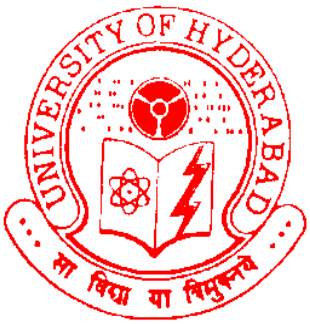
Molecular analysis of resistance responses of *Arachis diogoi* to infection by *Phaeoisariopsis personata* and characterization of some pathogen induced genes

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

By

Dilip Kumar

(Reg. No. 08LPPH01)



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September 2013

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Declaration

This is to declare that I, Dilip Kumar, have carried out the research work embodied in the present thesis entitled **"Molecular analysis of resistance responses of *Arachis diogoi* to infection by *Phaeoisariopsis personata* and characterization of some pathogen induced genes"** and submitted for the degree of Doctor of Philosophy under the supervision of Prof. P. B. Kirti, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad-500046. 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 from any University.

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Certificate

This is to certify that the present thesis entitled **"Molecular analysis of resistance responses of *Arachis diogoi* to infection by *Phaeoisariopsis personata* and characterization of some pathogen induced genes"** is a bonafide research work done by **Mr. Dilip Kumar**, Ph.D student, Department of Plant Sciences, School of Life Sciences, University of Hyderabad. This thesis has been submitted by Mr. Dilip Kumar for the degree of Doctor of Philosophy in Plant Sciences and has not been submitted previously in part or in full or by any other University or Institution for the award of any degree or diploma.

Prof. P. B. Kirti

(Supervisor)

Head

Department of Plant Sciences

Dean

School of Life Science

To my parents and brother

Acknowledgements

With immense respect and gratitude, I take this opportunity to thank Prof. P. B. Kirti my research supervisor, for his constant support, expert guidance and constant encouragement throughout my Ph. D work.

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

My sincere thank to Prof. R. P. Sharma, Dean, School of Life Sciences, and former Dean Prof. A. S. Raghavendra for the necessary facilities provided to the school and department for the research work.

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

I would like to thank Mrs. Monica and Mrs. Kalpana for their help in proteomic studies.

I also would like to thank Mrs. Madhurekha for helping me in cDNA-AFLP analysis, particularly during fluorescent scanning.

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

I am very much thankful to the former and present lab members, Dr. Srinivasan, Dr. Beena, Dr. Divya, Dr. Rajesh, Dr. Vijayan, Dr. Ashraf, Dr. Pushyami, Dr. Jinu, Dr. Uday, Dr. Upender, Naveen, Akanksha, Pawan, Jyotsna, Triveni, Ahan, Deepanker, Israr, Sakshi, Prasanna, Trishla, Kumari, Satish, Achala and Moin for their support and timely help.

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

I acknowledge the help of Dr. Syed Maqbool Ahmed, Principal Scientific Officer and Ms Nalini, technician, CIL for confocal microscopic studies.

I am highly grateful to my friends Shubha Narayan, Abhay Kumar, Abhay Pratap, Anirudh, Anirban, Sumit, Bimolata, Zahoor, Suresh, Kamal, Prateek, Subhasini for their affection and support throughout my research tenure.

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

I thank ICRISAT, Hyderabad for providing the germplasm of wild Arachis for research work.

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

Finally, I thank my family members for their love, patience, support and encouragement.

Dilip Kumar

Abbreviations

ABA	Abscisic acid
APS	Ammonium persulfate
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
BAP	6-Benzylaminopurine
BSA	Bovine serum albumin
CAMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
CTAB	Cetyl trimethylammonium bromide
DEPC	Diethyl pyrocarbonate
DEGs	Differentially Expressed Genes
DPI	Day post inoculation
dNTPs	Deoxy nucleotide triphosphates
DTT	Dithiothretol
EDTA	Ethylene diamine tetraacetic acid
ESTs	Expressed sequence tags
GFP	Green Fluorescent Protein
GSP	Gene Specific Primer
HPI	Hours post inoculation
HR	Hypersensitive response
IEF	Isoelectric focussing
MALDI-TOF MS	Matrix-assisted laser desorption ionization/time of flight mass spectrometry
MES	2-(N-Morpholino)-ethane sulfonic acid
MeJa	Methyl jasmonate
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
O.D	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCD	Plant cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PMSF	Phenylmethylsulfonylfluoride
pI	Iso electric point
PVPP	Polyvinyl polypyrrolidone
qRT	Quantitative Real Time
RACE	Rapid amplification of cDNA ends
SA	Salicylic acid
SNP	Sodium nitroprusside
SDS	Sodium dodecyl sulphate
TDFs	Transcript derived fragments
TEMED	N,N,N',N'- Tetramethylethylenediamine
UTR	Untranslated regions
UPM	Universal primer mixture

Contents

Chapter 1
General Introduction

General Introduction

All the organisms sense the environmental cue in their own way and responding to environmental stresses is crucial in survival. Being sessile in nature, plants are incapable of moving away from several stresses that occur due to changes in the environment. In spite of this disadvantage, plants provide a major contribution to our planet's biomass production. It could be conceived that plants must have, therefore, developed efficient strategies to adjust their physiology to threatening environmental conditions. Stresses can be abiotic (non-living), biotic (living) including weather, soil conditions, insects and disease incidence. Biotic stresses caused, especially by plant pathogenic fungi represents one of the most devastating problems for plants. Studying the mechanism that enables plants to sense and resist fungal attack, therefore, helps to understand how plants became such vital players in the biosphere. It will greatly improve the plant performance against various stresses using biotechnological approaches.

Groundnut is a member of the genus *Arachis* of the family Leguminosae. The only species in the genus of significant economic importance is *A. hypogaea* L., an annual herb that forms underground fruits. The botanical name is derived from the Greek word, *Arachis* meaning 'legume' and *hypogaea* meaning 'below ground', referring to the formation of pods in the soil. The cultivated groundnut, (*Arachis hypogaea* L.) (2n = 40), is an allotetraploid species native to South America, originated between Southern Bolivia and Northern Argentina, and presently cultivated in more than 100 countries. Groundnut was introduced in India by around 16^{th} century by the Portuguese. It is grown under a wide range of the environmental conditions encompassing latitudes between 40° South and 40° North of equator. All other species of the genus *Arachis* are wild, perennial and are mostly used for grazing.

Groundnut, *Arachis hypogaea* is one of the most important oil seed crops in the world and widely cultivated for its oil and protein, particularly in the Semi Arid Tropical region. Groundnuts are an excellent source of plant protein and contain 45-50% oil, 27-33% protein as well as essential minerals and vitamins, and are consumed either raw or roasted. Groundnut oil is composed of mixed glycerides and contains a high proportion of unsaturated fatty acids, in particular, oleic (50-65%) and linoleic (18-30%). Groundnut oil low in saturated fat and cholesterol and high in monounsaturated fat, when

included in a diet, will lower the triglyceride levels. The oil is also used to make margarines and mayonnaise (Hui 1996, Sanders et al. 2003).

In India, it is grown over an area of 8 million hectares with an annual production of 7.5 million tonnes. A production of 83.32 lakh tonnes has been reported during 2003-04 (Hegde 2005). Because of rain-fed nature of the crop, its yield depends on the vagaries of nature in the form of biotic and abiotic stresses. Biotic stresses include diseases caused by fungal pathogens such as early leaf spots caused by *Cercospora arachidicola* and late leaf spot caused by *Phaeoisariopsis personata* Berk. and M.A. Curtis (earlier known as *Cercospora personatum*) and the rust caused by *Puccinia arachidis*. Late leaf spot disease is the most devastating of these and can lead to yield losses up to 70% under favourable conditions (Grichar et al. 1998).

Groundnuts are susceptible to various fungal, viral and bacterial pathogens that can cause considerable losses. Early leaf spot disease is one of the most important foliar diseases of groundnuts in India and can cause considerable yield losses, particularly when the infection appears early in the season. Abundant moisture and high minimum (18-23 °C) and maximum (31-35 °C) temperatures are ideal conditions for an epidemic. Though fungicides are effective in controling of ELS, the most cost effective control measure is the developmentof resistant lines. Late leaf spot is similarly important. The disease causes severe defoliation of plants and adversely affects yields. Resistant cultivars are available but need to be evaluated for resistance to the other foliar diseases as well. Jacobi and Beckman (1995) and Kokalis-Burelle et al. (1997) reported that LLS infection is optimal at 20°C and a high relative humidity lasting more than 12 hours per day and that rust infection will be the highest at 20-25°C with a relative humidity ≥87%. Because of both LLS and rust often occur simultaneously on the same leaf. Establishment of the rust disease early in the growing season reduce pod fill and necessitates early harvesting. In India, late leaf spot is more severe than early leaf spot disease (Ghewande 1990, Anonymous 1993). It causes severe defoliation and reduces pod yields by more than 50%, if crop is not chemically protected (Shew et al. 1988)

Foliar disease- Late leaf spot

Late leaf spot (LLS) is caused by the fungus *Phaeoisariopsis personata* (Berk. and Curt.). The LLS pathogen is seen primarily in its imperfect state, known as *C. personatum.* The perfect state (*Mycosphaerella berkeleyii* W.A. Jenkins) is classified

under the ascogeneous fungi and, both asci and spermatogonia occur on debris where the fungus over-winters (Pattee and Young 1982). Jenkins (1938) described the imperfect state as follows: conidiophores (10-100 x 3-6.5μm) are mostly hypophyllous, arising in more or less distinctly concentric reddish-brown tufts, generally with hyaline tips. Conidia (20-70 x 4-9 μm) are generally cylindrical, pale brown, with somewhat attenuated tips and one or more septates.

Disease cycle and dissemination

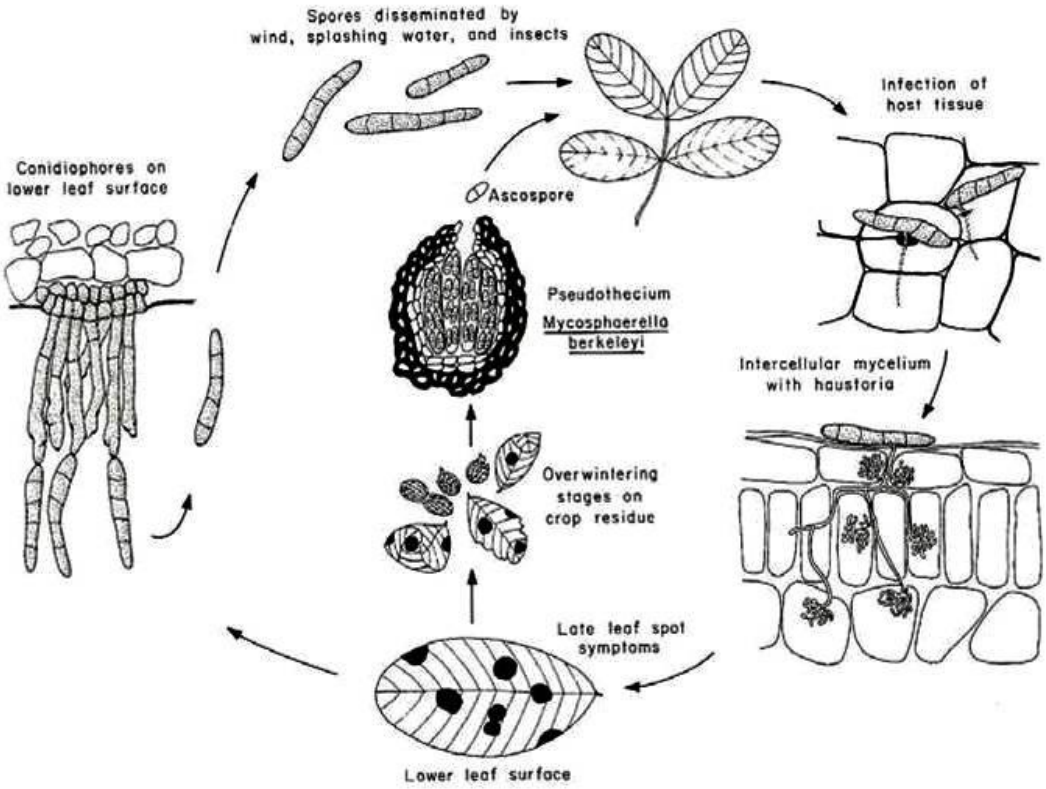


Figure 1.1 Disease cycle for late leaf spot caused by *Phaeoisariopsis personatum* (Berk. and Curt.) (Source: Porter et al. 1990).

High relative humidity and an increase in atmospheric temperatures in spring cause an increase in fungal activity. The optimum temperature range for growth and sporulation for *P. personata* is 25–30 °C. Conidia, produced by conidiophores on groundnut residue in the soil and off-season groundnut plants, serve as the principal source of initial inoculum. Intercellular haustoria are produced at temperatures from 25–31 °C and lesions develop within 12–18 days. The lesion forming cycle (Figure 1.1) starts all over again and the conidia are dispersed by insects, farm implements, water splashing (from

overhead irrigation or rain) and wind. The pathogen perpetuates from season to season only on volunteer groundnut plants and infected plant debris, building up an inoculum reservoir for the following season (Subrahmanyam et al. 1992).

According to Woodroof (1933) and Jenkins (1938), the late leaf lesions are very similar in size and form to those of early leaf spot. These lesions are, however, darker brown and without a definite chlorotic halo. On the adaxial side of the leaflets, lesions are almost black, in contrast to the lighter coloured lesions of ELS. Late leaf spot generally occurs later in the season and is often seen as a complex with other leaf spots. Pattee and Young (1982) reported that *P. personatum* produced cellulolytic and pectolytic enzymes that altered the starch, sugar and amino acid content of leaf tissue resulting in reduced leaf efficiency and premature abscission. Cercosporin, a biologically active red phytotoxin was also isolated from *P. personatum.* In a study conducted by Pattee and Young (1982), severe leaf spot damage reduced the leaf area index by 80%, the carbon dioxide uptake by 85% and the canopy carbon exchange rate by 93%. Photosynthesis of diseased canopies was reduced not only by defoliation, but also by inefficient fixation of carbon dioxide by diseased attached leaves. Late leaf spot fungus produces haustoria that penetrate individual plant cells and leaves infected with the fungus showed a marked increase in respiration (Horne et al. 1976).

The comparisons of early and late leaf spot of groundnut

Character	**Early leaf spot**	**Late leaf spot**
Stage of infection	Early infection	Late infection
Shape of spot	Circular to irregular	Usually circular
Leaf surface on which spores are produced	Upper surface	Lower surface
Arrangement of spores	Random	In concentric rings
Color of spot on upper leaf surface	Light brown to black, tending towards brown with some yellow halo	Brown to black, tending towards black
Color of spot on lower leaf surface	Brown	Black

Economic importance

In India, LLS can cause extensive defoliation and substantial yield losses. The intensity of the disease varies year to year depending on rainfall and the irrigation methods used. It is enhanced in groundnut monocultures and especially if plant residues are left in the field (Swanevelder 1998). Ghuge et al. (1980) found that reduced disease development resulted in an increase in the dry matter content of the plant, a higher number of mature pods, heavier nuts (as expressed in 100-kernel weight) and enhanced pod yield. The planting of resistant cultivars will reduce the use of fungicides, maintenance of equipment will be less costly, less fuel will be needed to run the tractors and less labour will be needed to apply the fungicides. Thus, farmers will benefit economically from planting resistant cultivars (Johnson and Beute 1986).

Fungicides

The fungicides are the most common tools for controlling disease losses. In India, Bavistin, Dithane Z-72, Tebuconazole, Chlorothalonil, Folicur, Mancozeb, Difolaton and Blitox are the commonly used fungicides to control the late leaf spot disease (Nath et al. 2013). In recent years, there has been growing concern in indiscriminate use of fungicides, because they are potentially hazardous to environment and chemical residues in the soil aggravate pollution. These factors have led to the search for new and innovative approaches for plant disease management.

Breeding for resistance

Some wild species do have resistance to some of the diseases, but interspecific hybridisation between *Arachis hypogaea* and the wild species is very difficult to achieve. Crosses between different wild species are of particular importance because they might reveal which diploid species are progenitors of the tetraploid *A. hypogaea.* Raman and Kesavan (1962) and Gibbons and Turley (1967) produced the first interspecific hybrid with fertile progenies, between wild species (Pattee and Young, 1982). Hybrids between the tetraploid cultigen and diploid species of section *Arachis* produced functionally sterile triploids.

Plant defense responses

Upon pathogen attack, plant induces coordinated up-regulation of defense related genes that contribute to disease resistance. Plant defense mechanism involve the expression of R-gene encoded proteins that recognize, directly or indirectly, pathogen avirulence gene (Avr) products and trigger immune responses to protect themselves (Dangl and Jones 2001). If plant possesses a resistance (R) gene product corresponding to the pathogen avirulence gene products, then interaction is said to be incompatible and no disease symptom develops. Furthermore, if plant does not possess a matching R- gene to pathogen avirulence gene (Avr), then the pathogen is compatible and the disease proceeds. Resistance and avirulence recognition event initiates a signalling cascade where Salicylic acid (SA) and Jasmonic acid (JA) are synthesized in two different pathways. Salicylic acid mediated defences are used to play major role in regulating resistance against biotrophic pathogens, while methyl jasmonate mediated defenses control resistance against necrotrophic pathogens (Glazebrook 2005). Methyl jasmonate is activated by herbivores, which signals the production of resistance associated molecules, while microbial infection induced the biochemical pathway to produce salicylic acid. Salicylic acid is an important signalling molecule to induce systemic acquired resistance leading to the production of pathogenesis-related (PR) protein (Ibeas et al. 2000) and other metabolites that contribute to defense (Durrant and Dong 2004). Jasmonic acid and salicylic acid are antagonistic, their biosynthesis suppresses each other (Thomma et al. 2001). Salicylic acid triggers plant resistance known as systemic acquired resistance (SAR), which induces hypersensitive response and localized cell death (Kombrink and Schmelzer 2001). The hypersensitive response is characterized by the collapse and death of tissue immediately surrounding the site of infection. One of the earliest events in incompatible plant pathogen interaction is oxidative burst during which reactive oxygen species such as H_2O_2 and O_2^- molecules are produced (Sutherland 1991). Hydrogen peroxide triggers production of phytoalexins, pathogenesis-related protein and other HR related events.

Wild groundnut- a source of resistance gene

There were no reports on the availability of sources of resistance genes in the cultivated genotypes of groundnut. However, wild relatives of the Genus *Arachis* are a rich source of genes for disease resistance and can be exploited by cloning disease resistance genes

through genomic approaches. In the Genus *Arachis*, there are many wild species at diploid and allo-tetraploid levels that possess resistance to various biotic and abiotic stresses making them a rich repository of genes of commercial importance. There were many attempts aimed at transferring the genes for disease resistance from the wild species to the cultivated accessions through conventional breeding programs. However, these attempts proved to be unsuccessful as the introgression of genes from these wild species also resulted in a linkage drag transferring unnecessary gene blocks carrying the desired genes and making the introgressed material unsuitable for use in subsequent breeding programs. Strategy to improve resistance is to characterize and clone novel resistance gene homologs from the resistant wild relatives. Several diploid wild species of the genus *Arachis*, Viz., *A. diogoi, A. stenosperma, A. cardenasii, A. duranensis* etc. show very high levels of resistance to fungal and rust pathogens (Pande and Narayana Rao 2001). These will constitute an ideal material to study the differences at molecular level involved in conferring resistance or susceptibility.

(**a**) *Arachis diogoi* (Resistant) (**b**) *Arachis hypogaea* L. (Susceptible)

Figure 1.2 Representative disease symptoms of *Phaeoisariopsis personata* infection at (**a**) wild type *Arachis diogoi* and (**b**) susceptible cultivar variety, *Arachis hypogaea* L., after 24 days post inoculation. No disease symptoms were observed in wild groundnut (*Arachis diogoi*).

The objective of this study is to investigate the defense responses of the wild groundnut, *Arachis diogoi* when challenged with the strictly biotrophic fungal pathogen, *Phaeoisariopsis personata* and we have identified genes and proteins differentially expressed during the incompatible interaction in wild groundnut by cDNA-AFLP and 2D electrophoresis. The data presented in this doctoral thesis relate to the elucidation of perception mechanisms and the regulation of defense responses of wild groundnut challenged by late leaf spot pathogen using cDNA-AFLP and 2D electrophoresis to explore host genes and proteins involved in the incompatible interaction and its comparison with the compatible interaction, and further characterization of some pathogen induced genes.

The specific output of this study was achieved through the following objectives:

Objectives

1. Analysis of differential gene expression in wild groundnut, *Arachis diogoi* challenged with late leaf spot pathogen using cDNA-AFLP and its quantitative comparison with susceptible groundnut.

2. Comparative proteomic analysis of the host responses in resistant and susceptible genotypes of groundnut infected with *Phaeoisariopsis personata*.

3. Characterization of pathogen induced genes:

    a) Defense response- hypersensitive like cell death of cysteine protease inhibitor (CPI) & hydroxyprostaglandin dehydrogenase (15-PGDH).
    b) Characterization of vacuolar processing enzyme of a wild groundnut (*AdVPE*) in tobacco.
    c) Functional characterization of a wild groundnut *AdSGT1* (suppressor of G2 allele of SKP1) in tobacco as well as in groundnut for disease resistance.

Chapter 2

Materials and Methods

2.1 Plant Material

Wild species, *Arachis diogoi* (accession number ICG 8962) supplied by the International Crops Research Institute for Semi Arid Tropics, Patancheru, India was used for the cDNA-AFLP analysis of an incompatible interaction followed by proteomics study in both wild groundnut (*Arachis diogoi*) as well as cultivar groundnut (*Arachis hypogaea* L.). For the semi quantitative RT-PCR and real time PCR analysis of some candidate genes, cultivar *Arachis hypogaea* cv. JL-24 (susceptible) was used to study the compatible interaction, in comparison with wild species to validate some of the genes identified in the cDNA-AFLP analysis. Plants were maintained under greenhouse condition. Shoots from wild and cultivated materials were detached from 60 day old plants with a sterile blade, washed thoroughly with sterile distilled water and maintained in plastic trays on moist filter paper with ends of the cuttings wrapped in water-soaked cotton. Trays were covered with polythene covers to maintain high humidity and kept in growth room at 25±1 °C with a photoperiod of 14 h of light and 10 h of dark, to enable the cuttings to get stabilized/acclimatized with the formation of adventitious roots at the stem cut points.

2.2 Experimental treatments

Initially, the germinability of the conidia of *Phaeoisariopsis personata* was checked in conidia germination solution. After checking, the inoculation was prepared by suspending the conidia at a concentration of 10^5/ml in 0.02% Tween-20 and spread onto the adaxial surface of detached leaves homogeneously by using a paint brush and the treated rooted twigs were maintained under high humidity in the growth room. Control plants corresponding to each time point were brushed with 0.02% Tween-20. Leaf tissues of treated and control of both plant species, *Arachis diogoi* (resistant) & *Arachis hypogaea* (susceptible) were harvested at 0, 24, 48, 72 and 96 hours post inoculation (hpi) and quickly frozen in liquid nitrogen, and stored at –80 °C prior to total RNA extraction. Plants were observed for pathogenecity of spores and symptom development 25 days post inoculation (dpi).

2.3 RNA Extraction and double stranded cDNA synthesis

Total RNA from control and treated, *P. personata* infected groundnut leaves was extracted according to method of Chang, Puryear & Cairney (1993). To avoid DNA contamination, total RNA was treated with RNase free DNase1 (Sigma-Aldrich)

according to the manufacturer's instructions. The quality of RNA was checked using a spectrophotometer (NanoDrop, Technologies Inc.) at two wavelength ratios of $A_{260/230}$ and $A_{260/280}$ nm. The integrity of total RNA was determined by running samples on ethidium bromide stained 1.2% agarose gel electrophoresis using Tris boric acid EDTA (TBE) buffer. For cDNA synthesis 20 µg of total RNA was used for first strand synthesis, followed by second strand synthesis using superscript^{TM} double stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions.

2.4 Plasmid DNA vectors used in study

pTZ57R (MBI Fermentas, Germany)

Most commonly used vector for cloning all PCR amplicons amplified by *Taq* polymerases except high fidelity polymerase. It has gene encoding β-lactamase, a bacterial selection marker for ampicillin resistance. This vector allows α-complementation for visual selection based on blue/white colonies.

pRT100 (Topfer et al. 1987)

This is a plant expression vector for cloning complete ORFs at a multiple cloning site, that was flanked by CaMV 35S promoter and poly-adenylation signal. It has gene encoding β-lactamase, a bacterial selection marker for ampicillin resistance.

pCAMBIA2300 (CAMBIA, Australia)

pCAMBIA2300 is a binary vector for plant transformation. This vector contains *nptII* gene conferring resistance to kanamycin for bacterial selection and as well works as a plant selectable marker.

pCAMBIA1302 (CAMBIA, Australia)

The vector is used to obtain ORF, N-terminally tagged translational fusion with GFP for localization studies. This vector contains *nptII* gene conferring resistance to kanamycin for bacterial selection and as well as plant selectable marker.

pER8 (Zuo et al. 2000)

An estrogen receptor-based transactivator XVE mediates estradiol-inducible system. Used for transient expression for a typical hypersensitive cell death phenotype study. This vector confers resistance to spectinomycin for bacterial selection with a plant

selectable marker. (A kind gift from Prof. Nam-Hai Chua, Rockefeller University, New York, USA).

2.5 Genomics

2.5.1 cDNA–AFLP analysis

About 500 ng double stranded cDNA was used for standard AFLP template according to Vos et al. (1995) and Bachem et al. (1996). cDNA-AFLP was performed using the AFLP core reagent kit and the AFLP PreAmp primer mix 1 (Invitrogen, Life Technologies, Merelbeke, Belgium). In brief, the double stranded cDNA samples were digested with the restriction enzymes, *Eco*RI (Rare cutter) and *Mse*I (Frequent cutter). The digested products of cDNA were ligated to adapters as provided in the kit and the sequences of these adapters are as follows:

*Eco*RI adopter, 5'- CTCGTAGACTGCGTACC-3' and 3'-CTGACGCATGGTTAA-5'
*Mse*I adopter, 5'- GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5'

The ligated products were then preamplified using *Eco*RI 5'-GACTGCGTACCAATTC-3' and *Mse*I 5'-GATGAGTCCTGAGTAA-3' preamplification primers corresponding to the *Eco*RI and *Mse*I adapters with one base pair extension. Preamplification was performed for 20 cycles at 94 °C for 30 sec, 56 °C for 60 sec and 72 °C for 60 sec followed by 5 min at 72 °C. Specific amplification was done using pre-amplified products as template with one primer labelled with Cyanine-5 (Cy5) fluorescent dye. Selective amplification primers have been provided with selective nucleotides at their 3' ends. The primers were used for this study are as follows:

*Eco*RI primers	E-AAG,	5'-GACTGCGTACCAATTCAAG -3'
	E-AGC,	5'-GACTGCGTACCAATTCAGC -3'
	E-ACG,	5'-GACTGCGTACCAATTCACG -3'
	E-AAC,	5'-GACTGCGTACCAATTCAAC -3'
	E-ACA,	5'-GACTGCGTACCAATTCACA -3'
	E-ACT,	5'-GACTGCGTACCAATTCACT -3'
	E-ACC,	5'-GACTGCGTACCAATTCACC -3'
	E-AGG,	5'-GACTGCGTACCAATTCAGG -3'

*Mse*I primers	M-CAA,	5'-GATGAGTCCTGAGTAACAA-3'
	M-CAC,	5'-GATGAGTCCTGAGTAACAC-3'
	M-CAG,	5'-GATGAGTCCTGAGTAACAG-3'
	M-CAT,	5'-GATGAGTCCTGAGTAACAT-3'
	M-CTA,	5'-GATGAGTCCTGAGTAACTA-3'
	M-CTC,	5'-GATGAGTCCTGAGTAACTC-3'
	M-CTG,	5'-GATGAGTCCTGAGTAACTG-3'
	M-CTT,	5'-GATGAGTCCTGAGTAACTT-3'

These 16 primers generates a total of 64 different combinations. *Eco*RI series (E-series) primers were labelled with fluorescent dye, Cy5 for visualization of the bands upon fluorescent scanning. Selective *Mse*I primer and fluorescent (Cy5) labelled *Eco*RI primer combinations were then subjected to amplification of preamplified cDNA in thermal cycler following touchdown PCR conditions; 2 min denaturation at 94 °C, followed by 30 s denaturation at 94 °C, 30 s annealing at 65 °C, 60 s extension at 72 °C (13 cycles, scale down of 0.7 °C per cycle); 30 s denaturation at 94 °C, 30 s annealing at 56 °C, 60 s extension at 72 °C (23 cycles) and 7 min at 72 °C. Six microlitre volume of the amplified products were mixed with an equal volume of loading buffer (Bromophenol Blue in 25 mM EDTA and 1.5 mM formamide 1:5), heat denatured prior to gel loading and then immediately placed on ice.

2.5.2 Denatured Polyacrylamide Gel Electrophoresis

Amplified fragments were resolved on 8M urea/6% denaturing polyacrylamide sequencing gel containing acrylamide: bisacrylamide (20:1) run with 1X TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA) electrophoresis buffer. For each gel, 100 ml casting solution was prepared and mixed with 200 µl 10% (w/v) ammonium persulphate and 30 µl TEMED. The gel solution was poured into 44.5 x 34.5 cm casting unit, in which one of the gel glass plates was treated with bind Silane M-6514 (Sigma-Aldrich) to stick the gel, while Sigma cote (Sigma-Aldrich) was applied on the other glass plate to repel the gel. The resulting glass-backed polyacrylamide gel has been used to perform silver staining for the visualization of the amplification products. Spacers and combs were 0.4 mm thick. A 12 µl sample of each reaction mixture was loaded on the gel. Gels were run using power pack at a constant power of 45 W and maximum voltage of 1000 V.

2.5.3 Fluorescent Scanning and Silver Staining

Sandwich gel was scanned on Typhoon imager (Typhoon Trio and Typhoon 9410) for the detection of bands having Cy5-labelled primers, applying a laser excitation at 633 nm and an emission filter at 670 nm. The gel was silver stained then in a 45 × 35 cm plastic tray on a shaker as described by Creste et al. (2001) with modifications. In brief, the Sigma Cote coated gel glass plate was carefully removed and the gel attached with other glass plate was fixed for 5 min in 500 ml fixing solution [10% (v/v) ethanol and 1% (v/v) acetic acid] by shaking gently. After staining for 5 min in 500 ml staining solution (10% ethanol, 1% acetic acid and 0.2% $AgNO_3$), the gel was rinsed with 500 ml distilled water and subsequently developed for 5–10 min in 200 ml of 2% (w/v) NaOH and 0.3 ml 37% (v/v) formaldehyde. The developing reaction was stopped in 500 ml 10% (v/v) ethanol and 5% (v/v) acetic acid for 3–5 min and finally, the gel was transferred to water.

2.5.4 Isolation of Transcript-derived fragments (TDFs)

Silver stained, visualized differentially expressed TDFs were excised from the gel with a surgical blade, and eluted in 100 µl of sterile double distilled water. They were heated at 95 °C for 15 min and then hydrated overnight at 4 °C, according to Baisakh et al. (2006). Five µl of eluted DNA was used for re-amplification by using same primer combination and identical PCR conditions. The PCR products were examined on a 1.2% agarose gel and visualized on a UV transilluminator using ethidium bromide stain. The products were further purified using gel extraction kit (Sigma-Aldrich, USA).

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

A single colony of *E. coli* DH5α cells were inoculated into 5 ml of LB broth and incubated overnight with constant shaking at 37 °C, 200 rpm. From the overnight culture, 1 ml was taken and inoculated in 50 ml of fresh LB medium with vigorous shaking until the OD reached 0.6 at 600 nm. The cells were cooled on ice for 20 min and pelleted by centrifugation at 4000 rpm for 4 min at 4 °C. The pellet was thoroughly suspended in 30 ml of ice cold 100 mM $CaCl_2$ and incubated on ice for 30 min and further centrifuged at 4000 for 4 min at 4 °C. The pellet was finally resuspended in 3 ml ice-cold 100 mM $CaCl_2$ with 50% sterile glycerol to a final concentration of 15% (v/v) 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 then 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 for 1 hr. After growth recovery, the cells were centrifuged at 4000 rpm for 2 min and resuspended in 100–200 µl LB medium, and were spread on LA plates with appropriate antibiotics and incubated at 37 °C overnight.

2.7 Cloning and sequence analysis of TDFs

The eluted amplicons were cloned into the pTZ57R/T cloning vector (Fermentas, Germany) and sequenced commercially. Sequence information of cloned TDFs was analysed by searching for homologous sequences in non-redundant and EST databases of NCBI using basic local alignment search tools (Blastn and Blastx). Annotation was based on the best match found in blastx alignment. The putative functions of the identified genes were assigned based on their similarity with other genes available in the database. The sequences were submitted to genbank (NCBI) and the accession numbers were released in public database.

2.8 Plasmid DNA isolation from *E. coli* (mini preparation)

Transformed *E. coli* single colony was inoculated in 5 ml of LB medium containing appropriate antibiotics and allowed to grow overnight with shaking at 200 rpm at 37°C. Cells from 4-5 ml of the overnight bacterial culture were harvested in 1.5 ml micro tubes by centrifugation at 12,000 rpm at room temperature for 1 min. The pellet was resuspended in 200 µl ice-cold solution-I (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg RNase A) and resuspend pellet thoroughly using vortex. The cells were lysed by adding 200 µl freshly prepared solution-II (200 mM NaOH, 1% (w/v) SDS) and mixed by gentle inversion till the solution became clear. To this lysate, 300 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, air dried and dissolved in 50–100 µl of TE buffer and stored at –20 °C.

2.9 Semi-quantitative RT-PCR and Real Time PCR Analysis

Semi-quantitative and Real Time PCR were carried out for some selected gene fragments at different time points after treatment with the fungal pathogen to validate the differential gene expression data obtained from cDNA-AFLP and 2-DE proteomics analysis. Leaf tissues of resistant (*Arachis diogoi*) and susceptibe (*Arachis hypogaea*) plants were challenged with *Phaeoisariopsis personata* and the samples collected at 0, 24, 48, 72, 96 hpi as well as mock inoculated plants. Gene specific primers were designed for 17 TDFs chosen for cDNA-AFLP and four were chosen for proteomics validation, using oligo analyzer software (IDT). Primers were used for studies are as follows:

Table 2.1 Primers used in study- For quantitative real time PCR analysis

TDFs	Primer Abbreviation	Primer Sequence (5'-3')	Primer Size	GC in %	Tm in ºC	Amplicon Size (bp)
Lea Protein	AdRTLEA-F AdRTLEA-R	GGCTTTGCATTGTGGGACATGA TCACTCCTCGTCGTCATCGTC	22 21	50.0 57.1	58.5 58.4	189
Cystatin	AdRTCPI-F AdRTCPI-R	GGAGCAAGTAGTTGCTGGAAGC AGTGCTCTAGACACTGAAGCAGC	22 23	54.5 52.2	58.3 58.8	189
Zincfinger protein	AdRTZFP-F AdRTZFP-R	CTGTGTTGCCTTGTGGGCATAC AGGTTTCTGGCATTGGAGTTGAGG	22 24	54.5 50.0	58.9 59.4	162
RacGTPase	AdRTRACG-F AdRTRACG-R	CCT TGCTTCTGCTGAGTGTAAGGA CTTCGGCCAACTCCGTTCTTG	24 21	50.0 57.1	58.8 58.6	167
LRR-RLK	AdRTLRR-F AdRTLRR-R	CGGAAATGATGTCAAGTGTGGTGG TGAAGTGTCAATTCTGCCCTCCAG	24 24	50.0 50.0	58.3 59.1	164
CC-NB-LRR	AdRTCNLRR-F AdRTCNLRR-R	GTGTGGAGACGTTGGAATGCAAG CTGACATCGTTTGGTCAGCAAAGG	23 24	52.2 50.0	58.6 58.5	164
Cysteine protease	AdRTCP-F AdRTCP-R	TGTCCAGGGGACGAGACATG CCAGCATTCTGGAGGCAGAGTC	20 22	60.0 59.1	58.9 59.8	170
SGT1	AdRTSGT-F AdRTSGT-R	GCTTGATGGCGATGCAGCTC GGACTTCCCTCCACCTTCTTCG	20 22	60.0 59.1	59.4 59.3	162
Fatty acid β-xidation	AdRTPFBO-F AdRTPFBO-R	GCTTCAAGGAAACCCTCTGTTGC GTCGCTGTGTTCCTCCAAAGC	23 21	52.2 57.1	58.7 58.8	163
HSP70	AdRT70HSP-F AdRT70HSP-R	GGACCAAGGCACCATGTAAGAGC CTGCTCCCATGGCAACTGC	23 19	56.5 63.2	60.0 59.5	183
Serine/threonine kinase	AdRTSPK-F AdRTSPK-R	GAAGAAGGGGAACAGATGCTGGT GATTGGAGGATCGGCGTGTTC	23 21	52.2 57.1	59.1 58.2	168
Cytochrome P450	AdRTCYP450F AdRTCYP450R	AGCACTTGCAATGCTTGTTAGACG ACGAGGGCACAATTGGAGGT	24 20	45.8 55.0	58.3 59.2	149
Protein kinase-6	AdRTPK6-F AdRTPK6-R	GGACTGTGGACCTCCCTAAGC CGAGACCAAAGCGAGACCTCTC	21 22	61.9 59.1	59.3 59.3	193
15-PGDH	AdRTPGDH-F AdRTPGDH-R	CTCCACTCAGGCAAAAGTGTTGG GCTGCAAACTCGAGCTCTCC	23 20	52.2 60.0	58.4 58.5	158
Vacuolar processing enzyme	AdRTVPE-F AdRTVPE-R	GGGATGAAGGAAGGACCTAATTGC CAAGGGAAGAGAACCTATCTGCCT	24 24	50.0 50.0	57.5 58.1	148
Thaumatin like protein	AdRTTLP-F AdRTTLP-R	CGTCGCGAGTTGTCCAGCTA GTGATCGCCGGTGCAACAA	20 19	60.0 57.9	59.5 58.9	157

Sedoheptulosebisphos-phatase	AdRTSBP-F AdRTSBP-R	TGCAAGTATGCATGTTCCGAGGAA CACGCCAAAGATTGTTCCAACTGT	24 24	45.8 45.8	58.9 58.3	144
Dihydroflavonol-4-reductase	AdRTDHFR-F AdRTDHFR-R	ACTTCAAGTGGCTCCGCTGTC CACAGCCTTCTCAGCCAAAGTC	21 22	57.1 54.5	59.8 58.3	147
Glyceraldehyde-3-phosphate dehydrogenase	AdRTGAPDH-F AdRTGAPDH-R	CAGTGGACAGAGGTGCACTGC CCGACGATTCAGACACAACTGGAG	21 24	61.9 54.2	60.6 59.4	155
F-box family protein	AdRTFbox FP-F AdRTFbox FP-R	ACGTGCATGGACGACAACTGC GAGTGCAAGGGACCTGACCTCA	21 22	57.1 59.1	60.6 61.0	120
Oxygen-evolving complex	AdRT-OEChl-F AdRT-OEChl-R	GGTGTGGCTGCCAAACTCATAG GTCCTGTCACCGTGTAGAGTCTG	22 23	54.5 56.5	58.4 58.6	157
Alcohol dehydrogenase-3	AdRTADH3-F AdRTADH3-R	GACGCTTGGCGAGATCAACA AACCGGACAACCACCACATG	20 20	55.0 55.0	57.9 58.1	140
60S Ribosomal protein	AdRT60SRibP-F AdRT60SRibP-R	TGGAGTGAGAGGTGCATTTG TCTTTTGACGACCAGGGAAC	20 20	50.0 50.0	55.0 54.9	155

The total RNA (2 µg) treated with DNase I was reverse transcribed to first strand cDNA with oligo dT (18 mer) using SMART™ MMLV Reverse Transcriptase (Clontech, Becton Dickinson, USA). First strand cDNA samples were diluted to 10 times and 1µl of the diluted reaction mixture was taken as qRT-PCR template in a 20 µl total reaction volume containing 0.4 µM gene-specific primers and 10 µl SYBR Premix Ex Taq with ROX (TAKARA BIO INC.) and the samples were appraised in three technical replicates including three non-templates as the negative control. PCR analysis was carried out in Realplex (Eppendorf) Amplifier with the following cycle parameters: 95 °C for 5 min; 40 cycle of 95 °C for 20 s, 58 °C for 25 s, 72 °C for 25 s followed by a melting curve to ensure that each amplicon was a single product. Alcohol dehydrogenase class III (*adh3*) and 60S Ribosomal protein gene were used as internal control for calculating relative quantification of gene expression as these are the most stable reference genes for *Arachis* to normalize the real time amplification data (Brand and Hovan 2010, Morgante et al. 2011). Relative fold change in RNA expression was estimated using threshold cycle (C_T) to calculate the relative fold changes (RFC) in each time point of infected sample compared to control conditions by $\Delta\Delta C_T$ method with the following formula: $RFC = 2^{-\Delta\Delta CT}$ where, $\Delta\Delta C_T = (C_{T\ target} - C_{T\ adh3})_{treated\ sample} - (C_{T\ target} - C_{T\ adh3})_{\ control\ sample}$. (Livak and Schmittgen 2001).

2.10 Proteomics

Plant materials, growth conditions and pathogen inoculums were the same as described in cDNA-AFLP (Genomics).

2.10.1 Total protein extraction

Proteins were extracted from sampled leaf tissue by phenol extraction method as described by Sarvanan and Rose (2004) with some modifications. One gram of groundnut leaf tissues were ground to a fine powder with pestle in a mortar containing liquid nitrogen and suspended in 10 ml of the extraction buffer (0.5 M Tris–HCl at pH 7.5, 0.7 M sucrose, 0.1 M KCl, 50 mM EDTA, 2% β-mercaptoethanol and 1 mM PMSF). Equal volume of phenol saturated with Tris-HCl (pH 7.5) was added, mixed for 30 min at 4 °C and centrifuged at 6,000 g for 25 min at 4 °C. The upper phenolic phase was collected and an equal volume of extraction buffer was added to it. The above step was repeated and the upper phenolic phase was re-extracted.

Four volumes of 0.1 M ammonium acetate in methanol was added to the collected phenolic phase and kept overnight at -20 °C for protein precipitation. The samples were then centrifuged at 12,000 g at 4 °C for 20 min and the precipitate was washed three times each in ice cold methanol as well as in ice cold acetone and air dried. The precipitates were resuspended in 500 µl of rehydration buffer [7 M (w/v) urea, 2 M (w/v) thiourea, 4% (w/v) CHAPS, 30 mM DTT, 0.8% (v/v) immobilized pH gradient (IPG) buffer pH range 4–7 (GE Healthcare, Uppsala, Sweden)], insoluble material was removed by centrifugation and the protein concentration was estimated by Bradford assay using BSA as a standard.

2.10.2 Bradford (1976) method for protein quantification

Bradford dye was prepared by dissolving 100 mg Coomassie Brilliant blue G-250 in 50 ml of 95% ethanol. Concentrated 100 ml phosphoric acid was added into it and the volume was made up to 200 ml with H_2O. Bradford dye concentrate can be stable upto 6 months at 4 °C. The Bradford dye concentrate was diluted five times (5x) with double distilled water and 1.0 ml was added to each sample. BSA samples were prepared with 10, 20, 30, 40, 50, 75 and 100 µg/100 µl of BSA in the same buffer solution in which the protein samples were extracted. The dye turns blue after binding to protein and is

allowed to develop color for at least 5 min. Absorbance at 595 nm was taken and a linear standard curve was prepared to calculate the concentration of unknown protein. The results were cross confirmed by using Lowry method (Lowry et al. 1951).

2.10.3 Two dimensional gel electrophoresis (2D-GE)

A solution containing 800 µg of total protein in rehydration buffer in a total volume of 320 µl was used for passive rehydration of 18 cm immobilized pH gradient (IPG) strips (18 cm, 4-7 pH linear gradient; Amersham, GE). Active rehydration of protein was done on immobilized pH gradient strips for 12 h at 50 V. Rehydration and focusing was carried out in Ettan IPGphor II (GE Healthcare) at 20 °C, using the following program: 30 minutes at 500 V, 3 h to increase from 500 to 10,000 V and 6 h at10,000 V (a total of 60,000 Vh). After IEF, strips were equilibrated twice to reduce the protein followed by alkylation for 25 minutes with gentle rocking at room temperature (25 ± 2 °C) in the equilibration buffers. The first equilibration was performed in a solution containing 6 M urea, 50 mM Tris-HCl buffer (pH 8.8), 30% (w/v) glycerol, 2% (w/v) SDS and 2% DTT and the second equilibration was performed by using 2.5% (w/v) iodoacetamide (Sigma Aldrich, USA) instead of DTT. The proteins were separated in the second dimension SDS-PAGE (12% vertical polyacrylamide slab gels) at 10 mA gel^{-1} for 1 h and then 38 mA gel^{-1} for 6 h, using an EttanDalt6 chamber (GE Healthcare). Gels were stained with modified coomassie staining (Wang et al. 2007b). Protein gels were scanned by a calibrated densitometric scanner (GE Healthcare) and spot detection, normalization, gel matching, expression analyses and statistics were conducted with ImageMaster 2D Platinum v. 6.0 image analysis software (Amersham Biosciences). Proteins that displayed one and half fold or greater changes in the spot's relative volume (spot volume/total spot volume x 100) were considered as differentially expressed proteins.

2.10.4 Staining and image analysis

Coomassie Brilliant blue R-250 dye was used to stain the 2 Dimensional electrophoresis gels. The gel with electrophoretically separated protein was incubated for staining in Coomassie brilliant blue solution (0.025% Coomassie Brilliant blue R-250 in 45% methanol and 10% acetic acid) for overnight. Destaining was performed with a destaining solution (45% methanol and 10% acetic acid) to remove background stain.

The destaining solution was replaced at every 10-15 min with a fresh solution, until the protein spots were clearly visible.

2.10.5 In gel digestion and mass spectrometry (MS)

Interested protein spots which showed significant changes after treatment were excised from two or three coomassie-blue stained replicated gels. The excised protein spots were destained with 200 µL of 50% acetonitrile (ACN) in 50 mM of ammonium bicarbonate (NH_4HCO_3) until the gel was completely destained. Thereafter, the gel pieces were treated with 10 mM DTT in 50 mM NH_4HCO_3 and incubated at 56 ºC for 1 h. This was followed by treatment with 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 45 min in dark at room temperature (25 ± 2 ºC). The gel pieces were then washed with 25 mM NH_4HCO_3 and ACN, dried in Speed Vac at ambient temperature and rehydrated in 15 µl of 25 mM NH_4HCO_3 solution containing 25 ng μl^{-1} trypsin at 4 ºC for 10 minutes and then digested at 37 ºC overnight (sequencing grade, Promega, Wisconsin, USA). After incubation, a short spin was given and the supernatant was collected in a fresh eppendorf tube. The left gel pieces were further sonicated for 10 minutes followed by frequent vortexing for 5 min in 10 µl of 0.1% trifluoroacetic acid (TFA) and 100% ACN (1:1) to extract the remaining peptides. This extraction step was repeated twice to improve the extraction yield. The supernatants were pooled together and dried using Speed Vac and were reconstituted in 5 µl of 100% ACN and 0.1% TFA (1:1 v/v). The above sample (1 µl) was mixed with 1 µl of a cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 2 µl of samples were spotted onto a MALDI plate and dried at room temperature for mass spectrophotometry.

Matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) analysis was carried out using MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III Smartbeam, Bruker Daltonics, Bremen, Germany) according to the protocol of Shevchenko et al. (1996) with minor modifications. Mass data acquisitions were piloted by FlexControl 3.0 (Build 100) software using batched-processing and automatic switching between MS and MS/MS modes. All MS survey scans were acquired over the mass range of 800–3500 m/z in the reflectron positive-ion mode and accumulated from an average of 2500 laser shots with acceleration of 19 kV. Peptide precursor ions corresponding to contaminants including keratins and trypsins autolytic products were excluded in a mass tolerance of ± 0.5 Da. The filtered precursor ions with a user defined

threshold were selected for the MS/MS scan. Fragmentation of precursor ions was performed using MS-MS 1kV positive mode.

2.10.6 Protein identification through peptide mass fingerprinting and MS/MS analysis

The MALDI-TOF/TOF data were loaded into the MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics) and protein identification was performed against the NCBInr and Swiss Prot databases using a combination of MS (peptide mass fingerprint approach) with MS/MS. The taxonomic category was set to *Viridiplantae* (Green plants). The other search parameters were: monoisotopic peptide mass (MH^+); one missed cleavage per peptide; enzyme, trypsin; precursor-ion mass tolerance on an average 200 ppm; MS/MS fragment-ion mass tolerance, 0.6 Da; variable modifications, carbamidomethylation (C) for cysteine and oxidation for methionine (M) were allowed. Contaminating peptides like trypsin and keratins were excluded from the peak lists before database searching. Top hit for each protein search were reported. Only proteins with a minimum of two matched peptides were considered to be positively identified. If a protein spot matched multiple proteins under different accession numbers, the candidate protein with the maximum Mascot score with highest peptides matched were selected. The nearest experimental MW (molecular weight) and PI (isoelectric point) values to the theoretical values (having the same Mascot score) were given equal weightage in spot selection. The identified proteins were named according to the corresponding annotations in NCBI and Swiss Prot.

2.11 Restriction endonuclease treatments

All the restriction endonucleases used in this study are from Fermentas, Germany and standard molecular biology laboratory methods were adopted from Sambrook et al. (1989). DNA digestion was carried out in a reaction volume of 20 µl with an appropriate reaction buffer (10 X) and 2U of restriction enzyme was used per 0.5 µg of DNA to be digested, while for double digestions the restriction digestions were carried out sequentially.

2.12 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 (λ DNA *Hin*dIII+*Eco*RI or λ DNA *Hin*dIII). After the extraction, purification of DNA from the agarose gel pieces was done with gel extraction kit (Sigma, Aldrich) following to the manufacturer's instructions.

2.13 Dephosphorylation

The single digested DNA fragments were dephosphorylated at their 5'-ends with calf intestine alkaline phosphatase (Fermentas, Germany) 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 20 µl comprising 2 µl dephosphorylation buffer (10X), 0.5 µl (0.5 unit) of calf intestine alkaline phosphatase and appropriate µg of plasmid DNA and incubated at 37 °C for 30 min, followed by heat inactivation at 85 °C for 15 min.

2.14 Ligation

For plasmid DNA constructs, different DNA inserts were ligated in to the cloning and expression vectors according to 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.

2.15 5'/3' RACE-PCR, isolation of full length cDNA and transient conditional over-expression of pathogen induced genes (VPE, SGT1, 15-PGDH and Cystatin)

Rapid amplification of cDNA ends was performed to make full length of vacuolar processing enzyme, suppressor of G2 allele of Skp1, hydroxyprostaglandin dehydrogenase and cystatin, by using SMART^{TM} RACE cDNA Amplification kit (Clontech, USA) following manufacturer's instructions.

Primers for first-strand cDNA synthesis for 3'/5' RACE are as follows-

SMART II ^{TM} A Oligonucleotide For 5' RACE

5'–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3'

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

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

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

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

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

Universal primers for 3'/5'-RACE PCR are as follows-

10X Universal Primer A Mix (UPM)

Long (0.4 μM):

5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3'

Short (2 μM): 5'–CTAATACGACTCACTATAGGGC-3'

Nested Universal Primer A (NUP; 10 μM)

5'–AAGCAGTGGTATCAACGCAGAGT-3'

The gene specific primers used for 5'/3' RACE-PCR were designated as follows:

Cystatin	5'AdCPI-GSP1:	5'- GCAGCTCCTTGAAGTTCAACCATGGC -3'
VPE	5'AdVPE-GSP1:	5'- CCAGAAGCATGCTTCTTCTTCAAG-3'
	3'AdVPE-GSP1:	5'- TTCTTCTTCTTCAATGGAGATTGG-3'
SGT1	5'AdSGT1-GSP1:	5'- CTTGTTGGTTTTGAGGATGGGTAAG-3'
	3'AdSGT1-GSP1:	5'- CATTTGTGGAGTCTAATGGGACAG-3'
15-PGDH	5'Ad15-PGDH-GSP1:	5'- TCCTCACTATTCTGGTAGCTTTCC-3'
	3'Ad15-PGDH-GSP1:	5'- CTGTCAGTGACTTGAAGGTTGG-3'

Full length cDNA sequence of these TDFs were obtained by aligning 5'/3' RACE product and its partial sequence obtained during interaction in *A. diogoi* through cDNA-

AFLP analysis. Open reading frames of these sequences were amplified with primers incorporating specific restriction enzymes for cloning in pER8 vector by using Phusion^{TM} High Fidelity DNA polymerase (Finnzymes, NEB). Primers sequences are as follows:

SGT1: AdSGT1-ApaI-F: 5'- CGGGCCCATGGCTTCTGATCTGGAAG -3'
AdSGT1-SpeI-R: 5'- CACTAGTCTAATATTCCCATTTCTTCAACTC-3'

15-PGDH: AdPGDH-ApaI-F: 5'- AGGGCCCATGGAGATCAAACCTGG -3'
AdPGDH-SpeI-R: 5'- CACTAGTTCATAATTTGGCCACTTGCTTC-3'

VPE: AdVPE-XhoI-F: 5'- GCTCGAGATGGAGTCCCTTCTAAGGA- 3'
AdVPE-SpeI-R: 5'- CACTAGTTTATGCACTAAAACCCCTCTC - 3'

Cystatin: AdCPI-XhoI-F: 5'- TCTCGAGATGGCTACACTAGGTGGCA -3'
AdCPI-SpeI-R: 5'- CACTAGTCTAGATAAGTGCTCTAGACACTG -3'

All the above amplified PCR products were cloned in pTZ57R/T vector and sequenced for the confirmation of reading frame of the sequences. Open reading frame of the cystatin and vacuolar processing enzyme were flanking with *Xho*I and *Spe*I sites while SGT1 and 15-PGDH were possessed *Apa*I and *Spe*I as forward and reverse restriction enzyme sites for cloning into pER8 vector (a kind gift from Prof. Nam-Hai Chua, Rockefeller University) to obtain the recombinant clones. Both the empty and recombinant vectors were mobilized into *Agrobacterium tumefaciens* strain LBA4404 and used for agroinfiltration.

2.16 *Agrobacterium* competent cell preparation and transformation

Agrobacterium tumefaciens (EHA105 and LBA4404) competent cells were prepared as described for *E. coli* except that cells were grown at 28 °C. Transformation of *Agrobacterium* 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 incubation at 37 °C in a water bath for 5 min. To this, 1 ml of LB medium was added and cells were further incubated at 28 °C for 2-4 h with shaking. Then, cells

were pelleted at 4000 rpm for 5 min and plated on LB agar medium with rifampicin and the corresponding selectable antibiotics of the plasmid DNA used for transformation.

2.17 Agroinfiltration, chemical treatment and cell death measurement

Recombinant and empty binary vectors were mobilized into *Agrobacterium* strain LBA4404 using freeze thaw method. *Agrobacterium* strains harboring different binary vector constructs were grown in LB broth in the presence of respective antibiotics, pelleted, resuspended and infiltrated as described earlier (Kumar and Kirti 2011). We have used *Agrobacterium* suspension OD of 0.6 for transient expression in the study. The agroinfiltrated leaves were chemically induced by the application of 30 µM 17β-estradiol, 48 h post infiltration. Cell death phenotype was observed and samples were collected, quick frozen in liquid nitrogen and stored at -80 °C for further analysis.

Baker and Mock (1994) method has been followed for quantifying cell death. In detail, one cm^2 leaf discs were cut out of the infiltrated regions from both recombinant and empty vector and submerged in 0.25% (w/v) Evans blue solution for 30 min followed by application of a 15 min vacuum pressure. The stained leaf discs were washed with distilled water to remove unbound stain. The washed discs were cut out of the infiltrated regions from both recombinant and empty vector and submerged in 0.25% (wt/vol) Evans blue solution for 30 min followed by application of a 15 min vacuum. The leaf discs were ground in 1% SDS and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and OD was measured at 600 nm. Data from three experimental replicates were plotted.

2.18 Hormonal treatments and expression of pathogen induced genes

Twigs of two month old wild groundnut plants were cut and kept in a tray with a moist cotton saturated with sterile distilled water at the base of twigs and covered with a polythene bag to maintain humidity and left for 1-2 week until adventitious roots developed. For various chemical treatments, rooted twigs were kept in the corresponding solution. The treatments given were 100 µM salicylic acid (SA), 100 µM methyl jasmonate (MeJA), 100 µM abscisic acid (ABA), 250 µM ethephon, 100 µM sodium nitroprusside (SNP) and treatment with water served as control. The treatments were carried out at different time periods upto 24 h and incubated in a growth room at 27 ± 1 °C under 14/10 h photoperiod provided by light intensity of 30 µmol m^{-2} s^{-1}. Samples

were collected at regular intervals, quick frozen in liquid nitrogen, and stored at –80 °C until use. The chemicals used for treatments were purchased from Sigma-Aldrich, USA.

2.19 Plasmid construction of Vacuolar processing enzyme (VPE) and Suppressor of G2 allele of Skp1 (SGT1) and *Agrobacterium* mediated genetic transformation in tobacco

The open reading frame of the cDNA of *AdVPE* and *AdSGT1* were amplified using Phusion^{TM} High Fidelity DNA polymerase (Finnzymes, NEB) with primers containing corresponding restriction site are as follows-

AdVPE: AdVPE-XhoI-F: 5'- GCTCGAGATGGAGTCCCTTCTAAGGA - 3'
AdVPE-SacI-R: 5'- CGAGCTCTTATGCACTAAAACCCCTCTC -3'

AdSGT1: AdSGT1-NcoI-F: 5'- GCCATGGCTTCTGATCTGGAAGC -3'
AdSGT1-SacI-R: 5'- GCGAGCTCCTAATATTCCCATTTCTTCAAC -3'

The ORF of *AdVPE* was cloned as an *Xho*I-*Sac*I fragment into a plant expression cassette containing vector pRT100 and *AdSGT1* as an *Nco*I-*Sac*I fragment in the same vector. The *AdVPE* expression cassette containing 35S CaMV promoter and polyA signal was released with *Hin*dIII digestion and cloned in binary vector pCAMBIA2300 in the sense orientation. The *AdSGT1* expression cassette with 35S CaMV promoter and nos terminator was released with *Pst*I and cloned in binary vector pCAMBIA2300. Recombinant binary vectors were mobilised into virulent *Agrobacterium* strain EHA105 by the freeze thaw method. A single colony of *Agrobacterium* containing recombinant binary vector was inoculated and culture with bacterial OD 0.6-0.8 was used for transformation of tobacco following leaf disc method (Horsch et al. 1985).

In detail, *Agrobacterium* carrying desired recombinant binary vector was grown at 28 °C in 50 ml of LB medium containing 100 mg/l rifampicin and 50 mg/l kanamycin to exponential phase of OD 0.6-0.8. The bacterial suspension was pelleted at 5000 rpm 5 min and supernatant was discarded and the cells were resuspended with sterile double distilled water and used for explant co-cultivation. Fully developed leaves of two month old tobacco plants were surface sterilized for 7 min with 0.1% (w/v) aqueous solution of $HgCl_2$ (mercuric chloride) and subsequent washes with sterile double distilled water.

Leaf discs of 2 cm^2 diameter were excised and infected in *Agrobacterium* suspension for 10 min and co-cultivated in dark at 28 °C for 3 days. Then the leaf discs were cultured on the regeneration medium containing MS medium supplemented with 2 mg/l benzyl aminopurine (BAP), 0.1% naphthalene acetic acid (NAA), 250 mg/l cefotoxime and 125 mg/l kanamycin for two subcultures with two week duration each. Healthy kanamycin resistant elongated shoots of 4–5 cm were cut out and cultured on a rooting medium containing MSH with 1 mg/l NAA, 250 mg/l cefotoxime and 125 mg/l kanamycin for two weeks. All the cultures were maintained at 27±1 °C under a 14/10 h photoperiod with light intensity of 30 µmol $m^{-2}s^{-1}$. The rooted shoots were acclimatized in culture room conditions, transferred to soil and grown in greenhouse. Vacuolar processing enzymes (VPE) harbouring binary vector was genetically transformed in *Nicotiana tabacum* cv. xanthi while suppressor of G2 allele of Skp1 (SGT1) was transformed in to *Nicotiana tabacum* cv. samsun. The seeds from these plants were collected and were germinated on MSH containing 100 mg/l kanamycin to select for transgenic T_1 seedlings.

2.20 Molecular analysis of tobacco and groundnut transgenic plants

DNA was isolated from two month old T_0 transgenic plants using CTAB method (Doyle and Doyle 1990), and around 100ng of DNA was used for PCR amplifications. Putative transgenics were confirmed by amplifying the genomic DNA with *nptII* forward and reverse primers and also by 35S-F as the sense primer designed against the CaMV35S promoter region and gene specific primer as the antisense. Southern analysis for transgenic plants was performed in which the genomic DNA was digested with *Eco*RI and hybridization was done using [α-32P] dATP labelled *nptII* fragment obtained by the amplification of neomycin phosphotransferase gene with nptII F and nptII R primers.

VirD2-F:	5'- TGCCAGGAGGTGGAACCAAGA -3'
VirD2-R:	5'- CGATTGACTGAGGTCCCGACGA -3'
NptII-F:	5'- AGATGGATTGCACGCAGGTTCTC -3'
NptII-R:	5'- ATCGGGAGCGGCGATACCGTA -3'
AdDefensin-F:	5'- GGGTACCATGGAGAAGAAATCACTAGC -3'
AdDefensin-F:	5'- GGGATCCTTAACATCTTTTAGTACACCA -3'
CaMV35S-F:	5'- ACGACACTCTCGTCTACTC -3'

2.21 Genomic DNA extraction and purification from leaf tissue (Murray and Thompson, 1980; Doyle and Doyle, 1990)

Healthy leaf of young plants were freshly collected and frozen in liquid N_2 and stored at –70 °C. One gram of leaf tissue was homogenized to a fine powder using a motor and pestle in liquid nitrogen along with a pinch of PVPP (Polyvinyl Polypyrrolidone). About 1 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 a micro tube, pre-warmed to 65 °C and the fine powder was added to the buffer. The homogenate was mixed uniformely with the buffer by repeated inversion of the tubes and incubated in a water bath at 65 °C for 30 min. 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, the phases were separated by centrifugation at 12,000 rpm for 15 min at room temperature. The clear aqueous phase in the top layer was collected in a fresh tube with cut tips and 0.6 volume of isopropyl alcohol was added. After gently inverting for several times, the DNA which appear as threads, was spooled out with a bent thin glass rod and transferred to a fresh microtube. DNA was washed with 1 ml of 70% ethanol. The tubes were centrifuged at 10,000 rpm for 5 min at room temperature. Ethanol was decanted carefully, the pellet was briefly air dried and dissolved in 50 µl TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0).

For further purification, DNA was treated with RNase (1mg/ml) for 3 h at 37 °C. 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 gently 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 (pH 5.2) and two volumes of absolute ethanol or one volume of isopropanol followed by centrifugation at 12,000 rpm for 10 minutes at 4 °C. The pellet was washed with 70% ethanol, briefly air dried and dissolved in minimum volume (30-50 µl according to the pellet size) of deionized water or TE and stored at 4 °C for short term and at –20 °C for long-term use.

2.22 Extraction of total RNA from tobacco

About 100 mg of fresh leaf tissue was grounded with mortar and pestle to a fine powder 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 and quantified.

2.23 Quantification of RNA and DNA

The quality and concentration of RNA and DNA samples were examined by ethidium bromide stained agarose gel electrophoresis and spectrophotometric analysis. The quality of RNA and DNA were checked using a spectrophotometer (NanoDrop, Technologies Inc.) at two wavelength ratios of $A_{260/230}$ and $A_{260/280}$ nm. The integrity of total RNA and DNA were determined by running samples on ethidium bromide stained 1.0% agarose gel electrophoresis using Tris-boric acid-EDTA (TBE) buffer and Tris-acetic acid-EDTA (TAE) buffer respectively. For the purity of RNA, the value of $A_{260/280}$ must be between 1.9 and 2.1. For pure DNA, the value of $A_{260/280}$ must be between 1.8 and 2.0. A value below 1.8 means the contamination of DNA with proteins and phenolic compounds.

2.24 Southern blot analysis

For southern analysis, 10-15 µg total genomic DNA was digested with the appropriate restriction enzymes, as *Eco*RI and *Hin*dIII enzyme were the most frequently used enzyme for Southern analyses and incubating overnight at 37 °C 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 °C until use. For resolving the digested DNA, 0.8% agarose gel was prepared by melting 0.8 g agarose in 100 ml of 1X TAE buffer (diluted from 50X TAE: 2M Tris, 1M Acetate, 100mM EDTA, pH 8.1) and pouring onto a casting tray with a comb. After the gel got 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 12 cm from the well. As a reference 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 DNA *Hin*dIII+*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 α-32P dATP radiolabelled DNA using Prime-a-gene labeling system of Promega, USA. After hybridization, the membranes were washed twice with 2X 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.

2.25 Preapration of α-32P 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 α-32P 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 boiling at 95-100 °C for 5 min, chilling on ice and adding EDTA to 20 mM. This was used directly in a hybridization solution.

2.26 Bioassay of *AdVPE* and *AdSGT1* transgenic tobacco plants against phytopathogenic fungus

Detached leaf assay was performed to analyze the disease resistance response of *AdVPE* and *AdSGT1* transgenic plants against fungal pathogen *Phytophthora parasitica* var. *nicotianae and Alternaria alternata var. nicotianae.* Whole plant assay was carried out to analyse disease resistance of *AdVPE* and *AdSGT1* transgenic plants against the fungal pathogen *Rhizoctonia solani*. 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 and transgenic plants were placed on moist filter papers for fungal inoculation. A 0.5 cm diameter plug of *Phytophthora parasitica* var. *nicotianae* fungal mycelium grown on PDA was placed on the middle of the adaxial side of leaf after damaging the leaf with a sterile blade to promote fungal infection (Tedford et al. 1990). In case of *Alternaria alternata var. nicotianae*, a 10^5 spores/ml suspension solution was inoculated on leaf petiole cut point covered with water soaked cotton (Lorito et al. 1998). The leaves were placed in growth room with 16h: 8h of light: dark photoperiod. Leaf damage and symptoms were recorded after seven and ten days. All the experiments were performed in duplicate. To execute whole plant assay, one month old seedlings of wild type and transgenic seedlings were used to analyse resistance against fungal pathogen *Rhizoctonia solani* (Anderson 1982). Fully grown fungal mycelium on PDA were cut into number of pieces of 0.5 cm diameter and 4-5 pieces were placed in each pot containing seedling, which was pretreated with 3% sucrose for better growth of fungal mycelium. The seedlings were placed in growth room with 16h: 8h of light: dark photoperiod and symptoms were recorded after fifteen days post inoculation.

2.27 *In Planta* genetic transformation of *Arachis hypogaea* cv. JL-24 with pCAMBIA2300 harboring *AdSGT1* gene

Arachis hypogaea cv. JL-24 seeds were surface sterilized by rinsing with 70% (v/v) ethanol for one min and then with 0.1% (w/v) aqueous $HgCl_2$ for 7 min followed by several washes with sterile double distilled water. Seeds were kept for germination on autoclaved filter paper soaked in sterile double distilled water on 110 mm Petri plate.

We have used non tissue culture approach, the *in planta* method to generate transgenic groundnut plants as reported by Rohini and Sankara Rao (2000) with minor modifications. *Agrobacterium* strain EHA105 containing *AdSGT1:*pCAMBIA2300 was grown overnight at 28 °C in LB medium containing 100 mgl^{-1} rifampicin and 50 mgl^{-1} kanamycin to log phase of OD 0.6-0.8. The bacterial suspension was pelleted at 5000 rpm 5 min and supernatant were discarded and resuspended in 100 ml of Winans' AB medium pH 5.2 (Winans et al. 1988) grown for 18 h. For *vir* gene induction, 150 µM acetosyringone was added to the *Agrobacterium* suspension in Winans' AB medium, 5 h before infection (Cheng et al. 1996). 4-6 day old germinating seedlings were infected by separating the cotyledons and removing one of them without damaging plumule and then pricked at the meristem region with a sterile needle and subsequently dunked in the

culture of *Agrobacterium* and infection was carried out by gentle agitation at 28 °C for 3 hours. Seedlings were blot dried and transferred to autoclaved soilrite moistened culture bottle and allowed to grow under aseptic conditions, which were maintained at 27±1 °C under a 14/10 h photoperiod with light intensity of 30 µmol $m^{-2}s^{-1}$. They were grown under growth room conditions for at least 10 days and then transferred to the greenhouse.

2.28 *Phaeoisariopsis personata* fungal bioassay of *AdSGT1* groundnut transgenic plants

Transgenic groundnut T_2 plants in the green house were evaluated for the disease resistance against *P. personata* infection. Conidia were collected from infected leaf samples and were allowed to germinate in sterile double distilled water and germinability was checked under microscope.

2.28.1 Detached leaf assay

Fungal assay was performed using healthy leaves of one month old T_2 plants. The concentration of conidial suspension was adjusted to 10^5 conidia/ml using haemocytometer and assys was carried out using conidial suspension with 0.1% of Tween-20 (v/v) as a surfactant. Conidial suspension was inoculated using atomizer and leaflets were placed on moist filter paper inside 110 mm Petri dishes sealed with parafilm to maintain the humid conditions (≥95 % RH) and placed in growth room with 16h: 8h of light:dark photoperiod and symptoms were recorded after 18 and 21 days post inoculation.

2.28.2 Whole plant assay

Forty days old healthy T_2 transgenic plants in glass house were inoculated uniformly by spraying conidial suspension of concentration 10^5 per ml, using an atomizer. Plants were irrigated and covered with thin plastic sheet to maintain high humidity (≥95 % RH) for 2-3 days at 24 °C. Symptoms were recorded after 21 days post inoculation and the experiment was repeated twice.

2.29 Subcellular localization of *AdSGT1*

AdSGT1 cDNA was amplified from reverse-transcribed RNA using primers AdSGT1302NcoI-F and AdSGT1302SpeI-R incorporated with *Nco*I and *Spe*I restriction

sites respectively. The resulting fragment was cloned into pCAMBIA1302 vector digested with appropriate restriction enzymes to make an in-frame N-terminally fusion with GFP to obtain *AdSGT1*:pCAMBIA1302. The pCAMBIA1302 control vector and *AdSGT1*: pCAMBIA1302 constructs were mobilized into *A. tumefaciens* strain EHA105 by the freeze thaw method (Holsters et al. 1978). In order to determine the subcellular localization of GFP-AdSGT1 fusion protein, leaves from 4 week old *N. benthamiana* plants were used for transient gene expression by agroinfiltration as described by Yang et al. (2000). In detail, *Agrobacterium* strains harboring the corresponding clones were grown overnight at 28 °C in the presence of appropriate antibiotics, pelleted at 4000 rpm for 5 min and diluted to an OD600 of 0.8 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). Primers used for subcellular localization study of *AdSGT1* are as follow:

AdSGT1302NcoI- F 5'- GCCATGGCTTCTGATCTGGAAGC-3'
AdSGT1302SpeI- R 5'- CACTAGTATATTCCCATTTCTTCAACTCCA -3'

2.30 Sequence and Statistical analysis

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) (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). All the primers were designed using IDT oligoanalyzer (www.idtoligoanalyzer). GraphPad Prism software version 5 was used to make all bar diagrams.

Chapter 3

Analysis of differential gene expression in wild groundnut, *Arachis diogoi* challenged with late leaf spot pathogen using cDNA-AFLP and its quantitative comparison with susceptible groundnut

3.1 Introduction and Background

Defense responses are induced in plants to protect themselves against impending pathogen attack. These responses include cell wall fortification, production of phytoalexins and secondary metabolites followed by defense related responses such as hypersensitive response (Dangl et al. 1996, Dangl and Jones 2001), production of reactive oxygen species (Sutherland 1991), production of antimicrobial compounds (Osbourn 1999) and pathogenesis related protein (Ibeas et al. 2000). Groundnut, *Arachis hypogaea* is one of the most important oil seed crops in the world, particularly in the Semi Arid Tropical region and is widely cultivated for its high quality edible oil and high protein content in the seed. Because of the rain-fed nature of the crop, its yields depend on the vagaries of nature in the form of biotic and abiotic stresses. Biotic stresses include diseases caused by fungal pathogens such as early leaf spots caused by *Cercospora arachidicola* and late leaf spot caused by *Phaeoisariopsis personata* (Berk & Curtis, previously known as *Cercospora personatum*) and the rust caused by *Puccinia arachidis*. Late leaf spot disease is the most devastating of these and can lead to yield losses up to 70% under favourable conditions (Grichar et al. 1998).

The groundnut genome (2,800 Mb/1C, Young et al. 1996) is large in comparison to other plant models, *Arabidopsis* (128 Mb), rice (420 Mb), *Medicago* (500 Mb) and soybean (1,100 Mb). However, the groundnut research community has reached the level of 252,832 expressed sequence tags (ESTs) in the public NCBI database till March 2012 in comparison to closely related soybean, which is represented by 1,461,624 ESTs (Feng et al. 2012). It has also been reported that an analysis of the groundnut transcriptome by RNA-seq using next-generation Illumina sequencing during seed development has generated a large number of unigenes and about four thousand SSR primers from three different varieties of groundnut (Zhang et al. 2012). Guo et al. (2008) constructed cDNA libraries for groundnut gene expression profiling in developing seeds at different reproductive stages during *Aspergillus parasiticus* infection.

Despite these transcriptome analyses, there were no reports on the availability of sources of resistance genes in the cultivated genotypes of groundnut. However, wild relatives of the Genus *Arachis* are a rich source of genes for disease resistance, which can be exploited by cloning through genomic approaches. In the genus *Arachis,* there are many wild species at diploid and allo-tetraploid levels that possess resistance to various biotic

and abiotic stresses making them a rich repository of genes of commercial importance. There were many attempts aimed at transferring the genes for disease resistance from the wild species to the cultivated accessions through conventional breeding programs (Wynne et al. 1991, Singh et al. 1997). However, these attempts proved to be unsuccessful as the introgression of genes from these wild species also resulted in a linkage drag transferring unnecessary gene blocks carrying the desired genes, and making the introgressed material unsuitable for use in subsequent breeding programs.

Strategy to improve resistance is to characterize and clone novel resistance gene homologs from the resistant wild relatives. Several diploid wild species of the genus *Arachis*, Viz., *A. diogoi, A. stenosperma, A. cardenasii, A. duranensis* etc. show very high levels of resistance to fungal and rust pathogens (Pande and Narayana Rao 2001). These will constitute ideal material to study the differences at molecular level involved in conferring resistance or susceptibility. Nobile et al. (2008) group elucidated the defence strategies of groundnut by using the approach of suppression subtractive hybridization (Guo et al. 2011) and used cDNA microarray strategy to identify the gene(s) for resistance to *Aspergillus flavus* in groundnut. Payton et al. (2009) compared gene expression profile in a variety of groundnut tissue using high density oligonucleotide microarrays.

Recently, a study on the differential gene expression in *Arachis diogoi* upon infection from the late leaf spot pathogen was reported by using Genefishing DEG premix kit in a differential display-reverse transcription PCR study (Kumar and Kirti 2011) and differentially expressed groundnut genes were identified and analyzed in response to challenge with bacterial wilt disease caused by *Ralstonia solanacearum* (Ding et al. 2012). Peng et al. (2011) identified 119 TDFs from resistant and susceptible cultivars of groundnut (Spanish type) using cDNA-AFLP after inoculation with the bacterial pathogen, *Ralstonia solanacearum* that causes wilt disease and studied their expression patterns.

Several methods are available for studying differential gene expression and cDNA-AFLP is an extremely efficient, sensitive and reproducible technique for the detection of differentially expressed genes (Vos et al. 1995, Bachem et al. 1996). It is a genome-wide expression analysis technique, which does not require prior sequence information, which makes it an excellent tool for gene discovery (Ditt et al. 2001). In relation to

hybridization-based techniques, such as macro- and microarrays, cDNA-AFLP can discriminate between homologous genes belonging to gene families that are very common in plants. Besides, the sensitivity of the technique is very high resulting in an excellent detection of low-abundance genes and, both induced and repressed genes can be easily detected (Fukumura et al. 2003). There are many examples of the successful use of the cDNA-AFLP as a genome-wide expression analysis tool of genes involved in various biological processes ranging from plant development to responses to environmental stimuli. Wang et al. (2009a, 2010d) revealed differential gene expression in incompatible and compatible interaction of wheat challenged with stripe rust fungus using cDNA-AFLP while Cheng et al. (2010) identified differentially expressed genes induced by bamboo mosaic virus infection in *Nicotiana benthamiana* by the same technique. Studies on abiotic stresses like response to salt in a halophyte, *Spartina alterniflora* (Baisakh et al. 2006), drought stress in *Popolus hopeiensis* (Song et al. 2012) and heat stress in rice (Liao et al. 2012) also revealed differentially expressed genes in transcriptome profiling by cDNA-AFLP leading to the identification of the candidate genes.

The objective of this study is to investigate the molecular responses of the wild groundnut, *Arachis diogoi* when challenged with the fungal pathogen, *Phaeoisariopsis personata* and we have identified genes differentially expressed during the incompatible interaction in wild groundnut by the cDNA-AFLP technique. We have validated the expression patterns of some of the genes and the novelty of this study lies in a comparative analysis of the expression of these genes in compatible (susceptible) and incompatible (resistant) interactions at different time points through qRT-PCR. Here, we report a number of gene fragments that were found to be induced or repressed during incompatible interaction between the wild groundnut and *P. personata*. Differentially expressed genes were discussed with emphasis on genes involved in defense, signal transduction and others cellular metabolic processes.

3.2 Results

We have investigated pathogenesis pattern of *P. personata* in groundnut during infection by light microscope and found that leaf spot disease appeared on the susceptible host plant (*Arachis hypogaea* cv. JL-24) after 10-12 day post inoculation of fungal spores while no such disease symptoms were found on the resistant wild species (*Arachis diogoi*). An early stage was chosen for analysis, as the conidia of *P. personata* were observed to germinate after 12-24 hpi and their germ tubes enter the plant cells directly via the epidermis or more frequently through stomata, allowing the intracellular mycelial growth (Abdou et al. 1974, Shokes and Culbreath 1997). Spots were fully developed after 20-24 day post inoculation in compatible (susceptible) plants, but no such lesions were observed on incompatible (resistant) plants. We chose to indentify changes in gene expression analysis in incompatible interaction at 24, 48, 72 and 96 hpi during the course of infection and its comparison to compatible interaction.

3.2.1 Identification of differentially expressed genes as TDFs during *A. diogoi* and *P. personata* interaction

We carried out a cDNA-AFLP analysis on the RNA samples of *Arachis diogoi* a wild accession as control and treated samples, which were pooled from different stages such as 24, 48, 72, 96 hpi of infection with *P. personata* along with a mock treatment. A total 64 primer combinations were used to visualize 4047 TDFs in control and treated samples. The number of amplified fragments varied from 30 to 55 per lane and their sizes ranged from 75 to 700 bp depending upon primer combinations. A total 233 differentially expressed gene fragments were selected on the basis of their intensity differences between control and treated sample, of which 125 were upregulated, 64 downregulated and 44 point expressed indicating that genes were expressed during interaction and the point expression TDFs were classified under upregulated gene fragments. These selected TDFs were recovered from gels, re-amplified, sub-cloned and sequenced commercially.

3.2.2 cDNA-AFLP Gel Picture:

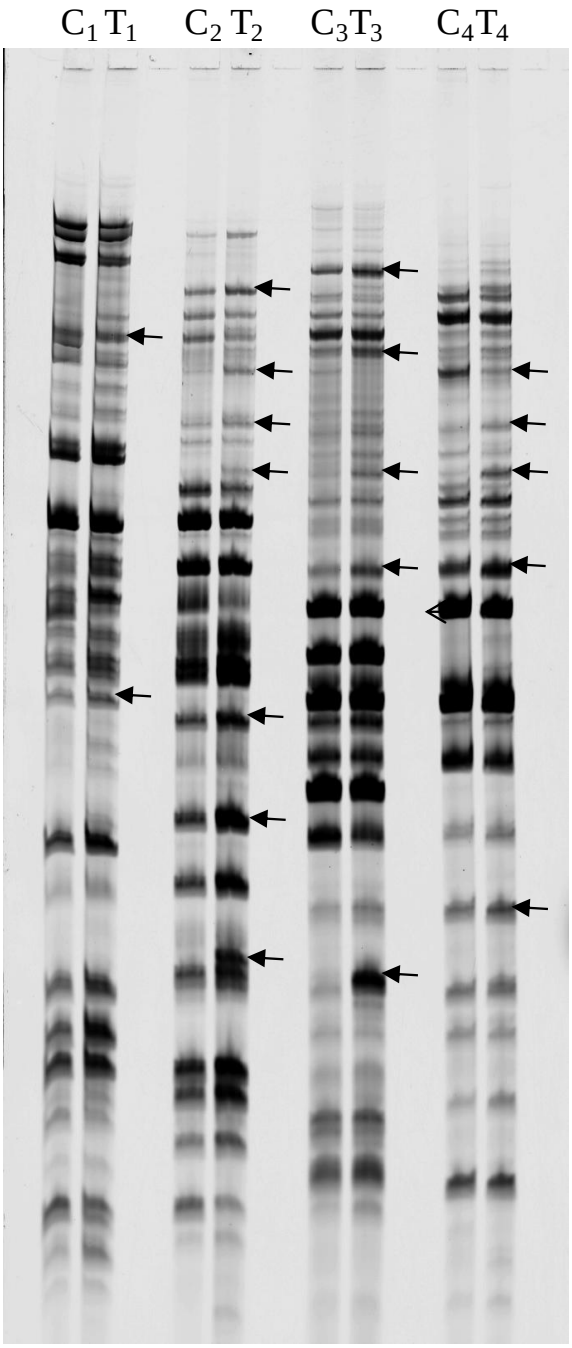


Figure 3.1 A representative picture of cDNA-AFLP gel showing differential expression of TDFs upon pathogen challenge in *Arachis diogoi*. C- represent the pool sample of mock inoculated at 24,48,72 and 96 hrs, while T- represent the pool sample of pathogen inoculated at 24,48,72 and 96 hrs. The primers combination used were; Lane C_1T_1: M-CAG/E-AGG, C_2T_2: M-CAG/E-ACG, C_3T_3: M-CAG/E-AGC, C_4T_4: M-CAG/E-ACC. Arrow indicate differentially expressed TDFs selected for further analysis.

Table 3.1 Important gene fragments and their significant similarity; U- Upregulated, D- Downregulated

TDF No.	Accession	Length	U/D	Annotation (BlastX), Organism	E-value
				Signal transduction	
1.	GU011970	309	D	Zinc finger protein, putative [*Ricinus communis*]	6e-44
2.	FJ581437	541	D	Receptor kinase, putative [*Ricinus communis*]	6e-99
3.	GU320766	120	U	DNA-binding SAP; Zinc finger, MIZ-type; Zinc finger, FYVE/PHD-type [*Medicago truncatula*]	2e-07
4.	GU473166	309	U	Protein kinase, putative [*Aspergillus flavus* NRRL3357]	0.001
5.	GQ922055	358	U	Serine/threonine-protein kinase PBS1, putative [*Ricinus communis*]	7e-58
6.	GU592825	231	U	Flag-tagged protein kinase domain of putative mitogen-activated protein kinase kinase kinase	2e-23
7.	GU592827	420	D	DNAJ heat shock N-terminal domain-containing protein [*A. thaliana*]	1e-68
8.	GU133626	243	U	Chaperone protein DnaJ-like [*Glycine max*]	6e-06
9.	GU062406	213	U	Sister chromatid cohesion 1 protein, putative [*Ricinus communis*]	1e-18
				Defense	
10.	EU935215	104	U	Cystatin [*Spinacia oleracea*]	9e-20
11.	GQ922057	351	U	SGT1-2 [*Glycine max*]	6e-53
12.	GQ922059	429	U	Heat shock 70 kDa protein, mitochondrial-like [*Glycine max*]	6e-89
13.	GU592820	351	U	CC-NB-LRR type disease resistance protein Rps1-k-2 [*Glycine max*]	4e-34
14.	GQ466607	666	U	Thaumatin-like protein 1a-like [*Glycine max*]	7e-93
15.	JN160607	240	U	Vacuolar-processing enzyme-like [Glycine max]	9e-41
16.	FJ581436	298	U	rac GTPase activating protein 1 [*Lotus japonicus*]	2e-24
17.	GU785018	285	U	15- Hydroxyprostaglandin dehydrogenase [*Medicago truncatula*]	1e-47
				Metabolism	
18.	GU223572	408	U	Isoamyl acetate-hydrolyzing esterase, putative [*Ricinus communis*]	1e-57
19.	GU223575	201	U	Late embryogenesis abundant protein Lea14-A, putative [*Ricinus communis*]	6e-11
20.	GU223576	142	U	Similar to beta-glucosidases [*Arabidopsis thaliana*]	1e-11
21.	GU011969	440	D	Ribonucleoprotein, chloroplast, putative [*Ricinus communis*]	2e-52
22.	GU223577	288	D	Nucleotide binding protein, putative [*Ricinus communis*]	3e-52
23.	GU011971	150	U	Fructose-1,6-bisphosphatase [*Fragaria x ananassa*]	2e-28
24.	GU223578	310	U	Exostosin-like [*Medicago truncatula*]	3e-27
25.	EU935216	362	U	Adenosine 5'-phosphosulfate reductase [*Glycine max*]	4e-74
26.	GU320767	115	U	Putative beta-galactosidase [*Glycine max*]	5e-15
27.	GU320768	246	U	Putative mutator sub-class protein [*Arachis hypogaea*]	8e-17
28.	FJ231268	206	U	Methionine synthase [*Glycine max*]	2e-32
29.	GU320771	669	D	Amine oxidase, putative [*Ricinus communis*]	6e-148
30.	FJ621571	498	D	Similar to cysteine protease Cp5 [*Vitis vinifera*]	1e-61
31.	FJ621572	426	D	Polygalacturonase precursor [*Glycine max*]	5e-63
32.	GU326969	261	D	Endo beta n-acetylglucosaminidase, putative [*Ricinus communis*]	6e-12
33.	GU326970	126	U	F-box family protein [*Populus trichocarpa*]	6e-06
34.	GU326971	300	U	Polyprotein [*Sorghum bicolor*]	1e-18
35.	GU326972	213	U	Retrotransposon gag protein [*Arachis hypogaea*]	8e-29
36.	GQ922058	432	U	Dihydroflavonol-4-reductase [*Medicago truncatula*]	8e-62
37.	GU473169	288	U	Probable NADH dehydrogenase-like[*Glycine max*]	2e-29

38.	GU473170	267	D	Cellulose synthase catalytic subunit [*Gossypium hirsutum*]	3e-57
39.	GU473171	315	U	Microtubule-associated protein, putative [*Ricinus communis*]	1e-27
40.	GU576547	552	U	GIGANTEA [*Glycine max*]	2e-99
41.	GU576549	285	U	Peroxisomal fatty acid beta-oxidation multifunctional protein [*Glycine max*]	9e-42
42.	GU062405	240	U	Glycine-rich protein [*Arabidopsis thaliana*]	5e-23
43.	GU576554	150	U	Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase [*Pisum sativum*]	1e-26
44.	GU592818	198	U	Phytochrome A1 [*Glycine max*]	1e-28
45.	GQ979706	226	U	Nucleic acid binding protein, putative [*Ricinus communis*]	4e-47
46.	GU592826	633	D	Granule-bound glycogen (starch) synthase [*Astragalus membranaceus*]	1e-123
47.	GU785014	135	U	N-alpha-acetyltransferase 35, NatC auxiliary subunit-like [*Glycine max*]	3e-15
48.	GU785017	153	U	Short-chain dehydrogenase/reductase [*Medicago truncatula*]	1e-16
49.	GU785019	168	U	Gag-pol polyprotein [*Phaseolus vulgaris*]	0.024
50.	FJ226754	338	U	ATP-dependent Clp protease regulatory subunit CLPX [*Arabidopsis thaliana*]	3e-66
51.	GU326968	255	U	Gag-pol polyprotein [Phaseolus vulgaris]	9e-19
				Photosynthesis	
52.	EU935214	355	U	Thylakoid lumen protein, chloroplast precursor [*Arabidopsis thaliana*]	2e-57
53.	FJ226755	210	U	Photosystem II type I chlorophyll a/b-binding protein[*Glycine max*]	2e-38
54.	GQ979704	187	D	Mg chelatase subunit (46 kD) [*Glycine max*]	1e-18
55.	GQ922056	591	U	Cytochrome P450 monooxygenase CYP97C10 [*Glycine max*]	2e-125
				Transport	
56.	FJ231267	300	D	Nodulin 26-like protein [*Medicago truncatula*]	1e-32
57.	GQ293093	129	D	AAA ATPase; ABC transporter, transmembrane region, type 1 [*Medicago truncatula*]	6e-08
58.	GU062403	204	D	Protein alx, putative [*Ricinus communis*]	2e-17
59.	GU576551	132	U	ATP/ADP transporter [*Populus trichocarpa*]	0.91
60.	GU062404	130	U	Cytochrome c biogenesis [Medicago truncatula]	5e-17
61.	GU592822	147	U	Nucleobase ascorbate transporter [*Populus trichocarpa*]	0.065
62.	GQ466606	465	D	Glutathione-regulated potassium-efflux system protein kefB, putative [*Ricinus communis*]	2e-63
				Transcription Factors	
63.	GU320773	207	D	ATP-dependent RNA helicase eIF4A, putative [*Phytophthora infestans* T30-4]	5e-35
64.	GQ293095	236	U	Transcriptional regulator, LysR family[*Burkholderia phytofirmans* PsJN]	2.0
65.	GU473167	237	U	Squamosa promoter-binding protein, putative [*Ricinus communis*]	1e-19
66.	GU062402	354	D	Valine--tRNA ligase-like protein [*Arabidopsis thaliana*]	5e-67
67.	GU592821	201	U	Pentatricopeptide repeat-containing protein, putative [*Ricinus communis*]	0.025
68.	GU320772	306	U	U3 small nucleolar RNA (U3 snorna) associated protein	3e-53

3.2.3 Gene sequence analysis

The sequences of the 233 transcript derived fragments were annotated by similarity search using the basic local alignment search tool (BLASTx & BLASTn) program against the non-redundant (nr) public database of the NCBI-GenBank. About half of the TDFs were identified to be coding for hypothetical proteins with no significant similarity to existing sequences in the GenBank. This shows the importance of the detailed analyses of the hypothetical proteins in identifying novel genes involved in the tolerance to biotic and abiotic stresses. According to Bevan's method, the TDFs were grouped into functional categories based on their homology to known proteins. A major group of 75 sequences (32.2%) showed no significant similarity, while 55 (23.6%) sequences were designated as unknown/hypothetical proteins. Genes involved in metabolism were found to be 53 (22.7%) and 15 (6.4%) sequences shared high similarity with genes functioning in signal transduction. The genes involved in defence and transcription factors shared equal number of 11 (4.7%) sequences, while the rest of the sequences were a group of genes involved in photosynthesis 5 (2.2%) and transport 8 (3.4%). The differentially expressed upregulated, downregulated TDFs were listed in Table- 1 and their sequences were submitted to NCBI database with assigned accession numbers. Most of the sequences matched with *Glycine max* and *Medicago truncatula* with significant similarity.

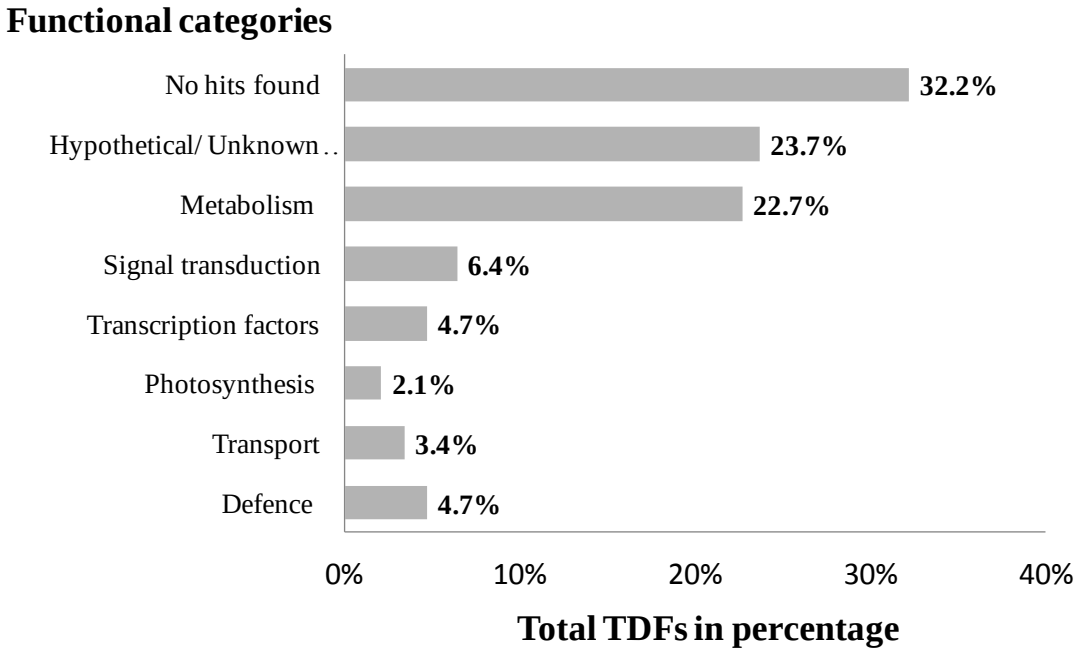
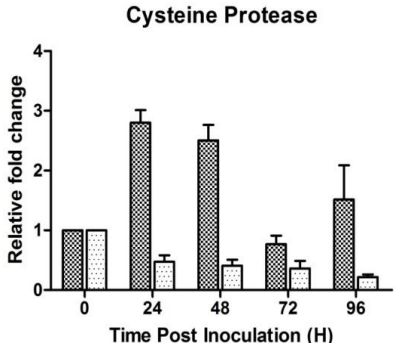
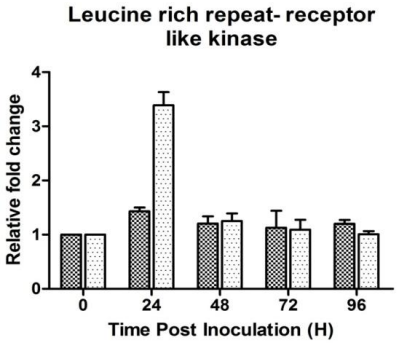


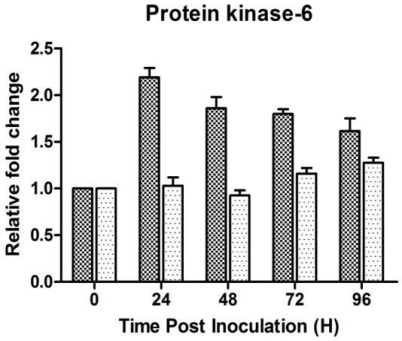
Figure 3.2 Classification of differentially expressed transcripts (TDFs) A total of 233 TDFs were classified based on the BLASTx homology search**.**

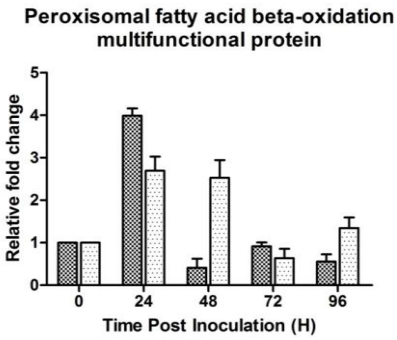
3.2.4 qRT-PCR analysis of different TDFs to validate cDNA-AFLP results

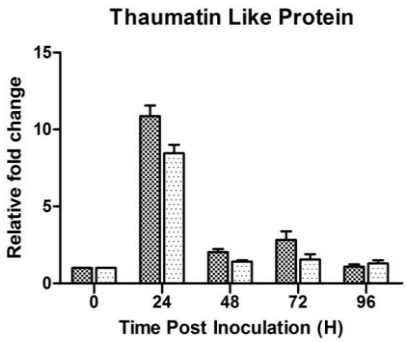
Seventeen differentially expressed TDFs, of which most of them were related to defense and signal transduction, were selected for validating the results obtained using the cDNA-AFLP analyses using qRT-PCR. In this analysis, a comparison has been made for the expression of the chosen genes between the resistant wild groundnut and the susceptible cultivated groundnut at similar time points (0, 24, 48, 72 and 96 hpi) after the pathogen challenge. This study showed that most of the gene fragments related to signal transduction and defense such as CC-NB-LRR, suppressor of G2 allele of Skp1 (*SGT1*), racGTPase activating protein, serine-threonine protein kinase, vacuolar processing enzyme (*VPE*), zinc finger protein, thaumatin like protein (*TLP*), Protein kinase-6, late embryogenesis abundant (*LEA*) protein and cysteine protease were found to be up-regulated within 24 hrs after the pathogen treatment in the resistant genotype compared to the susceptible cultivated genotype, which showed no such upregulation. 15-Hydroxyprostaglandin dehydrogenase was found to be upregulated constantly upto 96 hrs in the wild groundnut, while there was no such upregulation in the susceptible groundnut plants. Cysteine protease inhibitor has shown early response in the susceptible groundnut plants i.e, upregulted at 24 and 48 hrs, while the wild groundnut plants showed late response at 72 and 96. Leucine rich repeat- receptor like kinase (LRR-RLK) was found to be up-regulated in susceptible plants at 24 hrs, while no change was observed in the resistant plants, which was in agreement with our cDNA-AFLP data, where it was found to be downregulated. It is possible that it might be involved in downstream signaling in relation to pathogen recognition. Two TDFs of the cysteine protease showed contrasting expression patterns. While one was downregulated upon pathogen challenge, the other was upregulated. The upregulation of the cysteine protease has also been validated in the qRT-PCR analysis. Kumar and Kirti (2011) also observed the up-regulation of a cysteine protease in the same incompatible interaction with the DD-RT-PCR analysis using the SeeGene DEG Kit. Similarly, a zinc finger protein was found to be down-regulated in cDNA-AFLP analysis as against a second transcript that has shown up-regulation. Of total 17 gene fragments, gene expression patterns of fifteen were similar to those observed in cDNA-AFLP analysis. The results indicate that cDNA-AFLP technique and RT–PCR data are mostly concordant, confirming the reliability of the results with minimum discrepancies. The transcripts of a LEA protein, cysteine protease and the *Sgt1* showed very clear differences between the resistant and susceptible genotypes with the wild groundnut showing strong upregulation.

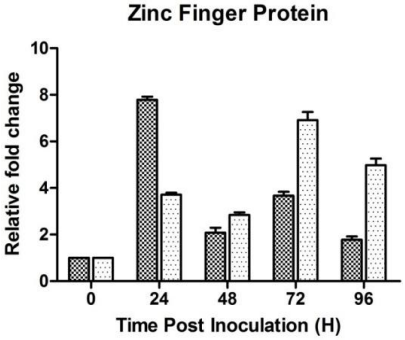

Cysteine Protease
Relative fold change
0
1
2
3
4
0
24
48
72
96
Time Post Inoculation (H)

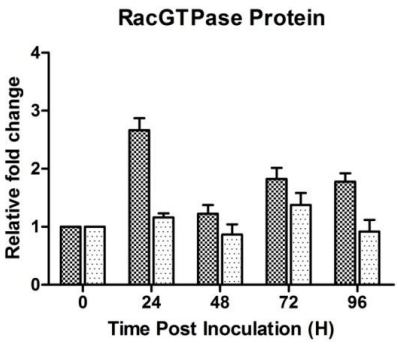

Leucine rich repeat- receptor like kinase
Relative fold change
0
1
2
3
4
0
24
48
72
96
Time Post Inoculation (H)

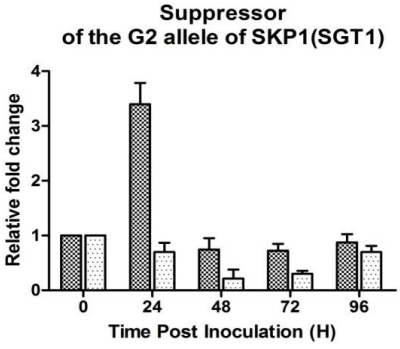

Protein kinase-6
Relative fold change
0.0
0.5
1.0
1.5
2.0
2.5
0
24
48
72
96
Time Post Inoculation (H)

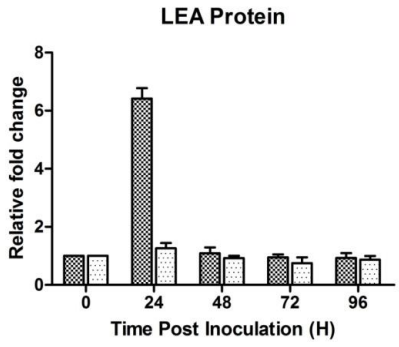

Peroxisomal fatty acid beta-oxidation multifunctional protein
Relative fold change
0
1
2
3
4
5
0
24
48
72
96
Time Post Inoculation (H)

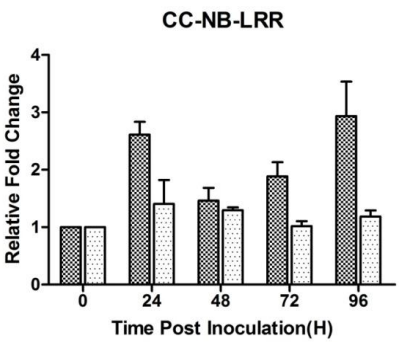

Thaumatin Like Protein
Relative fold change
0
5
10
15
0
24
48
72
96
Time Post Inoculation (H)


Zinc Finger Protein
Relative fold change
0
2
4
6
8
10
0
24
48
72
96
Time Post Inoculation (H)


RacGTPase Protein
Relative fold change
0
1
2
3
4
0
24
48
72
96
Time Post Inoculation (H)


Suppressor of the G2 allele of SKP1(SGT1)
Relative fold change
0
1
2
3
4
0
24
48
72
96
Time Post Inoculation (H)


LEA Protein
Relative fold change
0
2
4
6
8
0
24
48
72
96
Time Post Inoculation (H)


CC-NB-LRR
Relative Fold Change
0
1
2
3
4
0
24
48
72
96
Time Post Inoculation(H)

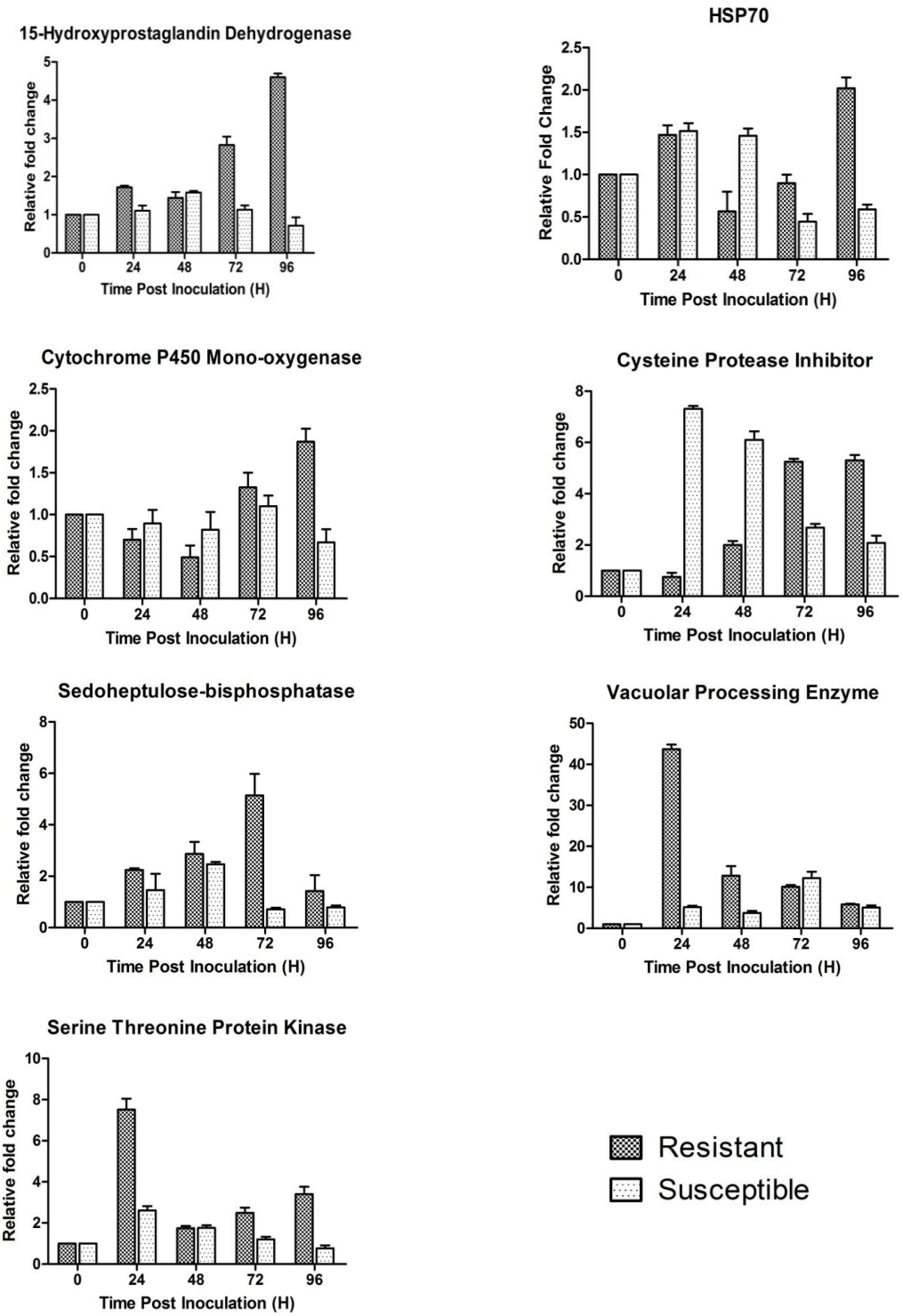


Figure 3.3 Quantitative real-time PCR (qRT-PCR) analyses of 17 selected TDFs- Leaf tissues were used for both inoculated and mock inoculated plants at 24, 48, 72 and 96 hpi, as well as mock-inoculated near 0 hpi. Relative gene quantification was calculated by comparative ΔΔCT method. All data were normalized to the Alcohol dehydrogenase-3 and 60S ribosomal protein expression level as these were used as internal reference gene and data was plotted from three independent experiment. *Arachis diogoi* (Resistant) and *Arachis hypogaea* cv. JL-24 (Susceptible).

Table 3.2 Quantitative validation of some TDFs which were differentially expressed in incompatible (Resistant = *A. diogoi*) interaction and compared with compatible (Susceptible = *A. hypogaea* L.) interaction upon *Phaeoisariopsis personata* inoculation at different time points. NC- No changes in expression profiling , + Transcripts >1 folds, ++ Transcripts > 2 folds, +++ Transcripts > 5 and < 10 folds, ++++Transcripts >10 folds, - Transcripts were repressed

Accession no.	Annotation	Blast score	Category	Incompatible interaction (in hpi)				Compatible interaction (in hpi)			
				24	48	72	96	24	48	72	96
EU935215	Cystatin [*Spinacia oleracea*]	9e-20	Defense	-	+	+++	+++	+++	+++	++	++
GQ466607	Thaumatin-like protein 1a-like [*Glycine max*]	7e-93	Defense	++++	+	+++	+	+++	+	+	+
GU592820	CC-NB-LRR type disease resistance protein Rps1-k-2 [*Glycine max*]	4e-34	Defense	++	+	++	++	+	+	NC	NC
JN160607	Vacuolar-processing enzyme-like [*Glycine max*]	9e-41	Defense	++++	++++	++++	+++	+++	++	++++	+++
FJ581436	rac GTPase activating protein 1 [*Lotus japonicus*]	2e-24	Defense	++	+	+	+	+	-	+	-
GQ922055	Serine/threonine-protein kinase [*Ricinus communis*]	7e-58	Signal transduction	+++	+	++	++	++	+	+	-
GU785018	15-Hydroxyprosta-glandin dehydrogenase [*Medicago truncatula*]	1e-47	Defense	+	+	++	++	NC	-	NC	-
FJ621571	Cysteine protease Cp5 [*Vitis vinifera*]	1e-61	Defense	++	++	-	+	-	-	-	-
GU011970	Zinc finger protein, putative [*Ricinus communis*]	6e-44	Signal transduction	+++	++	++	+	++	++	+++	++
FJ581437	LRR -receptor like kinase, putative [*Ricinus communis*]	6e-99	Signal transduction	+	NC	-	+	++	NC	-	NC
GU592825	Protein kinase -6 [*Glycine max*]	2e-23	Signal transduction	++	+	+	+	NC	-	NC	+
GQ922056	Cytochrome P450 monooxygenase CYP97C10 [*Glycine max*]	2e-125	Defense	-	-	+	+	NC	-	NC	-
GU223575	Late embryogenesis abundant protein Lea14-A, putative [*Ricinus communis*]	6e-11	Abiotic stress	+++	NC	NC	NC	+	NC	NC	NC
GU576549	Peroxisomal fatty acid β-oxidation multifunctional protein [*Glycine max*]	9e-42	Lipid metabolism	++	-	-	-	++	++	-	+
GU011971	Sedoheptulose-bisphosphatase [*Arabidopsis thaliana*]	2e-28	Metabolism	++	++	+++	+	+	++	-	-
GQ922055	Serine/threonine-protein kinase PBS [*Ricinus communis*]	7e-58	Signal transduction	+	-	NC	++	+	+	-	-
GQ922059	Heat shock 70 kDa protein, mitochondrial-like [*Glycine max*]	6e-89	Defense	+++	+	+	++	+	+	NC	-

3.3 Discussion

Groundnut is the most important oilseed-protein crop of semi-arid tropics with equal importance word-wide. This crop is highly susceptible to various foliar diseases particularly the late leaf spot disease and damage to the crop can reach up to 70% under severe epidemic conditions, particularly when the crop receives an extended rainy season. Molecular studies on the groundnut foliar diseases are very rare and the crop did not receive extensive attention that the crop deserves. There were only two reports on the characterization of genes involved in groundnut (*A. hypogaea*)–*P. personata* interaction (Luo et al. 2005, Nobile et al. 2008) and also two reports in the wild relatives of *Arachis* that are highly resistant to several pathogens including *P. personata* (Rao et al. 2003, Kumar and Kirti 2011). Kumar and Kirti (2011) has been reported several transcripts associated with phenylpropanoid pathway, such as phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, cinnamyl alcohol dehydrogenase and have significant role in first line of defense such as cell wall deposition and lignifications. Dirigent protein is another gene they have found induced upon pathogen challenge is involve in biosynthesis of lignans, which have antifungal, antibacterial and anti-insect activities. They have also reported two transcripts of differentially expressed cyclophilin and characterized it and found overexpressed transgenic tobacco harboring cyclophilin gene showed enhanced resistance against pathogen *R. solanacearum* and *P. parasitica* var. *nicotianae*.

Since the groundnut genome sequence has not been available in international GenBank, it is important to discover novel genes through alternative transcriptomic approaches. cDNA-AFLP method is a powerfull approach for differential gene expression analysis of plant-pathogen interaction and we used this technique to provide first large scale investigation of the genes expressed in the resistant wild groundnut in its incompatible interaction between *Phaeoisariopsis personata*. This technique has been used successfully to study plant-pathogen interaction in several plants (Polesani et al. 2008, Wang et al. 2009a, Sestili et al. 2011) and are highly reproducible (Jones and Harrower 1998) and have advantages over other commonly used gene display methods (Kuhn 2001).

In the present study, a total of 64 primer combinations were used to visualize 4,047 TDFs in the control and treated samples. Among the 233 differentially expressed gene

fragments between control and treated, 125 were upregulated, 64 downregulated and 44 point expressed. TDFs were grouped into functional categories based on their homology to known proteins. A major group of 75 sequences showed no significant similarity while 55 sequences designated unknown/hypothetical protein. The genes showing significant similarity to metabolism, photosynthesis, signal transduction related proteins were 53, 5 and 15 respectively. The genes involved in defense and transcription factors shared equal number of 11 sequences while rest of the genes are involved in transport mechanisms.

Seventeen differentially expressed TDFs were further validated by quantitative real time PCR. Of these, eight TDFs exhibited early response as they were up-regulated at 24 h time point after pathogen inoculation in resistance plants while there was no major up regulation in susceptible plants. This 24 h point also includes the time taken for the conidia to germinate. It may be mentioned that the conidia take 12-24 hrs for germination in conidial germination medium. Three TDFs (Cystatin, 15-PGDH, CyP450) exhibit late response in resistant plants and their transcripts were up-regulated at 72 and 96 hrs, while no changes were found in susceptible plants except cystatin. Cysteine protease inhibitor has shown early response as it up-regulated at 24 and 48 h after infection in compatible interaction.

3.3.1 Role of pathogen induced genes in plant defense

Thaumatin like protein

We have detected four transcripts with similarity to thaumatin like protein, a PR5 protein, which is involved in antifungal activity in vitro (Xu and Reddy 1997, Chan et al. 1999) as well as in vivo (Datta et al. 1999, Zhu et al. 1996). Cotton thaumatin like protein (GbTLP) in tobacco transgenic plants enhanced resistance against *Verticillium dahliae* (Munis et al. 2010). It showed differential expression profile in wild groundnut in cDNA-AFLP analysis but there was no significant difference between compatible and incompatible interaction in quantitative real time analysis.

Cysteine protease inhibitor

Another transcript similar to cystatin has been found up-regulated both in cDNA-AFLP as well as quantitative real time analysis and exhibit antifungal properties in several

plants (Rodriguez et al. 2010, Bangrak and Chotigeat 2011). Cystatin is well known antifungal protein and reported from several plants and animals of its antifungal properties against various phytopathogenic fungi such as *Trichoderma reesei, Botrytis, Claviceps, Helminthosporium, Curvularia, Alternaria Sclerotium, Rhyzoctonia* and *Fusarium species* (Joshi et al. 1998, Abraham et al. 2006, Valdés-Rodríguez et al. 2007).

CC-NB-LRR

We have observed three transcripts of a disease resistance protein similar to CC-NB-LRR protein in our analysis. It is a *R* gene and confers resistance to various plant fungal pathogens (Gao et al. 2005, Kohler et al. 2008) and has also been shown to impart leaf stripe resistance in barley (Bulgarelli et al. 2010). Quantitative real time analysis also indicated up regulation in wild groundnut which could not be observed in susceptible groundnut at different time point of pathogen inoculation.

15-Hydroxyprostaglandin dehydrogenase

Two TDFs of 15-Hydroxyprostaglandin dehydrogenase, a homolog of NADPH oxidoreductase have been found in cDNA-AFLP analysis and is involved in generating reactive oxygen species such as O_2^- & H_2O_2 for exhibiting hypersensitive response (Xing et al. 1997, Lherminier et al.2009). It has also been shown to be involved in the suppression of human breast cancer and also can modulate estrogen receptor pathway (Wolf et al., 2006). *Arabidopsis thaliana* 15-Hydroxyprostaglandin dehydrogenase plays a distinct role in plant anti-oxidant defense (Babiychuk et al. 1995). Our quantitative real time analysis showed its constant up-regulation at different time points in the resistant wild groundnut while there was no change in transcripts of susceptible plants. This protein might be playing an important role in preventing pathogen invasion through hypersensitive cell death.

Protein kinases

Protein kinases are known to play an important role in pathogen recognition through signaling and activation of plant defense mechanisms (Romeis 2001) through the phoshporylation of the target proteins. We have identified several TDFs encoding different protein kinases such as Leucine rich repeat receptor like kinase (LRR-RLK), Protein kinase-6, serine-threonine protein kinase (Nobile et al. 2008), flag-tagged protein

kinase domain of putative mitogen-activated protein kinase kinase kinase. Except the LRR-RLK, all other protein kinases were found to be induced in the wild groundnut, while LRR-RLK was repressed in both cDNA-AFLP as well as quantitative real time analysis. LRR-RLKs have been shown to have roles in downstream signaling pathway, particularly in relation to pathogen recognition.

Vacuolar processing enzyme and Cysteine protease

Moreover, we have identified cell death associated transcripts that are induced during pathogen infection and are involved in hypersensitive response (Swidzinski et al. 2002). Vacuolar processing enzyme has been reported as a cysteine proteinase responsible for the maturation of vacuolar proteins and exhibit caspase-1 like activity (Okamoto and Minamikawa 1999, Rojo et al. 2004). It exhibits endopeptidase activity and mediates, TMV and mycotoxin-induced cell death (Hatsugai et al. 2004, Kuroyanagi et al. 2005). It is strongly induced within 24 h of pathogen inoculation in the resistant wild groundnut, while there was no such induction found in susceptible groundnut variety in the real time analysis indicating its role in hypersensitive and responding to prevent pathogen spread. We have found a cysteine protease, another cell death associated gene fragment in our studies and it has been shown to be involved in senescence (Martinez et al. 2007) and various environmental stresses including hypersensitive cell death (Feller 2004).

Suppressor of G2 allele of skp1 (SGT1)

SGT1 is an essential component of signaling pathways leading to pathogen resistance and binds to the chaperone protein HSP70 in plants (Noel et al. 2007) and HSP90 in human, yeast, plants (Lee et al. 2004, Boter et al. 2007) indicating the role in regulating protein folding. SGT1 regulates defense responses triggered by various pathogens and interacts with RAR1 (Azevedo et al. 2002) and is essential for resistance conferred by multiple *R* genes (Austin et al. 2002, Muskett et al. 2002). It plays an important role in regulating process of cell death during compatible and incompatible plant-pathogen interaction (Wang et al. 2010b). We found that SGT1 was differentially expressed in cDNA-AFLP analysis and in quantitative real time analysis as well with its expression peaking at 24 hrs in resistant while there was no such strong expression in susceptible plant at any time point indicating its role in resistance of the wild groundnut against late leaf spot pathogen infection.

RacGTPase activating protein

In the present study, racGTPase was found to be differentially expressed, and in quantitative RT-PCR analyses, its expression reached the peak at 24 hpi in the resistant genotype, while no major changes in its expression were observed in susceptible variety. Rac GTPase protein plays an important role in plant defense against pathogen, as its role in the production of reactive oxygen species (ROS) such as O_2^- & H_2O_2 that are rapidly generated after infection leads to hypersensitive response, a form of programmed cell death in plants has been very clearly elucidated (Kawasaki et al. 1999). The expression of a rice racGTPase resulted in HR like response and resistance against a virulent strain of bacterial blight and blast fungus associated with an altered expression of defense related genes involved in the enhanced production of phytoalexins (Ono et al. 2001). However its function in *R* gene mediated disease resistance still needs to be established.

70 kDa heat shock protein

Most heat shock proteins (Hsp) function as molecular chaperones that help organisms to cope with stress of both an internal and external nature. Up-regulation of inducible Hsps is one important part of the cellular stress response, which includes molecular chaperones, antioxidases, proteases and DNA repair systems. A number of investigations have confirmed the importance of Hsps in resistance towards heat and cold and a range of other stresses including insecticides, heavy metals, desiccation, diseases, parasites and inbreeding (Parsell and Lindquist 1993, Bukau and Horwich 1998, Jaattela 1999).

Cytochrome P450 monooxygenase

Plant cytochromes P450 are involved in a wide range of biosynthetic reactions. Phytoalexins are low molecular weight antimicrobial compounds that are synthesized in response to pathogen attack. *PAD3* encodes a cytochrome P450 that might be directly involved in camalexin biosynthesis. PAD3 protein appears to be a cytochrome P450 monooxygenase, encodes an enzyme required for camalexin biosynthesis and involved in resistance to a fungal pathogen (Zhou et al. 1999).

Polygalacturonase precursor protein

Polygalacturonase protein (PGP) is a glycoprotein present in the cell wall of plants and inhibit the activity of fungal endopolygalacturonases (Desiderio et al. 1997). Transgenic plants with polygalacturonase protein were speculated to show enhanced level of protection. Expression of pear PGP in tomato plants showed enhanced resistance against *Botrytis* (Powell et al. 2000). Recently, Veronico et al. (2011) reported that pea polygalacturonase-inhibiting protein showed defense against the cyst nematode, *Heterodera goettingiana.*

Metabolism related proteins

Several metabolism related proteins were found to be differentially expressed such as sedoheptulose biphosphatase (SBP), LEA protein, methionine synthase, cellulose synthase, Exostosin like protein, glycine rich protein, NADH dehydrogenase, glyceraldehyde phosphate dehydrogenase, peroxisomal fatty acid β-oxidation multifunctional protein, dihydroflavonol reductase, UDP glucosyl transferases, GDSL-like Lipase/Acylhydrolase. ABC transporters etc. in the resistant genotype in comparison to the susceptible variety. We have quantitatively estimated the expression of lea protein, sedoheptulose-1,7- biphosphatase (SBP) and peroxisomal fatty acid β-oxidation and observed that LEA protein and peroxisomal fatty acid β-oxidation multifunctional protein were at peak at 24 hrs in resistant genotype while this was not the case in susceptile variety. SBP trsnscript accumulation was strong and constantaly increased upto 72 hpi in resistant genotype compared to susceptible one. SBP is a calvin cycle enzyme and stimulates photosynthesis and growth from an early stage of development in transgenic tobacco has been reported by Lefebvre et al. (2005). SBPase was found to be differentially expressed in *Arabidopsis thaliana* upon infection with tobacco etch virus (Agudelo-Romero et al. 2008). Hence, these TDFs might have significant role in the resistance phenomenon in the wild groundnut and need to be further investigated.

Most of these ESTs were not identified in previous studies of *Arachis hypogaea* and *Phaeoisariopsis personata* interaction at molecular level using subtractive suppression hybridization (Nobile et al. 2008), as well as ESTs libraries using microarray technique (Luo et al. 2005). Kumar and Kirti (2011) reported up regulation of phenylpropanoid pathway genes such as phenylalanine ammonia lyase, cinnamate 4-hydroxylase,

cinnamyl alcohol dehydrogenase and dirigent-like protein upon interaction between wild groundnut and *P. personata.* Previous reports showed the upregulation of serine threonine protein kinase, heat shock protein, ABC transporter protein in *Phaeoisariopsis personata* and *Arachis hypogaea* interaction (Nobile et al. 2008). Similarly, Kumar and Kirti (2011) reported on the expression of genes such as zinc finger protein, thaumatin like protein, methionine synthase, ATPase, nucleic acid binding protein, heat shock proteins, cysteine protease, oxygen evolving enhancer protein and a receptor kinase in the wild groundnut, *Arachis diogoi* challenged with *P. personata* using the approach of DD-RT-PCR. It is interesting to note that similar genes have been identified using the cDNA-AFLP approach in the present study showing the importance of these genes in the resistance phenomenon. Hence disease resistance associated genes such as CC-NB-LRR, SGT1, cystatin, protein kinases, racGTPase activating protein, Cytochrome P450 monoxygenase,vacuolar processing enzyme, heat shock 70 kDa protein and 15-Hydroxyprostaglandin dehydrogenase, which were not reported in the previous studies could serve as novel candidate resistance genes in the development of disease resistant variety of groundnut.

3.4 Conclusion

This is the first large-scale investigation into molecular basis of incompatible interaction between strictly biotrophic late leaf spot pathogen *P. personata* and a wild accession of groundnut and changes in transcriptome of the host. However, Kumar and Kirti (2011) have found up-regulation of phenylpropanoid pathway genes, which are involved in cell wall strengthening and lignifications. Here, we have reported several disease resistance genes along with some phenylpropanoid pathway genes, which are involved in various defense response including hypersensitive response like cell death. Differentially expressed genes in wild groundnut throw light on its resistance mechanism upon pathogen challenge and provide initial breakthrough of genes possibly involved in recognition events and early signaling responses to combat the pathogen through subsequent development of resistance. These data would provide potential candidate genes for improving groundnut varieties resistant to late leaf spot disease by genetic engineering or through breeding approaches and would give leads to the investigators, who are involved in this interaction.

3.5 Summary

A total of 233 reliable, differentially expressed genes were identified by using 64 combinations of cDNA-AFLP primers in *Arachis diogoi* after pathogen challenge, of which 125 transcript derived fragments (TDFs) showed upregulation, 44 downregulated and 64 represented point expression. About one third of the TDFs exhibit no significant similarity with the known sequences in the data bases. Expressed sequence tag data showed that the characterized genes are involved in conferring resistance in the wild groundnut to the pathogen challenge. Several genes for proteins involved in cell wall strengthening, hypersensitive cell death and resistance related proteins have been identified. Genes for other proteins appear to function in metabolism, signal transduction and defense. Seventeen TDFs based on the homology analysis of genes associated with defense and signal transduction were further validated by quantitative real time PCR (qRT-PCR) analyses in resistant wild species in comparison with a susceptible groundnut genotype in time course experiments. We have investigated the molecular basis of the incompatible interaction between the late leaf spot pathogen, *P. personata* and a wild accession of groundnut by analyzing the changes in transcriptome of the resistant host. We have identified several disease resistance genes involved in various defense responses including hypersensitive response like cell death. Differentially expressed TDFs in wild groundnut indicate its resistance mechanism upon pathogen challenge and provide initial breakthrough of genes possibly involved in recognition events and early signaling responses to combat the pathogen through subsequent development of resistivity. This is the first attempt to elucidate the molecular basis of the response of the resistant genotype to the late leaf spot pathogen, and its defense mechanism.

Chapter 4

Comparative proteomic analysis of the host responses in resistant and susceptible genotypes of groundnut infected with *Phaeoisariopsis personata*

4.1 Introduction and background

Plants have their own surveillance system to recognize attacking microorganisms and to induce effective defense mechanisms. However, a weaker encounter mechanism results in microbial invasion, causing deleterious effect, such as diversion of nutrients, metabolites and toxin production, which enhance the rate of disease progression followed by the death of the host tissues. Defense response is frequently controlled by interaction between plant resistance (R) genes and pathogen avirulence (avr) genes through gene-for-gene interaction (Hammond-Kosac and Jones 1997, Dangl and Jones 2001, Jones and Dangl 2006). Plant-pathogen interaction studies have the immense commercial importance as the pathogen attack can lead to massive yield penalties. Understanding compatible and incompatible plant-pathogen interaction mechanism by which plants resist infection or susceptible to microbial pathogen is very important from an agricultural standpoint.

The biotrophic pathogen *Phaeoisariopsis personata* causes the globally devastating late leaf spot disease in groundnut (*Arachis hypogaea* L.). Late leaf spot disease can lead to yield losses up to 70% under favorable conditions, as the crop is grown as rain-fed crop and its productivity depends on vagaries of environment (Mc Donald et al. 1985, Grichar et al. 1998). The wild diploid species, *Arachis diogoi* is reported to be highly resistant to this disease and totally asymptomatic to fungal and rust pathogens (Pande and Narayana Rao 2001). The resistant wild species and the susceptible cultivar variety will constitute an ideal material to study the differences at molecular level involved in conferring resistance or susceptibility.

Proteomic analysis reveals the translational product of gene expression of plant under stress conditions and its physiological state under particular conditions. Analysis of protein is a direct approach to define function of their associated genes as it can be linked to genome sequence information, which is important for functional genomics. There are scanty reports of proteome analysis that focus on study of stress response of groundnut genotypes against various stress conditions. Wang et al. (2010c) analysed differentially expressed groundnut seed proteins in resistant and susceptible groundnut cultivars in response to *Aspergillus flavus* and reported expression of several disease resistance associated proteins. Proteomic study of groundnut cotyledons in response to atoxigenic and toxigenic *A. flavus* strains reveals aflatoxin-triggered immune response (Wang et al.

2012). Kottapalli et al. (2008, 2009) have analysed seed protein of four groundnut cultivars that revealed differential expression of storage, allergenic proteins and also identified several physiologically significant candidate proteins associated with water-deficit stress tolerance mechanism in three groundnut genotypes. Katam et al. (2010) carried out proteomic study in groundnut leaf using a drought-tolerant variety and identified more than 200 proteins, predominantly carbohydrate metabolism and photosynthesis related proteins, which formed the basis for understanding the changes in groundnut leaf proteins under various physiological, developmental and environmental conditions.

Proteomic study of plant-pathogen interaction has been successfully reported in several plants. For example, Kaur et al. (2011) identified defense-related proteins, which are required for mounting a successful defense response in *Brassica juncea* against *Albugo candida*. Recently, Wu et al. (2013) analyses a plant-virus interaction in resistant and susceptible ecotypes of maize infected with the sugarcane mosaic virus and identified several defense and stress related proteins during both compatible and incompatible interactions. The modal organism, *Arabidopsis thaliana* differentially expressed proteins, related to oxidative stress and metabolism in response to treatments with fungal elicitors in *Arabidopsis* cell cultures (Chivasa et al. 2006). Castillejo et al. (2011) analyses pea root proteome in response to *Orobanche crenata* inoculation and identified several proteins with protease activity, which could play an important role in preventing the pathogen and some of metabolism and stress response protein.

Till date, there is no report of a proteome analysis that focuses on study of susceptible and resistant groundnut genotypes against late leaf spot disease. In the present study, we have carried out differential comparison of groundnut leaf proteomic response to pathogen *Phaeoisariopsis personata* infection between resistant and susceptible groundnut genotypes. The fold changes of differentially expressed proteins upon pathogen challenge were found to be high in case of *A. diogoi* (Resistant) in comparison to *A. hypogaea* (Susceptible). We demonstrate that the proteins involved in signaling and metabolism, stress and defense responses as well photosynthesis were differentially expressed. These observations may possibly reveal proteins, which make the resistant host plant to cope up the invading pathogen and provide new insights into the molecular mechanism of plant-fungal interaction.

4.2 Results

4.2.1 Late leaf spot infection analysis in groundnut leaves of resistant and susceptible cultivars

Pathogenesis pattern of *P. personata* in groundnut was investigated during infection under a light microscope and it was found that leaf spot disease appeared on the susceptible host plants (*Arachis hypogaea* cv. JL-24) after 10-15 day post inoculation of conidia, while no such disease symptoms were found on the resistant wild species (*Arachis diogoi*). An early stage was chosen for analysis, as the conidia of *P. personata* were observed to germinate after 12-24 hpi (Fig. 4.1) and their germ tubes enter the plant cells directly via the epidermis or more frequently through stomata there by allowing the intracellular mycelial growth (Abdou et al. 1974, Shokes and Culbreath 1997). Spots were fully developed after 20-25 day post inoculation in compatible (susceptible) plants, but no such lesions were observed on incompatible (resistant) plants (Fig. 4.1). Hence, we chose to indentify changes in protein expression in incompatible and compatible interaction at early stage of infection.

(**a**) Before infection After infection Conidia

(**b**) Before infection After infection Germinated conidia

Figure 4.1 Representative disease symptoms of *Phaeoisariopsis personata* infection at groundnut leaves (**a**) Disease symptom in *Arachis hypogaea* (Susceptible) before and after 24 dpi and morphology of conidia (**b**) Pathogen infection in *Arachis diogoi* (Resistant) before and after 24 dpi and conidia germination after 12-24 hrs.

4.2.2 2D gel electrophoresis and protein expression profiling

Proteomic approach was used to analyze the changes in protein profile during the interaction between wild and cultivated groundnut genotypes and *P. personata* as compatible and incompatible interactions. Mostly, resistance depends upon compatible or incompatible interaction between host and pathogen (Flor 1971). Host plants prevent disease development by inducing hypersensitive response in an incompatible interaction, where as compatible interaction does not induce HR resulting in disease development. In order to understand this mechanism, a comprehensive analysis is required. In this context, a proteomic approach was adopted to analyze the changes in protein profile during early stage of groundnut and *P. personata* interaction. Therefore, leaf samples were selected for proteomic analysis, which were pooled from different stages such as 24, 48, 72, 96 hrs after pathogen inoculation with *P. personata* along with a mock treatment**.** Triplicate gels were obtained from three independent experiments and the representative gels of *Arachis diogoi* (resistant) and *Arachis hypogaea* L. (susceptible) were illustrated in Fig 4.2 and Fig 4.3 respectively. We observed a total of nearly 350-400 protein spots on susceptible groundnut 2-DE gel while 450-500 spots were detected on wild groundnut 2-DE gel stained with Coomassie brilliant blue dye. Thereafter, we systematically screened the protein spots that were differentially regulated in response to pathogen challenge using Image Master 2-D platinum version 6 software. There was around 80–85% correlation between biological repeats, indicating the reliable reproducibility of the experiments.

Proteomic study indicated differential expression of proteins both in *A. diogoi* and *A. hypogaea* upon pathogen challenge. The fold change of differentially expressed proteins was found to be high in case of *A. diogoi* in comparison to *A. hypogaea*. In treated *A. diogoi* there were around 44 protein spots observed with more than 2 fold changes, while only 17 protein spot were found in *A. hypogaea* with more than 2 fold expression. There were 22 and 14 protein spots depicted with more than 3 and 5 fold higher expression respectively in treated *A. diogoi* in comparison to susceptible, where 15 and 7 spots were found similar expression. Five protein spots were detected to have more than 10 fold expression in *A. diogoi* while only two spots were found such fold changes in *A. hypogaea*. The fold change profiling of proteins are illustrated in Fig 4.4. Regulated proteins were determined based on the two basic criterion (1) reproducibility (2) fold change (% volume) at least $\geq$ 1.5 times.

4.2.3 2-DE Gel Expression Profile- *Arachis diogoi* (Resistant)

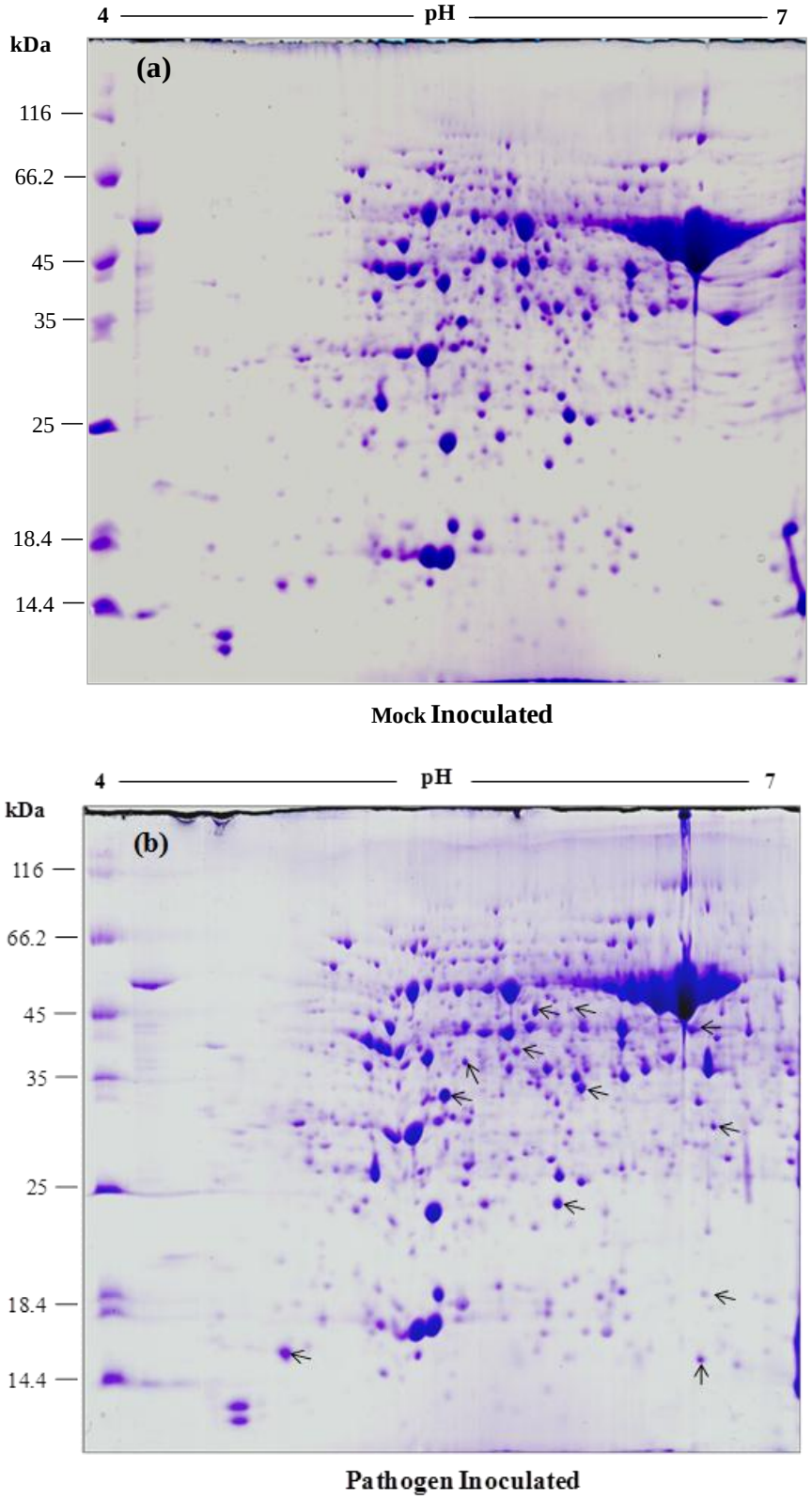


Figure 4.2 2-DE gel of groundnut leaf protein samples from a resistant (*A. diogoi*) in response to inoculation with *P. personata*, (**a**) mock inoculated, (**b**) pathogen inoculated. 800 µg of total leaf protein was loaded on 18 cm IPG strip with a linear gradient of pH 4-7, 12% SDS-PAGE gels were used for second dimension.

4.2.4 2-DE Gel Expression Profile- *Arachis hypogaea* L. (Susceptible)

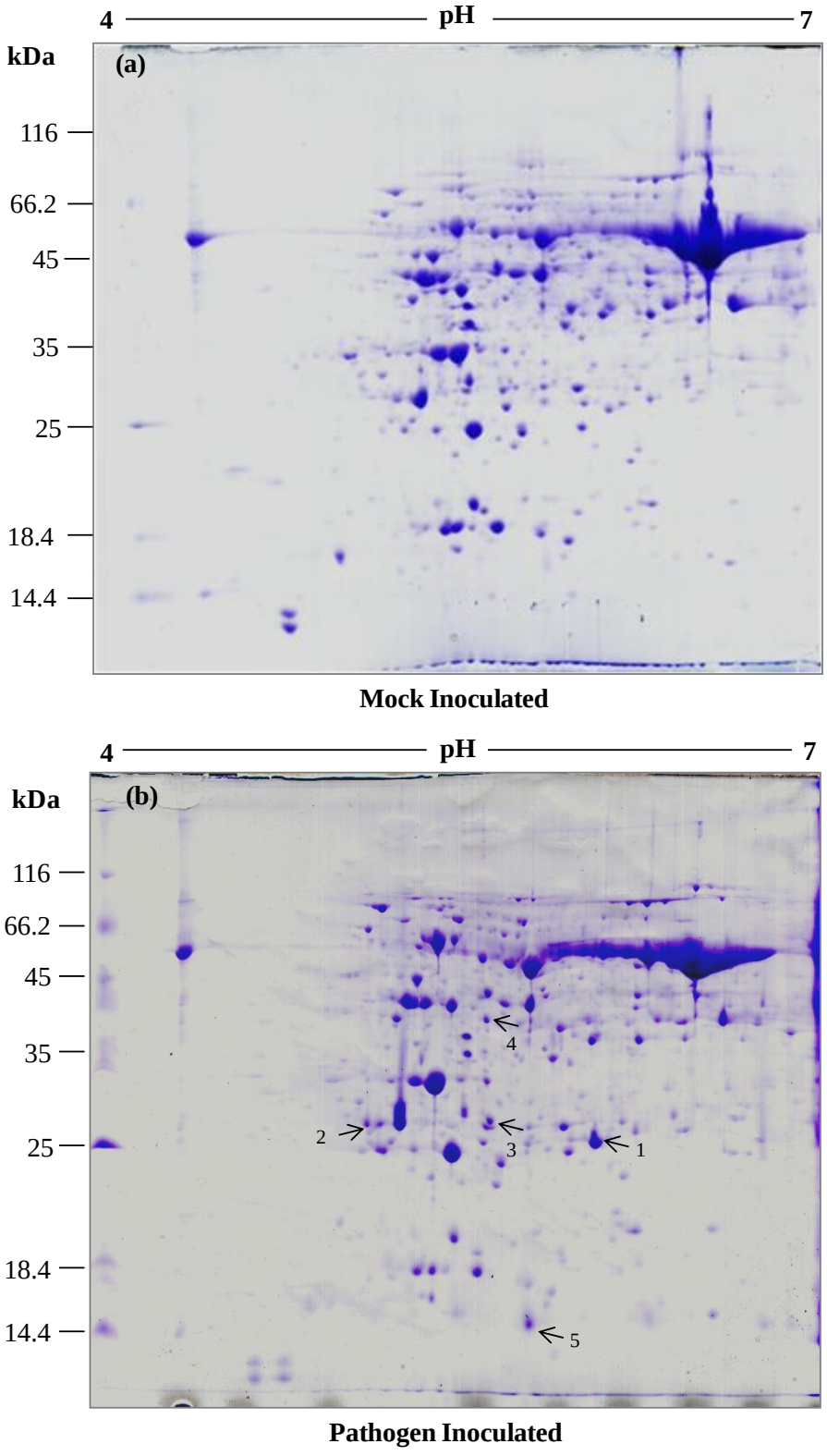


Figure 4.3 2-DE gel of groundnut leaf protein samples from a susceptible (*A. hypogaea* L.) in response to inoculation with *P. personata*, (**a**) mock inoculated, (**b**) pathogen inoculated. 800 µg of total leaf protein was loaded on 18 cm IPG strip with a linear gradient of pH 4–7, 12% SDS-PAGE gels were used for second dimension.

Table 4.1 Pathogen-induced proteins in wild groundnut and *Arachis hypogaea* L. identified by MALDI-TOF-TOF. Ad- *Arachis diogoi* (Resistant), Ah- *Arachis hypogaea* L. (Susceptible).

Spot No.	Protein identified	Accession no./ Protein match	Peptide sequence matched	Theoretical Mr/pI	Observed Mr/pI	S. C. (%)	MS/ MS score	Related function
Ad-1	Oxygen-evolving enhancer protein 2, chloroplastic (*Helianthus annuus*)	PSBP_HELAN	QYYFLSVLTR	28.2/8.67	31/6.1	3	47	Regulation of photosystem-II
Ad-2	Ribulose-bisphosphate carboxylase activase -common tobacco (fragment)	gi|100380	IVDTFPGQSIDFFGALR	26.0/5.01	36/5.7	7	83	Photosynthesis
Ad-3	Oxygen-evolving enhancer protein 2, chloroplastic (*Brassica juncea*)	gi|131390	SITDYGSPEEFLSQVNYLLGK	23.4/4.91	24/5.9	9	79	Regulation of photosystem-II
Ad-4	Chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase activase (*Morus alba*)	gi|119855475	VPIIVTGNDFSTLYAPLIR IVDTFPGQSIDFFGALR	27.3/4.76	16.5/4.8	11	90	Photosynthesis
Ad-5	Nucleoside diphosphate kinase (*Flaveria bidentis*)	NDKA_FLABI	GDFAIDIGR	16.1/5.93	19/6.6	6	44	Synthesis of nucleoside triphosphates
Ad-6	LRR receptor-like serine/threonine-protein kinase (*Arabidopsis thaliana*)	HSL2_ARATH	LSGEVPAR	111.6/5.8	37/5.9	1	28	Signal transduction
Ad-7	Defensin-like protein (*Solanum tuberosum*)	DF322_SOLTU	FSGGNCHGFRR MGPMRIAEAR	8.8/9.33	9.5/5.8	33	58	Defense
Ad-8	Terpenoid synthase (*Arabidopsis thaliana*)	TPS08_ARATH	DPQESNR FPPSEWTNR	69.5/6.15	29/6.7	5	68	Secondary metabolite biosynthesis
Ad-9	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic (*Hordeum vulgare*)	RCAB_HORVU	VPIIVTGNDFSTLYAPLIR LVDTFPGQSIDFFGALR	47.4/7.59	47/5.4	8	127	Photosynthesis
Ad-10	Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic (*Hordeum vulgare*)	RCAA_HORVU	IVDTFPGQSIDFFGALR	51.3/8.04	53/5.3	3	25	Photosynthesis

Ad-11	Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic (*Hordeum vulgare*)	RCAA_HORVU	IVDTFPGQSIDFFGALR	51.3/8.04	52.5/5.3	3	48	Photosynthesis
Ad-12	Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic (*Hordeum vulgare*)	RCAA_HORVU	IVDTFPGQSIDFFGALR	51.3/8.04	52/5.2	3	28	Photosynthesis
Ad-13	Sedoheptulose-1,7-Bisphosphatase, chloroplastic (*Arabidopsis thaliana*)	S17P_ARATH	LLFEALQYSHVCK GFPGTHEFLLLDEGKWQHVK	42.7/6.17	44/5.1	8	170	Metabolism
Ad-14	Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic (*Nicotiana tabacum*)	RCA2_TOBAC	VPIIVTGNDFSTLYAPLIR VPIIVTGNDFSTLYAPLIR	48.5/8.14	51/5.2	8	166	Photosynthesis
Ad-15	Putative F-box protein (*Arabidopsis thaliana*)	FB217_ARATH	LCLMACVKARDMR NQSKEDESR	49/5.83	47/5.9	4	68	Signal transduction & regulation of cell cycle
Ad-16	Phytochrome A (*Aristolochia tomentosa*)	gi|75674163	VRMICDCYAKPVK MICDCYAKPVKVYQDER	27/6.33	28/5.4	5	80	Signal transduction
Ad-17	DNA endonuclease I (*Chlamydomonas reinhardtii*)	DNE1_CHLRE	TTSETVR	18.7/9.45	17/5.3	4	31	Endonuclease
Ad-18	Glyoxalase I (*Picea sitchensis*)	gi|116781841	ITSFLDPDGWK	32.8/5.04	33/5.6	3	66	Metabolism
Ad-19	Dihydroflavonol reductase (*Medicago truncatula*)	gi|357458089	ETGFDVVMINPGTALGPLIPPR HLCVEAIR	35.2/5.63	34/5.8	9	56	Secondary metabolism
Ad-20	Formate dehydrogenase (Phaseolus vulgaris)	gi|270342112	HFRGEDFPEQNYIVK	41.5/6.47	43/6.7	4	44	Metabolism
Ad-21	Glyceraldehyde-3-phosphate dehydrogenase (*Populus trichocarpa*)	gi|224061855	VVAWYDNEWGYSQR GVLDVCDVPLVSVDFR	48.4/6.79	47/6.6	6	101	Metabolism/Defense
Ad-22	Malate dehydrogenase, cytoplasmic (*Beta vulgaris*)	gi|11133601	ELVADDAWLNGEFITTVQQR	35.8/5.89	37/6.3	6	60	Metabolism

Ad-23	Monodehydroascorbate reductase-like isoform 1 (*Glycine max*)	gi|50400859	AAEEGKTVEEYDYLPYFYSR	46.9/5.73	47/5.8	4	42	Defense
Ad-24	Photosystem II stability/assembly factor (*Medicago truncatula*)	gi|357473927	FIDDKKGFVLGNDGVLLR	43.7/7.74	43/5.4	4	64	Photosynthesis
Ah-1	Chlorophyll a/b-binding protein type III, partial, (*Alonsoa meridionalis*)	gi|7271947	WLAYGEIINGR GLGGSGDPAYPGGPFFNPLGF GKDEK	20.8/5.17	22/5.80	19	195	Photosynthesis
Ah-2	Light-harvesting chlorophyll a/b-binding protein (*Prunus persica*)	gi|556367	NRELEVIHSR NVSSGSPWYGPDR	28.3/5.3	27.5/4.9	8	83	Photosynthesis
Ah-3	Ferritin-3, chloroplastic (*Vigna unguiculata*)	FRI3_VIGUN	IAEYVTQLR FFKESSEEEREHAEK	28.5/5.54	28/5.4	9	90	Iron homeostasis, ferroxidase activity
Ah-4	Photosystem II stability/assembly factor (*Arabidopsis thaliana*)	gi|15237225	GFGILDVGYR GTGITEEFEEVPVQSR SAEMVTDEGAIYVTSNR	44.1/6.79	43/5.4	10	2442	Photosynthesis
Ah-5	Ribulose bisphosphate carboxylase,small chain (*Phaseolus vulgaris*)	gi|21050	EVDYLLR IIGFDNVR	20.3/9.16	16/5.8	8	82	Photosynthesis

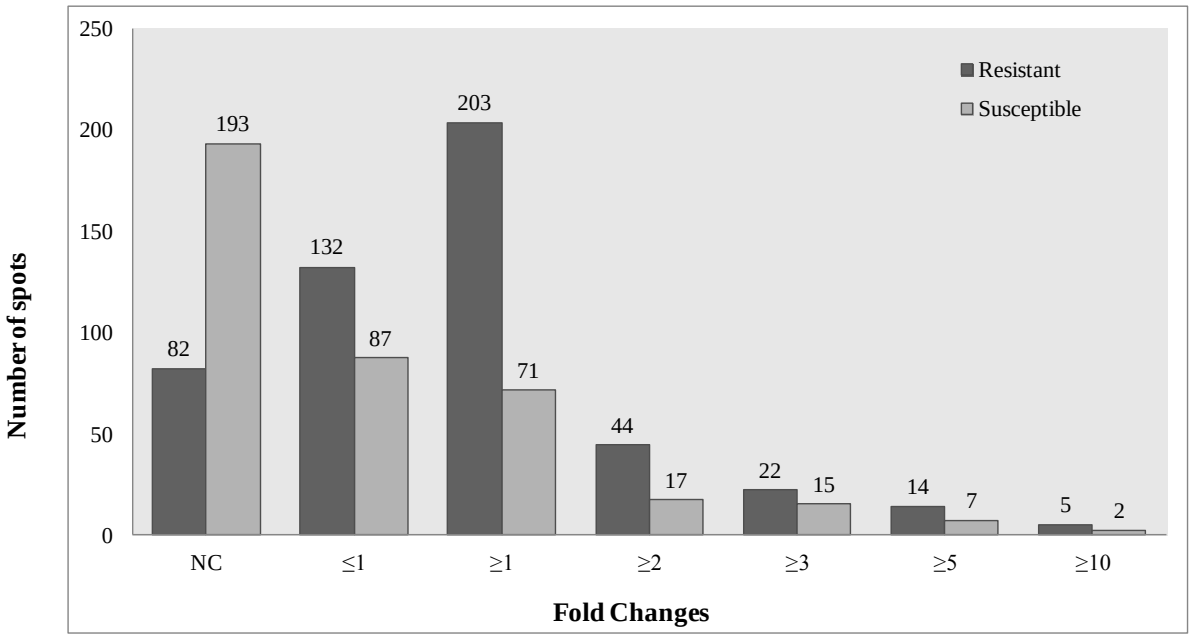


Figure 4.4 Bar diagram representing fold changes in leaf protein expression pattern in *Arachis diogoi* (Resistant) and *Arachis hypogaea* (Susceptible) upon pathogen challenge. NC- no change.

A total of 45 protein spots (37 spots from *Arachis diogoi* and 8 spots from *Arachis hypogaea* L.) including up-regulated and new spots representing point expression were selected, which were distinct, and were well separated with reliable expression pattern in groundnut and *P. personata* interaction for MALDI TOF-TOF analysis. Among 45 spots, only 29 proteins were successfully identified with putative function (Table 4.1) while rest 16 did not show any significant hits either in the NCBI or SwissProt database and hence were not considered. Out of 29 differentially expressed proteins, 24 identified proteins were found up-regulated in *Arachis diogoi* whereas 5 protein spots were found with enhanced expression in *Arachis hypogaea* L. We have studied the relative abundance of 29 differentially expressed proteins of resistant and susceptible variety of groundnut in comparison with the respective control.

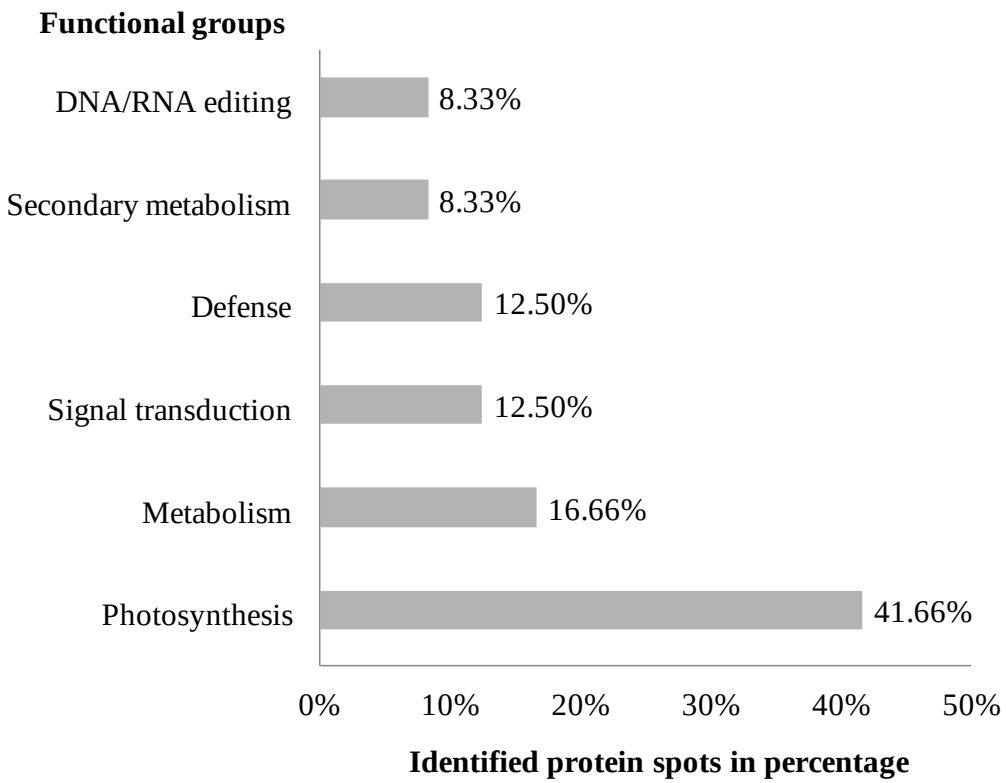


Figure 4.5 Classification of differentially expressed proteins identified- A total of 24 proteins of *Arachis diogoi* were classified based on homology search in database and their functional role in plant.

4.2.5 Identification and analysis of differentially regulated proteins

According to the method of Bevan et al. 1998, the identified proteins obtained from *Arachis diogoi* (resistant) were grouped into functional categories based on their homology to known proteins. A major group of 10 spots (41.66%) were photosynthesis related proteins, while 4 (16.66%) spots were designated as metabolism proteins. Proteins involved in signal transduction and defense related were found to be share equal number of 3 (12.5%) spots. The proteins involved in DNA/RNA editing and secondary metabolism were also found equal number of 2 (8.33%) spots (Fig. 4.5). The differentially expressed proteins from resistant and susceptible groundnut plants were listed in Table 4.1.

Differentially expressed proteins related to photosynthesis are Oxygen-evolving enhancer protein, Ribulose-bisphosphate carboxylase activase, Light-harvesting chlorophyll a/b-binding protein and Photosystem II stability/assembly factor. Metabolism related proteins are Sedoheptulose-1,7-bisphosphatase, Glyoxalase I,

Formate dehydrogenase and Malate dehydrogenase. Proteins involved in secondary metabolism are Dihydroflavonol reductase and Terpenoid synthase. Protein that are related to signal transduction and defense are LRR receptor-like serine/threonine-protein kinase, Putative F-box protein, Phytochrome A and Defensin-like protein, Monodehydroascorbate reductase, Glyceraldehyde-3-phosphate dehydrogenase respectively. Rest of the identified protein were involved in endonuclease and cell cycle regulation. The predicted molecular weight (MW) and pI of all these proteins were compared with their positions on the gel and were found consistent. The matched peptide sequence, reference organism, sequence coverage, experimental and theoretical molecular weight and pI, MS/MS score, related function of each protein were determined (Table 4.1). RuBisCO activase was identified as the most abundant differentially expressed protein, which could be due to the presence of different isoform of same protein or could be due to post translational modifications. The predicted molecular weight (MW) and pI of all these proteins were compared with their positions on the gel and were found consistent with few exceptions, which might be due to post translational modifications or due to polymeric nature of the protein.

4.2.6 Quantitative real-time PCR analysis

We examined transcripts levels of 4 selected differentially expressed proteins through quantitative real-time PCR, which were also found to be differentially expressed in the cDNA-AFLP analysis. In this analysis, a comparison has been made for the expression of the proteins between the resistant wild groundnut and the susceptible cultivated groundnut at similar time points (0, 24, 48, 72 and 96 hpi) after the pathogen challenge. We have found Glyceraldehyde-3-phosphate dehydrogenase to be up-regulated within 24 hrs after the pathogen treatment in the resistant genotype compared to the susceptible cultivated genotype, which showed no such up-regulation. Photosystem II regulated protein, Oxygen evolving enhancer complex was found to be up-regulated at 96 hrs in the wild groundnut, while there was no such up-regulation in the susceptible groundnut plants. A secondary metabolism protein, dihydroflavonol reductase did show early response in the wild as well as susceptible groundnut plants i.e, upregulted at 24 hrs, and gradually decline at 48, 72 and 96 hrs. F-box family protein regulates diverse cellular processes and was found to be up-regulated at different time point in both resistant as well as susceptible groundnut. The results indicate that 2-DE gel electrophoresis and

quantitative RT–PCR data are mostly concordant, confirming the reliability of the results. The transcripts of a glyceraldehyde-3-phosphate dehydrogenase and oxygen evolving complex showed very clear differences between the resistant and susceptible genotypes with the wild groundnut showing strong up-regulation.

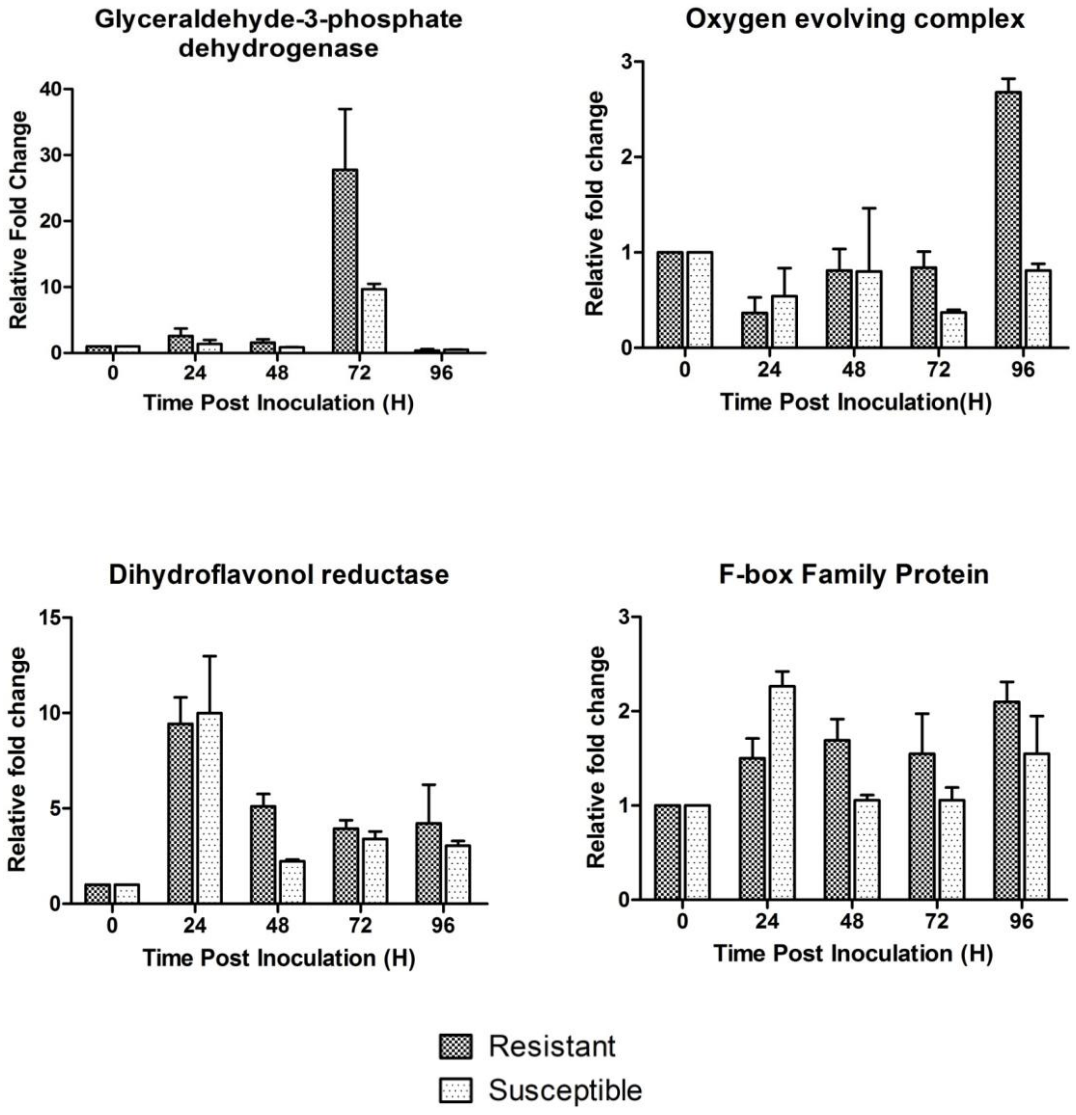


Figure 4.6 Quantitative real-time PCR analyses of 4 selected differentially expressed proteins- Leaf tissues were used for both pathogen and mock inoculated plants at 24, 48, 72 and 96 hpi, as well as mock-inoculated near 0 hpi. Relative gene quantification was calculated by comparative ΔΔCT method. All data were normalized to the Alcohol dehydrogenase-3 as it was used as internal reference gene and data was plotted from three independent experiment. *Arachis diogoi* (resistant) and *Arachis hypogaea* L. (susceptible).

4.3 Discussion

In order to study the proteins showing differentially expressed profiles in the resistant and susceptible genotypes of groundnut in response to the challenge from the late leaf spot pathogen *Phaeoisariopsis personata*, we have adopted a proteomic approach. We have successfully identified 24 protein spots with significant expression in wild groundnut and 5 protein spots in susceptible groundnut. Identified proteins could be divided into functional groups including photosynthesis, metabolism, secondary metabolism, signal transduction and defense related proteins.

RuBisCO activase is the key photosynthetic enzyme in C_3 plants which takes part in CO_2 fixation and photorespiration. The RuBisCO enzyme is located in the chloroplast play an important role in photosynthesis and is known to be reduced in infected plant cells because attack of pathogens lead to degradation of chloroplasts (Agrios 1997). In rice, activation of RcbL and other photosynthetic related proteins was found to be quite common in stress induced plants, which was observed both in susceptible/resistant hosts during plant-pathogen interaction (Mahmood et al. 2006, Yu et al. 2008). Wu et al. (2013) reported that Rubisco is differentially expressed in resistant and susceptible genotypes of maize infected with sugarcane mosaic virus. Ribulose bisphosphate corboxylase enzyme was also up-accumulated in wild groundnut, while it was also differentially up-regulated in the susceptible groundnut. Photosynthesis is carried out by two light dependent components, photosystem I and photosystem II in the thylakoid membranes of chloroplasts. The chlorophyll a/b binding protein stabilizes the photosystem I and II through balanced excitation energy. In our study, we have observed an increased expression of chlorophyll a/b binding protein in susceptible groundnut, in line with the findings of Wu et al. (2013), where it was induced in susceptible maize upon virus inoculation. We have also found differential expression of chlorophyll a/b binding protein upon pathogen challenge in wild groundnut. We have identified induced expression of photosystem II stability factor in both wild and susceptible groundnut indicating its possible role in disease resistance. According to Metha et al. (2008), photosystem II has emerged as a target of resistance signaling in plant-pathogen interaction. Oxygen evolving enhancer protein is another photosystem II associated protein, which is involved in the regulation of photosystem II and was differentially up-regulated upon pathogen challenge in wild groundnut in proteomic study as well as in

cDNA-AFLP analysis. Quantitative real-time analyses validated the up-regulation of oxygen evolving enhancer protein in wild groundnut, while no such up-regulation was found in the susceptible groundnut plants. Recently, it has been reported differentially expressed in resistant maize upon virus infection (Wu et al. 2013). The results indicates that the efficiency of photosynthesis in resistant and susceptible groundnut genotypes might be different as the chloroplasts in the sampled cells might be affected by the infection. Hence, photosynthetic activity might increase possibly to compensate the loss.

F-box proteins regulate diverse cellular processes, including cell cycle transition, transcriptional regulation and signal transduction by playing roles in Skp1p-cullin-F box protein (SCF) complexes**.** F-box proteins have also been reported to be expressed during panicle and seed developmental stages and therefore, appear to be involved in regulating plant growth and development. Wang et al. (2004) have demonstrated the role of F-box protein during various stress responses like water deficit, salts, wounding, and elicitation. In our study, we have found the F-box protein differentially up-regulated in wild groundnut upon pathogen inoculation and this was quantitatively validated by real-time analyses in resistant as well as susceptible groundnut plants.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a central glycolytic protein with pivotal role in energy production. Recent studies, in animal systems indicated the role of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in apoptosis or oxidative stress (Ishitani et al. 1998, Dastoor and Dreyer 2000). GAPDH is also involved in various diseases especially neurodegenerative disorders and cancers (Tristan et al. 2011). We have found differential up-regulation of GAPDH in wild groundnut upon pathogen challenge in proteomic study as well as cDNA-AFLP analysis. Quantitative real-time analyses also showed the strong up-regulation of GAPDH at 72 hrs of pathogen inoculation in wild groundnut, while trivial up-regulation was observed in the susceptible groundnut plants. Recently, GAPDH was also found up-regulated in proteomic study during plant-virus interaction in maize genotypes with sugarcane mosaic virus (Wu et al. 2013).

In plants, ascorbic acid plays an important role in stress responses as well as growth and development and act as an antioxidant, due to its ability to scavenge reactive oxygen species (ROS) generated during physiological processes (Conklin 2001). Ascorbic acid is also involved in defense mechanisms against pathogen attack and environmental

oxidative stresses, and has been implicated in the regulation of cell division and expansion (Smirnoff 1996). Ascorbic acid is readily oxidized to monodehydroascorbate, which dissociates to form unstable dehydroascorbate at alkaline pH. Monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) are known to play protective roles in plants as enzymes that maintain ascorbate in its reduced form (Eltayeb et al. 2006, Stevens et al. 2008). Monodehydroascorbate reductase uses NAD(P)H as a reductant and maintains reduced pools of ascorbate by recycling the oxidized form of ascorbate and serve as an important antioxidants (Noctor and Foyer 1998). Yoon et al. (2004) demonstrated expression of *Brassica campestris* monodehydroascorbate reductase mRNA increased in response to oxidative stress invoked by hydrogen peroxide, salicylic acid, paraquat, and ozone. In our study, monodehydroascorbate reductase was induced upon pathogen challenge in highly resistant wild groundnut genotype. Therefore, we can assume that MDAR also might be involved in controlling the balance amount of antioxidants in the cell during pathogen challenge, which has to be further validated for better understanding.

Plant defensins are a family of small, basic proteins that contain 4-5 disulfide bonds and known to possess potent antifungal activity. The majority of characterized plant defensins show a constitutive pattern of expression, with an induction in expression in response to pathogen attack, wounding and some abiotic stresses (Bahramnejad et al. 2009, Padovan et al. 2010). Plant defensins are best known for their antimicrobial activity against a broad spectrum of plant pathogens that include bacteria, yeast and a number of pathogenic fungi (Weerden and Anderson 2013). We observed induced expression of defensin protein in wild groundnut upon pathogen challenge while unable to detect in susceptible groundnut. It could be one of the important reasons for resistance of wild groundnut against micro-organisms.

Genomic DNA degradation has been observed in PCD, which is associated with the hypersensitive response in plants (Mittler and Lam 1995, Mittler et al. 1997, Tada et al. 2001). There are several kinds of nucleases reported with increased activity during programmed cell death in plants (Sugiyama et al. 2000). Ryerson and Heath (1996) observed nuclear DNA fragmentation during cell death induced upon fungal infection. Ito and Fukuda (2002) demonstrated that an S1-type nuclease, Zinnia endonuclease I, functions directly in nuclear DNA degradation during programmed cell death (PCD) of

tracheary elements. We found induced expression of endonulease I during plant-pathogen interaction in wild groundnut upon pathogen challenge in the proteomic study. Hence, it might be involved in resistance mechanism of wild groundnut during plant-pathogen interaction through DNA degradation lead programmed cell death in plants.

Sedoheptulose-1,7-biphosphatase (SBP) was found up-accumulated in the resistant genotype of groundnut. SBPase is a Calvin cycle enzyme and stimulates photosynthesis and growth from an early stage of development in transgenic tobacco upon overexpression (Lefebvre et al. 2005). It was found to be differentially expressed in *Arabidopsis thaliana* upon infection with tobacco etch virus (Agudelo-Romero et al. 2008). We found that proteins involved in secondary metabolism, such as Terpenoid synthase and Dihydroflavonol reductase were up-regulated along with other metabolism related proteins like glyoxalase I, malate dehydrogenase and formate dehydrogenase. We have also observed proteins involve in signal transduction such as phytochrome A and LRR receptor-like serine/threonine-protein kinase that were differentially up-regulated in wild groundnut upon pathogen challenge. Hence, these proteins through metabolism and siganaling, might have significant roles in the resistance phenomenon in the wild groundnut and need to be further investigated.

Taken together cDNA-AFLP as well as 2D analysis data, we observed that the oxygen-evolving enhancer protein, F-box protein, Sedoheptulose-1,7-biphosphatase, Glyceraldehyde-3-phosphate dehydrogenase, Dihydroflavonol reductase, Phytochrome A and Photosystem-II chlorophyll a/b binding protein were up-regulated upon pathogen challenge. This is the first approach to elucidate the molecular basis of the response of the resistant genotype to the late leaf spot pathogen, and its defense mechanism. How plants regulate the photosynthetic apparatus and increase their systemic pathogen resistance during defense however, remains unclear and further studies are required. These proteins might have significant role in the resistance phenomenon in the wild groundnut and need to be further characterize. Our results anticipate the cloning resistance genes for tikka disease from wild groundnut *Arachis diogoi* that would facilitate generation of pathogen-resistant groundnut cultivars.

4.4 Summary

Proteomic study indicated differential expression of proteins both in *A. diogoi* and *A. hypogaea* upon pathogen challenge. The fold change of differentially expressed proteins was found to be high in case of *A. diogoi* in comparison to *A. hypogaea*. A total of 29 reliable, differentially expressed proteins were identified by using 2-D gel electrophoresis in *Arachis diogoi* and *Arachis hypogaea* after pathogen challenge. About one third of the protein spots exhibit no significant hits with the known protein sequences in the data bases. Several identified proteins showed that the characterized genes are involved in conferring resistance in the wild groundnut to the pathogen challenge. Proteins involved in defense mechanism are glyceraldehyde-3-phosphate dehydrogenase, monodehydroascorbate reductase, endonuclease I and defensin like protein identified in wild groundnut upon pathogen challenge. Other identified proteins appear to function in metabolism, signal transduction and photosynthesis. We have validated four selected differentially expressed proteins through quantitative real time PCR in resistant wild species in comparison with a susceptible groundnut genotype in time course experiments, which were also found to be differentially expressed at cDNA-AFLP analysis. Differentially expressed proteins in wild groundnut indicate its resistance mechanism upon pathogen challenge and provide initial breakthrough of genes possibly involved in recognition events and early signaling responses to combat the pathogen through subsequent development of resistance. This is the first attempt to elucidate at the protein level of the response of the resistant and susceptible genotype to the late leaf spot pathogen, and its defense mechanism.

Chapter 5

Defense responses- hypersensitive like cell death of cysteine protease inhibitor (CPI) and 15- Hydroxyprostaglandin dehydrogenase (15-PGDH)

5.1 Introduction and Background

Hypersensitive response is an important phenomenon of plant defense mechanism to restrict the pathogen invasion by causing a localized programmed cell death at the site of infection. Plant cell death activation plays an important role during hypersensitive disease resistance response against pathogen attack and its process regulated by cysteine proteases and protease inhibitor (Solomon et al. 1999). Cystatin is a well known antifungal protein and reported from several plants and animals. Its antifungal properties against various phytopathogenic fungi such as *Trichoderma reesei, Botrytis*, *Claviceps, Helminthosporium, Curvularia, Alternaria Sclerotium, Rhyzoctonia* and *Fusarium species* were reported (Joshi et al. 1998, Abraham et al. 2006, Valdés-Rodríguez et al. 2007). Because of the protease inhibitor activity, expression of the barley cystatin enhanced resistance against phytophaguos aphids in *Arabidopsis* and mites in maize, and suppressed the phenomenon of cysteine protease induced programmed cell death against an avirulent pathogen (Solomon et al. 1999, Belenghi et al. 2003, Carrillo et al. 2011a, 2011b). Apart from its antifungal activity and protease inhibitor properties, we report here a novel role of pathogen induced wild groundnut cystatin in defense mechanism through hypersensitive response like cell death under conditional over-expression in tobacco.

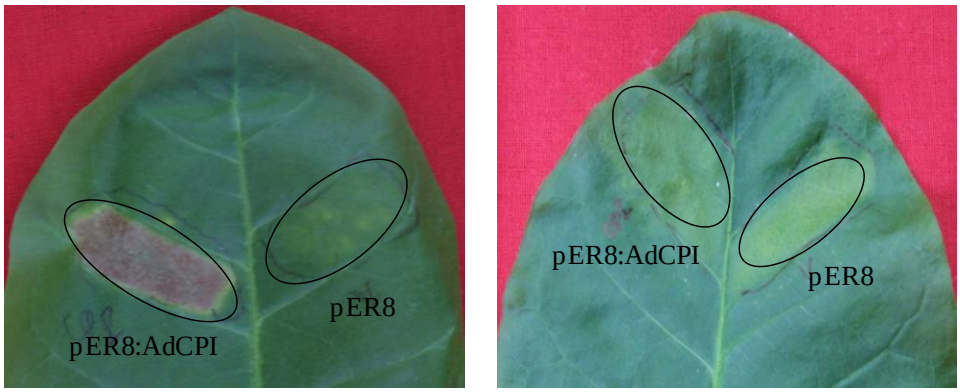
15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is a key metabolic enzyme of prostaglandin E_2 (PGE_2), which is synthesized from arachidonic acid by cyclooxygenase pathway. Overexpresion of PGE_2 enhances cell proliferation, invasion, metastasis, angiogenesis, and inhibits apoptosis. Various studies have reported the pro-inflammatory and pro-tumorigenic actions by induced expression of COX-2 and PGE_2 in various human and animal cancers (Wu 2005, Wang and Dubois 2010a). Hydroxyprostaglandin dehydrogenase is a tumor suppressor of human breast cancer as it is a key regulator of COX-2 and PGE_2 and can modulate the estrogen receptor pathway (Wolf et al. 2006). Its homolog in plant system plays a distinct role as an anti-oxidant defense mechanism (Babiychuk et al. 1995) and generates O_2^- & H_2O_2 sufficient for PCD in HR (Xing et al. 1997). 15-PGDH acts as a tumor suppressor in lung and its down regulated in colorectal cancer as well (Ding et al. 2005, Tai et al. 2007). Of late, Lu et al. (2013) reported that 15-PGDH-mediated 15-keto-PGE2 signaling cascade interacts with PPAR-γ, Smad2/3 and TAp63 to inhibit cholangiocarcinoma cell growth. Till date there is no detailed study

of 15-Hydroxyprostaglandin dehydrogenase in a plant system. To our knowledge, this is the first report on plant-pathogen interaction in wild groundnut using cDNA-AFLP to explore host genes involved in an incompatible interaction and expression of cystatin and 15-Hydroxyprostaglandin dehydrogenase leading to cell death.

5.2 Results

5.2.1 3'/5' RACE-PCR, Isolation of full lenth *AdCystatin* and *Ad15-PGDH* and its conditional expression in tobacco resulting HR-like cell death

Cystatin and hydroxyprostaglandin dehydrogenase were found differentially up-regulated in cDNA-AFLP analysis upon interaction between *Arachis diogoi* and late leaf spot pathogen *P. personata* and quantitatively validated real time analysis indicated their importance in plant defense mechanism. We have extended cystatin by using 5' RACE to get full length cDNA sequence. The ORF was 336 bp long encoding a single polypeptide of 111 amino acids and was designated as *AdCystatin* under accession number EU935215. 15-Hydroxyprostaglandin dehydrogenase was made full length by using 3'/5' RACE and its ORF was 1902 bp long encoding a polypeptide of 633amino acid and was designated as Ad15PGDH with assigned accession number GU785018. To study the role of *AdCystatin* and *Ad15-PGDH* in defence response, we transiently overexpressed *AdCystatin*-pCAMBIA2300 and *Ad15-PGDH*-pCAMBIA2300 in detached leaves of tobacco under constitutive promoter and observed hypersensitive response like cell death in infiltrated area within 3-4 day post infiltration. Following this, we investigated the phenomenon further.



(a) With Estradiol **(b)** Without Estradiol

Figure 5.1 AdCystatin induced cell death in tobacco leaf upon conditional expression. Tobacco leaf was transiently expressed with empty vector pER8 and the AdCystatin-pER8 using agroinfiltration. (**a**) Transgene expression was induced by application of 30 µM estradiol after 48 h post infiltration.(**b**) No induction has been given and picture was taken 96 h post induction.

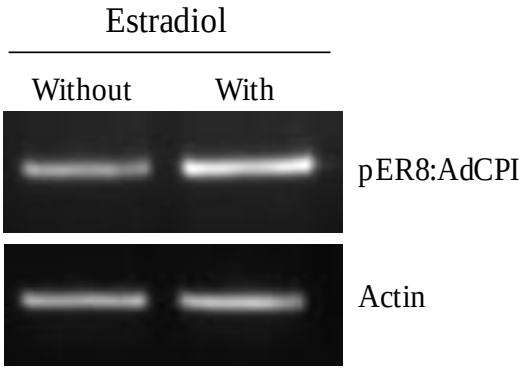


Figure 5.2 A semi-quantitative RT-PCR analysis of conditional expression of AdCystatin. RNA was extracted from leaves agroinfiltrated area with AdCystatin:pER8 vector, 24 h post treatment with or without estradiol and used for cDNA synthesis. Gene specific primers were used for the amplification of AdCystatin, with Actin serving as internal control. Transcript accumulation of AdCystatin was found to be high in estradiol treated sample in comparison to untreated sample.

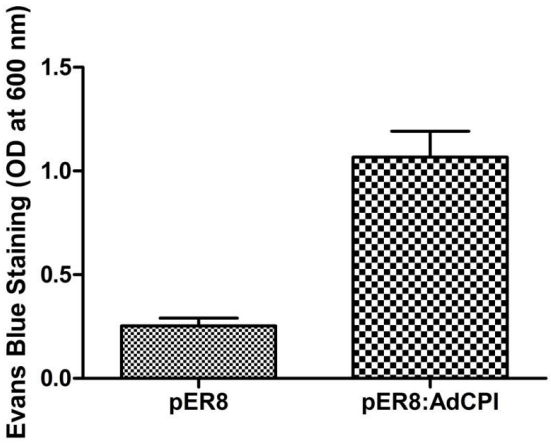
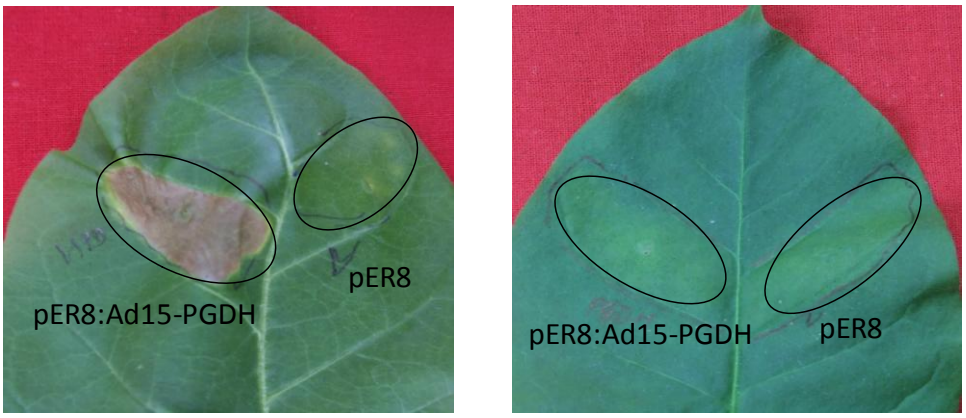


Figure 5.3 Cystatin (AdCPI-pER8) induced cell death in comparison to control (pER8) was quantified using Evans blue. The uptake of Evans blue 72 h post estradiol was quantified using spectrophotometry. Three independent experiment mean value ± SD data was plotted.

For HR- like cell death phenomenon, we cloned *AdCystatin* and *Ad15-PGDH* under an estradiol inducible promoter (XVE) Zuo et al. (2000) and transiently expressed in tobacco leaves using agroinfiltration method as described by Kumar and Kirti (2010, 2012). The *Agrobacterium* strains harboring *AdCystatin*-pER8, *Ad15-PGDH*-pER8 and the empty vector pER8 were infiltrated into tobacco leaves and estradiol was applied to induce gene expression at 48 hpi. After chemical induction, infiltrated area expressing the *AdCystatin* and *Ad15-PGDH* showed cell death but not the empty vector infiltrated region. Induced expression of *AdCystatin* and *Ad15-PGDH* was observed in the infiltrated regions by semi-quantitative RT-PCR, 24 h post estradiol application. Cell death was quantified by using Evans blue dye and *AdCystatin*-pER8 and *Ad15-PGDH*-pER8 induced cell death was high in the corresponding regions.



(a) With Estradiol **(b)** Without Estradiol

5.4 15-Prostaglandin dehydrogenase induced cell death in tobacco leaf upon conditional expression. Tobacco leaf was transiently expressed with empty vector pER8 and the Ad15-PGDH:pER8 using agroinfiltration. (a) Transgene expression was induced by application of 30 µM estradiol after 48 h post infiltration.(b) No induction has been given and picture was taken 96 h post induction.

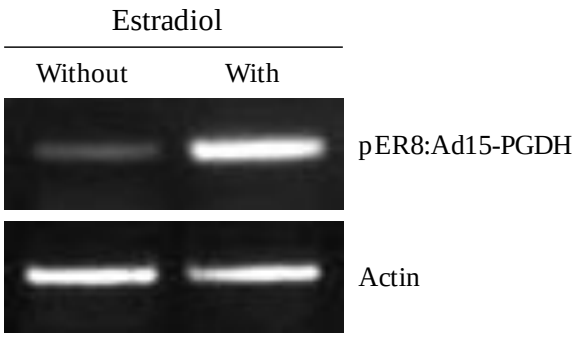


Figure 5.5 A semi-quantitative RT-PCR analysis of conditional expression of Ad15-PGDH. RNA was extracted from leaves agroinfiltrated area with Ad15-PGDH:pER8 vector, 24 h post treatment with or without estradiol and used for cDNA synthesis. Gene specific primers were used for the amplification of 15-Prostaglandin dehydrogenase, with Actin serving as internal control. The expression of Ad15-PGDH was found to be high in estradiol treated sample in comparison to untreated sample.

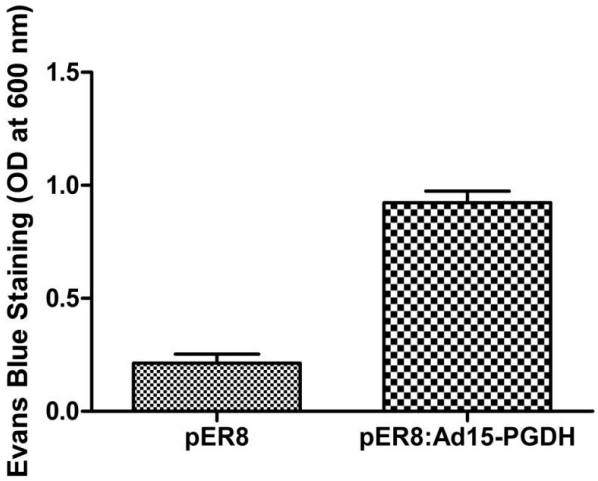


Figure 5.6 15-Prostaglandin dehydrogenase (pER8:Ad15-PGDH) induced cell death compared to control (pER8) was quantified using Evans blue. The uptake of Evans blue 72 h post estradiol was quantified using spectrophotometry. Three independent experiment mean value ± SD data was plotted.

5.2.2 Expression analysis of *AdCystatin* and *Ad15-PGDH* in response to various stresses

We carried out a study on the expression pattern of *AdCystatin* and *Ad15-PGDH* in response to various treatments of the signal molecules through a semi-quantitative RT-PCR was using RNA samples harvested at various time points. The results indicate that a basal level of *AdCystatin* and *Ad15-PGDH* is maintained in leaves, which got upregulated upon treatment. Salicylic acid and methyl jasmonate are the important signaling molecules in systemic acquired resistance (SAR) and wound signaling, respectively and the expression of *AdCystatin* showed upregulation at 6 and 12 hrs of methyl jasmonate, while trivial upregulation was observed at 12 and 24 hrs of salicylic acid treatments. *Ad15-PGDH* showed strong upregulation at 12 and 24 hrs of salicylic acid treatment while methyl jasmonate treated sample showed slight change in transcript levels. Nitric oxide (NO) is an emerging essential component of plant defense signaling and SNP treatment was used at different time intervals. We observed increase in both *AdCystatin* and *Ad15-PGDH* transcript levels at different time point and reaching a peak by 6 h of SNP treatment. ABA is the major signaling molecule for abiotic stress responses and we observed slight increase in transcript levels of *Ad15-PGDH* at 6 h of treated sample, while no such changes were found in transcripts of *AdCystatin*. In case of ethephon treatment, there were no changes found in transcripts of *AdCystatin* as well *Ad15-PGDH* and treatment with water was served as control.

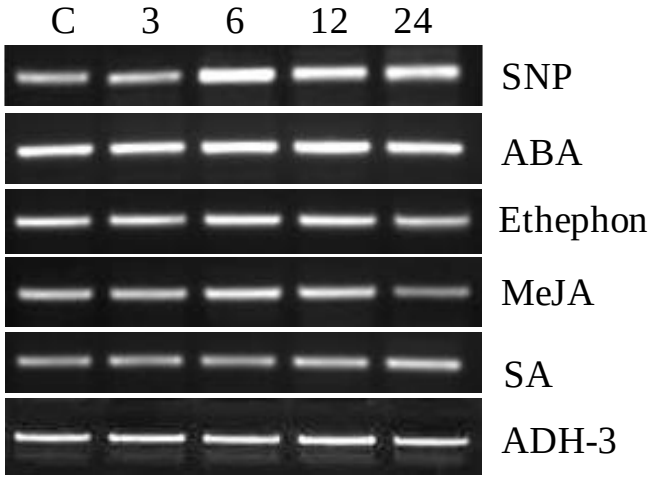


Figure 5.7 Transcriptional regulation of *AdCystatin* in response to stress hormones. A semi-quantitative RT-PCR was performed using total RNA isolated from samples collected at different intervals (in hours) involving treatments with Salicylic acid (SA), Methyl jasmonate (MeJA), Abscisic acid (ABA), Ethephon and Sodium nitropruside (SNP). *Adh-3* served as internal control.

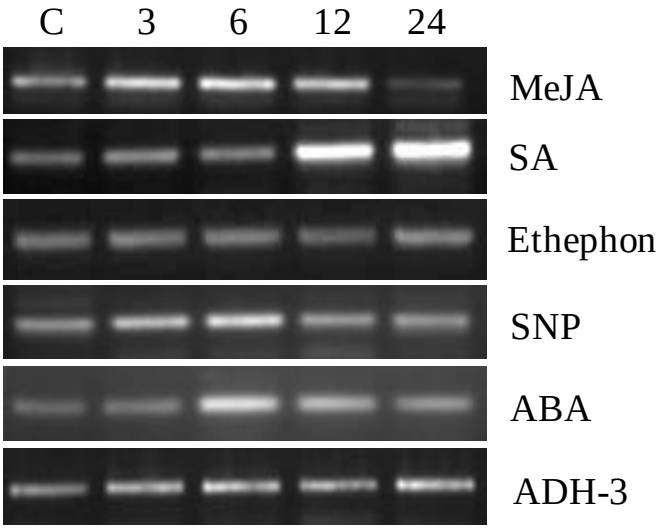


Figure 5.8 Transcriptional accumulation of 15-Hydroxyprostaglandin dehydrogenase in response to stress hormones. A semi-quantitative RT-PCR was performed using total RNA isolated from samples collected at different intervals (in hours) involving treatments with salicylic acid (SA), Methyl jasmonate (MeJA), Abscisic acid (ABA), Ethephon and Sodium nitroprusside (SNP). *Adh-3* served as internal control

5.3 Discussion

Cystatin is a potent antifungal protein againgst various phyopathogenic fungi (Rodriguez et al. 2010, Bangrak and Chotigeat 2011). We have found it differentially expressed in cDNA-AFLP during plant pathogen interaction and quantitative real time analysis confirmed its up-regulation at different time point upon pathogen challenge in both resistant as well as susceptible groundnut plants indicating its role in defense responses. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is a key enzyme to metabolize prostaglandin E_2 (PGE_2) that is synthesized from arachidonic acid in the cyclooxygenase pathway. Overproduction of PGE_2 leads to cell proliferation, invasion, metastasis, angiogenesis, and inhibits apoptosis. Various studies have reported the pro-inflammatory and pro-tumorigenic actions by induced expression of COX-2 and PGE2 in various human and animal cancers (Wu 2005, Wang and Dubois 2010a). Thus 15-PGDH acts as a rate limiting enzyme for COX-2 and PGE2 and its induced expression can suppress the COX-2 and PGE2 and this could be the most successful approach of cancer prevention in animal and human systems.

15-Hydroxyprostaglandin dehydrogenase is another disease resistance associated gene, which is differentially expressed in cDNA-AFLP analysis and we have found its constant upregulation at different times point upon pathogen challenge in resistance plants in real time analysis where as there was no such response observed in susceptible groundnut plants. Its homolog generates reactive oxygen species such as O_2^- and H_2O_2, sufficient for programmed cell death in the hypersensitive response (Xing et al. 1997, Lherminier et al. 2009). Hence, this protein might be playing an important role in plant defense mechanisms through hypersensitive cell death.

We have undertaken heterologous transient expression in *N. tabacum* and *N. benthamiana* to study the hypersensitive response of *AdCystatin* and *Ad15-PGDH* as stable transformation and agroinfiltration are not established in the wild groundnut *Arachis diogoi*. As reported by Kumar and Kirti (2010, 2012), we have expressed conditionaly, under transient and constitutive promoters, *AdCystatin* and *Ad15-PGDH* in tobacco and observed a typical phenotype of hypersensitive response like cell death after 48-72 h post chemical induction. AdCystatin and *Ad15-PGDH* expression were found to be high at 24 h post estradiol application in comparison to control. To our knowledge there were no previous reports of cystatin and 15- Hydroxyprostaglandin dehydrogenase homologs inducing cell death in plants. It seems, cystatin and 15-Hydroxyprostaglandin dehydrogenase might be involved in altering the expression of HR-like cell death associated genes, which needs to be further investigated. It shows that *AdCystatin* and *Ad-15PGDH* positively regulate defense responses and their induced expression is sufficient for HR- like cell death.

Pathogen and herbivore attack induces plant signaling molecules and establishes the development of two different pathways in plants involving methyl jasmonate and salicylic acid (Kessler and Baldwin 2001, Thomma et al. 2001). Herbivores attack activates jasmonic acid (JA), which signals the production of a number of resistance associated molecules, while the pathogen infection influences the biochemical pathaway leading to the production of salicylic acid, which is an important signaling molecule, which activates SAR resulting in the production of pathogenesis- related (PR) proteins and other metabolites that contribute to resistance (Durrant and Dong 2004). We found that *AdCystatin* transcript was slightly increased while *Ad15-PGDH* expression was highly upregulated at 12 and 24 h in response to salicylic acid (Botella et al. 1996),

which indicates these genes are involved in resistance mechanisms against plant pathogens. Methyl jasmonate, an important plant hormone that is involved in the signal transduction pathway and mediating the expression of several defence related genes such as proteinase inhitor, thionins and the production of secondary metabolites (Creelman and Mullet 1997). In response to methyl jasmonate, transcript accumulation of both the genes was found at an early stage declining at later stages (24 hpi). Cystatin accumulation in response to JA has also been reported from chestnut, potato, tomato and soybean (Pernas et al. 2000, Botella et al. 1996).

Abscisic acid is a major signaling hormone in abiotic stress signaling pathway and we have not observed induced expression in *AdCystatin*, which is in agreement with soybean cystatin, where no transcript accumulation were detected (Botella et al. 1996), while *Ad15-PGDH* was marginally induced at 6 h of treatment. We did not find any transcriptional changes in response to ethephon of both the genes, *AdCystatin* and *Ad15-PGDH*. Nitric oxide plays a signaling cascade in regulating plant responses to developmental processes, biotic and abiotic stress (Pernas et al. 2000, Belenghi et al. 2003, Delledonne et al. 2003, Courtois et al. 2008, Qiao and Fan 2008). We have found induced expression in the both *AdCystatin* and *Ad15-PGDH* transcript at different time point and reaching a peak by 6 h of SNP treatment. Recently, Keyster et al. (2013) proposed that soybean cystatin gene expression is regulated by nitric oxide, which mediates developmental processes and responses to abiotic stress. Thus, plants try to defend themselves from the pathogen and herbivory by activating a number of signaling molecules such as JA, SA and SNP, which share some common signaling components (Reymond and Farmer 1998).

5.4 Summary

Cystatin and hydroxyprostaglandin dehydrogenase were found differentially up-regulated in cDNA-AFLP analysis upon interaction between *Arachis diogoi* and late leaf spot pathogen *P. personata*. We have extended cystatin by using 5’ RACE to get full length cDNA sequence. 15-Hydroxyprostaglandin dehydrogenase was made full length by using 3'/5' RACE and its ORF was 1902 bp long encoding a polypeptide of 633 amino acids. We cloned *AdCystatin* and *Ad15-PGDH* under an estradiol inducible promoter (XVE) and transiently expressed in tobacco leaves using agroinfiltration. We have expressed conditionaly, under transient and constitutive promoters, *AdCystatin* and

Ad15-PGDH in tobacco and observed a typical phenotype of hypersensitive response like cell death after 48-72 h post chemical induction. *AdCystatin* and *Ad15-PGDH* expression were found to be high at 24 h post estradiol application in comparison to control. It shows that *AdCystatin* and *Ad-15PGDH* positively regulate defense responses and their induced expression is sufficient for HR- like cell death. We found that *AdCystatin* transcript was slightly increased, while *Ad15-PGDH* expression was highly upregulated in response to salicylic acid, which indicates these genes are involved in resistance mechanisms against plant pathogens. In response to methyl jasmonate, transcript accumulation of both the genes was found at an early stage. Abscisic acid is a major signaling hormone in abiotic stress signaling pathway and we have not observed induced expression in *AdCystatin* while *Ad15-PGDH* was marginally induced at 6 h of treatment. We did not found any transcriptional changes in response to ethephon of both the genes, *AdCystatin* and *Ad15-PGDH*. We have found induced expression in the both *AdCystatin* and *Ad15-PGDH* transcript at different time point and reaching a peak by 6 h of SNP treatment.

Chapter 6

Characterization of Vacuolar Processing Enzyme of a wild groundnut (*AdVPE*) in tobacco

6.1 Introduction and Background

Vacuolar processing enzyme (VPE) is synthesized on endoplasmic reticulum (ER) as a proprotein and then transported to the vacuole, where it is converted to its mature forms by self-endopetidase activity (Hara-Nishimura et al. 1985, Hara-Nishimura and Nishimura 1987). It is involved in both plant development and immunity. Vacuolar processing enzymes are also known as legumains or asparaginyl endopeptidases that comes under the C-13 family of proteases (Rawlings et al. 2012), which comprise caspases and metacaspases (family C14A, C14B). VPEs and Caspase-1 have several similarities such as structural and sequence identity. The caspases regulate programmed cell death in animals, while metacaspase are involved in PCD in plants and fungi (Tsiatsiani et al. 2011). Full genome sequence analysis of *Arabidopsis* revealed no such caspase-encoding genes present. Furthermore, VPEs are localized in the vacuoles while animal caspases are localized in cytosol as their cells lack of vacuoles (Hatsugai et al. 2006).

Vacuolar processing enzyme has been reported as a cysteine proteinase responsible for the maturation of vacuolar proteins (Okamoto and Minamikawa 1999) and exhibits caspase-1 like activity that contribute to defenses against pathogen attack (Rojo et al. 2004). Programmed cell death (PCD) occurs in animals and plants in various stresses (biotic and abiotic) and during development. Recently, a vacuolar processing enzyme was identified as an executioner of plant PCD exhibiting enzymatic properties similar to those of a caspase, which mediates the PCD pathway in animals (Cohen 1997, Lam et al. 2001). There are several reports showing that vacuolar processing enzyme exhibits caspase-1-like activity and regulates cell death in responses to pathogen infection both in resistant as well as susceptible genotypes (Hatsugai et al. 2004). Kuroyanagi et al. (2005) demonstrated that vacuolar processing enzyme is an executioner of plant cell death and involved in fungal toxin-induced cell death in the same way as hypersensitive response like plant cell death. Hiraiwa et al. (1997) reported that the VPE maturates by self-catalytic avtivity and is converted into the active enzyme by the removal of the C-terminal propeptide and subsequent removal of the N-terminal end too.

In *Arabidopsis*, vacuolar processing enzyme is encoded by four type of genes namely, *αVPE*, *βVPE*, *γVPE,* and *δVPE* (Gruis et al. 2002). *βVPE*, and *δVPE* are expressed in seed and play essential role in processing of storage proteins during seed development

and are called as seed type (Shimada et al. 2003). Rice vacuolar processing enzyme plays a crucial role in the crystalline structure and maturation of seed glutelins (Wang et al. 2009b, Kumamaru et al. 2010). The other forms, *αVPE* and *γVPE* are expressed in vegetative parts of the plant and found to be up-regulated upon pathogen infection, senescence, wounding and play important role in various type of plant cell death and are known as vegetative type (Kinoshita et al. 1999, Yamada et al. 2004, Hatsugai et al. 2004). Most recently, Misas-Villamil et al. (2013) reported that vacuolar processing enzyme activity was enhanced during the infection of oomycete fungus, an obligate biotroph, *Hyaloperonospora arabidopsidis* (Hpa) in *Arabidopsis*.

Our quantitative data have shown that vacuolar processing enzyme is strongly induced within 24 h of pathogen inoculation in the resistant wild groundnut, while there was no such induction found in the susceptible groundnut variety in the real time analysis indicating its role in defense response, which might be in form of hypersensitive response and responding to prevent pathogen spread. Previous research into plant VPEs has mostly focused on its role in seed development, self catalytic activity, senescence and pathogen-induced hypersensitive cell death. By, contrast apart from programmed cell death, its resistance property against various phytopathogenic fungi is poorly understood. Here, we report its antifungal activity against various plant pathogens by heterologous expression in tobacco including chemical induced hypersensitive cell death and its transcriptional regulation under various stress conditions.

6.2 Results

6.2.1 Isolation of full length *AdVPE*

Vacuolar processing enzyme was found differentially up-regulated in cDNA-AFLP analysis upon interaction between *Arachis diogoi* and late leaf spot pathogen *P. personata* and quantitatively validated real time analysis indicating its importance in the host plant defense mechanism. We have extended vacuolar processing enzyme by using 3'/5' RACE to get the full length cDNA sequence (Fig. 6.1). The ORF was 1446 bp encoding 487 amino acid, corresponding to a vacuolar processing enzyme that was cloned from wild groundnut (*Arachis diogoi*). Based on its high homology with other dicot plant VPEs, the cDNA was designated as *AdVPE* under accession number

JN160607. To study the role of *AdVPE* in defense response, we constitutively overexpressed *AdVPE:*pCAMBIA2300 in tobacco and generated transgenic plants.

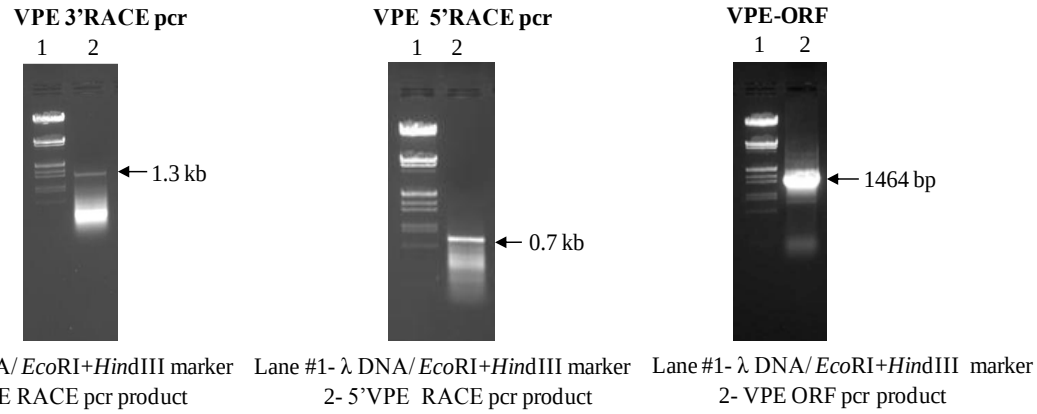


Figure 6.1 Gel pictures represent 3'/5' RACE PCR product of *AdVPE* and its ORF using gene specific primers.

6.2.2 Multiple sequence alignment and phylogenetic analysis

Sequence alignment of the predicted amino acid residues of AdVPE with closely related vacuolar processing enzymes.The AdVPE protein exhibited 82% sequence identity to GmVPE from *Glycine max*, 78% to HsLegumain from *Homo sapiens*, 77% to NtVPE from *Nicotiana tabacum*, 75% to SlVPE from *Solanum lycopersicum* and 74% to AtVPE of *Arabidopsis thaliana*. Conserved amino acids among different species are indicated which showed close relation to soybean and human legumain (Fig. 6.2). Subsequently, we have evaluated the molecular evolutionary relationships of AdVPE against other vacuolar processing enzymes, a phylogenetic tree was constructed. AdVPE exhibited the closest relationship with GmVPE (Fig. 6.3), belonging to a novel group of cysteine proteinases (C-13 family of proteases) and reported up-regulation in association with various types of cell death and under various stress conditions (Kinoshita et al. 1999, Hatsugai et al. 2004, Kuroyanagi et al. 2005). Furthermore, the result showed that the AdVPE shared 82% identity with GmVPE, and there were differences in the predicted protein sequences of these two genes. The predicted protein of AdVPE is made of 487 amino acid residues. However, the predicted protein of GmVPE consisted of 482 amino acid residues. Such differences would also have relation to in the functions of these vegetative type vacuolar processing enzymes.

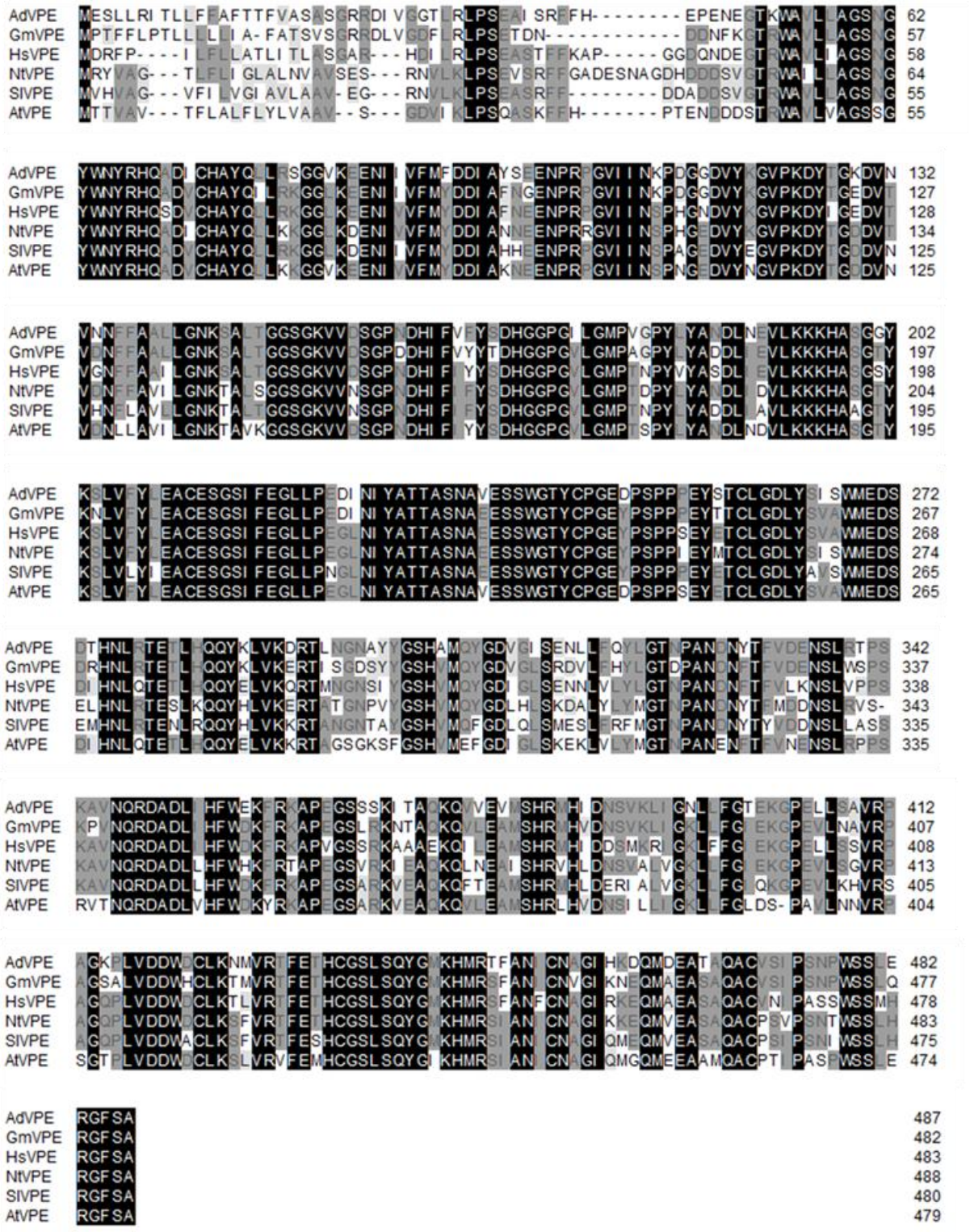


Figure 6.2 Multiple sequence alignment of *AdVPE* with closely related vacuolar processing enzymes from other organisms. Ad: *Arachis diogoi*, Gm: *Glycine max*, Hs: *Homo sapiens*, Nt: *Nicotiana tabacum*, Sl: *Solanum lycopersicum*, At: *Arabidopsis thaliana.*

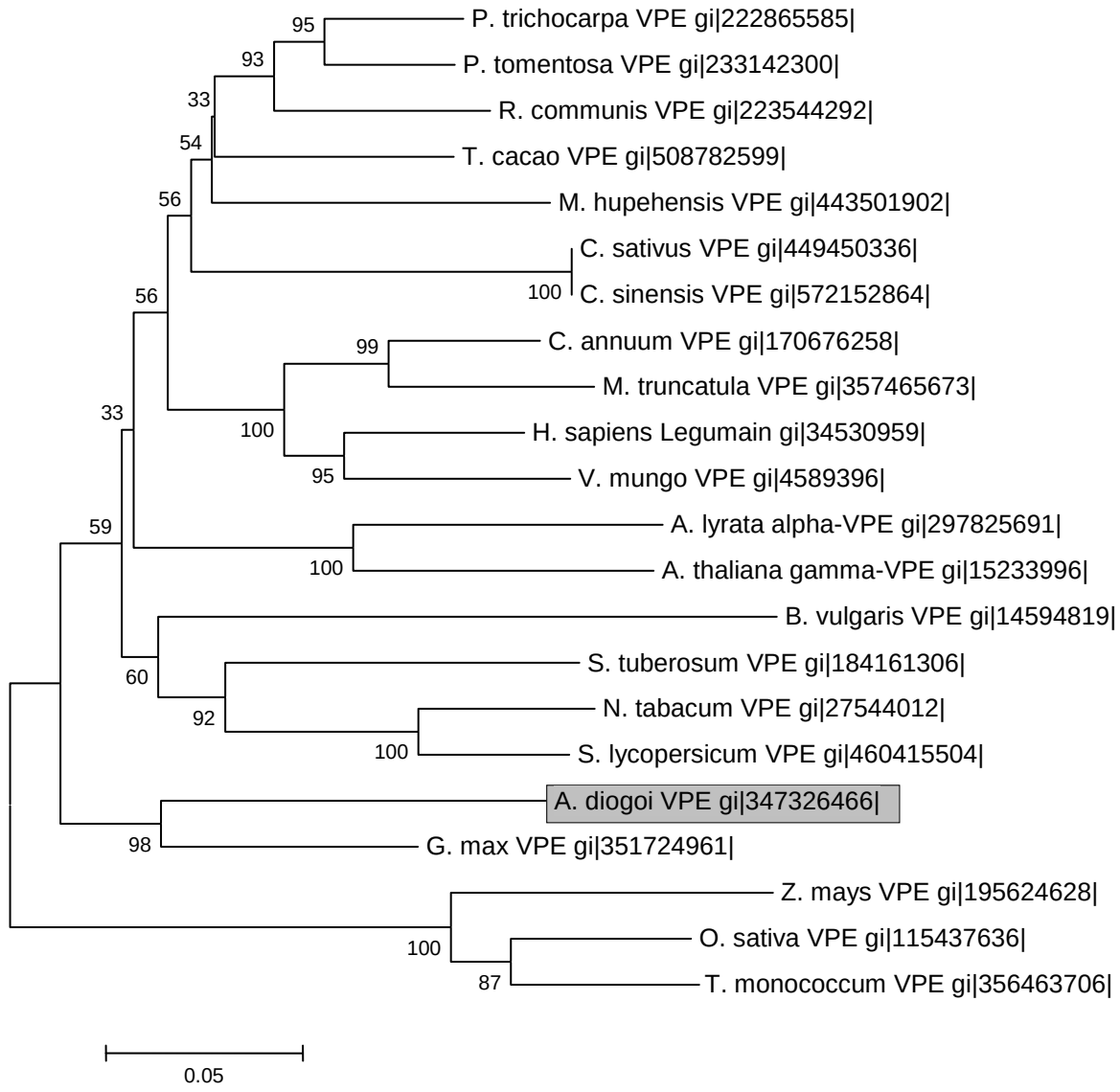


Figure 6.3 Phylogenetic relationship of *AdVPE* with other VPE family members from different plant species constructed by neighbor-joining algorithms of MEGA 4.0 software after the multiple protein sequences alignment using the Clustal W program. Bootstrapping was performed to obtain support values for each branch.

6.2.3 Expression analysis of *AdVPE* in response to various stresses

We carried out a study on the expression pattern of *AdVPE* in response to various treatments of the signal molecules through a semi-quantitative RT-PCR using RNA samples harvested at various time points. The results indicate that a basal level of *AdVPE* is maintained in leaves, which got upregulated upon stress treatment. Salicylic acid and methyl jasmonate are the important signaling molecules in systemic acquired resistance (SAR) and wound signaling, respectively and the expression of *AdVPE* showed

upregulation at 6 hrs of methyl jasmonate, while strong upregulation was observed at 3, 12 and 24 hrs of salicylic acid treatments. Nitric oxide (NO) is an essential component of plant defense signaling and SNP treatment was used at different time intervals. We observed an increase in *AdVPE* transcript levels at late stage and reaching a peak by 24 h of SNP treatment. ABA is the major signaling molecule for abiotic stress responses and we observed slight increase in transcript levels of *AdVPE* at 12 h of treated sample. In case of ethephon treatment, there was marginal upregulation was found in transcripts of *AdVPE* at 6, 12 hrs and vanished at 24 hrs of treated samples and treatment with water was served as control.

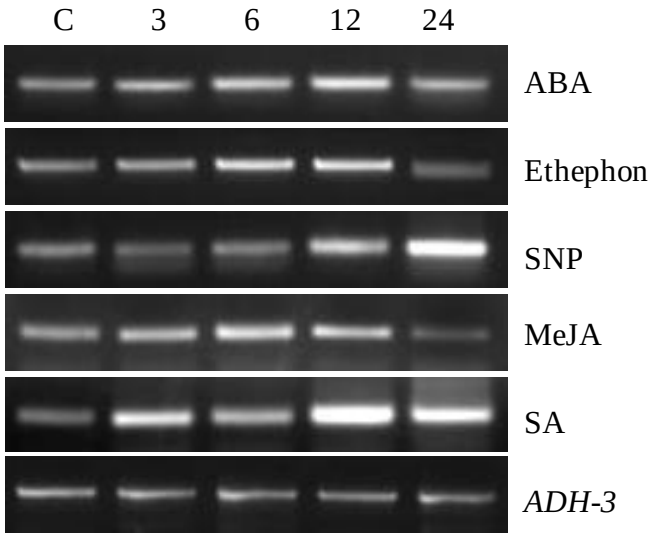
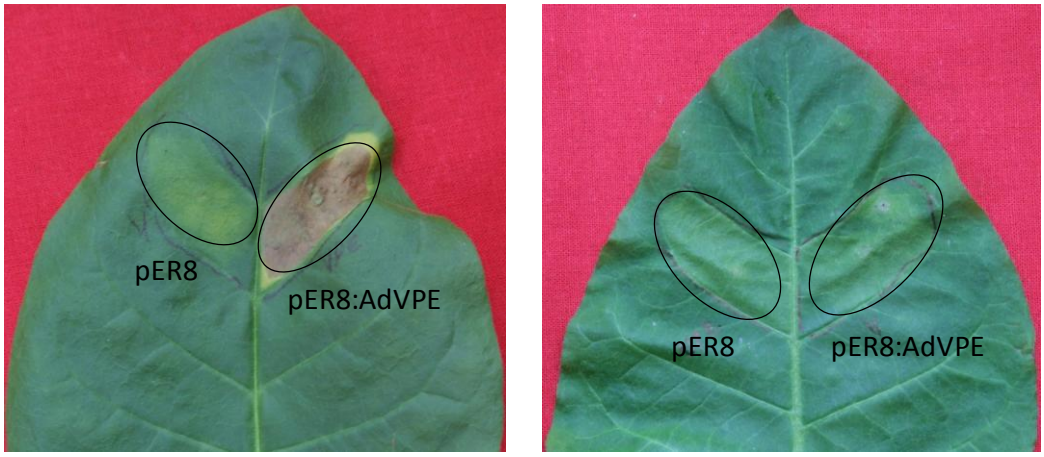


Figure 6.15 Transcriptional accumulation of vacuolar processing enzyme in response to various hormones. A semi-quantitative RT-PCR was performed using total RNA isolated from samples collected at different intervals (in hrs) involving treatments with salicylic acid (SA), Methyl jasmonate (MeJA), Abscisic acid (ABA), Ethephon and Sodium nitroprusside (SNP). *Adh-3* served as internal control.

6.2.4 Conditional expression of AdVPE in tobacco results in HR-like cell death

For studying the possible hypersensitive response of AdVPE, we transiently overexpressed *AdVPE*:pCAMBIA2300 in tobacco leaves under a constitutive promoter and observed hypersensitive response like cell death in infiltrated area within 3-4 day post infiltration. Following this, we investigated the phenomenon further. For HR- like cell death phenomenon, we cloned *AdVPE* under an estradiol inducible promoter (XVE) and transiently expressed it in tobacco leaves using agroinfiltration method as described in material and methods (Section 2.17). The *Agrobacterium* strains harboring *AdVPE*:pER8 and the empty vector pER8 were infiltrated into tobacco leaves and

estradiol was applied to induce gene expression at 48 hpi. After chemical induction, infiltrated area expressing the *AdVPE* showed cell death, but not the empty vector infiltrated region. Induced expression of *AdVPE* was observed in the infiltrated regions by semi-quantitative RT-PCR, 24 h post estradiol applications. Cell death was quantified by using Evans blue dye and pER8:*AdVPE* induced cell death was found high in the corresponding regions.



(**a**) With Estradiol (**b**) Without Estradiol

Figure 6.12 AdVPE induced cell death in tobacco leaf upon conditional expression. Tobacco leaf was transiently expressed with empty vector pER8 and the *AdVPE*-pER8 using agroinfiltration. **(a)** Transgene expression was induced by application of 30 µM estradiol after 48 h post infiltration, **(b)** No induction has been given and picture was taken 72-96 h post induction.

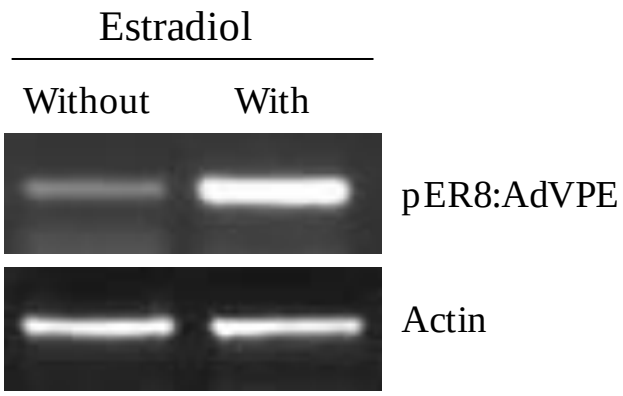


Figure 6.13 A semi-quantitative RT-PCR analysis of conditional expression of *AdVPE*. RNA was isolated from leaves agroinfiltrated area with pER8:*AdVPE* vector, 24 h post treatment with or without estradiol and used for cDNA synthesis. Gene specific primers were used for the amplification of *AdVPE*, with Actin serving as internal control. Transcript accumulation of *AdVPE* was experimentally high in estradiol treated sample in comparison to untreated sample.

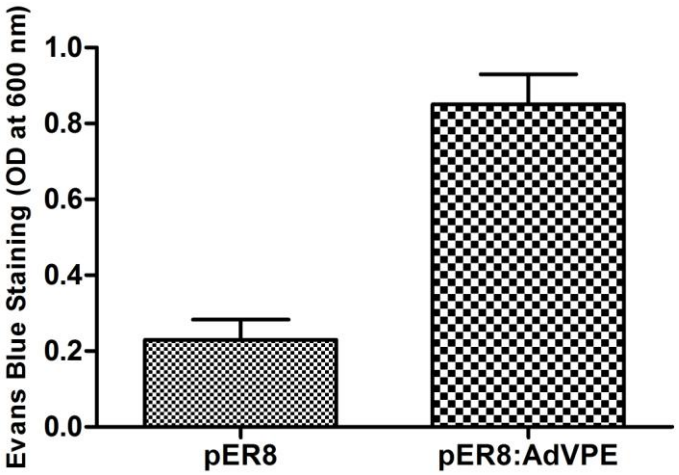


Figure 6.14 Vacuolar processing enzyme (pER8:*AdVPE*) induced cell death in comparison to control (pER8) was quantified using Evans blue. The uptake of Evans blue 72 h post estradiol was quantified using spectrophotometry. Three independent experiments mean value ± SD data was plotted.

6.2.5 Molecular analysis of putative transgenic plants

Several putative transgenic plants were generated by *Agrobacterium* mediated genetic transformation in *Nicotiana tabacum* var. *xanthi* using *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA2300 containing the *AdVPE* expressed under the CaMV 35S promoter. Putative transgenic plants were screened by PCR based amplification of VPE gene specific primers and plant selection marker gene, *nptII.* The expression of the transgene *AdVPE* in the T_0 plants was analyzed by semi-quantitative RT-PCR of which plant no.5 and no.8 showed low expression while no.6, no.14 and no.25 showed relatively higher level of expression of *AdVPE*. One low (no.8) and another high (no.25) expression plants were selected for further analysis in comparison to untransformed control plant with reference to their capacity to counter the attack from plant pathogens.

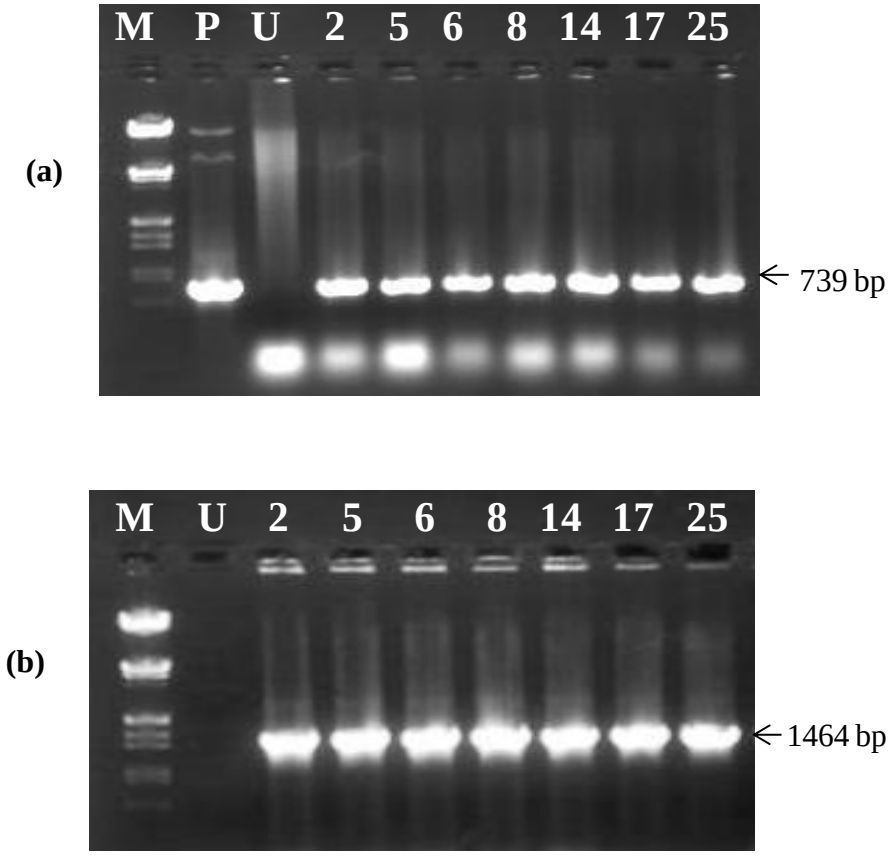


Figure 6.4 (a) A representative gel picture showing 739 bp amplified PCR product of *nptII* gene from genomic DNA of putative T_0 transgenic tobacco plants expressing VPE, **(b)** Represent gel picture showing 1464 bp amplified PCR product of genomic DNA from putative T_0 transgenic tobacco expressing VPE using gene specific primers. Letter, M- λ DNA/*Eco*RI+*Hin*dIII ladder, P- positive control, U- untransformed plant.

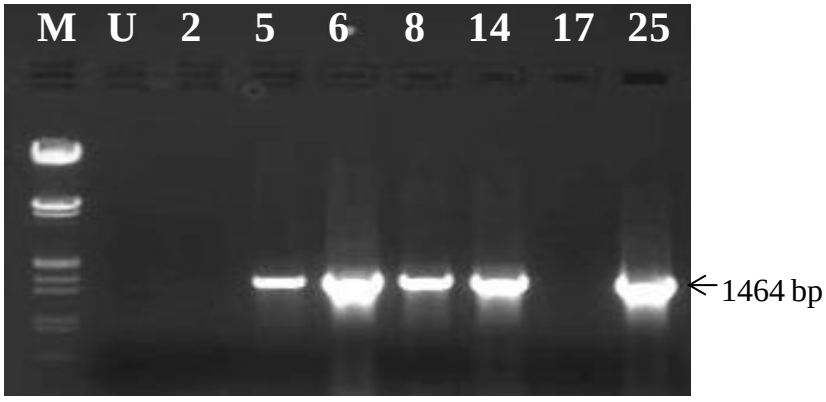
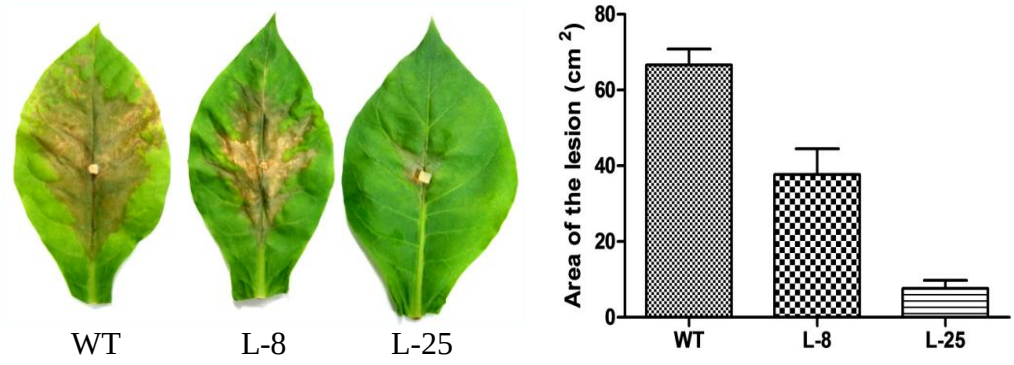


Figure 6.5 Molecular analyses of T_0 *AdVPE* transgenic tobacco plants. Semi-quantitative RT-PCR analysis of *AdVPE* expression in untransformed and transgenic plants. cDNA was synthesized from total RNA of untransformed and transgenic plants and amplified with AdVPE-XhoI-F and AdVPE-SacI-R primers.

6.2.6 Enhanced resistance of transgenic tobacco plants over-expressing *AdVPE* to fungal infection

Leaves of wild type and transgenic plants 8 and 25 were used for resistance analysis. These *AdVPE* transgenic tobacco plants of T_1 and T_2 generation exhibited enhanced resistance against black shank disease causing pathogen, *Phytophthora parasitica* pv. *nicotianae.* These plants were also challenged to *Alternaria alternata* pv. *nicotianae*, a causal agent of brown or leaf spot disease and the transgenic plants were found to be resistant to this pathogen also. Whole seedling assay of T_2 generation of transgenic plants was carried out using the soil born fungus *Rhizoctonia solani* that causes root rot disease, and transgenic plant progeny exhibited enhanced level of resistance. Transgenic plants 25 with high *AdVPE* expression levels exhibited enhanced resistance against *P. parasitica, Alternaria alternata and Rhizoctonia solani* compared to WT plants. Whereas transgenic line 8, which is a low expressing line displayed moderate resistance against these pathogens as represented by bar diagrams. (Fig. 6.6, 6.7, 6.8, 6.9 and 6.10)

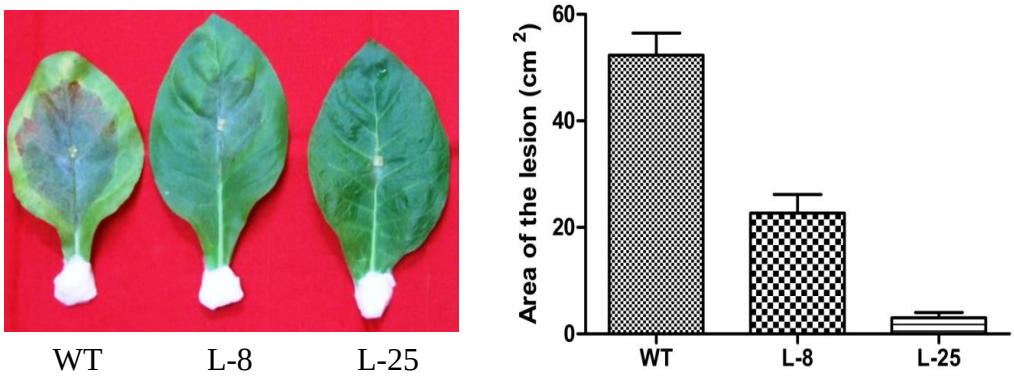
Detached leaf assay with *Phytophthora parasitica* pv. *nicotianae* (T_1-transgenic AdVPE plants)



(a) **(b)**

Figure 6.6 Detached leaf assay with *Phytophthora parasitica* pv. *nicotianae*, (**a**) T_1 transgenic plants L-8 (Low expression) and L-25 (High expression) lines showed enhanced resistance to infection with *Phytophthora parasitica* pv. *nicotianae* compared to WT plants 7 days post inoculation. (**b**) Bar diagram represent symptoms are expressed as diameter (cm) of the diseased lesions area 7 days after infection. Data represent the mean of lesion sizes from three different leaves.

Detached leaf assay with *Phytophthora parasitica* pv. *nicotianae* (T_2-transgenic AdVPE plants)



(a) **(b)**

Figure 6.7 Detached leaf assay with *Phytophthora parasitica* pv. *nicotianae*, (**a**) T_2 transgenic plants L-8 (Low expression) and L-25 (High expression) lines enhanced resistance to infection with *Phytophthora parasitica* var. *nicotianae* compared to WT plants 7 days post inoculation. (**b**) Bar diagram represent symptoms are expressed as diameter (cm) of the diseased lesions area 5 days after infection. Data represent the mean of lesion sizes from three independent experiments.

Alternaria alternata* pv. *nicotianae

Figure 6.8 Detached leaf assay with *Alternaria alternata* var. *nicotianae*, T_2 transgenic plants L-8 (Low expression) and L-25 (High expression) lines enhanced resistance to infection with *Alternaria alternata* var. *nicotianae* compared to WT plants 10 days post inoculation.

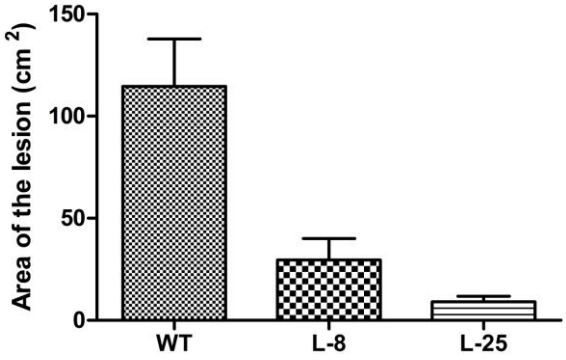
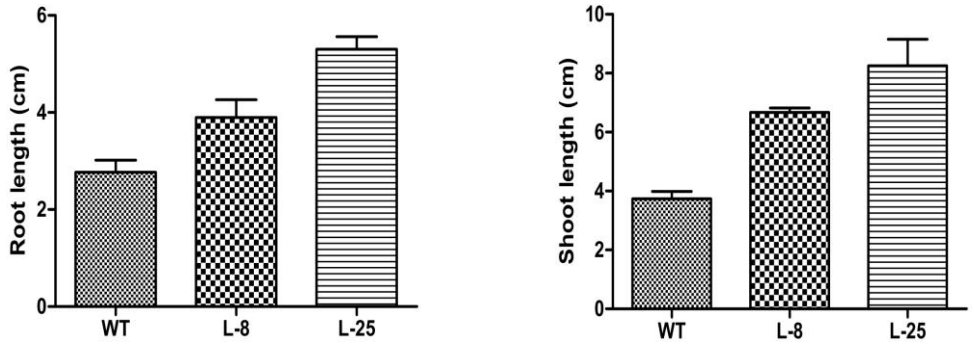


Figure 6.9 Bar diagram represent T_2 transgenic tobacco leaf symptoms are expressed as diameter (cm) of the diseased lesions area 10 days after infection with *Alternaria alternata* var. *nicotianae*. Data represent the mean of lesion size from three different leaves.

Rhizoctonia solani

Figure 6.10 *Rhizoctonia solani* whole plant assay with T_2 transgenic seedlings of L-8 (Low expression) and L-25 (High expression) lines. Transgenic lines showed enhanced resistance compared to wild type plants 15 days post inoculation. One month old seedlings were used for the bioassay.



(a) **(b)**

Figure 6.11 Bar diagram represent comparative study of enhanced resistance of T_2 transgenic tobacco seedlings, **(a)** root length, **(b)** shoot length of the transgenic and wild type plant after 15 days post inoculation of *Rhizoctonia solani*.

6.3 Discussion

Vacuolar processing enzyme is a cysteine proteinase responsible for the maturation of vacuolar proteins and exhibit caspase-1 like activity, which contribute defense againgst pathogen through hypersensitive cell death. It is also otherwise called as legumain. We identified it to be differentially expressed in cDNA-AFLP during a plant pathogen interaction and quantitative real time analysis confirmed its transcript accumulation at early stage upon challenge with biotrophic pathogen *P. personata*, in resistant wild type groundnut plan,t while no such up-regulation was detected in susceptible cultivated groundnut plants indicating its role in defense responses against the pathogen attack.

Plant signaling molecules are induced upon pathogen and herbivore attack and the mechanism of two pathways in plants involving methyl jasmonate and salicylic acid were established (Kessler and Baldwin 2001, Thomma et al. 2001). Methyl jasmonate activated by herbivores signals the production of a number of resistance associated molecules, while microbial infection induces the biochemical pathway to produce salicylic acid, which is an important signaling molecule to induce systemic acquired resistance leading to the production of pathogenesis-related (PR) proteins and other metabolites that contribute to resistance (Durrant and Dong 2004). We found that *AdVPE* transcript was highly upregulated at 3, 12 and 24 h in response to salicylic acid, which indicates that this gene is involved in resistance mechanisms against the late leaf spot pathogen. Vacuolar processing enzyme from *Arabidopsis* has been reported to be up-regulated in salicylic acid treated leaves and its wound-induced expression depends on the action of SA (Kinoshita et al. 1999, Yamada et al. 2004). Recently, Kang et al. (2013) reported that wheat vacuolar processing enzymes were accumulated upon SA treatment. Methyl jasmonate, an important plant hormone involved in signal transduction pathway which mediates several defense related genes such as proteinase inhitor, thionins and secondary metabolites (Creelman and Mullet 1997). In response to methyl jasmonate, trivial expression was found at 6, 12 hrs which declined at later stage 24 h of treatment, and this is in agreement with earlier reports (Kinoshita et al. 1999, Yamada et al. 2004). Abscisic acid is a major signaling hormone and related to many abiotic stress responses (Wasilewska et al. 2008). We observed induced expression of *AdVPE* at 6 and 12 hrs of treatment, which is in agreement with sugarcane legumain, where Santos-Silva et al. (2012) found highly induced expresion of sugarcane legumain upon ABA treatment. We have also found slight up regulation in *AdVPE* transcript at 6 and 12 hrs of

ethephon treatment which disappeared at 24 hrs. Nitric oxide, is becoming a very important signaling molecule acting to induce a signalling cascade regulating plant responses to developmental processes, biotic and abiotic stress (Pernas et al. 2000, Belenghi et al. 2003, Delledonne et al. 2003, Courtois et al. 2008, Qiao and Fan 2008). *AdVPE* was detected to be strongly induced at 12, 24 hrs of SNP treatment. Neill et al. (2002) and Bright et al. (2006) have shown that NO is a key regulator of ABA-induced stomatal closure in pea and *Arabidopsis* respectively. Zhang et al. (2010a) also indicated that tobacco VPE with caspase-1 like activity mediated elicitor- triggered stomatal closure via NO signaling. Late leaf spot disease of groundnut caused by a biotrophic pathogen, *Phaeoisariopsis personata* in which the germ tubes of the pathogen enter the plant cells directly via. the epidermis or more frequently through stomata, leading to intracellular mycelial growth (Abdou et al. 1974, Shokes et al. 1997). Depending upon these finding, it can be postulated that over-expression of VPE upon SA, NO and ABA treatment may enhance resistance in host plant through systematic acquired resistance, elicitors-triggered immunity and stomatal closure.

Plant immune system has the ability to develop different strategies to combat against different types of pathogens (Jones and Dangl 2006). Hypersensitive response is one of the important strategies which confers broad-spectrum disease resistance in plants. Localized programmed cell death is a form of hypersensitive response, which occurs at the site of infection to prevent the growth and spread of pathogens into healthy tissue (Goodman and Novacky 1994, Greenberg 1997). Several types of cell death have been reported from vacuolar processing enzymes such as vacuole destructive, non-destructive, developmental and pathogen induced (Hara-Nishimura and Hatsugai 2011). Hatsugai et al. (2004) reported that VPE mediates virus induce cell death involved rapid and localized cell death at infection site and prevents spread of pathogen. In *Arabidopsis thaliana*, Kuroyanagi et al. (2005) suggested that VPE is involved in both toxin-induced cell death as pathogen strategy and the hypersensitive cell death as plant defense strategy. The fungus, *Alertnaria alternata* f. sp. *lycoperisici* produces AAL- toxin, which was shown to induce PCD in tomato plants (Wang et al. 1996). In a very recent report, Misas-Villamil et al. (2013) found the enhanced activity of *Arabidopsis* VPE, during compatible interaction of obligate biotroph oomycete pathogen *Hyalopernospora arabidopsidis* (*Hpa*).

Hence, VPE might be playing an important role in plant defense mechanisms in groundnut also as its enhanced expression was observed during plant-pathogen interaction. We have undertaken heterologous transient expression in *N. tabacum* and *N. benthamiana* to study the hypersensitive response of *AdVPE* as stable transformation and agroinfiltration are not established in the wild groundnut *Arachis diogoi*. As reported by Kumar and Kirti (2010, 2012), we have expressed conditionaly, under transient and constitutive promoters, *AdVPE* in tobacco and observed a typical phenotype of hypersensitive response like cell death after 48-72 h post chemical induction. *AdVPE* expression was found to be high at 24 h post estradiol application in comparison to the control. Cell death was quantified by using Evans blue dye and the cell death was found to be high in the tobacco cells expressing the pathogen induced gene *AdVPE*. To our knowledge there were no previous reports of chemical induced cell death of VPE in plants. It appears that VPE might be involved in altering the expression of HR-like cell death associated genes positively regulating defense responses and their induced expression is sufficient for HR- like cell death which needs to be further investigated.

Till date, over-expression of plant vacuolar processing enzyme in tobacco for disease resistance has not been reported. Here we report, its enhanced disease resistance against several phytopathogens including a broad host range pathogen, *R. solani* along with chemical induced hypersensitive like cell death. *AdVPE* transgenic tobacco plants of T_1 and T_2 generation exhibited enhanced resistance against black shank disease causing pathogen *Phytophthora parasitica* pv. *nicotianae.* These plants were also challenged with *Alternaria alternata* pv. *nicotianae,* a causal agent of brown or leaf spot disease and the transgenic plants were found to be resistant to this pathogen. Whole seedling assay of T_2 generation of transgenic plants was carried out using the soil born fungus *Rhizoctonia solani* that causes root rot disease, and transgenic plant progeny exhibited enhanced level of resistance. Hence, transgenic plants expressing the cDNA of *AdVPE* in tobacco showed enhanced resistance against the pathogens, *Phytophthora parasitica* pv. *nicotianae, Alternaria alternata* pv. *nicotianae* and *Rhizoctonia solani.* It appears that the vacuolar processing enzyme might be involved in altering the expression of disease resistance gene along with HR-like cell death associated genes, which needs to be further investigated.

6.4 Summary

Vacuolar processing enzyme was found differentially up-regulated in cDNA-AFLP analysis upon interaction between *Arachis diogoi* and the late leaf spot pathogen *P. personata*. We have made full length cDNA using 3'/5' RACE and its ORF was 1446 bp encoding a polypeptide of 487 amino acid. We found that *AdVPE* expression was highly upregulated in response to salicylic acid, which indicates that the gene is involved in resistance mechanisms against plant pathogens, while in response to methyl jasmonate, marginal expression was detected. Abscisic acid is a major signaling hormone in abiotic stress signaling pathway and we have observed induced expression of *AdVPE* under abscisic acid treatment, while slight up regulation found in response to ethephon. We have also detected induced expression in *AdVPE* transcript at a late stage of SNP treatment. We cloned *AdVPE* under an estradiol inducible promoter (XVE) and transiently expressed in tobacco leaves using agroinfiltration. We have expressed conditionaly under transient and constitutive promoters, *AdVPE* in tobacco and observed a typical phenotype of hypersensitive response like cell death after 48-72 h post chemical induction. *AdVPE* expression was found to be high at 24 h post estradiol application in comparison to control. It shows that *AdVPE* positively regulate defense responses and their induced expression is sufficient for HR- like cell death. Transgenic tobacco plants ectopically expressing *AdVPE* exhibited enhanced resistance against *Phytophthora parasitica* var. *nicotianae, Alternaria alternata* pv. *nicotianae* and *Rhizoctonia solani.*

Chapter 7

Functional characterization of a wild groundnut AdSGT1 (suppressor of G2 allele of SKP1) in tobacco as well as in groundnut for disease resistance

7.1 Introduction and Background

Plants are regularly challenged by biotic stresses including viruses, bacteria, fungi, nematodes and insects in their natural surroundings. To cope with these parasites, plants have evolved an innate disease resistance mechanism involving the expression of R-gene encoded proteins that recognize, directly or indirectly, foreign invader and trigger immune response to protect themselves (Dangl and Jones 2001, Belkhadir et al. 2004). Upon protection, plants defend themselves by two ways, first inducing basal defense against virulent, avirulent pathogens, known as primary immune response, where plant responses in which, pathogen-associated molecular patterns (PAMP) are recognised by the plant pattern recognition receptors resulting in PAMP-triggered immunity (PTI) and systematic acquired resistance (SAR) against secondary pathogen. Second is the species specific or effector–triggered immunity (ETI) is induced when a specific protein from pathogen associates directly or indirectly with a plant R-protein (Dangl and Jones 2001, Chisholm et al. 2006). SGT1, RAR1 (required for Mla12- mediate resistance) and HSP90 proteins are the downstream component of effector-trigger immunity, physically interact with each other and make a complex which modulates the activity and stability of the essential components of signaling pathways leading to pathogen resistance recruited by R protein against variety of pathogens (Azevedo et al. 2002, Shirasu and Schulze-Lefert 2003, Takahashi et al. 2003, Hubert et al. 2003, Bieri et al. 2004). SGT1 is an essential signaling component for R-gene mediated resistance response against various plant pathogen including fungi, bacteria and viruses. (Austin et al. 2002, Azevedo et al. 2002, Liu et al. 2004).

SGT1 (suppressor of G2 allele of SKP1) is a conserved protein in all eukaryotes and is crucial for resisting pathogens by both plants and humans as well (Muskett & Parker 2003, Mayor et al. 2007). SGT1 contain three conserved domains: a tetratricopeptide repeat (TPR) domain, a CS (CHORD SGT1) motif and the SGT1- specific sequence (SGS). TPR domain is essential for cell cycle regulation, RNA biogenesis and heat shock response (Goebl and Yanagida 1991, Lamb et al. 1995), while CS motif of barley SGT1 binds to ATPase domain of HSP90 (Takahashi et al. 2003) and CS domain also interacts with CHORD-II domain of RAR1 and HSP90. Noel et al. (2007) reported that SGT1 can interact with HSP70 through SGS domain and regulates immune responses in *Arabidopsis*. The SGS motif of yeast SGT1 mediates binding with LRR domains

(Dubacq et al. 2002) and similarly, Bieri et al. (2004) reported that, barley SGT1 interacts with LRR domain of MLA1 via its SGS domain in yeast two hybrid assay. Nucleotide binding-leucine rich repeat (NB-LRR) proteins are a type of resistance (R) proteins which are involved in pathogen recognition in plants, stabilized and mediates by SGT1 (Lu et al. 2003, Boter et al. 2007, Zhang et al. 2010b). SGT1 was identified in yeast cells (*S. cerevisiae*) as an essential component of cell cycle progression at G1/S and G2/M transitions by Kitagawa et al. (1999), who have shown that it binds to SKP1**,** a component of SCF (Skp1-Cullin-F-box) ubiquitin ligase complex and mediates the regulation of plant disease resistance responses in both yeast and plants (Kitagawa et al. 1999, Azevedo et al. 2002). Hoser et al. (2013) suggested that phosphorylation of SGT1 by plant MAPK regulates nucleocytoplasmic distribution of N- receptor and is necessary for effective plant resistance response to TMV infection. Shapiro et al. (2012) established that HSP90 co-chaperone SGT1 regulates *C. albicans* morphogenesis and drug resistance, providing new therapeutic target for the treatment of life-threatening fungal infections.

In general, effective triggered immunity, a form of plant immune response involved in rapid plant cell death, known as hypersensitive cell death prevents the spread of pathogen infection of the host plant (Greenberg and Yao 2004). Several resistance (R) genes in plants and corresponding avirulence genes in pathogen have been identified and their interaction leading to HR, which confines the spread of the pathogen from infection site (Martin et al. 2003). Studies specify that SGT1 plays an important role in plant cell death during plant resistance response mediated by both PTI and ETI. However, SGT1 has been shown to be involved in cell death against necrotrophic pathogen, *Botrytis cinerea* and hemibiotrophic pathogen, *Fusarium culmorum* (EI Oirdi and Bouarab 2007, Cuzick et al. 2009). Moreover, Wang et al. (2010b) reported that SGT1 is an essential component, which regulates the process of cell death positively, during both compatible and incompatible plant-pathogen interaction. Fu et al. (2009) found that soybean RAR1 and SGT1 are required for basal, R-gene mediated and systemic acquired resistance.

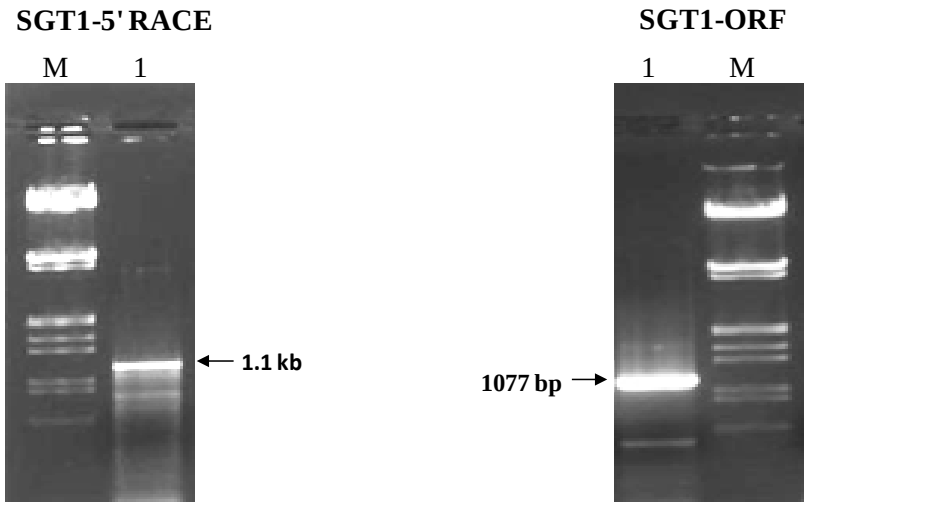
In our present study, quantitative real time analysis including cDNA-AFLP, showed that suppressor of G2 allele of Skp1 (SGT1) was strongly induced within 24 h of pathogen inoculation in the resistant wild groundnut, while there was no such induction found in susceptible groundnut variety indicating its role in defense response in the resistant

groundnut, which might be in the form of hypersensitive response or as regulator of R-gene and responding to prevent pathogen spread. Previous research into plant SGT1 has mostly focused on its role in R- gene regulation and its interaction with other proteins, pathogen-induced hypersensitive cell death. By contrast apart from programmed cell death and R- gene mediated resistance response, its modulation of resistance against various phytopathogenic fungi is poorly understood. Here, we report on the regulation of SGT1 under various treatments and its involvement in chemically induced hypersensitive cell death in transient expression in tobacco, and its protective role against challenge from pathogen in the heterologous system tobacco and the homologous system groundnut.

7.2 Results

7.2.1 Isolation of full length *AdSGT1*

Suppressor of G2 allele of Skp1 (SGT1) was differentially up-regulated in cDNA-AFLP analysis upon challenge from the late leaf spot pathogen *P. personata* and *Arachis diogoi* and the quantitative real-time analysis confirmed its importance in wild groundnuts defense mechanism. We have extended the partial SGT1 by using 5' RACE to get the full length cDNA sequence. The ORF was 1077 bp capable of encoding 358 amino acids, corresponding to a Suppressor of G2 allele of Skp1 from wild groundnut (*Arachis diogoi*). Based on its high homology with others dicot plant genes, the cDNA was disignated as *AdSGT1* under accession number GQ922057. To study the role of *AdSGT1* in defense response, we constitutively overexpressed *AdSGT1*-pCAMBIA2300 in tobacco as well groundnut, and generated transgenic plants.



Lane # M- λ DNA/*Eco*RI+*Hin*dIII marker
1- AdSGT1 5' RACE pcr product

Lane # M- λ DNA/*Eco*RI+*Hin*dIII marker
1- AdSGT1 ORF pcr product

Figure 7.1 Gel picture represent 5' RACE PCR product of *AdSGT1* and its ORF

7.2.2 Multiple sequence alignment and phylogenetic analysis

Sequence alignment of the predicted amino acid sequence of AdSGT1 with closely related suppressor of G2 allele of Skp1.The AdSGT1 protein exhibited 82% sequence identity to GmSGT1 from *Glycine max*, 79% to CaSGT1 of *Cicer aeritinum*, 73% to FvSGT1 from *Fragaria vesca*, 71% to SlSGT1 from *Solanum lycopersicum* and 70% to NbSGT1 from *Nicotiana benthamiana*. Conserved amino acids among different species are indicated which showed close relation to soybean and *Cicer aeritinum* (Fig. 7.2). Sequence analysis of SGT1 proteins from yeast, human, barley, rice, and *Arabidopsis* shows three conserved domains [tetratricopeptide repeat (TPR), CHORD-containing proteins and Sgt1 (CS), and Sgt1-specific (SGS)] domain (Azevedo et al. 2002). Subsequently, we have evaluated the molecular evolutionary relationships of AdSGT1 against other SGT1, a phylogenetic tree was constructed. AdSGT1 exhibited the closest relationship with GmSGT1 and CaSGT1 (Fig. 6.3). Furthermore, the result showed that the AdSGT1 shared 82% identity with GmSGT1, and almost similar in the predicted protein sequences of these two genes. The predicted protein of AdSGT1 is made of 358 amino acids, while the predicted protein of GmSGT1 consisted of 359 amino acid residues. GmSGT1 has been characterized by Fu et al. (2009) and found that soybean RAR1 and SGT1 are required for defense signaling pathway.

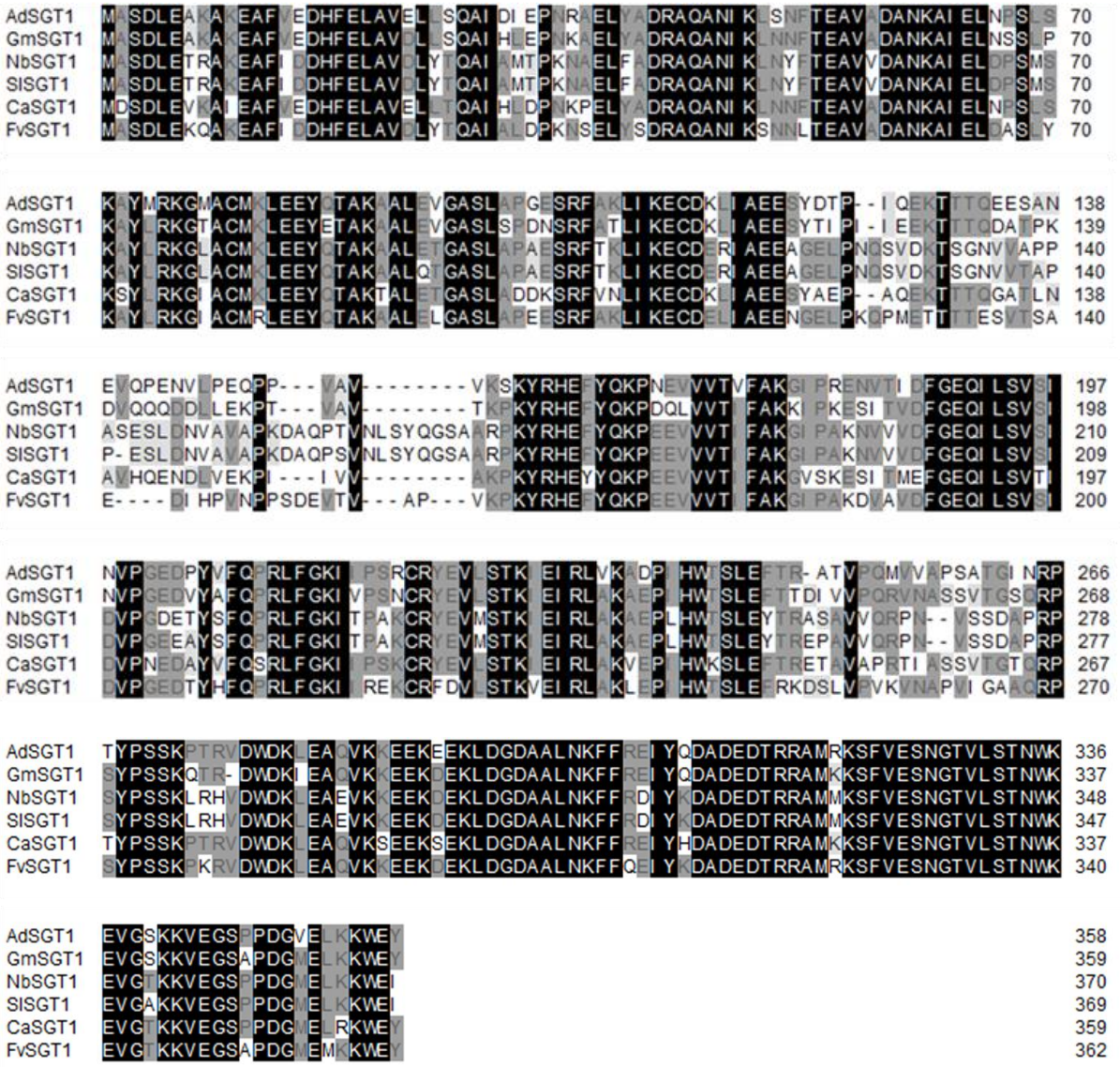


Figure 7.2 Alignment of deduced amino acid sequences of AdSGT1 with closely related SGT1 from other organisms. Ad: *Arachis diogoi*, Gm: *Glycine max*, Nb: *Nicotiana benthamiana*, Sl: *Solanum lycopersicum*, Ca: *Cicer arietinum*, Fv: *Fragaria vesca*

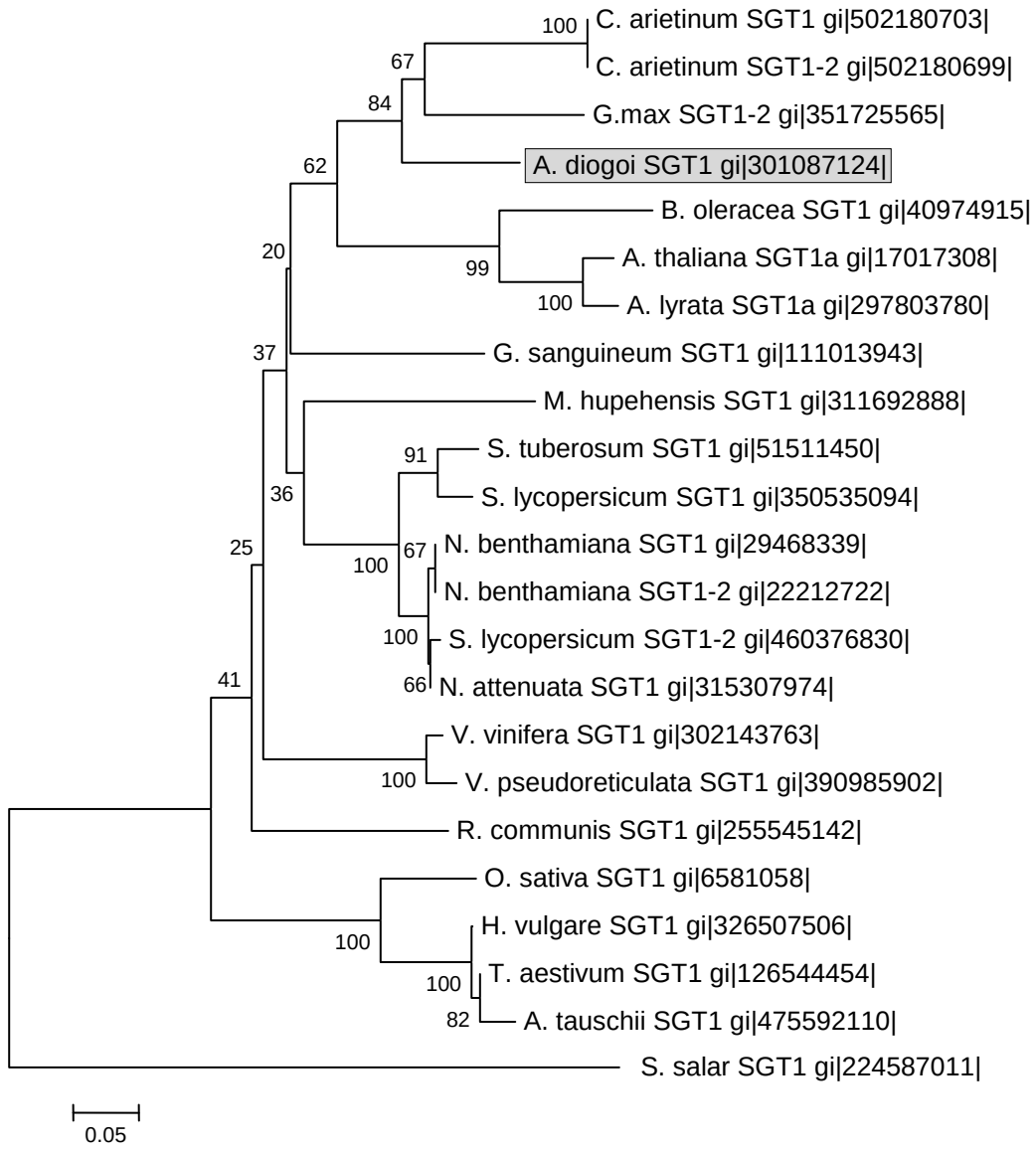
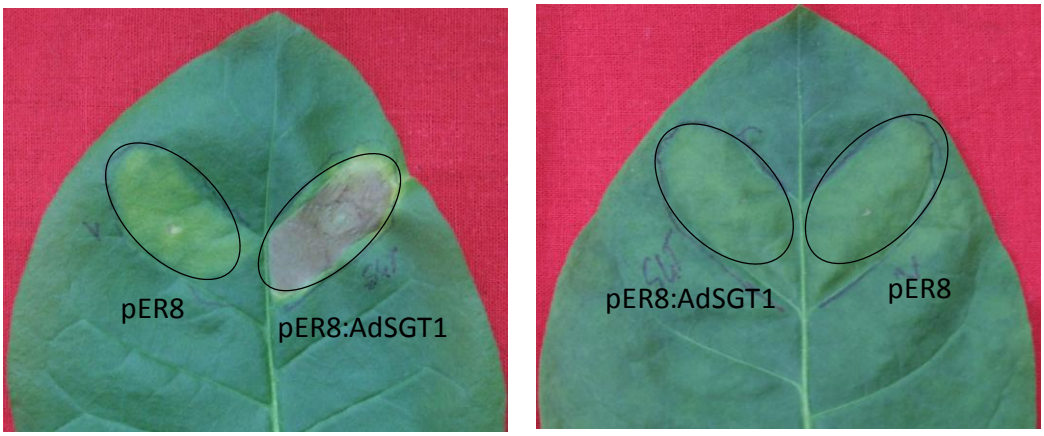


Figure 7.3 The phylogenetic relationship of *AdSGT1* with other SGT1 family members from different plant species constructed by neighbor-joining algorithms of MEGA 4.0 software after the multiple protein sequences alignment using the Clustal W program. Bootstrapping was performed to obtain support values for each branch.

7.2.3 Conditional expression of *AdSGT1* in tobacco results in HR-like cell death

For hypersensitive like cell death response of *AdSGT1*, we transiently overexpressed *AdSGT1:*pCAMBIA2300 in tobacco leaves under the constitutive promoter 35S and observed hypersensitive response like cell death in infiltrated area within 3-4 days post infiltration. Following this, we investigated the phenomenon further. For HR- like cell death phenomenon, we cloned *AdSGT1* under an estradiol inducible promoter (XVE) and transiently expressed in tobacco leaves using agroinfiltration method as described in material and methods. The *Agrobacterium* cells harboring *AdSGT1*-pER8 and the empty vector pER8 were infiltrated into tobacco leaves and estradiol was applied to induce the gene expression at 48 hpi. After chemical induction, infiltrated area expressing the *AdSGT1* showed cell death, but not the empty vector infiltrated region. Induced expression of *AdSGT1* was observed in the infiltrated regions by semi-quantitative RT-PCR, 24 h post estradiol application. Cell death was quantified by using Evans blue dye and *AdSGT1*-pER8 induced cell death was found significantly high in the corresponding regions.



(**a**) With Estradiol (**b**) Without Estradiol

Figure 7.4 *AdSGT1* induced cell death in tobacco leaf upon conditional expression. Tobacco leaf was transiently expressed with empty vector pER8 and the *AdSGT1*-pER8 using agroinfiltration. (**a**) Transgene expression was induced by application of 30 µM estradiol after 48 h post infiltration, (**b**) No induction has been given and picture was taken 72-96 h post induction.

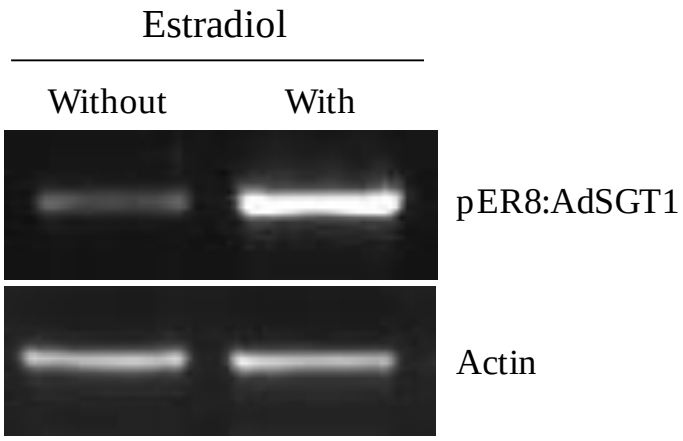


Figure 7.5 A semi-quantitative RT-PCR analysis of conditional expression of *AdSGT1*. RNA was extracted from leaves agroinfiltrated area with pER8:*AdSGT1* vector, 24 h post treatment with or without estradiol and used for cDNA synthesis. Gene specific primers were used for the amplification of *AdSGT1*, with Actin serving as internal control. Transcript accumulation of *AdSGT1* was found to be high in estradiol treated sample in comparison to untreated sample.

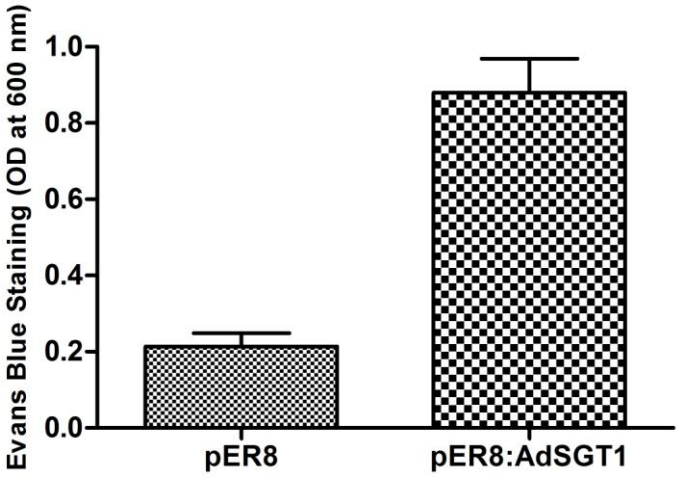


Figure 7.6 Supressor of G2 allele of Skp1 (pER8:*AdSGT1*) induced cell death in comparison to control (pER8) was quantified using Evans blue. The uptake of Evans blue 72 h post estradiol was quantified using spectrophotometry. Three independent experiments mean ± SD data was plotted.

7.2.4 Expression analysis of *AdSGT1* in response to various stresses

We carried out a study on the expression pattern of *AdSGT1* in response to various treatments of the signal molecules through a semi-quantitative RT-PCR using RNA samples harvested at various time points. The results indicate that a basal level of *AdSGT1* is maintained in leaves, which got upregulated upon stress treatment. Salicylic

acid and methyl jasmonate are the important signaling molecules in systemic acquired resistance (SAR) and wound signaling, respectively and the expression of *AdSGT1* showed upregulation at 6 hrs of methyl jasmonate and maintained upto 24 hrs, while strong up-regulation was observed at 3, 6, 12 and 24 hrs of salicylic acid treatments. ABA is the major signaling molecule for abiotic stress responses and we observed slight increase in transcript levels of *AdSGT1* at 6 h of treated sample. In case of ethylene treatment, a marginal up-regulation was found in transcripts of *AdSGT1* at 6, 12 hrs and vanished at 24 hrs in treated samples and treatment with water served as control.

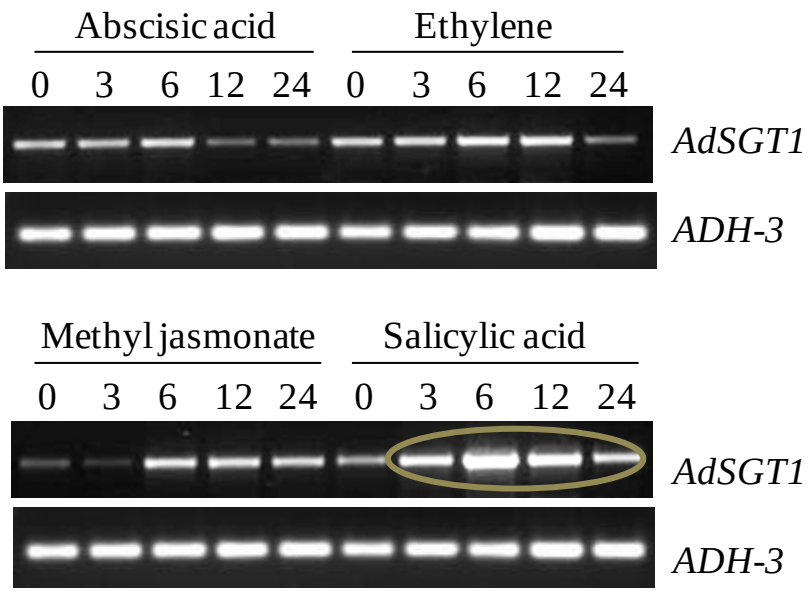


Figure 7.7 Transcriptional accumulation of *AdSGT1* in response to stress hormones. A semi-quantitative RT-PCR was performed using total RNA isolated from samples collected at different time (in hrs) intervals involving treatments with Salicylic acid (SA), Methyl jasmonate (MeJA), Abscisic acid (ABA) and Ethylene. *Adh-3* served as internal control.

7.2.5 Generation of transgenic tobacco plants and its molecular analysis

To study the role of *AdSGT1* in defense response, we constitutively over-expressed *AdSGT1*-pCAMBIA2300 in tobacco as well groundnut, and generated several transgenic plants. The putative tobacco transgenic plants were generated by *Agrobacterium* mediated genetic transformation leaf discs of *Nicotiana tabacum* var. *samsun* using *Agrobacterium tumefaciens* strain EHA105, harboring the binary vector pCAMBIA2300 containing the *AdSGT1* expressed under the CaMV 35S promoter. Putative plants were

screened by PCR based amplification of *AdSGT1* gene specific primers and plant selection marker gene, *nptII*. The expression of the transgene *AdSGT1* in the T_0 plants was analyzed by semi-quantitative RT-PCR of which 1, 5, 6, 7, 9 & 17 showed low expression while 2, 3, 8, 11, 12, 16 & 18 relatively higher level of expression. One low and another high expression plant were selected for further analysis in comparison to untransformed control plant.

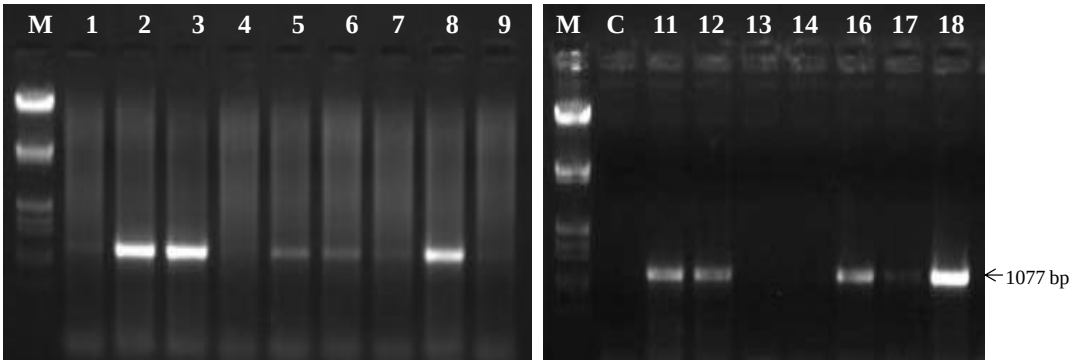


Figure 7.8 Molecular analyses of T_0 *AdSGT1* transgenic tobacco plants. Semi-quantitative RT-PCR analysis of *AdSGT1* expression in untransformed and transgenic plants. cDNA was synthesized from total RNA of untransformed and transgenic plants and amplified with *AdSGT1* ORF-F and ORF-R primers.

7.2.6 Enhanced resistance of transgenic tobacco plants over-expressing *AdSGT1* to fungal infection

Leaves of wild type and transgenic plants no. 6 and no.18 were used for resistance response analysis. These *AdSGT1* transgenic tobacco plants of T_1 and T_2 generation exhibited enhanced resistance against black shank disease causing pathogen *Phytophthora parasitica* pv. *nicotianae.* These plants were also challenged to *Alternaria alternata* pv. *nicotianae,* a causal agent of brown or leaf spot disease and the transgenic plants were also found to be resistant to this pathogen. Whole seedling assay of T_2 generation of transgenic plants was carried out using the soil born fungus *Rhizoctonia solani* that causes root rot disease, and transgenic plant progeny exhibited enhanced level of resistance. Transgenic plants no. 18 with high *AdSGT1* expression level exhibited enhanced resistance against *P. parasitica*, *Alternaria alternata and Rhizoctonia solani* compared to WT plants. Where as transgenic line 6, which is a low expressing line displayed delayed susceptibility against these pathogens as represented by bar diagrams.

a) *Phytophthora parasitica* var. *nicotianae*

Figure 7.9 AdSGT1 transgenic tobacco of T_2 generation plant leaves of low expression line 6 and high expression line 18 showed enhanced resistance to infection with *Phytophthora parasitica var. nicotianae* (Ppn) compared to WT plants.

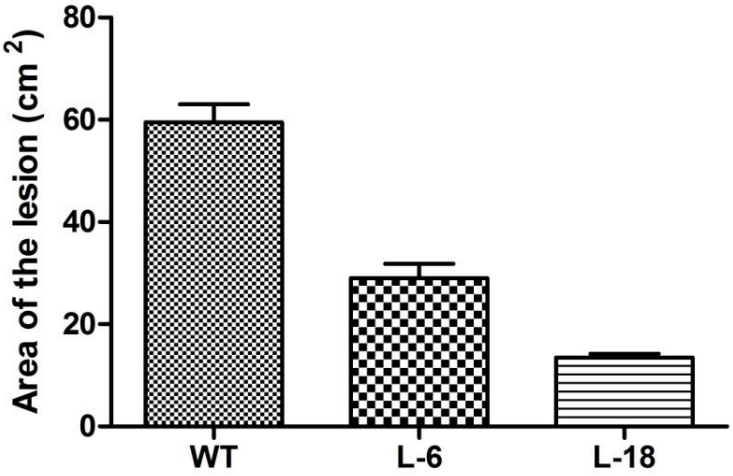


Figure 7.10 Bar diagram represents disease infection of low expression line 6 and high expression line 18 as well as compared to wild type control plant. Disease lesions expressed as (cm) of the infected area after 7 day post inoculation. Data represent the mean of lesion sizes from three different leaves.

b) *Alternaria alternata* pv. *nicotianae*

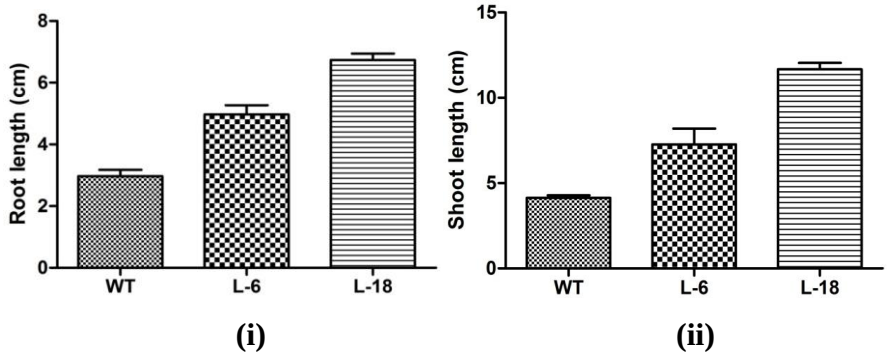
WT L-6 L-18

Figure 7.11 Detached leaf assay with *Alternaria alternata* var. nicotianae, Transgenic tobacco expressing AdSGT1-T_2 plants of low expression line 6 and high expression line 18 showed enhanced resistance to infection with *Alternaria alternata* var. *nicotianae* compared to WT plant. Photograph was taken after 7 days post infection.

c) *Rhizoctonia solani*

WT L-6 L-18 WT L-6 L-18

Figure 7.12 *Rhizoctonia solani* whole plant assay with T_2 transgenic seedlings of L-6 (Low expression) and L-18 (High expression) lines. Transgenic lines showed enhanced resistance compared to wild type plants 15 days post inoculation. One month old seedlings were used for bioassay.



(i) **(ii)**

Figure 7.13 Bar diagram represent comparative analysis of enhanced resistance of *AdSGT1*-T_2 transgenic tobacco seedlings against *Rhizoctonia solani*, (**i**) root length and (**ii**) shoot length of the transgenic and wild type plant after 15 days post infection.

7.2.7 *In planta* genetic transformation and molecular analysis of groundnut transgenic plants

We have generated a number of putative transgenic groundnut plants of *Arachis hypogaea* cv. JL-24 by employing *in planta* method using pCAMBIA2300 harboring *AdSGT1* gene. Putative transformants were confirmed by PCR based analysis of *nptII* fragment and integrated gene (*SGT1*) with promoter amplified by 35S forward and SGT1 reverse primer. VirD2 gene was amplified by using VirD2-F and VirD2-R primers to confirm that there was no contamination of EHA105 cells in the plants in the second generation. Relative expression of *AdSGT1* was examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) in transgenic *AdSGT1* over-expressing plants with putative transgenic plants designated as 33, 42 and 54 showing higher expression while the plant number 4 exhibited low expression.

7.2.8 Expression of the *nptII* gene

The plants were selected for PCR analysis using *nptII* gene specific primers to amplify the transgene. Amplification of the expected size of 739 bp was seen in some plants using *npt*II gene primers, as similar to that of vector control, while no such band was observed in the wild type under similar conditions. At least, two amplicon of *nptII* of the putative transgenic groundnut plants were cloned in pTZ57R, a cloning vector, sequenced commercially. This sequencing confirmed the presence of *nptII* gene in the genome of the putative transgenic plants.

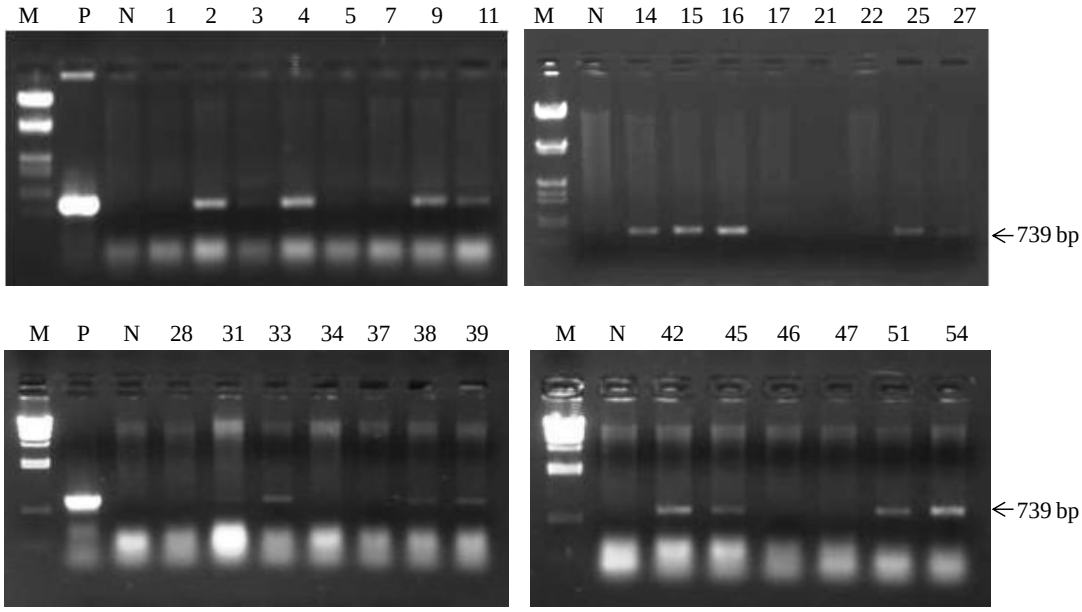


Figure 7.14 Analysis of the T_1 generation groundnut plants by PCR with *nptII* gene specific primers. Lane M: λ DNA/*Eco*RI+*Hin*dIII and λ DNA *Hin*dIII marker, P: Vector positive control, N: negative control (DNA from untransformed plants), Rest of the lanes are genomic DNA samples of putative transformants.

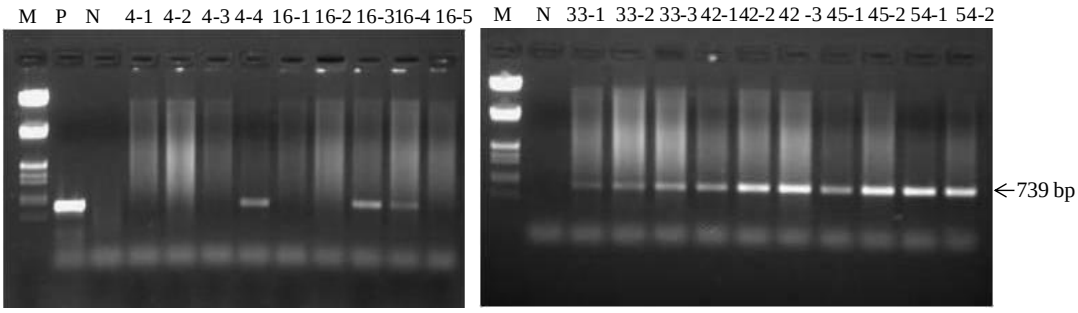


Figure 7.15 Analysis of the T_2 generation groundnut plants by PCR with *npt*II gene specific primers. Lane M: λ DNA/ *Eco*RI+*Hin*dIII marker, P- Vector positive control, N: Negative control (DNA from untransformed plants), Rest of the lanes are genomic DNA samples of putative transformants.

7.2.9 Integration and expression of the transgene in T_2 plants

T_2 plants were selected for PCR analysis using 35S-F and SGT-R primers to amplify the transgene. Amplification at the expected size of 1256 bp was seen in some plants, as similar to that of vector control, while no such band was observed in the wild type under similar conditions. The amplicons from two plants were cloned in pTZ57R, a cloning

vector, sequenced and confirmed by NCBInr BLASTx search. This observation clearly indicated the presence of the transgene in the genome of T_2 transformants.

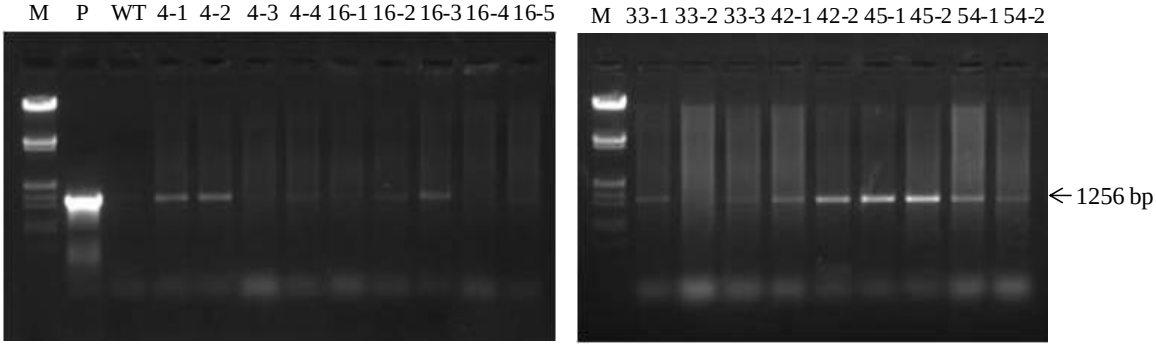


Figure 7.16 Analysis of the T_2 generation groundnut plants by PCR with 35S-F and SGT-R gene specific primers. Lane M: λ DNA/*Eco*RI+*Hin*dIII marker, P: Vector positive control, WT: Wild type (DNA from untransformed plants), Rest of the lanes are genomic DNA samples of putative transformants.

VirD2 genes of the Ti plasmid of *Agrobacterium* cells helps in the transfer and proper integration of T-DNA into the recipient genome. The amplification of VirD2 using VirD2-F and VirD2-R primers confirms the contamination or otherwise of agrobacterial cells in transformants. We have detected expected size of amplicon in positive control using *Agrobacterium* cells and no such amplification was found in putative groundnut transformants of T_1 generation plants, which clearly indicates that the amplification of the target *SGT1* observed in the putative transgenic plants is due to the integration of the T-DNA and not due to the contamination of agrobacterial cells.

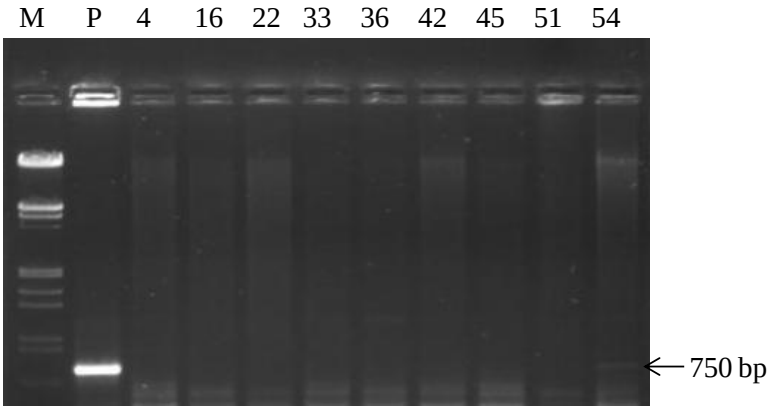


Figure 7.17 Represents putative T_1- SGT1 transgenic groundnut plants were tested by PCR amplified VirD2-F & VirD2-R primers using gDNA as template. M- λ DNA/*Eco*RI+*Hin*dIII marker, P-positive control (pCAMBIA2300 in EHA105), Rest of the lanes are genomic DNA samples of putative transformants.

7.2.10 Southern blot analysis of groundnut transgenic plants

The genomic DNA samples of groundnut T_1 transgenic plants were digested with *Eco*RI enzyme and the blot was probed with PCR amplified *nptII* fragment. Different sizes of the hybridizing bands indicate that these plants represent to individual transformation events. Transgene integration was detected in all the 6 plants, of which 33, 42, 45 and 54 have shown single copy number while 4 and 39 exhibit two copies of integrated T-DNA.

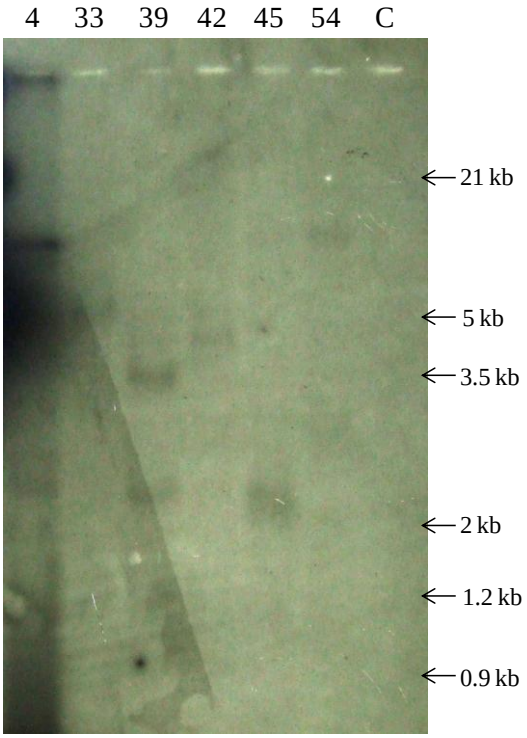


Figure 7.18 Southern hybridization analysis of groundnut T_1 transgenic plants expressing *AdSGT1* gene. DNA samples were digested by *Eco*RI, which has single site on the T-DNA and blots were probed by PCR amplified fragment of *nptII*. Plants 33, 42 and 45 represent single copy integration.

7.2.11 Relative Expression Analysis of *AdSGT1* Transgenic T_1 groundnut plants

Relative transcription levels of *AdSGT1* transgenic T_1 groundnut plants was determined by real-time RT-PCR in the bar diagram shown below. Transcripts of *AdSGT1* were found 3-4 times higher in 33, 42 and 54 number of transgenic plants while plant number 4 did not show significant change at transcript level in comparison to the wild type untransformed plant.

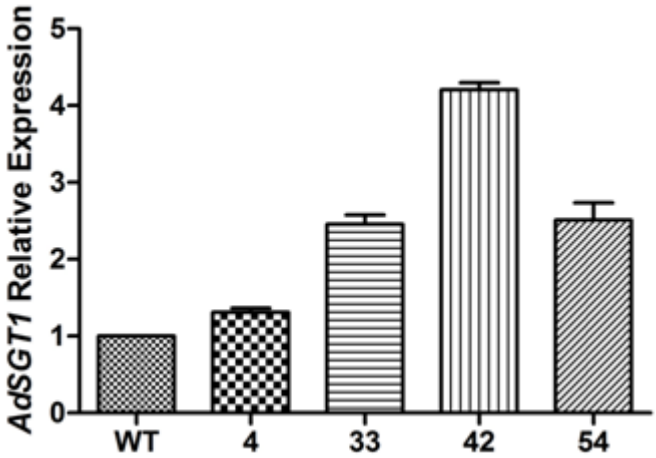


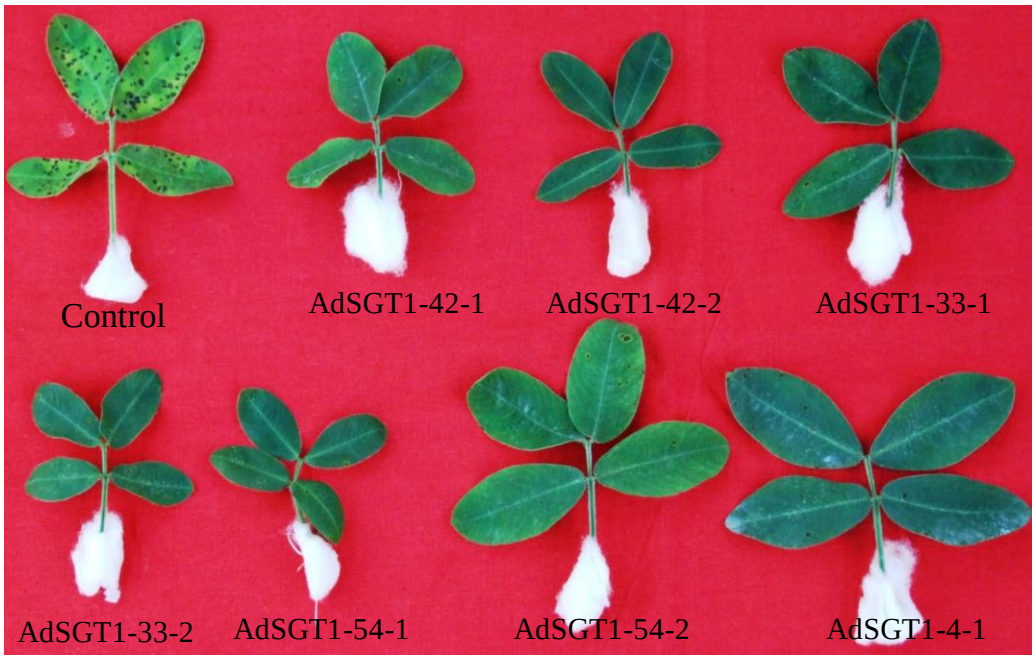
Figure 7.19 Relative expression of *AdSGT1* examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) in groundnut T_1 transgenic *AdSGT1*-overexpressing lines. Data were plotted from three technical replicates.

7.2.12 Enhanced disease resistance response of T_2 transgenic groundnut plants against late leaf spot disease

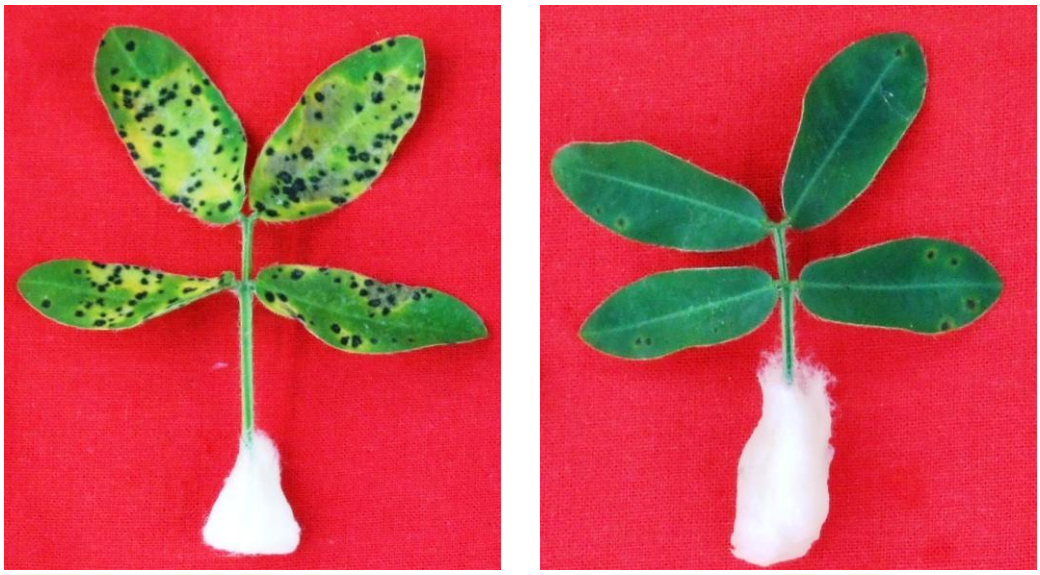
Detached leaf assay

Transgenic plants confirmed by Southern analysis were assayed for their resistance towards the late leaf spot pathogen *P. personata*. Detached leaf assay was conducted using conidial spray and disease symptoms started appearing on the control and transgenic plants after 10–12 days as small specks, which later developed into full sized lesion in control plants. Data was scored after 17 and 21 days post inoculation and leaves were assessed for leaf spots. On control untransformed plants the numbers of spots were high while on the resistant transgenic plants only few late leaf spots were found even after 21 days post inoculation. All the transgenic lines showed increased resistance to *P. personata* as measured by the average number of lesions.

(a)



(b)



Wild Type **AdSGT1-42-2 transgenic**

Figure 7.20 *Phaeoisariopsis personata* fungal conidia bioassay of (**a**) control and *AdSGT1*-T_2 transgenic detached leaves after 17 days post inoculation, (**b**) enlarge view of wild type and AdSGT1-42-2 transgenic plant leaf after 20 days post infection.

Fig 7.21 Detached leaf assay for the late leaf spot disease using conidial spray of Control and AdSGT1-T_2 transgenic plants in petri dishes after 21 days post infection.

Whole plant assay

We have carried out whole plant assay to further confirm our results on the analysis in detached leaf assay and we obtained data consistent with our data on detached leaf assay. Late leaf spot symptoms started appearing after 12-15 days post inoculation. Transgenic lines showed increased resistance to *P. personata* as measured by the average number of lesions in comparison to wild type. The average number of lesions was found to be significantly less in transgenic plants compared to the controls and the number of lesions were found to be significantly lower in transgenics.

Control AdSGT1-42-1 AdSGT1-33-1

Figure 7.22 *Phaeoisariopsis personata* whole plant bioassay performed on T_2-transgenic plants under green house conditions. 10^5 conidia/ml were sprayed on plants and symptoms were developed after 15 days post inoculation and photograph were taken after 25 dpi. Note the development of late leaf spots on transgenic plants resisted in comparison to control untransformed plant.

Spot Number Analysis

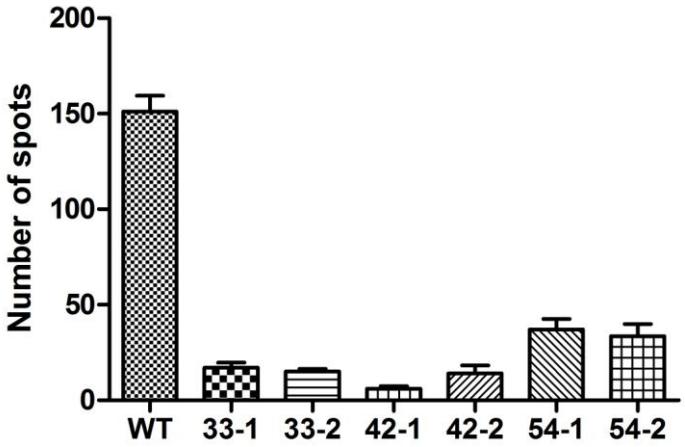


Figure 7.23 Bar diagram represent number of lesions present in control and transgenic plants after conidial spray on the detached leaf assays. A significant decrease in the number of lesions on transgenic plants were detected compared to wild type.

Cell death assay

Cell death was quantified by using Evans blue dye in infected leaves of *AdSGT1* transgenic plants and untransformed control plant after 21 days post infection. We observed elevated cell death in wild type compared to transgenics.

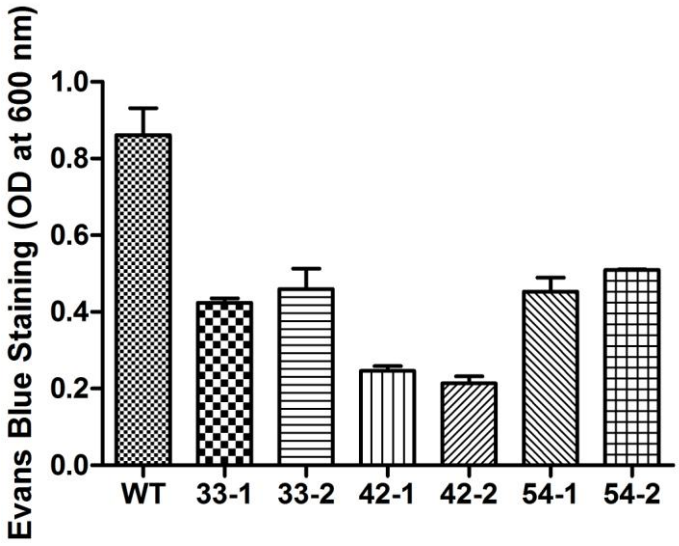


Figure 7.24 Cell death was quantified in wild type and transgenic groundnut plants using Evans blue with spectrophotometry. Three independent experiment mean value ± SD data was plotted.

7.2.13 SGT1 role in R-gene accumulation

Semi-quantitative RT-PCR analysis was performed on the transgenic *AdSGT1* groundnut plants and wild type plant to examine its role in R- gene regulation. As *AdSGT1* transgenic plants displayed enhanced resistance against *P. personata*, transcript levels of various defense related genes were analyzed in transgenic plants using semi-quantitative RT-PCR. *AdSGT1* transgenic plants exhibited higher transcript levels of R-gene CC-NB-LRR, LRR-RLK, Serine-threonine protein kinase, Protein kinase-6 and chaperone protein HSP70. However, the transcript level for defensin was similar in both WT and transgenic plant.

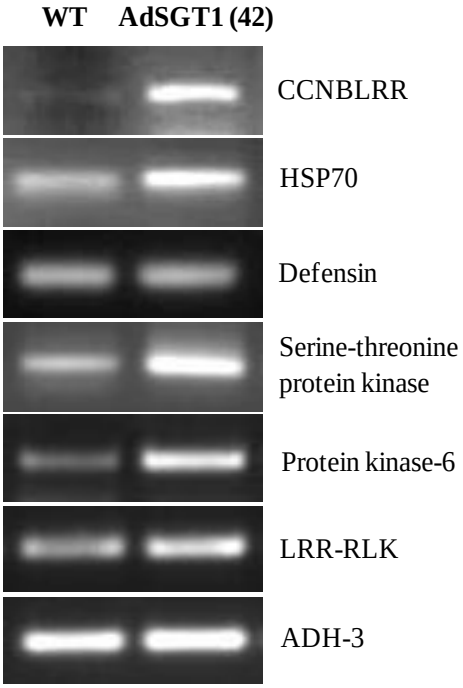


Figure 7.25 Transcript profiles of defense response genes in WT and groundnut transgenic plant (42). Semi-quantitative RT-PCR was performed using total RNA of WT and transgenic plant. The genes were found differentially expressed in cDNA-AFLP analysis except defensin, CC-NB-LRR: Coiled coil nucleotide binding leucine rich repeat, HSP70: Heat shock protein 70, LRR-RLK: Leucine rich repeat receptor like kinase. ADH-3 was used as an internal control.

7.2.14 Identification of differentially up-regulated proteins in *AdSGT1* transgenic groundnut plants

Proteomic approach was used to analyze the changes in protein profile between wild and transgenic groundnut plants to elucidate the role of SGT1 in R- gene accumulation. We observed a total of nearly 200-250 protein spots on wild type and transgenic (*Arachis*

hypogaea cv. JL-24) plants. **S**tudy indicated differential expression of proteins in transgenic plants and several proteins spots were found to be upregulated in comparison to wild type. Eighteen major protein spots showing more than 1.5 fold up-regulation were subjected to MALDI-TOF-TOF analysis and among them, we could successfully identify 10 proteins, while the rest eight proteins showed no significant hits in the database. Protein identification of the 10 up-regulated spots was performed based on their mass signals using an algorithm based homology search with protein databases such as NCBInr and SwissProt through MASCOT search engine.

We found that proteins involved in energy metabolism, such as Phosphoglycerate kinase and Ferredoxin-NADP reductase were up-regulated along with other metabolism related protein like Alanine aminotransferase. Moreover, most of the identified leaf proteins were found to be photosynthesis related. Three up-regulated spots showed similarity to Rubisco activase and one was similar to Chlorophyll a/b binding protein. One of the up-regulated protein spot was identified as cytosolic ascorbate peroxidase, which has been shown to exhibit anti-oxdative properties and protect cellular components such as mitochondria and chloroplast against oxidative stress. Identified proteins showed involvement in defense and stress tolerance, energy metabolism, photosynthesis and protein synthesis under stress conditions. The details of the ten identified proteins with their matched reference organism, observed as well as theoretical molecular weight (Mr) and isoelectric point (pI), the peptide sequences matched, peptide masses, MS/MS scores, sequence coverage (%) and related function are given in Table-7.1.

2-DE Gel Expression Profile - Wild type and *AdSGT1* transgenic groundnut plant

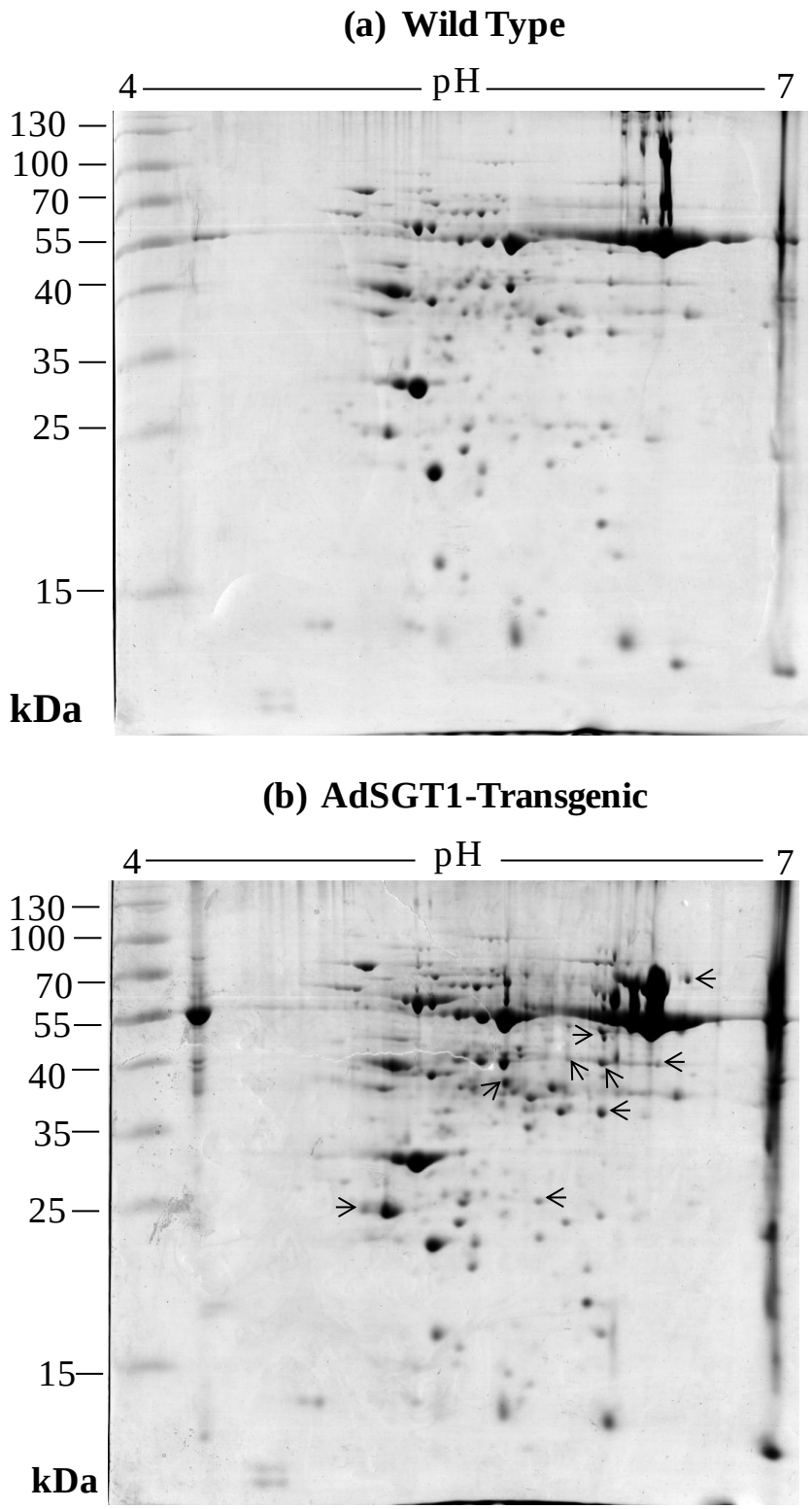


Figure 7.26 Coomassie-stained 2D gel analyses of groundnut leaf protein samples from, (**a**) Wild type and (**b**) *AdSGT1* transgenic groundnut plants. 800 µg of leaf protein was loaded on 18 cm IPG strip with a linear gradient of pH 4-7, 12% SDS-PAGE gels were used for second dimension.

Table 7.1 Identification of differentially upregulated proteins in *AdSGT1* transgenic groundnut plants by MALDI-TOF-TOF

Spot No.	Protein identified	Accession no.	Peptide sequence matched	Theoretical Mr/pI	Observed Mr/pI	S. C. (%)	MS/ MS score	Related function
3.	Chlorophyll a-b binding protein 2, (*A. thaliana*)	CB1A_ARATH	FGEAVWFK	28.2/5.29	28/5.15	2	30	Photosynthesis (LHC)
6.	Ferredoxin-NADP reductase, isozyme, (*Nicotiana tabacum*)	FENR1_TOBAC	ITGDDAPGETWHMVFSTEG-EVPYR DPNATVIMLATGTGIAPFR	40.7/8.37	38/6.2	11	51	Regulating cyclic and non-cyclic electron flow i.e. energy metabolism
9.	Ribulose-bisphosphate carboxylase activase (*Nicotiana tabacum*)	gi|100380	VPIIVTGNDFSTLYAPLIR IVDTFPGQSIDFFGALR LLEYGNMLVQEQENVKR	26/5.01	40/5.8	22	195	Photosynthesis
10.	Phosphoglycerate kinase, cytosolic (*Populus trichocarpa*)	gi|224109060	FSLAPLVPR LVASLPDGGVLLLENVR	50.2/8.25	51/6.1	5	72	Catalyzes high-energy phos-phate group of 1,3-BPG to ADP, forming ATP and 3-phosphoglycerate
13.	Phosphoglycerate kinase, cytosolic (*T. aestivum*)	gi|129916	LASVADLYVNDAFGTAHR KLASVADLYVNDAFGTAHR	42.1/5.64	42/6.4	4	118	Energy Metabolism
14.	RUBISCO (*Jasione laevis*)	gi|194400642	VTPEPGVPPEEAGAAVAAES STGTWTTVWTDGLTSLDR	49.2/6.15	82/6.6	8	93	Photosynthesis
15.	RUBISCO activase, (*C. sativus*)	gi|266893	VPIIVTGNDFSTLYAPLIR LVDTFPGQSIDFFGALR	45.9/7.57	40/5.9	8	224	Photosynthesis
16.	Cytosolic ascorbate peroxidase (*V. Unguiculata*)	gi|1420938	YAADEDAFFADYAAAHQR SGFEGAWTNNPLIFDNSYFK	27/5.64	27/5.9	7	72	Cellular antioxidant
17.	Phosphoglycerate kinase, chloroplastic (*Chlamydomonas reinhardtii*)	gi|1172455	KLAANADLYVNDAFGTAHR LAANADLYVNDAFGTAHR	49.2/8.84	49/6.2	8	188	Energy Metabolism
18.	Alanine aminotransferase 2 (*Glycine max*)	gi|351724369	IIFTNVGNPHALGQKPLSFPR MVIINPGNPTGQCLSEANLR NVVCNFTEGAMYSFPQIR	53.8/5.42	54/6.3	12	157	Metabolism

7.2.15 Subcellular localization of *AdSGT1*

Localization of *AdSGT1* was analyzed by constructing *AdSGT1* protein N-terminally tagged with GFP and transiently expressed in tobacco leaves using agroinfiltration. Previous studies showed that *NbSGT1* localized simultaneously in nucleus and cytoplasm depending upon phosphorylation by kinases to modulate the R-protein for defense response to pathogen infection (Hoser et al. 2013). *Arabidopsis* SGT1 and HSC70 (heat shock cognate 70) proteins together colocalized in the cytosol as well as in nucleus (Noel et al. 2007). We have found that *AdSGT1* was predominantly localized in the nucleus in the localization studies.

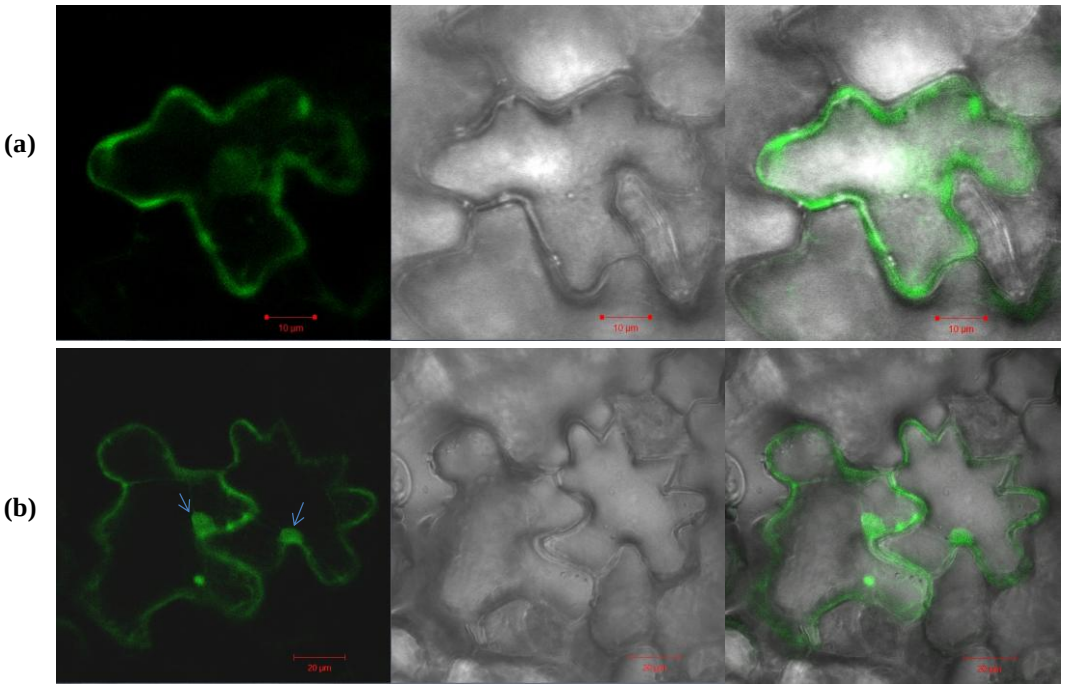


Figure 7.27 Subcellular localization of *AdSGT1*. GFP was visualized using confocal images of representative *Nicotiana benthamiana* leaf epidermal cells transiently expressed through agroinfiltration, (a) Control vector pCAMBIA1302, (b) pCAMBIA1302:*AdSGT1*, arrowheads mark the nucleus and indicate predominantly localized in nucleus.

7.3 Discussion

We identified SGT1 as one of the differentially expressed genes in cDNA-AFLP analysis during plant pathogen interaction and quantitative real time analysis confirmed its transcript accumulation at early stage upon challenge with biotrophic pathogen *P. personata* in resistant wild type groundnut plant, while no such up-regulation was detected in susceptible groundnut plants indicating its role in defense responses against pathogen. This has prompted us to investigate its role in resistance against the pathogen. SGT1 protein is conserved throughout eukaryotes, and is shown to play important role in immune response triggered by pathogen elicitors in plants and humans as well (Mayor et al. 2007, Shirasu 2009). Immune system in plants have different strategies to combat against different types of pathogens (Jones and Dangl 2006). One of the important strategy that confers broad–spectrum disease resistance in plants is hypersensitive response and it is a form of localized programmed cell death, which prevents the pathogen growth at the infection site (Greenberg 1997). Peart et al. (2002) have shown that SGT1 mediated cell death occur by Avr- R protein interactions and several effectors are involved in inducing cell death. Wang et al. (2010b) showed that *NbSGT1* overexpression and accelerated cell death induced by nonhost and host pathogens during ETI and PTI, which was not observed in absence of pathogen suggesting that tobacco SGT1 is a component of signaling cascade and positively regulates the process of cell death during both compatibale and incompatble interactions. However, El Oirdi and Bouarab (2007) reported that silencing tobacco SGT1 results the hypersensitive response induced by necrotrophic pathogen, *Botrytis cinerea*.

Hence, *AdSGT1* might be playing an important role in plant defense mechanisms in groundnut as its expression upregulated during plant-pathogen interaction. We have expressed *AdSGT1* in *N. tabacum* and *N. benthamiana* to study its hypersensitive response as agroinfiltration method could not be established in the wild groundnut *Arachis diogoi* because of its leathing leaf architecture. As reported by Kumar and Kirti (2010, 2012), we have expressed conditionaly, under transient and constitutive promoters, *AdSGT1* in tobacco and observed a typical phenotype of hypersensitive response like cell death after 48-72 h post chemical treatment. *AdSGT1*expression was found to be high at 24 h post estradiol application in comparison to control. Cell death was quantified by using Evans blue dye and the cell death was found to be high in the transiently expressed *AdSGT1* tobacco infiltrated region in relation to control. To date,

there were no previous reports of chemical induced cell death of SGT1 in plants. SGT1 might be involved in altering the expression of HR-like cell death associated genes. These finding indicate that *AdSGT1* positively regulates defense responses and its induced expression is sufficient for HR- like cell death, which is in agreement with Wang et al. (2010b), who observed a similar phenomenon.

Induction of plant signaling molecules upon pathogen and herbivore attack established the mechanism of two pathways in plants involving methyl jasmonate and salicylic acid (Kessler and Baldwin 2001, Thomma et al. 2001). Salicylic acid mediated defenses are used to play major role in regulating resistance against biotrophic pathogens, while methyl jasmonate mediated defenses control resistance against necrotrophic pathogens (Glazebrook, 2005). Moreover, methyl jasmonate is activated by herbivores, which signals the production of a number of resistance associated molecules, while microbial infection induced the biochemical pathway to produce salicylic acid, which is an important signaling molecule to induce systemic acquired resistance leading to the production of pathogenesis- related (PR) proteins and other metabolites that contribute to resistance (Durrant and Dong 2004). We found that *AdSGT1* transcript was highly up-regulated at 3, 6 and 12 h in response to salicylic acid and was peaking at 6 h of treatment, which indicates that the gene is involved in resistance mechanisms against the pathogen. Tobacco SGT1 has been reported to be up-regulated by the biotrophic pathogen, *Botrytis cinerea* through salicylic acid pathway and it has been hypothesized that the pathogen attack promotes its expression to exploit the antagonistic effect between SA and JA (El Oirdi and Bouarab 2007, Meldau et al. 2011). Methyl jasmonate, an important plant hormone involved in signal transduction pathways which mediate several defense related genes such as proteinase inhibitor, thionins and secondary metabolites (Creelman and Mullet 1997). In response to methyl jasmonate, *AdSGT1* up-regulation was found at 6, 12 and 24 hrs of treatment. In addition, Meldau et al. (2011) showed that tobacco SGT1 is a key regulatory element of plant that deploys defense responses against herbivores and is required for herbivory induced SA and JA homeostasis. Abscisic acid is a major signaling hormone and related to many abiotic stress responses (Wasilewska et al. 2008) and we have not found altered expression of *AdSGT1* in treated samples. We have also not found up regulation in *AdSGT1* transcript of ethephon treatments. This clearly indicates that *AdSGT1* might be not regulated by ABA and Ethylene signaling molecules. These observations also indicate that expression

of *AdSGT1* upon SA and JA treatment may enhance resistance in host plant through systematic acquired resistance, elicitor-triggered immunity and herbivory.

There are limited reports on heterologous expression of plant SGT1 in tobacco for disease resistance (Wang et al. 2010b) and here we report the enhanced disease resistance in tobacco transgenics expressing *AdSGT1* against several phytopathogenic fungi in addition to chemical induced hypersensitive like cell death. *AdSGT1* transgenic tobacco plants of T_2 generation exhibited enhanced resistance against black shank disease causing pathogen *Phytophthora parasitica* pv. *nicotianae.* These plants were also challenged with *Alternaria alternata* pv. *nicotianae,* a causal agent of brown or leaf spot disease and the transgenic plants were found to be resistant to this pathogen too. Whole seedling assay of T_2 generation of transgenic plants was carried out using the soil born fungus *Rhizoctonia solani* that causes root rot disease, and transgenic plant progenies exhibited enhanced level of resistance. Hence transgenic plants expressing the cDNA of *AdSGT1* in tobacco showed enhanced resistance against the pathogen *Phytophthora parasitica* pv. *nicotianae, Alternaria alternata* pv. *nicotianae* and *Rhizoctonia solani.* These results indicate that *AdSGT1* might be involved in altering the expression of genes involved in disease resistance along with HR-like cell death associated genes, which needs to be further investigated with respect to the mode of action against fungal pathogens.

After demonstrating the enhanced defense response of *AdSGT1* in tobacco against several phytopathogenic fungal species, we have overexpressed it in the nearly homologous system, the groundnut for combating the late leaf spot disease. Stable integration of transgene was confirmed by Southern blot analysis and relative expression of transgenic plants in relation to untransformed plant was analysed by quantitative real time polymerase chain reaction (Wang et al. 2010b). Several attempts have been made to overcome this disease through transgenic approach using defense related genes. Anuradha et al. (2008) reported enhanced disease resistance against tikka disease in transgenic groundnut expressing Brassica defensin. Vasavirama and Kirti (2012) have shown that overexpression of *SniOLP and Rs-AFP2* genes in groundnut elevated disease resistance to late leaf spot pathogen, while Rohini and Rao (2001) reported expression of tobaco chitinase gene in groundnut enhanced disease resistance to early leaf spot disease caused by *Cercospora arachidicola.* Similarly, Sundaresha et al. (2010) overexpressed

toabcco β-1,3-glucanase in groundnut for resistance against two major fungal pathogens of groundnut, *Cercospora arachidicola* and *Aspergillus flavus*. Several earlier studies showed that SGT1 regulates R-gene signaling, R-protein accumulation, mediates defense response in plants and also regulates the process of cell death in both compatible and incompatible interaction (Muskett and Parker 2003, Azevedo et al. 2006, Wang et al. 2010b). We have assayed *AdSGT1* overexpressing transgenic groundnut plants against conidial suspension of *Phaeoisariopsis personata* and found enhanced level of resistance in T_2 transgenic plants compared to wild type in both detached as well as whole plant assay. A significant decrease in number of lesions was found on transgenic plants in comparison to untransformed control plants, which was in line with the earlier published reports in the same plant by using defense related genes (Rohini and Sankara Rao 2001, Anuradha et al. 2008, Sundaresha et al. 2010, Vasavirama and Kirti 2012). Cell death was quantified by using Evans blue dye and the cell death was found to be high in wild type infected plant in comparison to *AdSGT1* expressing transgenic groundnut plants, which further indicate that late leaf spot were found more on wild type plants.

SGT1 has been shown to have functions in R-gene regulation, protein accumulation and mediate resistance responses in plants (Muskett and Parker 2003, Azevedo et al. 2006, Wang et al. 2010b). We analysed some defence related genes in *AdSGT1* transgenic groundnut plants which were found to be differentially expressed in cDNA-AFLP analysis during plant-pathogen interaction. We have found transcript accumulation of CC-NB-LRR, LRR-RLK, serine-threonine protein kinase and protein kinase-6, which belong to different classes of R-genes (Tor et al. 2003). Plants respond to pathogen attack by expressing disease resistance R-gene products for recognising and countering pathogen-derived molecules (Dangl and Jones, 2001) and these are usually recognised through nucleotide-binding domain and leucine rich repeat containing (NLR) protein (Bieri etl al. 2004, Leister et al. 2005). We have not found changes in transcript levels of defensin, while chaperone protein HSP70 was upregulated in transgenic plant in comparison to the wild type, which suggests that *AdSGT1* might be involved in interaction with HSP70 playing an important role in folding and stability of the protein complexes. Noel et al. (2007) reported that SGT1 can interact with HSP70 through the SGS domain, which is supporting our analysis. Hence, transcript accumulation of these R-proteins and chaperone proteins in transgenic groundnut plants expressing *AdSGT1*

indicates it role in R-gene mediated response and might be one of the important contributors in enhancing disease resistance against the late leaf spot disease.

Proteomic study was also taken in to account to analyze the changes in protein profiles between wild type and transgenic groundnut plants to reveal the role of SGT1 in R-protein accumulation. We have found several photosynthesis related proteins like Rubisco activase, Chlorophyll a/b binding protein and Ferredoxin-NADP reductase up-regulated in the transgenic plant. Rubisco is the abundant enzyme of plant cell and plays important role in photosynthetic carbon fixation. Wu et al. (2013) identified its up-regulation during plant-virus interaction in resistant and susceptible ecotypes of maize infected with sugarcane mosaic virus. Chlorophyll a/b binding protein provides excitation energy between photosystem I and II, which balances the photosystem (Kundu et al. 2011). Chlorophyll a/b binding protein upregulation was also reported in *Brassica juncea* and maize during plant-pathogen interaction (Kaur et al. 2011, Wu et al. 2013). Ferredoxin-NADP reductase transfers electrons between the electron carriers ferredoxin and NADP(H) in the photosynthetic electron transport system and up-regulated upon biotic stress (Wu et al. 2013). Phosphoglycerate kinase catalyzes the reaction of 1,3-Biphosphoglycerate and ADP to produce 3-Phosphoglycerate and ATP. This method for ATP production is known as substrate-level phosphorylation because it produces energy storing ATP molecules without the use of oxygen, NADH, or an ATPase. We have found up-regulation of three spots corresponding to phosphoglycerate kinase. Kaur et al. (2011) also observed the up-regulation of phosphoglycerate kinase in proteome analysis of *Albugo candida- Brassica juncea* pathosystem. Cytosolic ascorbate peroxidase, which possesses the anti-oxdative properties and protects cellular components such as mitochondria and chloroplast against oxidative stress was also found to be up-accumulated. Similarly, Wu et al. (2013) observed its up-regulation during plant-virus interaction in maize with sugarcane mosaic virus. Identified proteins were involved in defense and stress tolerance, energy metabolism, photosynthesis and protein synthesis under stress conditions. However, we were unable to get differential expression of any R-gene related protein as expected, as SGT1 is reported to be an R-gene regulator. It is likely that we should proceed with the analyses of more number of spots. We have observed *AdSGT1* predominantly localized in nucleus while previous report showed that it localized simultaneously in nucleus and cytoplasm depending upon phophorylation and interaction (Noel et al. 2007, Hoser et al. 2013).

7.4 Summary

SGT1 (suppressor of G2 allele of SKP1) was differentially up-regulated in cDNA-AFLP analysis upon plant-interaction between *Arachis diogoi* and *P. personata*. We have made full length using 3'/5' RACE and its ORF was 1077 bp encoding a polypetide of 358 amino acids. We cloned *AdSGT1* under an estradiol inducible promoter (XVE) and transiently expressed in tobacco leaves using agroinfiltration and expressed conditionaly, under transient and constitutive promoters, *AdSGT1* in tobacco and observed a typical phenotype of hypersensitive response like cell death after 48-72 h post chemical induction. *AdSGT1* expression was found to be high at 24 h post estradiol application in comparison to control. This shows that *AdSGT1* positively regulates defense responses and their induced expression is sufficient for inducing HR- like cell death. We found that *AdSGT1* expression was highly up-regulated in response to salicylic acid, which indicates that the gene is involved in resistance mechanisms against plant pathogens while in response to methyl jasmonate, induced expression was detected. Abscisic acid is a major signaling hormone in abiotic stress signaling pathway and we have observed altered expression of *AdSGT1* under abscisic acid and ethephon treatment. Transgenic tobacco plants ectopically expressing *AdSGT1* exhibited enhanced resistance against *Phytophthora parasitica* var. *nicotianae*, *Alternaria alternata* pv. *nicotianae* and *Rhizoctonia solani.*

We have generated several transgenic groundnut plants harboring *AdSGT1* gene in *Arachis hypogaea* cv. JL-24 by employing *in planta* method of transformation and transgene in groundnut transgenic was confirmed by Southern blot. Relative expression of *AdSGT1* in transgenic groundnut plants was verified by quantitative real time polymerase chain reaction. Transgenic groundnut plants harboring *AdSGT1* showed enhanced resistance against the fungal pathogen *Phaeoisariopsis personata* in both detached leaf assay as well as whole plant assay. *AdSGT1* transgenic plants exhibited higher transcript levels of *R*-gene CC-NB-LRR, LRR-RLK, Serine-threonine protein kinase, Protein kinase-6 as well as chaperone protein HSP70. We have identified proteins in transgenic groundnut plants that were shown to be involved in defense and stress tolerance, energy metabolism, photosynthesis and protein synthesis under stress conditions. We have found *AdSGT1* was predominantly localized in the nucleus.

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Literature cited

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