (Jung et al. 2003; Jang et al. 2004). LTPs have been shown to have *in vitro* antimicrobial activities against fungi and bacteria (Molina et al. 1993; Cammue et al. 1995; Wang et al. 2004b). Overexpression of LTP in transgenic plants conferred resistance against various pathogens (Molina and Garcia-Olmedo 1997; Ho et al. 2005).

Isolation of full length cDNA of *AdLTP* and sequence analysis

A partial cDNA clone designated as AdDR-51 encoding a lipid transfer protein was isolated in the previous study (Refer to section: 4.3.1), which was cloned as an induced transcript in *A. diogoi* upon infection with *P. personata*. Using RACE approach full length cDNA was isolated (Refer section 3.2.7 for methodology followed). LTP125R and LTP62R primers (Table. 6.2) were used in 5' RACE to obtain the full length cDNA sequence. The isolated full length cDNA is 756bp in length with an open reading frame of 351 bp and was designated as *AdLTP*. A 96 bp 5' UTR and a 309 bp 3' UTR flanked the ORF. It encodes a polypeptide of 116 amino acids (Fig 6.1). An N-terminal signal peptide of 24 amino acids was present suggesting that protein is secreted into apoplast (Fig 6.1). The signal peptide was predicted using SignalP 3.0 (Bendtsen et al. 2004).The theoretical *pI* and estimated molecular mass of AdLTP are 9.28 and 11537.68 Da respectively including the leader peptide. The basic *pI* exhibited by AdLTP is the typical characteristic of all plant LTPs.The full length *AdLTP* cDNA was submitted to Genbank under the accession number EU183364.

Multiple sequence alignment and Phylogenetic analysis

Performed as described earlier in sections: 3.2.6

Alignment of deduced amino acid sequence of *AdLTP* with *LTP*s from other plant species was performed, which showed that eight cysteine residues essential for forming four disulfide bridges were conserved in all LTPs from both dicot and monocot plant species (Fig 6.2). AdLTP also lacked the tryptophan like most other plant LTPs. Phylogenetic analysis showed that AdLTP belongs to LTP1 family and form a distinct clade compared to LTP2 family, which form a separate clade (Fig 6.3). AdLTP showed 92% similarity

with AhLTP whereas all other LTPs including legumes showed similarity between 50% and 59%, which suggested a lot of diversification within legumes.

```
1     gac caa att caa gct ttc aac act cca aaa cac act tag ctt tta   45

46    ttt cac ata cat cac cac ttc ctc ttc ttc ttc ttg ttc ata tta   90

91    taa cta ATG GCA GGC CTC AAG TTT GCA TTT GTG ATG CTT GTG TGC   135
              M   A   G   L   K   F   A   F   V   M   L   V   C    13

136   ATG GCC ATG GTG GGA GCA CCA ATG GTG AAT GCC CTA TCA TGT GGC   180
14     M   A   M   V   G   A   P   M   V   N   A   L   S   C   G    28

181   CAA GTG AAC AGT GCC CTA GCA CCA TGC ATC ACT TTC CTC ACA AAG   225
29     Q   V   N   S   A   L   A   P   C   I   T   F   L   T   K    43

226   GGT GGA GCT CCT TCT CCG CCT TGT TGT AGC GGA GTT AGA GGC CTT   270
44     G   G   A   P   S   P   P   C   C   S   G   V   R   G   L    58

271   CTC GGT GCT GCA AAA ACC ACC GCG GAC CGC CAG GCC GCC TGT AAC   315
59     L   G   A   A   K   T   T   A   D   R   Q   A   A   C   N    73

316   TGC CTC AAA GCC GCT GCC GGT TCC GTT CAT GGC CTC AAC CAA GGC   360
74     C   L   K   A   A   A   G   S   V   H   G   L   N   Q   G    88

361   AAC GCC GCC GCC CTC CCT GGA AGA TGC GGT GTC AGC ATT CCT TAC   405
89     N   A   A   A   L   P   G   R   C   G   V   S   I   P   Y   103

406   AAG ATC AGC ACC TCC ACC AAC TGT GCT ACC ATT AAG TTC TGA gga   450
104    K   I   S   T   S   T   N   C   A   T   I   K   F   *

451   gag gaa gat gaa gaa ggt ggc tct agc cag cac tgg aca caa caa   495

496   gta gtt atg tga aag cag ctt ata tta att att aat taa tga gaa   540

541   taa aca tga ggg tga tga tga ggg cta tat ata tac tta tat ata   585

586   tat ata tgc ccc tct cct ctt gta gtc ttt gta tga ggt gga aat   630

631   gga ttc tct tat ttc ttt ttt ttt ttg tta tgc ata tgg agt tgt   675

676   tac ttg ttt caa ctt cca act acc tat agc aat caa tga agc tgc   720

721   ttt tat ttg gtt aaa aaa aaa aaa aaa aaa aaa aaa   756
```

Figure 6.1 Nucleotide and deduced amino acid sequence of *AdLTP*. A 24 amino acid N-terminal signal peptide is underlined. Letters in upper case represent ORF. Nucleotides and amino acids are numbered. (*) indicates a stop codon. Letters in lower case represent UTR regions.

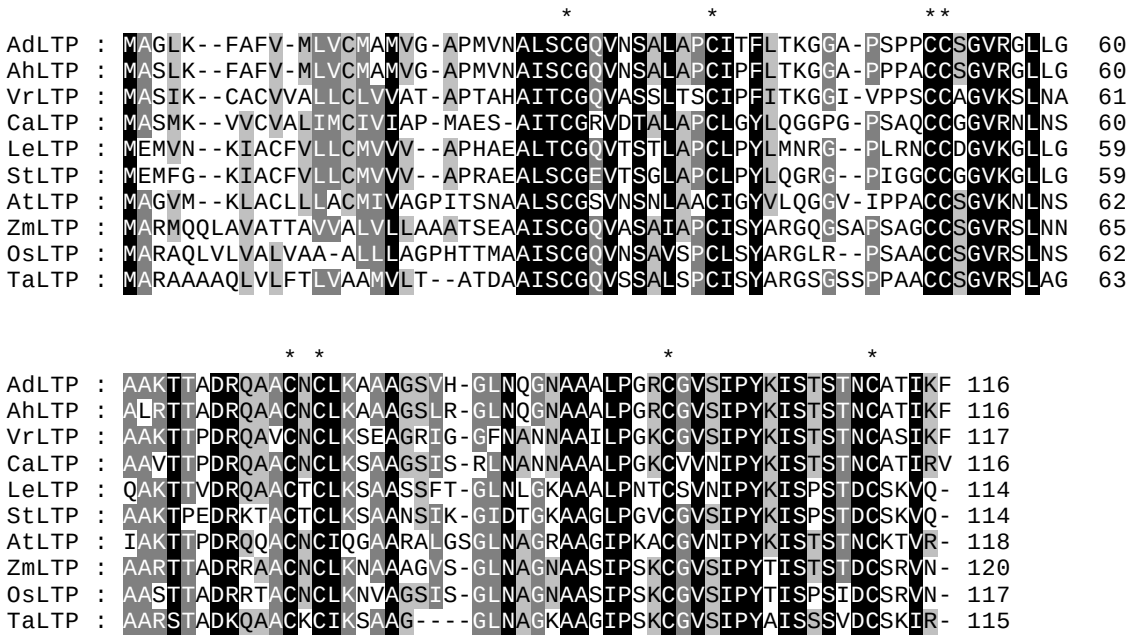

Figure 6.2 Multiple sequence alignement of AdLTP with other LTP1 family members from both dicots and monocots. Asterisk (*) marks all the eight cysteine residues, which form four disulfide bridges. The prefix designates the plant taxonomical name and the corresponding LTPs accession numbers are as follows. Ad: *Arachis diogoi* (EU183364); Ah: *Arachis hypogaea* (EU159429); Vr: *Vigna radiate* (AY300806); Ca: *Cicer arietinum* (AJ002958); Le: *Lycopersicon esculentum* (AM051295); St: *Solanum tuberosum* (EU057717); At: *Arabidopsis thaliana* (NM_129411); Zm: *Zea mays* (DQ147204); Os: Oryza sativa (Z23271) and Ta: *Triticum aestivum* (DQ286561).

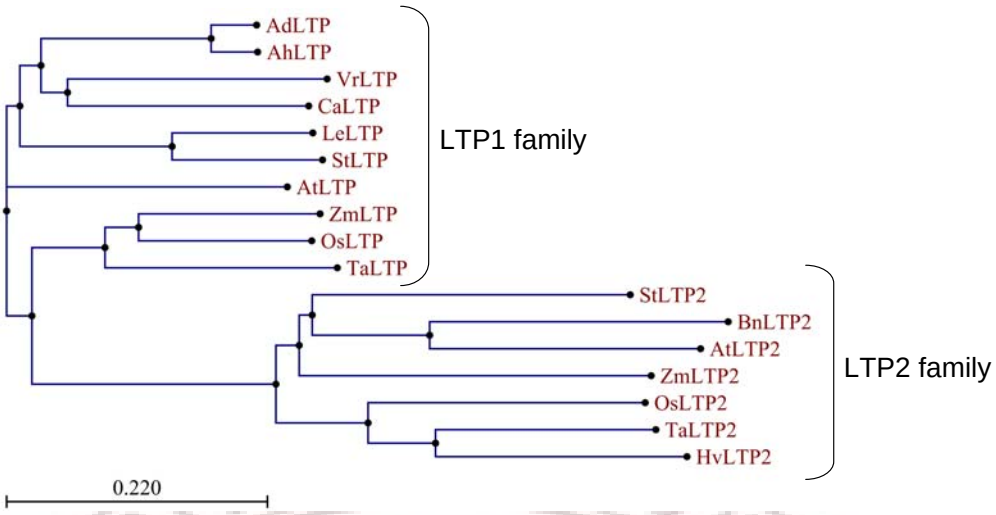

Figure 6.3 phylogenetic relationship of AdLTP with LTP1 and LTP2 family members. AdLTP(EU183364); AhLTP (EU159429); VrLTP(AY300806); CaLTP(AJ002958); LeLTP (AM051295); StLTP (EU057717); AtLTP (NM_129411); ZmLTP(DQ147204); OsLTP (Z23271) and TaLTP (DQ286561); StLTP2(BAC23052); BnLTP2(AY570248); AtLTP2(NM_123177); ZmLTP2(EU976089); OsLTP2(OSU16721); TaLTP2(AJ852554); HvLTP2(X15257). Ad: *Arachis diogoi*; Ah: *Arachis hypogaea*; Vr: *Vigna radiata*; Ca: *Cicer arietinum*; Le: *Lycopersicon esculentum*; St: *Solanum tuberosum*; At: *Arabidopsis thaliana*; Zm: *Zea mays*; Os: *Oryza sativa*; Ta: *Triticum aestivum*; Bn: *Brassica napus*; Hv: *Hordeum vulgare.*

Expression pattern of *AdLTP* in response to *P. personata* infection

Transcript levels of *AdLTP* were analyzed using semi-quantitative RT-PCR as described in sections: 4.2.3 and 4.2.7. *AdLTP* ORF-F and ORF-R primers were used to amplify *AdLTP*. *AdLTP* transcripts were induced as early as 36 hpi upon treatment with *P. personata* (Fig 6.4). Such an early regulation suggests a possible regulatory role in plant defense. Most of the reports on LTPs were on *in vitro* antimicrobial activity of these proteins (Cammue et al. 1995; Wang et al. 2004). There were reports suggesting its regulatory role in systemic acquired resistance (SAR) as evidenced by the *dir1* mutant which fails to develop SAR against virulent *Pseudomonas syringae* in *Arabidopsis thaliana.* It was found that DIR1 encodes a putative apoplastic LTP and proposed to

interact with a lipid-derived molecule to promote long-distance signaling involved in SAR (Maldonado et al. 2002). Recombinant LTP1 from *N. tabacum* was shown to bind jasmonic acid (JA), and the complex between LTP1 and JA is able to bind the elicitin receptor (Buhot et al. 2004).

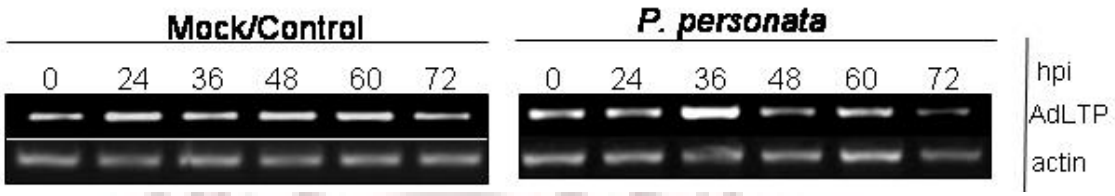

Figure 6.4 Semi-quantitative RT-PCR analysis of *P. peronata* induced *AdLTP* transcript levels. Actin served as internal control.

All these reports suggest that LTPs hold a lot more regulatory role in plant defense other than antimicrobial activity. Further work on *AdLTP* is in progress to address its role in *A. diogoi* and *P. personata* interaction.

6.2 Thaumatin-like protein

Thaumatin-like proteins (TLPs) belong to PR-5 family of pathogenesis related proteins and are commonly found in both monocotyledonous and dicotyledonous plant species. TLPs show primary sequences and structural arrangements similar to the sweet tasting protein thaumatin isolated from the fruit of African shrub *Thaumatococcus danielli* (van der Wel and Loeve 1972).

Most of the known PR-5 proteins play a defensive role against pathogenic fungi (Koiwa et al. 1997b; Ng 2004). The antifungal activity observed was correlated with plasma membrane permeabilization as in case of PR-5 proteins like zeamatin (Roberts and Selitrennikoff 1990) and tobacco osmotin (Abad et al. 1997). The precise mechanism by which PR-5 proteins exert their antifungal activity has not been clearly understood. The defensive role was also attributed to α-amylase inhibitor or endo β-1, 3-glucanase-like activities (Van Loon et al. 2006). The basic tobacco PR-5c (osmotin), which is inducible by pathogens and osmotic stress, and its homologs in tomato and potato displayed *in vitro* antioomycete activity against *P. infestans*, and transgenic tobacco and potato plants have enhanced resistance against this pathogen, but not against *P. parasitica* var. *nicotianae* (Van Loon et al. 2006). Osmotin induces apoptosis in the yeast, *Saccharomyces cerevisiae*, apparently by binding to phosphomannans in the cell wall (Ibeas et al. 2000). There are several reports on overexpression of PR-5 genes in transgenic plants conferring resistance against pathogens, like in rice (Grover and Gowthaman 2003), Wheat (Chen et al. 1999), and tobacco (Velazhahan and Muthukrishnan 2003).

Isolation of full length cDNA of *AdTLP* and sequence analysis

A partial cDNA clone designated as AdDR-11 encoding a putative thaumatin-like protein was isolated in the previous study as described in section: 4.3.1. 5' RACE-PCR approach was used (Refer section 3.2.7 for methodology followed) to obtain the full length cDNA sequence. The full length cDNA was showing maximum homology with plant thaumatin-like proteins and was designated as *AdTLP*. The full length cDNA is 988 bp in length with an open reading frame of 726 bp potentially encoding a polypeptide of 241 amino acids (Fig 6.5). The theoritical *pI* and the molecular mass of AdTLP are 4.71 and

25005.63 Da respectively including the signal peptide. An N-terminal signal peptide of 21 amino acids was present suggesting that protein will get secreted into apoplast (Fig 6.5). The signal peptide was predicted using SignalP 3.0 (Bendtsen et al. 2004). The full length cDNA of *AdTLP* was submitted to the Genbank under the accession number FJ481982.

Multiple sequence alignment

Alignment of deduced amino acid sequence of AdTLP with other TLPs from other plant species shows that there are sixteen cysteine residues involved in the formation of eight cysteine disulfide bridges and are conserved across the species (Fig 6.6). The AdTLP shows maximum similarity of 59% with PpTLP and PaTLP of *Pyrus pyrifolia and Prunus avium* repectively. AtTLP from *Arabidopsis thaliana* shares 54% similarity with AdTLP. The most interesting observation was, there is not even a single TLP from any legume which has a similarity of 40% with AdTLP in the non-redundant protein databases, suggesting that it is a novel protein at least within legumes. This is very surprising in the light of available genomic resources for legumes like *Medicago truncatula* and *Lotus japonicus.*

P. personata* induced expression of *AdTLP

Transcript levels of *AdTLP* were analyzed using semi-quantitative RT-PCR as described in sections: 4.2.3 and 4.2.7. *AdTLP* ORF-F and ORF-R primers were used to amplify *AdTLP*. A slight upregulation of AdTLP was observed as early as 24 hpi which gradually reached the basal level by 48 hpi and again rebounding to a very high level by 72 hpi (Fig 6.7). Induction of TLP upon pathogen treatment was observed in several plant-microbe interactions (Ferreira et al. 2007; Van Loon et al. 2006).

Considering the novelty of the protein coded by the *AdTLP,* further complete characterization is in progress, which will be useful in depicting its role in *A. diogoi* and *P. personata* interaction. Due to the lack of nearest homologs within legumes, its evolutionary divergence would also be of interest.

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1     tgc tat aat att ttc ttg atc act aag cta aca aca ata ATG GCG   45
                                                        M   A     2

46    ATT ACT CGT GTT GTT CTC TCC CTA AGC TTT GCA TTC TTC CTT TGT   90
3      I   T   R   V   V   L   S   L   S   F   A   F   F   L   C   17

91    GTT GCT CAT GGA GCT CAA ATA ACC CTT ACA AAC AAG TGT TCA TAC   135
18     V   A   H   G   A   Q   I   T   L   T   N   K   C   S   Y   32

136   ACA GTG TGG CCA GGA TCA CAA GCC AAC GCC AAT AGT GCT CAA CTA   180
33     T   V   W   P   G   S   Q   A   N   A   N   S   A   Q   L   47

181   TCA ACA ACC GGT TTT GAA TTG CCA ACT GGC CAA TCC AAA ACA GTT   225
48     S   T   T   G   F   E   L   P   T   G   Q   S   K   T   V   62

226   GAT GTC CCA GCA CCA TGG TCC GGG AAG TTT TGG GCT AGA ACA GGA   270
63     D   V   P   A   P   W   S   G   K   F   W   A   R   T   G   77

271   TGC TCC AAC AAT AAC GGA GTA TTC TCT TGT GCT ACT GCA GAC TGT   315
78     C   S   N   N   N   G   V   F   S   C   A   T   A   D   C   92

316   GGC AAC CAC CTT GAA TGC AGT GGG GCC GGC GAA GCC ACA CCA GCA   360
93     G   N   H   L   E   C   S   G   A   G   E   A   T   P   A   107

361   TCT CTA ATG GAA TTT ACC ATT GCA TCT AAC GGT GGA CAA GAC TTC   405
108    S   L   M   E   F   T   I   A   S   N   G   G   Q   D   F   122

406   TAC GAT GTT AGC AAC GTT GAC GGA TTC AAC GTT CCC TCT TCA ATT   450
123    Y   D   V   S   N   V   D   G   F   N   V   P   S   S   I   137

451   ACC CCA CAG GGT GGA TCT GGT GCC TGT AAC GTC GCG AGT TGT CCA   495
138    T   P   Q   G   G   S   G   A   C   N   V   A   S   C   P   152

496   GCT AAC ATC AAT GCT GCT TGT CCC GCC GCA TTG CAA TTT AAG GGA   540
153    A   N   I   N   A   A   C   P   A   A   L   Q   F   K   G   167

541   TCT GAT GGA AGC GTT ATT GGT TGC AAG AGT GCT TGC GTG GAA TTT   585
168    S   D   G   S   V   I   G   C   K   S   A   C   V   E   F   182

586   GGC ACC CCC GAG TAT TGT TGC ACC GGC GAT CAC AAC ACA GCG GCG   630
183    G   T   P   E   Y   C   C   T   G   D   H   N   T   A   A   197

631   ACT TGC CCG GCG ACA AAC TAC TCC GAA TTC TTT AGC AAC CAG TGC   675
198    T   C   P   A   T   N   Y   S   E   F   F   S   N   Q   C   212

676   CCT AAT GCT TAT AGC TAT GCT TAT GAT GAC AAA AGA GGA ACT TTC   720
213    P   N   A   Y   S   Y   A   Y   D   D   K   R   G   T   F   227

721   ACT TGT TCA GGA AGC CCT AAT TAT GCT ATC AAC TTC TGT CCA TGA   765
228    T   C   S   G   S   P   N   Y   A   I   N   F   C   P   *

766   act taa att aat gca tac att ata tat ata tgg cat taa tat taa   810

811   tta atc aat aat ttt gct atg aaa aaa taa gtg cat ata tca atg   855

856   cat aaa tag att cat agc aca tgc tat ata tat ggc att gtt ata   900

901   gtg ttt gtc atg aaa tgt aat aaa aga tat ata att ttt ctt ctt   945

946   cta tat ata tat gat ttt tca aca gca aaa aaa aaa aaa aaa a 988
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Figure 6.5 The nucleotide and deduced amino acid sequence of *AdTLP*. Letters in upper case represent ORF. Nucleotides and amino acids are numbered. (*) indicates a stop codon. Letters in lower case represent UTR regions. A 21 amino acid N-terminal signal peptide is underlined.

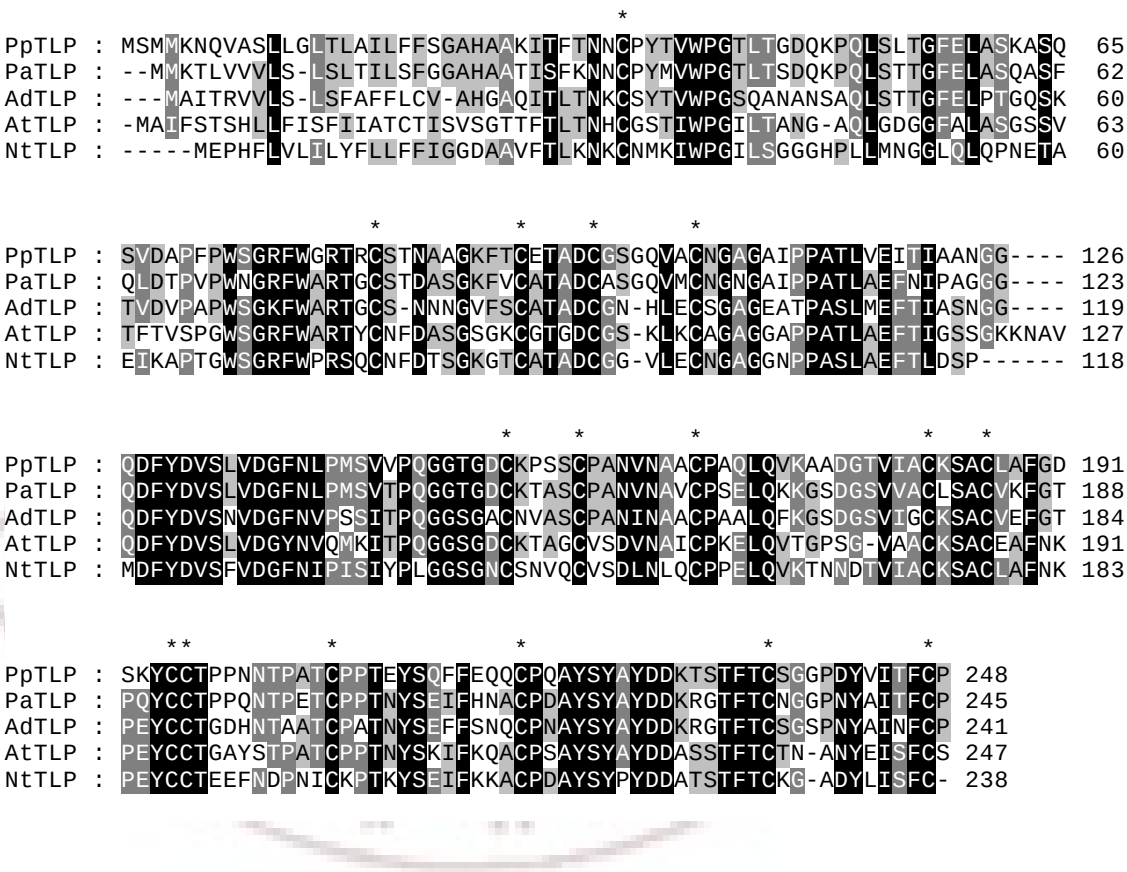

Figure 6.6 Sequence alignment of AdTLP with TLPs from other plant species. The sixteen cysteine residues required for the formation of eight disulfide bridges are conserved in AdTLP also and are indicated by asterisk (*). PpTLP (FJ197337), PaTLP (U32440), AtTLP (NM_101789), NtTLP (AB000834). Pp: *Pyrus pyrifolia*, Pa: *Prunus avium*, At: *Arabidopsis thaliana*, Nt: *Nicotiana tabacum*.

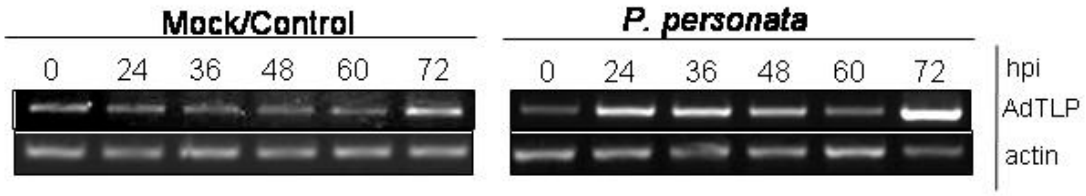

Figure 6.7 Semi-quantitative RT-PCR analysis of *P. peronata* induced *AdTLP* transcript levels. Actin served as internal control.

6.3 TIR-NB-LRR protein

While over 40 resistance (R) genes have been isolated from plants (Martin et al. 2003), only two have been isolated from legumes. Isolation of R genes from legumes has been slow due to lack of detailed genetic maps, appropriate mapping populations, and chromosome walking tools. In addition, the polyploid genomes of many crop legumes makes obtaining mutants difficult, and high frequency transformation systems for legumes are limited. With the active development of genetic and genomic tools for model and crop legumes, isolation of more legume R genes should be forthcoming. Numerous R gene homologs have been identified in legume species by sequence identity of conserved motifs with the known R genes (Yu et al. 1996). The challenge is to identify the specific genes conferring resistance to a particular pathogen. For example the Rpg1-b gene from soybean (*Glycine max*) confers resistance to *Pseudomonas syringae* pv *glycinea* (causing bacterial blight) carrying the avrB gene in a classic gene for gene specific manner (Ashfield et al. 2004).

Majority of R proteins contain a central nucleotide-binding (NB) subdomain as part of a larger entity called the NB-ARC domain, which is present in the human apoptotic protease-activating factor 1 (APAF-1), R proteins, and the CED-4 protein of *Caenorhabditus elegans* (Van Der Biezen and Jones 1998b). C-terminal to the NB-ARC domain lies a leucine-rich repeat (LRR) domain, which is sometimes followed by an extension of variable length. Hence, this group of R proteins is collectively referred to as NB-LRR proteins. These NB-LRR proteins are divided into two classes based on their N-terminal region. If the N-terminal shows homology with a protein domain found in the

Drosophila Toll and human Interleukin-1 Receptor (IL-1R), it is called the TIR domain (Whitham et al. 1994) and these proteins are referred to as TIR-NB-LRR or TNL proteins (TNL class). The other class with out TIR domain and some contain predicted coiled-coil structures in their N-terminal, and Non-TIR NB-LRR proteins are collectively referred as CC-NB-LRR or CNL proteins(CNL class) (Ooijen et al. 2007).

Resistance mediated by R proteins is often associated with the appearance of localized cell death at the infection site, a phenomenon called the hypersensitive response (HR). Some of the best studied TIR-NB-LRR class R genes are N-gene of tobacco and RPS4 of *Arabidopsis*. Tobacco N gene confers resistance to Tobacco Mosaic virus (Whitham et al. 1994), whereas RPS4 confers resistance to *P. syringae* pv. *tomato* strain DC3000 expressing the AvrRps4 effector (Gassmann et al. 1999) in *Arabidopsis*. Other genes which were studied from this family include L gene and M gene of *Linum* (Martin et al. 2003).

Isolation of full length cDNA of *AdTIR* and its analysis

A partial cDNA clone designated as AdDR-10, showing similarity with LRR protein of *Medicago* was identified in previous study (as described in section: 4.3.1). Full length cDNA was obtained using combination of 5' RACE and Genome walking approaches (Refer to section 3.2.7 for RACE and 5.2.4 for genome walking methodologies). Alignment of the sequences of various RACE and genome walking products was used to deduce the full length cDNA. This was further confirmed by using primers designed against the ORF region and amplification of complete ORF and its sequencing. The gene was designated as AdTIR and was submitted in the Genbank under the accession number FJ481983. AdTIR is 3,491bp length including the ORF of 3,288 bp and UTR region (Fig.6.8). It encodes a polypeptide of 1096 amino acids with a predicted molecular mass of 125909.05 Da and a predicted *pI* of 8.13 respectively. Multiple sequence alignment showed that it exhibit conserved regions or domains with known R proteins (Fig 6.9). AdTIR as a complete protein does not have more than 31% similarity with any protein in the non-redundant protein database. Northern analysis of *AdTIR* showed that it gets induced at 36 hpi in response to *P. personata* treatment, whereas no induction was observed in other time periods or in the control treatment (Fig 6.10). There were several

reports of induced R gene expression. For example Rice *Xa1* gene was induced on pathogen inoculation and wounding (Yoshimura et al. 1998). In soybean, the R gene candidates DD6 and KR1 were also induced during the HR (Seehaus et al. 1998; He et al. 2003). Further characterization of this protein is in progress.

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1     ATG TTT CAA AAT CCA AGG ACA TTA GCT TGT AAA ATG AAA TAT ATT   45
1      M   F   Q   N   P   R   T   L   A   C   K   M   K   Y   I    15

46    GGA TTA ATT GTT TGT GTA ATA GCA ATG TGT ATT GCA ATT ATT GCT   90
16     G   L   I   V   C   V   I   A   M   C   I   A   I   I   A    30

91    CAG AAA TTT GAT GCA GAG ACA CCG GTA GAA GAA AAT ACT CCT CCT   135
31     Q   K   F   D   A   E   T   P   V   E   E   N   T   P   P    45

136   TCG TAT CCA GAG GCA TCA TCA CCA ACT CAT GAC ACA AAG TTT GGT   180
46     S   Y   P   E   A   S   S   P   T   H   D   T   K   F   G    60

181   GTG TTC ATC GGC TTC AGT GGC AAA GAC ATT CGG GAA GGT TTG CTC   225
61     V   F   I   G   F   S   G   K   D   I   R   E   G   L   L    75

226   AGC CAT CTC GCC AAG GCA TTA CGG CAG AAG CAA ATC TTC ACC TTC   270
76     S   H   L   A   K   A   L   R   Q   K   Q   I   F   T   F    90

271   GTT GAC ACG AAG CTC GAG CAA GGC GGA GAA ATC TCG CAA GAG CTT   315
91     V   D   T   K   L   E   Q   G   G   E   I   S   Q   E   L   105

316   CTC CAA GCA ATT GAA AAG TCG TTG ATC TCG TTA GTT GTG TTC TCA   360
106    L   Q   A   I   E   K   S   L   I   S   L   V   V   F   S   120

361   GAA AAC TAT GCG TTT TCG ACT TGG CGT TTG GAT GAG CTG GTC AAG   405
121    E   N   Y   A   F   S   T   W   R   L   D   E   L   V   K   135

406   ATT ATG GAG TGT AGA AGA GAA AAA GGA CAA ATT GTT TTA CCG GTT   450
136    I   M   E   C   R   R   E   K   G   Q   I   V   L   P   V   150

451   TTC TAC AGA GTG GAA CCG TCT CAT GTA AGA CAC CAA AAG GGC GTT   495
151    F   Y   R   V   E   P   S   H   V   R   H   Q   K   G   V   165

496   TTT TCT ACT GCC TTT GCT AAA CAA GAG AGA AGG TTT GGT AAG GAA   540
166    F   S   T   A   F   A   K   Q   E   R   R   F   G   K   E   180

541   AAG GCA CAA ACA TGG AGA TCT GCT TTT CAG GAA GCT GCT AAT ATT   585
181    K   A   Q   T   W   R   S   A   F   Q   E   A   A   N   I   195

586   TCA GGC TTT CAT TCA GCA AAA TTC GGG AAT GAT GCT GAG CTT ATT   630
196    S   G   F   H   S   A   K   F   G   N   D   A   E   L   I   210

631   GAA GAA ATC ATC CAA TCA GTG AAC ACT AGG TTG AAA AAT ATG CGC   675
211    E   E   I   I   Q   S   V   N   T   R   L   K   N   M   R   225

676   CAG TTT TCC TCA AAA GGA CTT TTC GGA ATT GCT AAA TCA ATT TCT   720
226    Q   F   S   S   K   G   L   F   G   I   A   K   S   I   S   240

721   CGT GTT GAA TCA TTG CTT CGC CAA GAG CCA GAG AGC GTC CGT GTC   765
241    R   V   E   S   L   L   R   Q   E   P   E   S   V   R   V   255

766   ATT GGA ATT TGG GGC ATG GGT GGT TTC GGA AAG ATA ACC GTT TCA   810
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256    I   G   I   W   G   M   G   G   F   G   K   I   T   V   S    270

811   GAA GTA GTT TAT AAC CTA CTT CGT GAT GAA TAC GAA AGC GTT GTT   855
271    E   V   V   Y   N   L   L   R   D   E   Y   E   S   V   V    285

856   TTT CTT CGA AAC GTA AGG GAA GTA TCA TTG AGA CAT GGA ATT ATT   900
286    F   L   R   N   V   R   E   V   S   L   R   H   G   I   I    300

901   TAC TTG AAG AAT GAA CTC TTT TCT AAA CTC TTA GGC GAA AAT CTT   945
301    Y   L   K   N   E   L   F   S   K   L   L   G   E   N   L    315

946   GAA ATT GAC ACA CAA AAT GGA TTG CCT ACT TAT GTT GAG AAG AGA   990
316    E   I   D   T   Q   N   G   L   P   T   Y   V   E   K   R    330

991   ATT GGC CGC ATG AAG GTT CTT ATT GTT CTT GAT GAT GTT AAT CAA  1035
331    I   G   R   M   K   V   L   I   V   L   D   D   V   N   Q    345

1036  TCA GAG CAG TTT GAA ATT CTA GTT GGA ACC CCG CAA AGT TTT GGA  1080
346    S   E   Q   F   E   I   L   V   G   T   P   Q   S   F   G    360

1081  TCA GGT AGT AGA ATT ATT GTA ACT ACC AGA GAT AGG CAA GTG CTT  1125
361    S   G   S   R   I   I   V   T   T   R   D   R   Q   V   L    375

1126  GCG AAA TAT GCT CAT GCT AAT GAT ACA TAC AAG GTT GAA CCA TTG  1170
376    A   K   Y   A   H   A   N   D   T   Y   K   V   E   P   L    390

1171  GAA TCT GAT GAA GCA CTT CAG CTT TTC AAT TTG ATT GCG TTT CAA  1215
391    E   S   D   E   A   L   Q   L   F   N   L   I   A   F   Q    405

1216  CAA AAT GAA GTT GTT GAA AAG GAG TAT CGT GCG TTA GCA GAG AGG  1260
406    Q   N   E   V   V   E   K   E   Y   R   A   L   A   E   R    420

1261  GTG GTG GAT CAT GCC AAA GGG ATT CCA CTG GTT CTT AAG ACT TTG  1305
421    V   V   D   H   A   K   G   I   P   L   V   L   K   T   L    435

1306  GGT CAT TTA CCT CAT GAA AAA GAA AAG TGG ATA TGG GAA AGT GAA  1350
436    G   H   L   P   H   E   K   E   K   W   I   W   E   S   E    450

1351  TTA GAG AAA CTT GGG AAG ATT CCG AAT AAG AAG GTT TTT GAT ATG  1395
451    L   E   K   L   G   K   I   P   N   K   K   V   F   D   M    465

1396  ATG AGG CTG AGT TAT GAT GAG TTG GAT CGC CAA GAG AAA TCA ATG  1440
466    M   R   L   S   Y   D   E   L   D   R   Q   E   K   S   M    480

1441  CTT TTG GAC ATT GCA TGC TTT TTT GAT GGA ATG AAG TTG AAG GTG  1485
481    L   L   D   I   A   C   F   F   D   G   M   K   L   K   V    495

1486  AAG TAC TTA GAG AGT TTG TTA AAG CAT GGG GAT TTT CCA GTT CCT  1530
496    K   Y   L   E   S   L   L   K   H   G   D   F   P   V   P    510

1531  GCA GCA TTG AAA AGA CTT GAA GAT ATA TCT TTC ATA ACC ATT TCT  1575
511    A   A   L   K   R   L   E   D   I   S   F   I   T   I   S    525

1576  AAA GAG GAT GTA GTG ACA ATG CAT GAT ATC GTA CAA GAA ATG GCT  1620
526    K   E   D   V   V   T   M   H   D   I   V   Q   E   M   A    540

1621  TGG GAG ATT GTT CGC CAA GAA TCT ATT GAA GAC CCG GGT AAC TAT  1665
541    W   E   I   V   R   Q   E   S   I   E   D   P   G   N   Y    555

1666  AGT CGA ATT TGG AAT CCT GAG GAC ATT TAT CAA GTA CTG AAA AAT  1710
556    S   R   I   W   N   P   E   D   I   Y   Q   V   L   K   N    570

1711  AAT CAG GGG AGT GAG GCC ATT AGA AGC ATA AAC TTC AGC TAT TCC  1755
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571    N   Q   G   S   E   A   I   R   S   I   N   F   S   Y   S    585

1756  AAA GCA ACA GTT AGG AAC ATG CAG TTA AGT CCT CAA GTA TTT TCT   1800
586    K   A   T   V   R   N   M   Q   L   S   P   Q   V   F   S    600

1801  AAG ATG AGT AAG CTG CGA TTT CTC GAC TTT TAT GGA GAA CGA CAC   1845
601    K   M   S   K   L   R   F   L   D   F   Y   G   E   R   H    615

1846  CTC TTA CAC TTT CCT GAG GGG CTT CAA CAG TTG CCA AGT CGA CTC   1890
616    L   L   H   F   P   E   G   L   Q   Q   L   P   S   R   L    630

1891  AGA TAT CTT CGT TGG ACT TAT TAC CCT CTC AAA TCA TTA CCA AAG   1935
631    R   Y   L   R   W   T   Y   Y   P   L   K   S   L   P   K    645

1936  AAA TTT TCT GCT GAG AAG CTA GTT ATA TTG GAA CTA CCT TAT AGT   1980
646    K   F   S   A   E   K   L   V   I   L   E   L   P   Y   S    660

1981  CAA GTG GAA AAG CTT TGG TAT GGG ATC CAG AAT CTT GTG AAT TTG   2025
661    Q   V   E   K   L   W   Y   G   I   Q   N   L   V   N   L    675

2026  AAA GTC CTT AAG GCT CCA TAT TCC TCA CAG TTA AAG GAG TTT CCA   2070
676    K   V   L   K   A   P   Y   S   S   Q   L   K   E   F   P    690

2071  GAC TTA TCA AAA GCC ACC AAT CTT GAG ATA TTG GAT TTC AAA TAT   2115
691    D   L   S   K   A   T   N   L   E   I   L   D   F   K   Y    705

2116  TGT CTC CGC TTG ACT CGT GTC CAT CCA TCT GTC TTC TCC CTC AAC   2160
706    C   L   R   L   T   R   V   H   P   S   V   F   S   L   N    720

2161  AAG CTT GAG ACA TTG GAT CTA AGC TGG TGC TCT CAA CTT GCC AAA   2205
721    K   L   E   T   L   D   L   S   W   C   S   Q   L   A   K    735

2206  CTT GAA ACT AAT GCT CAC TTG AAA TCC CTT CGT TAT CTG AGC CTC   2250
736    L   E   T   N   A   H   L   K   S   L   R   Y   L   S   L    750

2251  TAT CAC TGC AAA AGA TTG AAC AAA TTT TCA GTG ATA TCA GAA AAC   2295
751    Y   H   C   K   R   L   N   K   F   S   V   I   S   E   N    765

2296  ATG ACA GAA CTG GAT TTG CGA CAC ACA TCT ATC CGA GAA TTG CCC   2340
766    M   T   E   L   D   L   R   H   T   S   I   R   E   L   P    780

2341  TCG TCC TTC GGA TGT CAA AGC AAA CTC GAA AAG CTT CAT CTA GCA   2385
781    S   S   F   G   C   Q   S   K   L   E   K   L   H   L   A    795

2386  AAC TCT GAA GTA AAG AAG ATG CCA GCT GAT AGC ATG AAG CTT CTC   2430
796    N   S   E   V   K   K   M   P   A   D   S   M   K   L   L    810

2431  ACA AGT CTG AAA TAT CTT GAC ATA AGT GAT TGC AAG AAT CTT CAA   2475
811    T   S   L   K   Y   L   D   I   S   D   C   K   N   L   Q    825

2476  ACT CTG CCG GAG CTT CCC CTC TCC ATA GAA ACA TTA GAT GCA GAT   2520
826    T   L   P   E   L   P   L   S   I   E   T   L   D   A   D    840

2521  AAC TGT ACA TCA TTG AAG GCT GTG CTT TTC CCA AAT GCG AGT GAA   2565
841    N   C   T   S   L   K   A   V   L   F   P   N   A   S   E    855

2566  CAG CTG AAG GAA AAC AAG AAA AAG GCT GTG TTC TGG AAC TGC TTG   2610
856    Q   L   K   E   N   K   K   K   A   V   F   W   N   C   L    870

2611  AAG TTG GAA AAT CAA TTT CTC AAT GCT GTC GCA TTG AAT GCT TAT   2655
871    K   L   E   N   Q   F   L   N   A   V   A   L   N   A   Y    885
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2656  ATC AAC ATG GTG AGA TTC TCA AAC CAA TAT TTG TCT GCA ATA GGA   2700
886    I   N   M   V   R   F   S   N   Q   Y   L   S   A   I   G    900

2701  CAT GAC AAT GTA GAC AAT TCT AAT GAA GAC CCT GAA GCC TCT TAT   2745
901    H   D   N   V   D   N   S   N   E   D   P   E   A   S   Y    915

2746  GTT TAT CCC AGG AGC AAA GTT CCA AAC TGG CTT GAG TAT CAA ACA   2790
916    V   Y   P   R   S   K   V   P   N   W   L   E   Y   Q   T    930

2791  AAT ATG GAT CAC CTT ACT GTT AAT CTC TCT TCA GCT CCA TAT GCA   2835
931    N   M   D   H   L   T   V   N   L   S   S   A   P   Y   A    945

2836  CCC AAA TTA GGC TTC ATT TTA TGC TTC ATT GTT CCT GCA GTA CCA   2880
946    P   K   L   G   F   I   L   C   F   I   V   P   A   V   P    960

2881  TCA GAG GGC TTT AGG CTG ATG TTT ACA ATC AGT GGC GAC GAC CAA   2925
961    S   E   G   F   R   L   M   F   T   I   S   G   D   D   Q    975

2926  GAG GAA GAC GAT GTC AAC GAA GTC AGG TTG TAC GTG GAC AGA CCA   2970
976    E   E   D   D   V   N   E   V   R   L   Y   V   D   R   P    990

2971  AGG AAG GAG ATT TCA TGG GAT CAT GTG ATT CTA ATA TAC GAC CAA   3015
991    R   K   E   I   S   W   D   H   V   I   L   I   Y   D   Q   1005

3016  CGT TGT AGT AGC TTC CTA AAT AAC AGA GGT CAA AAT CGA AGA ATG   3060
1006   R   C   S   S   F   L   N   N   R   G   Q   N   R   R   M   1020

3061  TTT AAC ATC AAG GTC TCA GTT GTG TCA CTA TCC ATG ACG TCA GAG   3105
1021   F   N   I   K   V   S   V   V   S   L   S   M   T   S   E   1035

3106  TAT GTG GCA GTG GAG TTG AAA GGG TTT GGA GTG CAC CCA GTA AAC   3150
1036   Y   V   A   V   E   L   K   G   F   G   V   H   P   V   N   1050

3151  CCA TTA GAA TAT CCA AGT TTC ATT AGT TTT ATT AAA AAA ATG GAA   3195
1051   P   L   E   Y   P   S   F   I   S   F   I   K   K   M   E   1065

3196  CAG TTG GGT TAT TAT ACT ACT CCT GCT ACT GTC AAC CCG GTT TCA   3240
1066   Q   L   G   Y   Y   T   T   P   A   T   V   N   P   V   S   1080

3241  CTG TGG AAT GGA ATT AGG TCG TGG TTT GGA GCT CAC AAT TGG GGA   3285
1081   L   W   N   G   I   R   S   W   F   G   A   H   N   W   G   1095

3286  TAA tat tca gga tat ttg tta tat ata tgg gtg tca tgt tag gtt   3330
1096   *

3331  att gta tgc gct taa tct tca aat taa gcc ctg caa ttg gga gag   3375

3376  tag tag aat aat agg acg aaa atg gat tct ctc aat ttt ttt aat   3420

3421  aat tga ggg aat aaa gtg taa ttt tta att ctt caa aaa aaa ttt   3465

3466  aga ttt tta gta aaa aaa aaa aaa aa   3491
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Figure 6.8 Nucleotide and deduced amino acid sequences of *AdTIR*. Letters in upper case represent ORF. Nucleotides and amino acids are numbered. (*) indicates a stop codon.

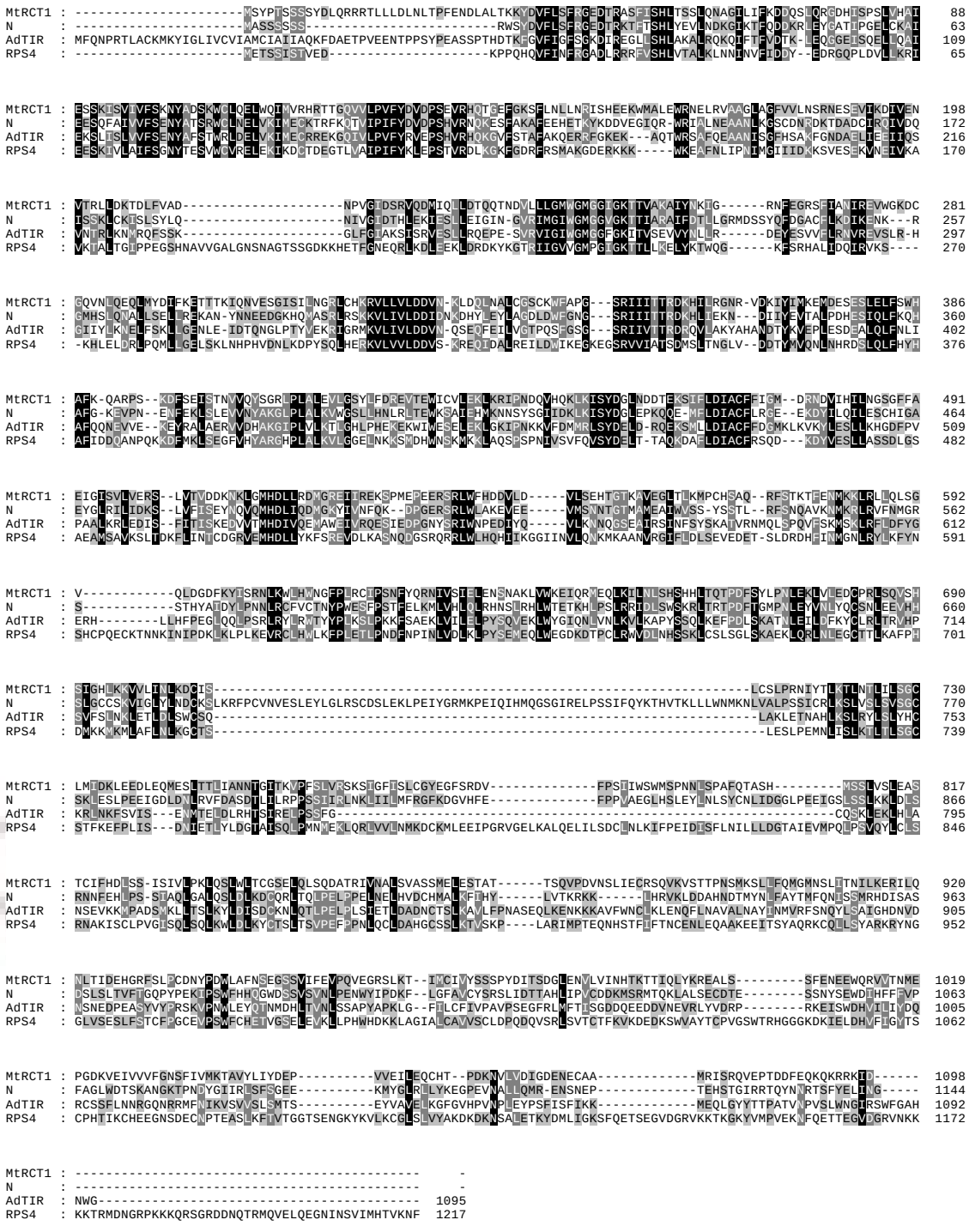

Figure 6.9 Multiple sequence alignment of AdTIR with other TIR-NB-LRR class R proteins. N-gene (U15605: *Nicotiana glutinosa*); MsRCT1 (EU812206: *Medicago truncatula*); RPS4 (AJ243468: *Arabidopsis thaliana*).

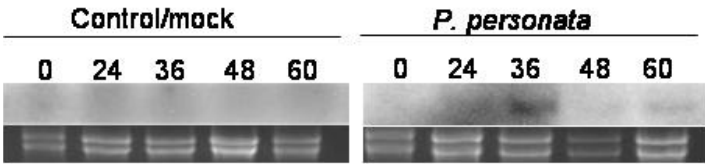

Figure 6.10 Northern analysis of *AdTIR* in response to *P. personata* infection. RNA samples (20 μg) were separated by denaturing formaldehyde-agarose gel electrophoresis, blotted, and hybridized with $^{\alpha\text{-}32}$P-labelled AdDR-10 partial cDNA as probe. Ethidium bromide stained ribosomal RNA bands are shown as loading controls.

Table 6.2 Primers used in the present study

Name of the Primer	Primer Sequence (5'-3')
LTP62 R	CAGTTGGTGGAGGTGCTGA
LTP125 R	TGTGTCCAGTGCTGGCTAG
LTP ORF-F	GGGTACCATGGCAGGCCTCAAGTTTG
LTP ORF-R	GGGATCCTCAGAACTTAATGGTAGCACA
TLP117R	GCCTCCTGAACAAGTGAAAG
TLP146R	CATGGACAGAAGTTGATAGC
TLP ORF-F	GGGATCCATGGCGATTACTCGTGTTGT
TLP ORF-R	CCTCGAGTCATGGACAGAAGTTGATAGC
Act F	TGGCATCACACTTTCTACAA
Act R	CAACGGAATCTCTCAGCTCC
TIR 1	GCCAATTCTCTTCTCAACATAAGTAGG
TIR 2	CCATGTCTCAATGATACTTCCCTTAC
TIR 3	GCCTATTATCAATCATAATCTAATCTTAAGG
TIR 4	GTTTAATCAAAGATACCAGCCCAGGC
TIR 5	AGCCTGAAATATTAGCAGCCTCCTGA
TIR 6	GTCTTACATGAGACGGTTCCACTCTG
TIR 7	GGG TTC CAA CTG GAA TCT C
TIR 8	CTC TTC TCA ACA TAA GTA GGC
TIR ORF- F	ATGTTTCAAAATCCAAGGACAT
TIR ORF- R	TTATCCCCAATTGTGAGCTC

6.4 Summary

Three AdDR partial cDNA clones encoding lipid transfer protein, thaumatin-like protein, and a resistance protein were isolated as early response genes from *A.diogoi* upon infection with *P. personata.* Three partial cDNAs were extended using 5' RACE to obtain the corresponding full cDNAs. The full length cDNA of lipid transfer proteins is 756 bp in length with an open reading frame of 351bp and was designated as *AdLTP*. It encodes a polypeptide of 116 amino acids with an N-terminal signal peptide of 24 amino acids and an estimated molecular weight 11537.68 Da. Eight cysteine residues essential for forming four disulfide bridges were conserved in AdLTP. Phylogenetic analysis showed that AdLTP belongs to LTP1 family. *AdLTP* transcripts were induced as early as 36hpi upon treatment with *P. personata.* The full length cDNA of thaumatin- like protein was 988 bp in length with an open reading frame of 726 bp potentially encoding a polypeptide of 241 amino acids and was designated as *AdTLP*. An N-terminal signal peptide of 21 amino acids was present suggesting that protein will get secreted into apoplast and the estimated molecular weight of AdTLP was 25005.63 Da. AdTLP harbors sixteen cysteine residues involved in the formation of eight disulfide bridges and are conserved across the species. Upon treatment with *P. personata* of *AdTLP* transcripts were induced to a very high level by 72 hpi. The full length cDNA of gene encoding a resistance protein was designated as *AdTIR* and it belongs to TIR-NB-LRR class of R proteins. Full length cDNA of *AdTIR* was 3,491 bp in length and encodes a polypeptide of 1096 amino acids with a predicted molecular weight of 125909.05 Da. AdTIR exhibits several domains/ regions conserved in TIR-NB-LRR class of R proteins. Northern analysis indicated that *AdTIR* transcripts were induced at 36 hpi in response to *P. personata* treatment.

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