Biochemical and molecular analyses of apoplast in tomato during non-host resistance

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

by

Battepati Uma

(Regd No. 07LPPH11)

Department of Plant Sciences

University of Hyderabad

Hyderabad-500046

India

March, 2013

University of Hyderabad

(A Central University established in 1974 by an act of parliament)

HYDERABAD-500 046, INDIA

DECLARATION

I, Battepati Uma, hereby declare that this thesis entitled **"Biochemical and molecular analyses of apoplast in tomato during non-host resistance"** submitted by me under the guidance and supervision of **Prof. Appa Rao Podile** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Battepati Uma

(07LPPH11)

Prof. Appa Rao Podile

(Research Supervisor)

University of Hyderabad

(A Central University established in 1974 by an act of parliament)

HYDERABAD-500 046, INDIA

CERTIFICATE

This is to certify that this thesis entitled **"Biochemical and molecular analyses of apoplast in tomato during non-host resistance"** is a record of bonafied work done by **Battepati Uma** a research scholar for Ph.D. programme in Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

Prof. Appa Rao Podile

(Research Supervisior)

Head,

Department of Plant Sciences

Dean,

School of Life Sciences

CONTENTS

Acknowledgement

This report would not have been possible without the essential and gracious support of many individuals.

I take immense pleasure in thanking Prof. Appa Rao Podile, MPMI Group Leader, who had been a source of inspiration and role model in any given way, for his constant moral and personal support and guidance throughout my doctoral research. The leadership and critical assessment capabilities of Prof. Podile, allowed me to mobilize my collective knowledge and capacity in the best way possible and created an enabling environment to complete my research work.

I thank the present and former Deans, School of Life Sciences, Prof. M Ramanadham and Prof. A. S. Raghavendra, the present and former Heads, Department of Plant Sciences Prof. A. R. Reddy and Prof. P. B. Kirti for allowing me to use the facilities of the School and the Department.

I sincerely acknowledge the infrastructural support provided by UGC-SAP, DST-FIST, DBT-CREBB, to the Dept. of Plant Sciences and DBT-CREBB for the research fellowship.

This thesis is not only the result of my scientific works but the outcome of a long road and I am glad to acknowledge everyone who has contributed to the build-up of my scientific personality and therefore, to the completion of this thesis.

My heartfelt thanks are also due to my former colleagues of MPMI group Dr. Anil Kondreddy, Dr. B. Sashidhar, Dr. V. L. Vasudev, Dr. Purushotham, and Dr. Swarnalee Dutta, for their timely help and constant encouragement.

I would like to mention my colleagues Sippy, Swaroopa, Sharma, Das, Manjeet, Madhu, Rambabu, Papa Rao, Durga, Sandhya, Sadaf, Suvarna, and Narayan Rao who have made the last few years so cheerful

I would like to thank all my dearest friends Suma, Neeraja, Sireesha, Vinod, Deb, and Sravani who made my stay always cheerful in HCU.

Words are inadequate in offering my thanks Dr. K. Satya Prasad and Mrs. Sailaja for their love and affection which made Hyderabad as my second home town.

This dissertation work at MPMI group could never run smoothly without the help I got from Narasimha, Malla Reddy, Devaiah and Seetaram for their assistance in the lab and in the green house.

I am thankful to all my batch mates and other research scholars of the School of Life Sciences for their timely help.

I highly acknowledge the efforts of Ramesh, Mohan Rao and Sudarshanam and other non-teaching staff of Dept. of Plant Sciences.

Saving the most important for the last, I would like to express my heartfelt thanks to my beloved parents Mr. Maheswar Rao and Mrs. Masthanamma and my lovely little sister Deepthi for their blessings, wishes, support and constant encouragement throughout my research career.

Battepati Uma

ABBREVIATIONS

2-DE	: 2-dimensional electrophoresis
2, 4-DNPH	: 2, 4-dinitrophenylhydrazine
4CL	: 4-coumarate-CoA ligase
µg	: microgram
µM	: micromolar
°C	: degree centigrade/degree Celsius
$O2^{\cdot-}$	: superoxide radicals
•OH	: hydroxyl radicals
AA	: ascorbic acid
AADC	: aromatic amino acid decarboxylase
Abs	: absorption
ACC	: 1-aminocyclopropane-1-carboxylate
ACN	: acetonitrile
AF	: apoplastic fluid
AO	: ascorbic oxidase
AOS	: allene oxide synthase
AcNH4	: ammonium acetate
APX	: ascorbate peroxidase
ATP	: adenosine tri phosphate
BSA	: bovine serum albumin
Bgt	: *Blumeria graminis* f. sp*. tritici*
Bgh	: *Blumeria graminis* pv*. hordei*
BPB	: bromophenol blue
Cw-Inv	: cell wall invertase
CAT	: catalase
CDPK	: Ca^{2+}-dependent protein kinases
cDNA	: complementary DNA
CHAPS	: 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-opanesulfonate
CHCA	: α-cyano-4-hydroxycinnamic acid
DEPC	: diethylpyrocarbonate
DOR	: deoxyribose
DMSO	: dimethyl sulfoxide
DNA	: deoxy ribonucleic acid
DNS	: 3,5-dinitrosalicylic acid
dNTPs	: deoxy nucleotide triphosphates
DTT	: dithiothreitol
CT	: coumaroyl tyramine
EDTA	: ethylene diamine tetra acetic acid
ET	: ethylene
FT	: feruloyl tyramine

g	: gram
GST	: glutathione S-transferase
GR	: glutathione reductase
h	: hour(s)
HSP	: heat shock protein
HR	: hypersensitive response
HXT	: hexose transporter
hpi	: hours post inoculation
H_2O_2	: hydrogen peroxide
IAA	: Indole-3-acetic acid
IBP	: ionically bound protein
IEF	: isoelectric focusing
IFR	: isoflavone reductase
JA	: jasmonic acid
kDa	: kilodalton
KCN	: potassium cyanide
L	: litre
LOX	: lipoxygenase
M	: molar
MALDI-TOF	: matrix-assisted laser desorption/ionization-time of light
MAPK	: mitogen-activated protein kinase
MDH	: malate dehydrogenase
mg	: milligram
min	: minute(s)
ml	: milliliter
mM	: millimolar
nm	: nanometers
MS	: mass spectrometry
NHR	: non-host resistance
NH_4HCO_3	: ammonium bicarbonate
NBT	: nitro-blue tetrazolium
NH_4HCO_3	: ammonium bicarbonate
OEE	: oxygen evolving enhancer
PAGE	: polyacrylamide gel electrophoresis
PAL	: phenylalanine ammonia-lyase
PCR	: polymerase chain reaction
PAMP/MAMP	: pathogen/microbe-associated molecular patterns
PDA	: potato dextrose agar
PPO	: polyphenol oxidase
PR proteins	: pathogenesis-related proteins
PRX	: extracellular peroxidase
rbohF	: respiratory burst oxidase
RNA	: ribonucleic acid
RNase	: ribonuclease

ROS	: reactive oxygen species
SAR	: systemic acquired resistance
SDS	: sodium dodecyl sulphate
SOD	: superoxide dismutase
Sec	: seconds
SA	: salicylic acid
SS	: soluble sugars
SUT	: sucrose transporter
TBARS	: thiobarbituric acid reactive substances
TEMED	: tetramethylethylenediamine
TFA	: trifluoroacetic acid
Tris	: tris-(Hydroxymethyl) aminoethane
TSP	: total soluble protein
Xag	: *Xanthomonas axonopodis* pv. *glycines*

List of figures

List of tables:

Introduction

1.1. Outcome of plant-pathogen interactions

Plants cannot move to escape environmental challenges. Biotic stresses result from a battery of potential pathogens like fungi, bacteria, nematodes and insects that intercept the photosynthate produced by plants. Although lacking an immune system comparable to animals, plants have developed a wide variety of constitutive barriers and inducible reactions. Preformed physical or chemical barriers constitutively present on the plant surface (wax layers, rigid cell walls, and antimicrobial secondary metabolites) may initially prevent establishment of infection structures. Plants do have induced cellular defenses that prevent further colonization of the tissue, once the structural barriers of the host have been breached. Inducible defense responses comprise of the synthesis and accumulation of antimicrobial reactive oxygen species (ROS), phytoalexins, and induction of *pathogenesis-related* (*PR*) genes as well as the localized reinforcement of the plant cell wall and hypersensitive cell death. These induced defenses are described as active defense mechanisms because they are a part of response to an invading pathogen and require host metabolism to function.

Disease occurs when a potentially parasitic microorganism circumvents the passive defenses of the plant and suppresses active defense responses. The host range refers to the plant species on which a pathogen is capable of causing disease. A plant species that does not succumb to disease, when infected by a pathogen, is referred to as a non-host plant for that pathogen, and the interactions as non-host interactions. When a pathogen is capable of causing disease on a particular host species, two outcomes are possible: a compatible interaction that results in disease, and an incompatible interaction that results in resistance (no disease).

1.2. Insights into non-host resistance in plants

The native resistance of most plant species against a wide variety of pathogens is known as non-host resistance (NHR), which confers durable protection to plant species (Uma et al 2011). NHR is polygenic and appears to be linked with basal resistance, a form of elicited protection. NHR is conditioned by at least two layers of defense response (Thordal-Christensen 2003; Lipka et al 2005). The layers that account for NHR can be broadly categorized

as pre- and post-penetration defenses, based on their engagement and efficacy at different stages of the infection sequence (Fig 1.1).

Many fungi have evolved specialized infection structures known as appressoria to facilitate the breach of the passive barriers like cuticle and cell wall through mechanical and/or enzymatic means. When a pathogen attempts to penetrate the cell wall, the first line of inducible plant defense is called into action (Fig 1.2A). Non-adapted fungi, that overcome penetration resistance and successfully develop haustoria, are subjected to a second layer of defense called post-penetration resistance. Post-penetration resistance is characterized by the hypersensitive response (HR)-like programmed cell death of invaded host cells (Fig 1.2B). Little progress has been made to identify key components of NHR, in some of the known plant cellular components. Disruption of any of these components leads to loss of NHR against certain pathogens.

1.2.1. Pathogen recognition and PAMP-triggered immunity

Plants have developed multiple layers of sophisticated surveillance mechanisms that recognize potentially dangerous pathogens. Basal resistance, also called innate immunity, is the first line of pre-formed and inducible defenses that protect plants against entire group of pathogens. Basal resistance can be triggered when plant cells recognize pathogen or microbe-associated molecular patterns (PAMPs/MAMPs) including specific proteins, lipopolysaccharides, and cell wall components commonly found in microbes. Pathogen recognition in non-host plants is brought about by PAMPs, also known as general or exogenous elicitors (Gomez-Gomez and Boller 2002; Montesano et al 2003). Recognition of PAMPs by cognate PRRs induces multiple defense responses referred to as PAMP-triggered immunity. Non-pathogens as well as pathogens are capable of triggering basal resistance in plants due to the widespread presence of these molecular components in their cells. A successful pathogen defeats this first line of defense with effectors that enhance their virulence.

Pathogens have developed counter measures that are able to suppress basal resistance in certain plant species. If a pathogen is capable of suppressing basal defense, plants may respond

with another line of defense called as HR. The HR is characterized by deliberate plant cell suicide at the site of infection. Although drastic, compared to basal resistance, the HR may limit pathogen access to water and nutrients by sacrificing a few cells to save the rest of the plant. The HR is typically more pathogen-specific than basal resistance and is often triggered when gene products in the plant cell recognize the presence of specific disease-causing effector molecules introduced into the host by the pathogen. Once the HR is triggered, plant tissues may become highly resistant to a broad range of pathogens for an extended period of time at places far away from the site of HR. This phenomenon is called as systemic acquired resistance (SAR).

1.2.2. Signal transduction during defense response

PAMP perception triggers activation of signaling cascade and transcriptional changes. Early events in the cascade involve ion fluxes, protein phosphorylation, and enhanced cytosolic Ca^{2+} concentrations, Ca^{2+}-dependent protein kinases, mitogen-activated protein kinase (MAPK), and ROS accumulation (Huckelhoven 2007). Ca^{2+} influx into the cytoplasm results in the decrease of apoplastic Ca^{2+} concentration which might influence cell wall rigidity as calcium plays a role in non-covalent cross-linking. This Ca^{2+} influx is essential for alkalization of apoplast and initial ROS accumulation (Blume et al 2000; Grant et al 2000). MAPKs are important in cross-talk during stress signaling that results in protection against microbial invasion (Nurnberger and Scheel 2001; Zhang and Klessig 2001; Gomez-Gomez and Boller 2002). In *Nicotiana benthamiana*, virus-induced gene silencing of NbSIPK and NbWIPK (orthologues of AtMPK6 and AtMPK3, respectively) allows multiplication of non-host bacterium *Pseudomonas cichorii* revealing the importance of MAPK in NHR (Sharma et al 2003). High-throughput over expression and NbMKK1-silencing in *N. benthamiana* has further confirmed the role of MAPKs in controlling NHR (Takahashi et al 2007).

Plant hormones like salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) appear to be involved in the later signaling events of induced immune responses. The genetic evidence about the role played by hormones in non-host interactions came from the mutant analysis of

Arabidopsis, defective in SA (sid2), JA (jar1) and ET-mediated defense signaling pathways (Knoester et al 1998; Zimmerli et al 2004). Sunflower rust fungus (*Puccinia helianthi*) failed to form haustoria on any of the *Arabidopsis* mutants lacking the production of SA and JA/ET. This was attributed to strong expression of wall-associated defense responses (Mellersh and Heath 2001). Mysore and Ryu (2004) showed the crucial importance of JA, ET and SA not only in cultivar specific resistance but also in the maintenance of NHR in specific plant-non-host pathogen interactions. The differential response of *Arabidopsis* mutants to different pathogens in NHR suggests the need to further dissect the role of hormones in this form of resistance.

1.2.3. Pre-penetration resistance

Molecular genetic analyses of *Arabidopsis*, showing altered pre-haustorial responses, led to the identification of genes encoding secretion-associated and efflux-associated proteins like PM-resident PEN1 syntaxin, PEN2 myrosinase, and PM-resident PEN3 ABC transporter (Collins et al 2003; Lipka et al 2005; Stein et al 2006) (Fig 1.2A).

All three *PEN* genes are involved in secretion and accumulation of anti-microbial components to the papillae. In pen1 mutants, the delay in papillae formation resulted in decreased penetration resistance but without disease development indicating that PEN1/HvROR2 is not the only component of complex NHR mechanisms (Assaad et al 2004). PEN2 initiates the metabolism of group of tryptophan-derived compounds known as indole glucosinolates (IGs), to release potential antimicrobial compounds. Glucosinolates together with their hydrolytic enzymes myrosinases are known to be involved in plant defense (Sonderby et al 2010). PEN3/PDR8 may be involved in exporting toxic materials to attempted invasion sites, and intracellular accumulation of these toxins in pen3 mutants may secondarily activate the SA pathway (Stein et al 2006). Vesicle trafficking-mediated extracellular accumulation of toxic antimicrobial compounds, though, could be contained within the papillary cell wall scaffold, and might form a fine-tuned chemical and structural barrier against microbial intruders together with chemical cross-linking of the newly synthesized cell wall polymers (Kwon et al 2008).

1.2.4. Cytoskeleton role in penetration resistance

During plant-pathogen interactions, polarized accumulation of defense compounds at the pathogen intruder site depends on cytoskeletal rearrangements and secretory processes (Schmelzer 2002; Lipka and Panstruga 2005). It is thought that the actin cytoskeleton plays a pivotal role for cell polarization process by providing tracks for organelle and vesicle traffic during compatible, incompatible and non-host interactions (Takemoto et al 2003). The formation of radial actin array in response to the attempted penetration by a pathogen is associated with extensive accumulation of endoplasmic reticulum and golgi bodies at the penetration site. Inhibition of actin polymerization using cytochalasins resulted in penetration and haustoria formation by *Erysiphe pisi* in the non-host plant barley but there was no disease development because of HR (Kobayashi et al 1997). This reveals that the penetration stage is not a critical point to determine success or failure of fungal penetration. The NHR is mostly operated by multiple mechanisms rather than a specific single mechanism.

PEN1/ROR2 syntaxins do not exert an entry-limiting function in pre-invasion resistance to the hemibiotrophic fungi like *Colletotrichum* and *Magnaporthe* species (Shimada et al 2006; Jarosch et al 2003). Papillary callose formation and pre-invasion resistance to C*olletotrichum* sp. requires actin cytoskeleton function. Cytochalasin E treatment allowed the non-adapted *Colletotrichum* to enter *Arabidopsis* epidermal cells. So, the pre-penetration resistance to non-adapted anthracnose fungi is dependent on actin cytoskeleton (Shimada et al 2006).

1.2.5. Genes involved in post-penetration resistance

During non-host pathogen interaction, some of the spores escape the pre-penetration resistance and form haustoria which are mopped up by the HR-mediated cell death. This form of resistance is dependent on several genes linked to *R*-gene-mediated resistance (Peart et al 2002; Kanzaki et al 2003). Genes like *EDS1*, *PAD4*, *SAG101*, and *NHO1* are likely to participate in various non-host responses after penetration resistance has been breached. Compromising of NHR is evident in pen2 pad4 sag101 triple and pen3eds1 double mutants, but not in pad4 sag101 double mutant (Zimmerli et al 2004; Lipka et al 2005;

Stein et al 2006). NHR is severely compromised in *Arabidopsis* against *Blumeria graminis* f. sp. *tritici* (*Bgt*) when the actin cytoskeleton functions in combination with eds1 (Yun et al 2003). The silencing of SGT1 (ubiquitin ligase-associated protein) also compromised NHR against *Pseudomonas syringae* pv. *maculicola* and *Xanthomonas axonopodis* pv. *vesicatoria* (Peart et al 2002). Silencing of heat shock proteins (Hsp70 and Hsp90) results in the reduction of NHR against non-host pathogen *P. cichorii* due to the decrease in expression and HR (Kanzaki et al 2003). Another mutational analysis of *Arabidopsis* indicated the involvement of specific gene *NHO1* (glycerol kinase) in NHR against bacteria (Kang et al 2003).

1.3. Apoplast-associated defenses during plant-pathogen interactions

Apoplast is a sub-cellular compartment which includes primary wall, middle lamella and intercellular space (Fig 1.3A). Cell wall with complex arrangement of carbohydrate polymers and proteins provides structural support to plant cell. Apoplast is one of the dynamic plant cell compartments which interacts with environment and responds to external cues like stress. The apoplast plays critical role in plant's interaction with environment in coping up against biotic and abiotic stresses (Fig 1.3B). The apoplast is a source of nutrition for pathogens with strictly apoplastic growth, or a launching pad for pathogens that attack host cells through the production of haustoria. Accumulation of ROS at apoplast restricts the growth of pathogens and also can influence other defense responses, e.g. direct antimicrobial action, lignin formation, stiffening of cell walls, phytoalexin production, HR and triggering of SAR. As a second layer of defense, HR-mediated cell death restricts the growth of pathogens due to accumulation of ROS.

1.3.1. Oxidative burst at apoplast restricts the entry and growth of pathogens

Rapid production of ROS at apoplast is one of the early events of plant-pathogen interactions. The attack of fungal pathogen at apoplast activates several defense responses like oxidative burst, signal transduction for defense gene activation and/or cell death. The production of ROS such as $O_2^{\cdot-}$, •OH and H_2O_2 at the cell surface, well known as the "oxidative burst" (Doke et al 1983) (Fig 1.4). Different enzymes contribute to the generation of apoplastic ROS. The major sources of ROS could be plasma membrane localized NADPH oxidases or cell wall-localized

peroxidase. Either NADPH oxidases or peroxidases that may exist singly or in combination in different plant species have been proposed for the generation of ROS. Apoplastic NADH-peroxidase was involved in production of $O_2^{\cdot-}$ during interactions of cotton- *X. campestris* pv. *malvacearum* (Martinez et al 1998) and in *Arabidopsis* in response to *Fusarium oxysporum* cell-wall preparation (Bindschedler et al 2006). Other than these two major mechanisms, poly (di) amine oxidase contributes to ROS production in apoplast. During the non-host interaction of tobacco-*P. cichorii*, accumulation of polyamines in apoplast and increased activity of poly (di) amine oxidase were observed (Yoda et al 2009).

Accumulation of extracellular ROS, beneath fungal germ tubes and appressoria, induces cell death and restricts pathogen growth. NHR of barley against wheat powdery mildew fungus is associated with H_2O_2 accumulation at sites of attempted penetration and under appressoria. During the non-host interaction of cereals with inappropriate formae speciales of *B. graminis*, accumulation of $O_2^{\cdot-}$ is associated with successful fungal penetration in attacked cells and a concomitant accumulation of H_2O_2 beneath fungal penetration sites (Trujillo et al 2004). Tissue necrosis caused by ROS during pathogen infection increases host susceptibility to necrotrophic but resistance to biotrophic pathogen. Consequently, elevation of antioxidant capacity of plants enhances their tolerance to development of necroses caused by necrotrophic pathogens. The Janus face nature of ROS and plant cell death on biotrophic and on necrotrophic pathogens is also supported by the experiments with BAX inhibitor-1 and the mlo mutation of *Mlo* gene in barley (Barna et al 2012).

Different plants exhibit different oxidative burst phases with an early production of ROS in both compatible and incompatible interactions (Grant et al 2000; Apel and Hirt 2004). In plant-fungal interactions, oxidative burst occurs in two or three phases, whereas biphasic accumulation of ROS occurs in plant–bacterial interaction (Baker and Orlandi 1995; Thordal-Christensen et al 1997; Huckelhoven et al 2000). Increase in extracellular peroxidase activity and H_2O_2 accumulation in lettuce- *P. syringae* pv. *phaseolicola* non-host interaction (Bestwick et al 1998) has been known for a long time. Antisense expression of a heterologous French bean (*Phaseolus vulgaris*) peroxidase (FBP1) cDNA in *Arabidopsis* was

diminishes the expression of two *Arabidopsis* peroxidases (PRX33 and PRX34), block the oxidative burst in response to a fungal elicitor, and cause enhanced susceptibility to a broad range of fungal and bacterial pathogens. Thus, it was demonstrated that the peroxidase-dependent oxidative burst plays an important role in *Arabidopsis* basal resistance mediated by the recognition of MAMPs (O'Brien et al 2012; Daudi et al 2012). Despite the similarities to host resistance, the involvement of ROS in non-host interactions may vary in terms of the intensity and timing of ROS bursts (Huckelhoven et al 2001; Able et al 2003).

1.3.2. Carbohydrates induce defense responses

Photosynthetic CO_2 fixation occurs in source leaves producing an excess of assimilates that are allocated, mostly in the form of sucrose, to sink tissues *via* the phloem. Carbohydrate partitioning between source and the different competing sink tissues is a highly dynamic process that is influenced by environmental and developmental cues. Plant pathogens like viruses, fungi, and bacteria are known to interfere with the source-sink balance (Bolton et al 2009) and in the case of a successful interaction, pathogens are believed to reprogram a plant's metabolism to their own benefit. The localized reduction in photosynthetic metabolism in conjunction with increased cellular demands during the resistance response initiates the transition from source status to sink status in infected tissue. This transition is often accompanied by an increase in cell wall (apoplastic or extracellular) invertase (cw-Inv) gene expression and activity (Roitsch et al 2003) (Fig 1.5).

Cw-Inv is an extracellular enzyme catalyzing the cleavage of the transport sugar sucrose into glucose and fructose. Cw-Inv activity increases the local hexose availability and is therefore thought to be a key enzyme for supplying sink tissues with carbohydrates. These hexoses are then transported into the cell by hexose transporters where they are believed to fulfill the energy and carbon requirements for the resistance response (Truernit et al 1996). This transport also reduces hexose concentration in the apoplast, thereby reducing potential nutrients for apoplast-colonizing pathogens (Fotopoulos et al 2003). Increase in the concentration of hexoses is believed to be a metabolic signal that induces the expression of

defense-related genes and repression of photosynthesis (Ehness et al 1997; Herbers et al 1996; Roitsch et al 2003; Scholes et al 1994). There were also differences in responses to pathogen attack regarding photosynthesis and the induction of PR gene expression, which were found to be faster and stronger in incompatible interactions (Berger et al 2007; Seo et al 2007).

A rapid accumulation of soluble sugars caused by an early induction of cw-Inv activity is thought to promote their utilization for host defense reactions, supporting the successful establishment of resistance (Scharte et al 2005; Swarbrick et al 2006; Essmann et al 2008). For instance, callose deposition and the production of phenolic compounds (including SA) are both known outputs of plant defense and require large amounts of metabolizable sugars (Herbers et al 1996; Scharte et al 2005). Thus, an early reprogramming and redirecting of carbon flow seems to support plant defense. This is in accordance with the model of "high-sugar resistance" (Horsfall and Dimond 1957), and with the observation that transgenic tobacco plants over expressing a yeast invertase in the apoplast were found to be resistant against potato virus Y infection (Herbers et al 2000). In contrast, the accumulation of sugars during later stages of infections might serve as nutrients for the invading pathogen, resulting in disease development (Seo et al 2007).

Cw-Inv repressed tomato plants hosted bacterial growth similar to the wild type during the compatible interaction with *X. campestris* pv. *vesicatoria* (Kocal et al 2008), suggesting that this bacterium utilizes nutrients besides hexoses during growth in plant. Indeed, pathogen effectors may actively suppress host cw-Inv activity during compatible interactions to prevent hexose-mediated defense signaling (Biemelt and Sonnewald 2006). Cw-Inv-repressed plants had normal development but defense-related callose deposition, PR protein activity, and the HR were delayed after infection with avirulent *Phytophthora nicotianae*, supporting the notion that cw-Inv is a component of plant defense (Essmann et al 2008).

1.4. Proteomics to dig into plant-pathogen interactions

Rapid progress was made in characterization of plant sub-cellular proteomes due to the advantage of dramatically reducing the complexity of crude cell or tissue extracts. Proteomic

approach to study apoplast proteins, compared to other sub-cellular compartments, is in its infancy, partly due to the difficulty in isolating a representative fraction of apoplast/cell wall bound proteins demonstrably free from intracellular contamination. In order to isolate more tightly bound proteins from the wall, it is often necessary to employ disruptive techniques to obtain purified walls for sequential extraction as suggested by Chivasa et al (2002). On the other hand, cell-disruptive methods could result in wall contamination through the electrostatic binding of intracellular proteins with the ECM, which is largely negatively charged (Shomer et al 2003). Several studies using disruptive techniques have, in fact, not only identified several classical cell wall proteins but also unexpected proteins that generally have been considered to be non-secretory or traditionally associated with organelles other than the wall (Ndimba et al 2003; Bayer et al 2006; Slabas et al 2004; Watson et al 2004). The presence of such non-canonical cell wall proteins at the surface of the cell has two explanations: either they are contaminants or there is an unknown secretion system for proteins devoid of a canonical signal peptide. The latter hypothesis has not yet been experimentally demonstrated.

A large proportion of the apoplast proteome resides in the apoplast fluid (AF) or ionically-bound to the wall matrix. The AF is important for the transport of essential solutes in extracellular spaces. The intercellular spaces are also likely to contain signaling proteins which can interact with specific receptors at the apoplast to initiate signal cascades in cell-cell communication and other events. Many studies revealed that several PR-proteins present in the AF and represent the major quantitative changes in soluble proteins during defense responses. The PR proteins are found in the apoplast where they serve as the first mediator of cell-to-cell communications. Apoplast has an important function during plant defense responses to pathogens like reinforcement of the cell wall, antimicrobial activity by PR proteins including chitinases, β-1, 3 glucanases, thionins, defensins, and lipid transfer proteins. Apoplast is also a place where cells recognize extracellular signals including PAMPs and effectors released from pathogens in diverse ways (Jones and Dangl 2006). Thus, plant secreted proteins might play essential roles in the early recognition and defense against pathogen attack. Other than revealing their primary purpose in the plant cell environment, identification and

characterization of secreted proteins, the secretome may provide clues for understanding the complex defense mechanisms initiated in plants due to plant-pathogen interactions.

Secretome is an emerging area that describes the global study of proteins secreted by a cell, tissue or organism at any given time or under certain conditions (Hathout 2007). Proteomic analysis of apoplast extracts from the leaves of *Arabidopsis*, *Triticum aestivum* and *Oryza sativa*, which revealed very different polypeptide patterns between the three species and led to the identification of proteins belonging to various families, reflecting the variety of functions of the apoplast (Haslam et al 2003).

Proteomic approaches have been used to identify secreted proteins in *Arabidopsis* suspension-cultured cells (Ndimba et al 2003; Oh et al 2005), maize (Chivasa et al 2005), and tobacco BY2 cell (Okushima et al 2000) in response to fungal elicitor. Several pathogen elicitor responsive proteins including lectin receptor-like kinase, endochitinase, xyloglucan endo-1, 4-β-D-glucanases, and peroxidase have been identified in culture filtrate extracts.

Proteomic studies have also been performed on the whole protein extracts to understand the plant-pathogen interactions and defense signaling in wheat (Rampitsch et al 2006), maize (Chivasa et al 2005), pea (Curto et al 2006), *Arabidopsis* (Jones et al 2006), oilseed rape (Floerl et al 2008) and rice (Kim et al 2003; 2004). Proteomic analysis of the cell wall of *Arabidopsis* treated with chitosan suggested involvement of cell wall kinases in defense responses against pathogen attack (Chivasa et al 2002). In *Arabidopsis*, it has been demonstrated that the stem cell-specific protein, CLAVATA3 (CLV3), is soluble in the extracellular space where it is required for the activation of the CLV1/CLV2 receptor complex at the plasma membrane of the underlying cells (Rojo et al 2002).

Extracellular accumulation of a specific subset of host proteins lacking traditional signal peptides during interaction of *Arabidopsis* with *Pseudomonas syringae* indicates the manipulation of host secretion system to promote successful invasion of plants (Kaffarnik et al 2009). Proteomic analysis of apoplast of *Arabidopsis* during interactions with *Verticillium longisporum* revealed that proteins related to defense and cell wall modification were induced.

Decrease in the abundance of a chitin-inducible lectin-like protein suggests that *V. longisporum* enhances its virulence by rapid down-regulation and delay of induction of plant defense genes (Floerl et al 2012).

1.5. Transcriptome analysis to understand plant–pathogen interactions

Transcriptional re-programming is a key step of plant defense in response to pathogen recognition. The activation of the defense transcriptome is a complex, multidimensional process. In the area of plant-pathogen interactions, transcriptome profiling has provided unparalleled perception into the mechanisms underlying gene-for-gene resistance, basal defense, host vs NHR, biotrophy vs necrotrophy, and pathogenicity of vascular vs nonvascular pathogens, among many others. Large-scale transcript profiling has uncovered key features of defense transcriptome and its regulation. In host-pathogen interactions, it is particularly useful to use a time course combined with alternate plant genotypes or pathogen isolates to elicit a response that will enable a hypothesis to be tested. NHR, a less studied defense phenomenon, was believed to be genetically complex of the fact that activation of any specific defense component may not be sufficient to render a plant resistance reaction. Microarray experiments played a key role in delineating the molecular mechanism of NHR.

A plant is resistant or susceptible to a specific pathogen depending on the speed and rate at which the same host defense molecules are produced, suggesting that the resistance is based on quantitative rather than qualitative differences on host defense (Eulgem et al 2004; Tao et al 2003). However, others have observed significantly divergent gene expression not only between compatible and incompatible interactions but also between incompatible interactions of the same pathogen-host combination that are mediated by different resistance (R) proteins. In non-host interaction between *Arabidopsis-P. syringae pv. phaseolicola* expression kinetics was slower than in incompatible interactions. A non-host pathogen weakly induces responses somewhat similar to those in incompatible interactions (Tao et al 2003). Total transcriptome analysis of barley during susceptible and non-host interactions against *Blumeria graminis* pv. *hordi* (*Bgh*) and *Bgt* did not show significant host- or non-host specific expression. About 30

genes showed significant differences at expression levels at 12 or 24 h post inoculation (hpi), respectively. Higher up-regulation of activated genes in the non-host interaction occurred at 12 hpi and in the compatible interaction at 24 hpi as well as for stronger down-regulation of repressed genes in the compatible interactions at 24 hpi (Eichmann et al 2006).

A majority of genes repressed by inoculations with non-host (*Bgh*) and host powdery mildews (*Bgt*) in *Arabidopsis* encoded components of photosynthesis and general metabolism. Down-regulation of housekeeping or development-related genes might also represent physiological requirements for allocation of resources for non-host defense (Zimmerli et al 2004). A number of defense-associated genes were induced during both interactions. These genes likely are components of basal defense responses, which do not effectively block host powdery mildew infections. In addition, genes encoding defensins, anti-microbial peptides whose expression is under the control of the JA/ET signalling pathway, were induced exclusively by non-host pathogens. Ectopic activation of JA/ET signalling protected *Arabidopsis* against two biotrophic host pathogens (Zimmerli et al 2004). *Arabidopsis* inoculated with *Bgh* (non-host) produced a more dramatic up- or down-transcript response than *E. cichoracearum* (host) because *Bgh* cannot suppress host basal defenses where as *E. cichoracearum* can suppress the basal defenses (Stein et al 2006). Microarray analysis of pathogen-responsive genes during NHR of hot pepper against *X. axonopodis* pv. *glycines* (*Xag*) led to identification of new transcription factor. The genes that encode CCR4-associated factor (CAF), AT Hook DNA binding protein and SPF1 were abundantly expressed during *Xag* infiltration. In addition, two genes encoding bZIP transcription factors showed reduced, implicating bZIPs as possible negative regulators in the non-host defense response of the hot pepper (Lee et al 2004).

The transcriptional responses of barley against three different pairs of adapted (host) and non-adapted (non-host) isolates of fungal pathogens, which belong to the genera *Blumeria* (powdery mildew), *Puccinia* (rust), and *Magnaporthe* (blast) were studied. NHR against each of these pathogens was associated with changes in transcript abundance of distinct sets of non-host-specific genes, although general (not non-host-associated) transcriptional responses to the different pathogens overlapped considerably. The powdery mildew- and blast-induced

differences in transcript abundance between host and non-host interactions were significantly correlated with differences between a near-isogenic pair of barley lines that carry either the Mlo wild-type allele or the mutated Mlo5 allele, which mediates basal resistance to powdery mildew. Similar patterns of over-represented and under-represented functional categories of genes during the interactions of barley with the different host or non-host pathogens were found. NHR and basal host defense of barley are functionally related and that NHR to different fungal pathogens is associated with more robust regulation of complex but largely non-overlapping sets of pathogen-responsive genes involved in similar metabolic or signaling pathways (Zellerhoff et al 2010).

1.6. Model system to study non-host interactions

NHR is broad spectrum including both constitutive and inducible defenses. Details of NHR are not clearly deciphered when compared to *R*-gene-mediated resistance because of its complex and multilayered nature. So far, the non-host interactions focused on barley and *Arabidopsis*, more against powdery mildew fungi (Schweizer 2007). Here, we have selected tomato (*Lycopersicon esculentum* cv. money maker) as non-host plant and *Magnaporthe grisea* as non-host pathogen to study the mechanisms involved in the NHR. *M. grisea* is a hemibiotrophic fungus and an important pathogen causing blast disease on different cereals like rice, rye, barley etc. which does not cause disease in dicot plants. For comparative studies, we selected *Alternaria alternata* f. sp. *lycopersici*, a causal agent of stem canker on tomato which is a necrotrophic fungus, as compatible pathogen that infects only dicots (Grogan et al 1975). *A. alternata* f. sp. *lycopersici* produces host specific toxin and symptoms are like dark brown to black cankers with concentric zonation on leaves, stems and fruits. In the present study, using this non-host pathosystem, we would like to address some questions related to NHR like, what are the defense pathways activated and responsible for restriction of growth of *M. grisea* in tomato? In the same way, how is tomato accessible to *A. alternata* f. sp. *lycopersici* but not to *M. grisea*? A better understanding of the underlying mechanisms is expected to provide answer to these questions. The most crucial point was to understand the importance of apoplastic defenses using different biochemical and molecular approaches. Here, we studied the changes

in apoplast proteins and the defense transcripts during the non-host and compatible interactions of tomato through proteomics and genomics approach. In line with these questions the fallowing objectives were set for this study:

1.7. Objectives

- ### Histochemical changes during non-host interactions of tomato

  We characterized tomato-*M. grisea* non-host pathosystem using cytological staining methods to know, how and at what stage *M. grisea* growth was restricted in tomato. We performed aniline blue and trypan blue staining methods to investigate callose deposition and cell death respectively during non-host interactions of tomato.

- ### Changes in the redox status of the leaf apoplast

  Apoplast is the first site attacked by the pathogens. So, we checked the role of apoplastic oxidative defenses in NHR. To understand the role of apoplastic defenses during NHR in tomato, we compared the ROS generation and antioxidative enzymes during non-host and compatible interactions.

- ### Proteome analysis of during non-host and compatible interactions

  Proteome analysis gives the direct evidence of changes in proteins during any biological process. So, we attempted to identify and characterize the changes in abundance of apoplast proteins during NHR and analyzed by 2-DE in conjunction with MALDI-TOF-MS.

- ### Total transcriptome analysis of tomato during non-host and compatible interactions

  Microarrays have become a powerful tool for simultaneously probing the expression levels of thousands of genes. The potential of capturing genome-wide transcriptional changes allows a better understanding of the molecular mechanisms involved in non-host interactions. We used this microarray to determine the transcriptional changes that underlie the interactions between tomato-*M. grisea* and tomato-*A. alternaria* f. sp. *lycopersici*

Materials & Methods

2.1. Fungal cultures

M. grisea and *A. alternata* f. sp. *lycopersici* were isolated from infected rice and tomato plants, respectively. Stock cultures were maintained on potato dextrose agar (PDA) at 24 ^{o}C. A conidial suspension was obtained by washing PDA slant cultures with distilled water. A suspension containing 1 x 10^{6} spores per mL was used to inoculate tomato leaves.

2.2. Plant material

Tomato (*Lycopersicon esculentum* cv. money maker) plants were grown in soil in a growth chamber with a 16 h photoperiod at 350 lE/m2 light intensity at 24 ^{o}C and at constant (70%) humidity. One month-old tomato leaves were inoculated by drop-inoculation method with *M. grisea* or *A. alternata* f. sp. *lycopersici* conidial suspension prepared in distilled water containing 0.02% Tween-20. Mock inoculation was done with 0.02% Tween-20 in distilled water. Inoculated leaves were incubated in an incubation chamber at 90% humidity and 24 ^{o}C.

2.3. Histochemical observations

2.3.1. Callose staining

A KOH-aniline blue fluorescence technique was used to observe callose deposition and appressorium formation during non-host interactions of tomato-*M. grisea* (Hood and Shew 1996). Briefly, pathogen inoculated leaf samples were boiled at 95 ^{o}C for 15 min in 1 M KOH solution and washed twice with double distilled H_2O. These leaf samples were mounted in stain solution (0.05% aniline blue in 0.067M K_2HPO_4 pH 9), and examined under UV- fluorescence microscope (Leica, Wetzlar, Germany).

2.3.2. Dead cell staining

Mock- and *M. grisea*-inoculated tomato leaves were stained with lactophenol- trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol and 0.1% trypan blue) (Koch and Slusarenko 1990). Leaf samples were boiled for ~1 min in the stain solution and then decolorized in 2.5% chloral

hydrate. Leaf samples were mounted in chloral hydrate and observed under light microscope (Leica, Wetzlar, Germany).

2.4. Preparation of apoplastic fluid (AF)

The AF was extracted using gravity extraction method (Jung et al 2008). Briefly, each leaf was cut with scissor into 3-4 pieces and AF was collected in 50 mL falcon tube containing a plastic sheet laid with holes followed by centrifugation at 1000g for 20 min at 10 °C. The AF collected at the bottom of the falcon tube was stored at -20 °C. With this procedure, we obtained 30-80 µL of AF for 2 g fresh weight tomato leaves. Cytosolic contamination of the AF was monitored by malate dehydrogenase (MDH; EC 1.1.1.37) assay (López-Millán et al 2000) and SDS-PAGE analyses. Protein concentration of the AF was determined by the Bradford reagent (Sigma, Aldrich, USA) using bovine serum albumin (Sigma, Aldrich, USA) as standard.

2.5. Measurement of extracellular ROS

2.5.1. Extracellular $O_2^{\cdot-}$

Extracellular production of $O_2^{\cdot-}$ was measured by the oxidation of epinephrine (Sigma, Aldrich, USA) to adrenochrome and determined spectrophotometrically at 480 nm (Misra and Fridovich 1972) from 3 to 48 hours post inoculation (hpi). Briefly, 25 mg leaf discs were incubated in 1 mL of 1 mM epinephrine in 50 mM Tris-HCl (pH 7.8) buffer for 30 min at 25 $^{\circ}$C. The concentration of $O_2^{\cdot-}$ was calculated by using adrenochrome extinction coefficient of 4020 M^{-1} cm^{-1}.

2.5.2. •OH radicals

The •OH were measured using deoxy ribose (DOR) sugar (Halliwell and Gutteridge 1981), which forms thiobarbituric acid-reactive substances (TBARS) from 6 to 48 hpi. Briefly, 25 mg of inoculated leaf disc was incubated in 550 µL of 1 mM DOR for 45 min in the dark. To a preheated mixture of 500 µL thiobarbituric acid (TBA, 1% w/v in 0.05 mM NaOH) and 500 µL trichloroacetic acid (TCA, 2.8% w/v), 500 µL of DOR solution was added, immediately boiled for 10 min, and cooled on ice for 10 min and A_{540} was recorded.

2.5.3. Extracellular H_2O_2

A xylenol orange assay was adopted to measure H_2O_2 concentration (Gay and Gebicki 2000). Working reagent contained 0.1 mL of reagent A (25 mM $FeSO_4$, 25 mM $(NH4)_2SO_4$ and 2.5 M H_2SO_4) and 10 mL of reagent B (125 mM xylenol orange (Sigma, Aldrich, USA) and 100 mM sorbitol). Tomato leaf discs (50 mg) were incubated in 1 mL double distilled water (pH 7.0) for 30 min under mild shaking in dark. After removing the leaf discs, 1 mL of the working reagent was added to 1 mL of double distilled water, incubated for 30 min and A_{560} was recorded. The H_2O_2 concentration was calculated using standard curve of H_2O_2.

2.5.4. Lipid peroxidation

Lipid peroxidation was measured in terms of TBARS (Heath and Packer 1968). Tomato leaf tissue (150 mg) was homogenized in 0.25% TBA in 10% TCA using mortar and pestle. The samples were heated at 95 ^oC for 30 min and quickly cooled in an ice bath for 10 min and centrifuged at 10,000g for 10 min. The A_{532} of the supernatant was read and unspecific turbidity was corrected by subtracting A_{600}. Total TBARS were expressed in terms of nmol g^{-1} fresh weight using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5.5. Protein carbonyl content

Protein carbonyl content was measured using 2, 4-dinitrophenylhydrazine (DNPH) (Levine et al 1990). Briefly, 0.5 mg of protein was taken in two tubes as test and control, 800 µL of 10 mM DNPH prepared in 2.5 M HCl was added to the test sample and 800 µL of 2.5 M HCl alone was added to the control sample and incubated for 1 h in the dark at 37 °C. To the reaction tubes, 20% TCA (w/v) was added, incubated on ice for 10 min and centrifuged at 1000g for 20 min to pellet the protein. Protein pellets were dissolved in 8 M guanidine chloride and incubated for 10 min at 37 °C. Carbonyl content in the supernatant was recorded as A_{380} and calculated by using an absorption coefficient of 22,000 $M^{-1}cm^{-1}$.

2.5.6. NADH-peroxidase

NADH-peroxidase was measured by monitoring the NADH oxidation at 340 nm (Patykowski and Urbanek 2003). Briefly, the reaction mixture contained 50 mM Tris-acetate buffer (pH 6.0), 0.2 mM NADH, 25 µM *p*-coumaric acid, 5 mM $MnCl_2$ and 5 µg of protein. The NADH-peroxidase activity was calculated from the molar extinction co-efficient of 6.3 M^{-1} cm^{-1} for NADH.

2.6. Measurement of antioxidant enzymes

2.6.1. SOD

The SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). Briefly, 1 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 63 µM NBT, 1.3 µL riboflavin, 0.1 mM EDTA, 10 µg protein. Riboflavin was added at the end. The reaction was initiated by switching on 15 W fluorescent lamps for 10 min. The tubes covered with a black cloth served as blanks. One unit of the SOD was defined as amount of enzyme required to inhibit reduction of NBT, recorded as $A_{560,}$ by 50%.

2.6.2. PRX

PRX was assayed by measuring the increase in absorbance at 470 nm due to oxidation of guaiacol to tetraguaiacol as described by Chance and Maehly (1955). The reaction mixture consisted of 0.1 mM acetate buffer (pH 5.0), 5 mM guaiacol, 0.03% H_2O_2 and 10 µg of protein. PRX activity was calculated using molar extinction co-efficient 26.61 $mM^{-1}cm^{-1}$for tetra-guaiacol at 470 nm.

2.6.3. APX

The APX was assayed by measuring decrease in absorbance at 290 nm due to reduction of ascorbate (Nakano and Asada 2001). Briefly, the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.5 mM $H_2O_{2,}$ and 10 µg of protein. The APX

activity was calculated using extinction co-efficient 2.8 mM^{-1} cm^{-1} for reduced ascorbate and expressed as mM min^{-1} mg^{-1} protein.

2.6.4. PPO

PPO was assayed by monitoring the oxidation of pyrogallol to purpurogallin (Kar and Mishra 1976). The reaction mixture contained 30 mM phosphate buffer (pH 6.8), 5 µM pyrogallol and 10 µg of protein. The reaction was allowed to proceed for 5 min at 25 °C and stopped by adding 0.5 mL 5% (v/v) H_2SO_4. Reaction tubes were centrifuged for 15 min at 14,000g and the absorbance of supernatant read at 420 nm.

2.6.5. Profiling of SOD and PRX isozymes

Isozymes of PRX in the AF were separated on 10% native PAGE was performed. To detect PRX after electrophoresis, the gel was incubated with 20mM guaiacol and 0.03% H_2O_2 in 100 mM potassium phosphate buffer (pH 6.5) and PRX isozymes were visualized as orange-brown bands.

To detect SOD isozymes, after electrophoresis, the gels were incubated in a reaction buffer containing 0.01 M EDTA, 0.098 mM NBT, 0.030 mM riboflavin and 2 mM TEMED in 50 mM potassium phosphate (pH 7.8) for 30 min in the dark, followed by washing with distilled water and illumination by 15 W fluorescent lamps for 15 min. SOD isozymes were visualized as colorless bands on purple colour background in gels (Beauchamp and Fridovich 1971). Cu/Zn-, Mn-and Fe-SODs were distinguished from each other by incubating the gel with 5 mM KCN and/or 5 mM H_2O_2 before staining. Cu/Zn-SOD was inhibited by KCN and H_2O_2 and Fe-SODs were inactivated by H_2O_2 whereas, Mn-SOD is resistant to both the inhibitors (Weisiger and Fridovich 1973; Yamahara et al 1999)

2.7. Ascorbate-related changes

2.7.1. AO

AO was assayed by measuring increase in absorbance at 265 nm (Moser and Kanellis 1994). The reaction mixture contains 66 mM potassium phosphate (pH 5.3), 37.5 µM ascorbic acid (AA),

and 10 µg AF protein. AO activity was calculated using a co-efficient of 14 $mM^{-1}cm^{-1}$ for ascorbate at 265 nm.

2.7.2. Ascorbate content

Ascorbate content was measured using bipirydyl method (Gillespie and Ainsworth 2007). The AF collected from 0.5 g tomato leaves was incubated with 500 mL 10% TCA, 400 mL 43% H_3PO_4, 400 mL 4% α-α-bipyridyl and 200 mL 3% $FeCl_3$. Reaction tubes were incubated at 37 °C for 1 h. Absorbance was read at 525 nm. The concentration of ascorbate in the apoplast was calculated using ascorbic acid standards.

2.8. Extracellular protease

Extracellular protease was measured using casein as substrate. The AF was mixed with 130 µL of casein solution (10 mg/1.5 mL) and incubated at 37 ^{o}C for 10 min. To the reaction tubes, 130 µL of 110 mM TCA was added and incubated at 37 ^{o}C for 20 min. Reaction tubes were centrifuged at 10,000g for 5 min and 250 µL of supernatant was mixed with 625 µL of 0.5 mM Na_2CO_3, 125 µL of Folin-Ciocalteau reagent and incubated at 37 ^{o}C for 30 min and read A_{660}. Protease activity was calculated as µmoles of L-tyrosine released during reaction (Tsuchida et al 1986).

2.9. Phenolic content

Phenolic content was measured using Folin-Ciocalteau reagent (Ainsworth and Gillespie 2007). The AF was extracted directly into methanol from 0.5 g of tomato leaves, and 200 µL of 10% Folin-Ciocalteau reagent was added and vortexed. To the reaction tubes, 800 mL of 700 mM Na_2CO_3 was added and incubated for 2 h at 30 ^{o}C and read A_{765}. The phenolic content was calculated using standard curve of gallic acid.

2.10. Changes in carbohydrate metabolism

2.10.1. Cw-Inv

A modified method of Harris and Jaffcoat (1974) was used for measuring of invertase activity. Briefly, 1 mL reaction mixture contained 20 mM acetate buffer (pH 4.8), 30 mM sucrose and 10 µg protein and reaction mixture was incubated at 37 °C for 30 min. After incubation 1 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added and boiled in water bath for 10 min. A_{560nm} was measured. Cw-Inv activity was expressed as mg sucrose hydrolysed min^{-1} mg^{-1} protein.

2.10.2. Extraction and measurement of total soluble sugars (SS)

The SS from leaf tissue (25 mg) were extracted with 1.0 mL of 80% (v/v) ethanol and incubated at 80 °C for 60 min. After centrifugation at 4 °C for 5 min at 12,000g, clear supernatants were transferred into new tubes and evaporated to dryness at 40 °C. The residue was resolved in 250 µL of water was used for the determination of SS by phenol-sulphuric acid method (Dubois et al 1956). Briefly, 200 µL 5% phenol was carefully added to 20 µL methanol extract in a test tube and mixed thoroughly. One mL of concentrated H_2SO_4 (analytical grade) was added very carefully and mixed the tubes thoroughly by agitation. The tubes were cooled at room temperature and A_{485nm} was recorded. The SS were measured and expressed as μM^{-1} g^{-1} fresh weight.

2.10.3. Collection and quantification of SS composition in apoplast

Sugars were separated by a amino column (Shodex NH2P-50 4E, 4.6X 250 mm), using a solvent of acetonitrile:water (75:25, v/v) in Shimadzu LC-10AT HPLC system, and the flow rate was 1 mL min^{-1}. Identification and quantification of the major sugars present in the samples was achieved by comparing each peak retention time and peak area with those of the standard. Sugar standards were made for glucose, fructose, and sucrose. A standard curve for each sugar was prepared by injecting different concentrations of the standard solution and plotting HPLC peak areas versus sugar concentration in the standards (Joosten et al 1990).

2.11. Apoplast proteome responses during non-host and compatible interactions

A large proportion of the apoplast proteome resides in the AF or as ionically bound to the wall matrix. Analyses of AF and ionically cell wall bound protein (IBP) fractions of the tomato leaf apoplast were performed during non-host and host pathogen interactions using a proteomics approach (Fig 2.2).

2.11.1. Extraction of IBP fraction

IBP fraction was extracted from tissue homogenates as described by Jackson et al (2001). Briefly, non-host, compatible pathogen, and mock-inoculated tomato leaves were collected at three different time points (12, 24, and 72 hpi). Leaf tissues were homogenised in 2 mL g^{-1} (fresh weight) of sodium acetate buffer (15 mM, pH 4.5) and crude cell wall fractions were isolated by centrifugation at 4500g for 5 min. The crude cell wall fraction was washed twice by centrifugation at 4500g in 1% TritonX-100, followed by three washes with sodium acetate buffer (pH 4.5). The resultant cell wall pellet was resuspended in of 1 M NaCl for a maximum of 5 min before centrifugation at 4500g to yield the saline extract. Collected saline supernatant, which contained IBP, was dialyzed against water for 12 h and concentrated in speed vac. Resultant pellets were solubilized in IEF buffer (9 M Urea, 2 M thiourea, 4 % CHAPS, 50 mM DTT, and 1% pH3-10 range ampholytes). Concentration of proteins (three repetitions for each sample) was measured using Bradford reagent (Sigma, Aldrich, USA).

2.11.2. Extraction and solubulization of AF fraction

The AF fraction from non-host and compatible pathogen-challenged tomato leaves, at three different time points (12, 24, and 72 hpi), was extracted using gravity extraction method as described in section 2.4. Extracted AF fraction was concentrated in speed vac. Pellet was resuspended in 0.8 mL of dense SDS extracting buffer (sucrose 30%, SDS 2%, 0.1 M Tris-HCl pH 8.0, 2-mercaptoethanol 5%), sonicated (3 pulses of 8 s each, with 10 s intervals) at 50% output on an Ultrasonic processor (Soincs, Vibra Cell 130W) and incubated 10 min on ice. One volume of saturated phenol pH 8.0 was added and incubated for 30 min at 30 ^{o}C under agitation and centrifuged at 5000g for 10 min at 37 °C. Phenol phase extractions were incubated for 14 h at

-20 °C with five volumes of 0.1 M ammonium acetate (AcNH4) in methanol. Samples were centrifuged at 12,000g for 15 min at 4 °C. Protein pellets were washed three times with 0.1 M AcNH4 in methanol, twice with acetone and once with ethanol 70%, and then the pellets were finally dried at room temperature (Pirovani et al 2008). The pellets were resuspended in IEF buffer and concentration of proteins (three repetitions for each sample) was measured using Bradford reagent.

2.11.3. Bradford assay for protein quantification

High concentration of urea, thiol-reducing agents and dithiothreitol (DTT) interfere with protein quantification assays. The modified Bradford protein assay described here takes advantage of the Bradford assay's insensitivity to many of the reagents in these mixtures, while correcting for the effects caused by basic reagents (Ramagli 1999). The use of dilute acid to neutralize the sample, prior to adding of the dye, produces a biphasic response over the concentration range of 0.5-50 µg. The nonlinearity over this entire range is inherent to all protein assays using Coomassie brilliant blue G-250. Bovine serum albumin (BSA) was prepared in IEF buffer to use as protein standard. Each protein sample (2 µL) and BSA standards (0, 2, 4, 6, 8, and 10 µg) were mixed with 20% Bradford reagent (Sigma, USA) containing 0.1 mM HCl in a final volume of 1 mL. The absorbance of sample mixture was read at 595 nm.

2.11.4. Purity assessment of IBP and AF fractions

2.11.4.1. Enzyme assays

Cytosolic contamination of AF and IBP was monitored by MDH and catalase (CAT; EC 1.11.1.6) activities, respectively. MDH activity was assayed by measuring decrease in absorbance at 340 nm (Lopez-Millan et al 2000). Briefly, 1 mL of reaction mixture consisted of 46.5 mM Tris-HCl buffer (pH 9.5), 0.1 mM NADH, 0.4 mM oxaloacetate and 10 µg of AF and total soluble protein (TSP). MDH activity expressed as nmol min^{-1} mg^{-1} protein. CAT activity was assayed by measuring decrease in absorbance at 240 nm due to decomposition of H_2O_2 (Aebi and Lester 1984). Briefly, the 1 mL reaction mixture consisted of 50 mM potassium phosphate buffer (pH

7.0), 10 mM H_2O_2 and 10 µg of IBP and TSP. The extinction co-efficient of H_2O_2 40 mM^{-1} cm^{-1} was used to calculate the CAT activity and expressed as mM^{-1} min^{-1} mg^{-1} of protein.

2.11.4.2. Western blot analysis

To check the cytosolic contamination of IBP and AF fractions, we used fructose 1,6-bisphosphatase as cytosolic marker protein. Proteins (20 µg) were separated on 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (Immobilon-NC, 0.45 µm, Millipore). The blotted membranes were incubated with fructose 1,6-bis phosphatase antibodies. Membranes were then incubated with secondary antibody conjugated with horse radish peroxidase. Signals were detected using the chemiluminescence (ECL) system as per manufacturer's protocol (Bio-Rad, Hercules, California, USA). All procedures were carried out at 30 ^oC ± 2.0 ^oC.

2.11.5. 2-DE

For IEF, the Ettan IPGphor II system was used (GE) with 3-10 non-linear (NL) pH gradient strips (IPG strips, GE Health Care). Proteins were loaded on strips in active rehydration method. IEF was carried out at 50 V for 12 h, followed by 250 V for 1 h, 500 V for 1.5 h, 1000 V for 1.5 h, a gradient to 5000 V over 1.5 h and for a further 4 h, all at 20 °C. Prior to the second dimension SDS-PAGE, the focused IPG strips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8) buffer containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS and a trace of bromophenol blue (BPB). DTT at 2% (w/v) was added to the first equilibration step and 2.5% (w/v) iodoacetamide to the second. IPG strips were then rinsed with cathode running buffer (0.025 M Tris, 0.192 M glycine and 0.2% (w/v) SDS) and placed onto 15% SDS-PAGE and overlaid with agarose solution (60 mM Tris-HCl (pH6.8), 0.5% (w/v) agarose, 0.01% (w/v) BPB). Molecular mass of the proteins was determined by running standard protein markers (Fermentas). For each sample, triplicate IPG strips were processed for electrophoresis under the same conditions.

2.11.6. Protein visualization and image analysis

Gels for image analysis were removed from the cassette and soaked in ultrapure water for 10 min, fixed for 1 h in methanol/water/acetic acid (5:4:1), and finally rinsed twice in ultrapure water for 5 min each rinse. Gels for protein analysis were removed from the cassette and soaked in ultrapure water for 10 min and then put directly into staining solution. Gels were stained for 16 to 24 h in Coomassie protein stain (20% [v/v] ethanol, 8% [w/v] ammonium sulfate, 0.08% [w/v] Coomassie brilliant blue G-250, and 0.35 M phosphoric acid) (Sarma et al 2008). Gels were destained for 24 h with several changes of ultrapure water using mild shaking. The gels were scanned in Image Scanner and analyzed with Image Master 2-D Platinum Software (GE Health Care). Automatic matching was complemented by manual matching.

2.11.7. Trypsin digestion of proteins and characterization by MALDI-TOF/TOF

Coomassie-stained protein spots were manually excised from three reproducible gels and transferred to sterilized eppendorf tubes (1.5 mL). The excised gel pieces were destained with 100 µL of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for five times. Thereafter, the gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56 °C for 1 h. This is followed by treatment with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at 25 ± 2 °C, washed with 25 mM NH_4HCO_3 and ACN, dried in speed-vac and rehydrated in 20 µL of 25 mM NH_4HCO_3 solution containing 12.5 ng μL^{-1} trypsin (sequencing grade, Promega, Wisconsin, USA). The above mixture was incubated on ice for 10 min and kept overnight for digestion at 37 °C. After digestion, a short spin for 10 min was given and the supernatant was collected in a fresh eppendorf tube. The gel pieces were re-extracted with 50 µL of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min with frequent vortexing. The supernatants were pooled, dried using speed vac and reconstituted in 5 µL of 1:1 ACN and 1% TFA. Two µL of the above sample was mixed with 2 µL of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 µL was spotted on target plate. Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) analysis was conducted with a MALDI-TOF/TOF mass spectrometer (Bruker

Autoflex III smartbeam, Bruker Daltonics, Germany) as described by Shevchenko et al (1996) with slight modifications.

2.11.8. Database queries and protein identification

Protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics). The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. The taxonomic category was set to Viridiplantae (green plants). The other search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT probability analysis (P<0.05), only significant hits were accepted for protein identification.

2.12. Total transcriptome analysis

2.12.1. Sample collection

All samples were collected in two independently repeated experiments at different time points (0, 6, and 24 hpi). For each sample, leaf material was harvested from three plants inoculated with non-host and host pathogens, pooled separately, and flash frozen in liquid nitrogen. Material harvested at 0 hpi was used as the reference sample to which all other samples were compared (Fig 2.3).

2.12.2. RNA isolation

Total RNA was prepared from 100 mg of the frozen plant material using NucleoSpin RNA plant kit (Machery Nagel, Duren, Germany). Quantification and purity analysis was done using the Nano Drop ND-1000UV-Vis spectrophotometer. Total RNA with OD 260/280 >1.8 and O.D260/OD270 ≥ 1.3 was used for microarray experiments. RNA was considered to be of good quality when the rRNA 28S/18S ratios were greater than or equal to 1.5, with the rRNA contribution being 30% or more and an RNA integrity number (RIN) was ≥7.0.

2.12.3. RNA labeling

A primer encoding a T7 RNA polymerase promoter sequence fused to (dT) 24 (Agilent Quick Amp Kit, USA) was used for double-stranded cDNA synthesis using MMLV-reverse transcriptase. The double-stranded cDNA was purified and used as a template in the subsequent *in vitro* transcription reaction. Agilent's Quick Amp Labeling Kit generated fluorescent complimentary RNA (cRNA) from cDNA for one-color processing. The amplification of cRNA was carried out in the presence of T7 RNA polymerase, cyanine 3-labeled CTP and NTPs mix.

2.12.4. Hybridization and data collection

The labeled target cRNA was purified, fragmented, and hybridized to a whole genome tomato 4X44K AMADID: 22270 gene chip arrays according to protocols provided by the manufacturer (Agilent, USA). Fragmentation of labelled cRNA and hybridization was done using the Gene Expression Hybridization kit of Agilent (Part Number 5188-5242). Hybridization was carried out in Agilent's surehyb chambers at 65 °C for 16 h. The hybridized slides were washed using Agilent's gene expresssion wash buffers (Part No. 5188-5327) and scanned using the Agilent microarray scanner G Model G2565BA at 5 µ resolution.

2.12.5. Data analysis

Normalization was done using Gene Spring GX v11.5 Software. Intra-array normalization which deals with variability within a single array was done among the controls using Percentile Shift Normalization method. In intra array normalization, gProcessed signal (dye normalized background subtracted signal intensity) is log transformed, and for each of the array, the 75th percentile value was calculated separately. In each sample, the log transformed intensity value for each probe was subtracted by the calculated 75th percentile value of the respective array and expression values were obtained.

Feature extracted data was analyzed using Gene Spring GX v11.5 software from Agilent. Signal quantification and data analysis were achieved using Gene Spring GX v11.5 software. Following local background subtraction, the signal for each spot was normalized based on the median

value of the median intensity of all the spots for each array. Only genes for which the hybridization signal was greater than the average value plus two standard deviations of the controls were analyzed. Each ratio was converted to its $\log_2$ value, and the average $\log_2$ value for each gene of the two independent arrays corresponding to each experiment was calculated. Statistical significance of the gene expression differential over the course of the replicate experiments was calculated by using a Student's t test analysis. Only genes with high levels of significance ($P < 0.01$) and a minimum absolute value of $\log_2 > 1$ were systematically considered in this study, to minimize the false positive as up- or down-regulated. Expression profiles from each time point were clustered based on their similarity in expression pattern using a hierarchical average linkage clustering algorithm and Pearson correlation distance.

2.13. Statistical analysis

Statistical analyses of the mean values in different treatments were performed using IBM SPSS statistics 20 software (tukey test). Differences were considered to be significant at $P<0.05$ and shown with different letters in the graphs.

For proteomic analysis, three independent experiments with three replications of both control and stressed samples of each time point with each replication comprising of 10-15 pooled plants were considered and the spots were analyzed using Image Master 2-D Platinum image analysis software (GE Healthcare). The normalized volume (% vol) of each spot was automatically calculated by the software as a ratio of the volume of a particular spot to the total volume of all the spots present on the gel.

Results

3.0. Biochemical and molecular analyses of NHR in tomato apoplast

A better understanding of the molecular mechanisms underlying plant NHR, which is strong and durable, promises the potential for targeted employment in agricultural practices. Since NHR operates at the plant-species level, it often has been beyond the reach of breeding programs and also difficult to decipher the mechanisms involved in execution of NHR. In the present study, we used histochemical and molecular analyses like proteomics and genomics in a selected non-host pathosystem (tomato-*M. grisea*) to study the NHR. We also studied tomato interactions with host pathogen (*A. alternata* f. sp. *lycopersici*) as a comparative pathosystem.

Apoplast is one of the dynamic plant cell compartments which interacts with environment and responds to external cues like stress. The apoplast is a source of nutrition for pathogens with strictly apoplastic growth, or a launching pad for pathogens that attack host cells through the production of haustoria. The attack of fungal pathogen at apoplast activates several defense responses like oxidative burst, signal transduction for defense gene activation and/or cell death. The importance of apoplast led us to select and study the biochemical and proteome analyses during non-host and compatible interactions of tomato.

3.1. Histochemical observations

Deposition of callose, which is a major component of cell wall reinforcement, could be correlated to resistance during non-host interactions. Tomato leaves inoculated with *M. grisea* spores were stained with aniline blue revealed that accumulation of callose occurred at 24 h post inoculation (hpi) (Fig 3.1). Melanised appressorium formation occurred at 12 hpi. Spindle shaped spores of *M. grisea* and germ tubes were also detected through aniline blue staining. Light microscopic observations of tomato leaf specimens, at different time points after inoculation, stained with trypan blue revealed that appressorium formation and cell death occured at 12 and 24 hpi, respectively (Fig 3.1).

3.2. Biochemical responses of apoplast during non-host and host interactions

Obtaining a population of representative apoplast proteins from tomato leaves devoid of cytosolic contamination is technically challenging. We adopted gravity extraction method which involved a direct centrifugation of leaf pieces to extract the AF. The AF obtained with this method, free from cytosolic contamination, was used for biochemical and proteomic analyses.

3.2.1. Purity assessment of AF

The cytosolic contamination of the AF was analyzed using SDS-PAGE. The total soluble protein (TSP) showed a major band of ~50 kDa in SDS-PAGE, probably corresponding to the Rubisco large subunit, but absent in the AF (Fig. 3.2). A cytosolic marker enzyme, MDH was measured in both TSP and AF to assess the purity of AF. The AF obtained through gravity extraction method had very low contamination of cytosolic proteins (0.26 μM of MDH) as against 18.12 μM in TSP (min^{-1} mg^{-1} protein).

3.2.2. Extracellular accumulation of ROS during non-host and host interactions

Inoculation of tomato leaves with either non-host (*M. grisea*) or host (*A. alternata* f. sp. *lycopersici*) pathogens resulted in rapid accumulation of ROS. *A. alternata* f. sp. *lycopersici*-challenged leaves accumulated $O_2^{\cdot-}$ earlier (3 hpi) than in *M. grisea*-challenged leaves and consistently increased up to 48 hpi. Such increase in $O_2^{\cdot-}$ content occurred in *M. grisea*-challenged leaves during late stages of interactions (48 hpi) (Fig 3.3A).

Accumulation of $O_2^{\cdot-}$ was 1.7-fold high in *A. alternata* f. sp. *lycopersici*-inoculated leaves and 1.9-fold in *M. grisea*-inoculated leaves at 48 hpi. Tomato leaves challenged with both *M. grisea* and *A. alternata* f. sp. *lycopersici* spores accumulated •OH radicals. *M. grisea*-challenged leaves accumulated •OH earlier (12 hpi) than in *A. alternata* f. sp. *lycopersici*-challenged leaves (48 hpi) (Fig. 3.3B). A 3.5-fold and 4.4-fold increase in accumulation of •OH in *M. grisea* or *A. alternata* f. sp. *lycopersici*-challenged leaves occurred at 12hpi and 48hpi, respectively, compared to mock-inoculated leaves.

H_2O_2 accumulated at 24hpi in tomato leaves during both non-host and compatible interactions. The increase in H_2O_2 was 1.8-fold and 1.5-fold in *M. grisea* or *A. alternata* f. sp. *lycopersici*-inoculated leaves, compared to mock-inoculated leaves (Fig. 3.3C). However, there was a continuous decrease in H_2O_2 in the non-host interactions with *M. grisea*, while it remained constant in *A. alternata* f. sp. *lycopersici*-inoculated leaves during the progression of infection.

3.2.3. Lipid peroxidation

The ROS produced during plant-pathogen interactions were often detected as TBARS. Lipid peroxidation increased consistently in both *A. alternata* f. sp. *lycopersici*- and *M. grisea*-challenged leaves up to 96 hpi (Fig 3.4A). The TBARS in *A. alternata* f. sp. *lycopersici*-inoculated leaves were significantly high (1.8-fold) due to ROS than in *M. grisea*- inoculated (1.3-fold) leaves, compared to mock-inoculated leaves.

3.2.4. Carbonyl content

Carbonyl content increased in both *M. grisea* and *A. alternata* f. sp. *lycopersici*-inoculated leaves up to 72 hpi. In *A. alternata* f. sp. *lycopersici*-challenged leaves, the carbonyl content increased significantly by 96 hpi, compared to *M. grisea*-inoculated leaves (Fig 3.4B). The increase in carbonyls in *M. grisea*- inoculated leaves was similar to *A. alternata* f. sp. *lycopersici*-inoculated leaves up to 72 hpi.

3.2.5. Antioxidant enzymes

3.2.5.1. SOD

SOD, that dismutates $O_2^{\cdot -}$ to H_2O_2, increased in both non-host and compatible interactions, compared to mock-inoculated leaves. But, *M. grisea*-inoculated leaves had high SOD activity compared to *A. alternata* f. sp. *lycopersici*-inoculated leaves (Fig 3.5A). SOD activity in *M. grisea*-inoculated leaves increased consistently up to 72 hpi. SOD activity increased 1.7-fold and 1.5-fold during non-host and compatible interactions, respectively, compared to mock-inoculated leaves.

3.2.5.2. PRX

PRX is involved in detoxification of H_2O_2 and oxidation of phenolics in apoplast. PRX activity consistently increased up to 72 hpi in *M. grisea*-inoculated leaves. But, in *A. alternata* f. sp. *lycopersici*-challenged leaves, increase in PRX activity was not significant compared to mock-inoculated leaves. A 2-fold increase in PRX activity was observed in *M. grisea*-inoculated leaves, at 72 hpi, compared to *A. alternata* f. sp. *lycopersici*-inoculated leaves (Fig 3.5B).

3.2.5.3. PPO

The *o*-hydroxylation of phenolics by PPO produces *o*-quinones which possess anti-microbial activity. The PPO activity did not increase significantly during early stages of interaction with either of the pathogens. In both non-host and compatible-interactions, there was an increase in PPO at 72 hpi. The PPO was high in *A. alternata* f. sp. *lycopersici*-inoculated leaves compared to *M. grisea*-inoculated leaves at 96 hpi (Fig 3.5C). There was a 5.0-fold increase of PPO in *A. alternata* f. sp. *lycopersici*-inoculated leaves and a 3.8-fold increase in *M. grisea*-inoculated leaves compared to mock-inoculated leaves.

3.2.5.4. APX

APX, which detoxifies H_2O_2, is one of the antioxidative enzymes in the apoplast. APX activity increased in both *M. grisea* and *A. alternata* f. sp. *lycopersici*-inoculated tomato leaves. *A. alternata* f. sp. *lycopersici*-challenged leaves had marginally high activity at 24 and 72 hpi compared to *M. grisea*-inoculated tomato leaves. Both non-host and compatible pathogen-inoculated leaves showed high APX activity at 96 hpi (Fig 3.5D). APX activity was 3.3-fold high in *M. grisea*-inoculated leaves and 2.5-fold in *A. alternata* f. sp. *lycopersici*-inoculated leaves at 96 hpi, compared to mock-inoculated leaves.

3.2.5.5. Profiling of SOD and PRX isozymes

In apoplast, four SOD isozymes were detected in non-host and compatible interactions of tomato (Fig 3.6A). H_2O_2 and KCN inhibited the activity of all four isozymes, suggesting that all

isozymes belong to Cu/Zn-SOD. Apart from these four isozymes, another new band of Cu/Zn-SOD transiently induced at 72 hpi in both the interactions but activity decreased at 96 hpi in non-host interactions and induced at 96 hpi in compatible interactions, new band. Four isozymes of PRX were detected in tomato apoplast during compatible and non-host interactions (Fig 3.6B)

3.2.6. NADH- peroxidase

NADH-peroxidase, that contributes to H_2O_2 accumulation, increased in both non-host/host pathogen-inoculated leaves. NADH-peroxidase increased early (12 hpi) in *M. grisea*-inoculated leaves than in *A. alternata* f. sp. *lycopersici*-inoculated leaves (72 hpi) (Fig 3.7A). The NADH-peroxidase gradually decreased in tomato during interaction with non-host pathogen. In contrast, there was a gradual increase of NADH-peroxidase during interaction with *A. alternata* f. sp. *lycopersici*.

3.2.7. Extracellular proteases

Extracellular proteases increased by 96 hpi of inoculation in both non-host and compatible interactions of tomato compared to mock-inoculated leaves (Fig 3.7B). Protease activity increased by 2.6- or 1.4-fold in *M. grisea* or *A. alternata* f. sp. *lycopersici*-inoculated leaves compared to mock-inoculated leaves.

3.2.8. Phenolic content

Phenolic content increased consistently in tomato leaves inoculated with either of the pathogens. However, the phenolic content was high in *M. grisea*-inoculated leaves compared to *A. alternata* f. sp. *lycopersici*-inoculated leaves, except at 72 hpi (Fig 3.7C).

3.2.9. AO activity

AO oxidizes ascorbate to dihydroxy ascorbate and contributes to the maintainance of redox status of the apoplast. Tomato leaves inoculated with either of the pathogens had elevated AO

activity at different time points. Tomato leaves challenged with *M. grisea* had significantly high activity at 12 and 24 hpi that decreased by 96 hpi (Fig 3.8A). In *A. alternata* f. sp. *lycopersici*-inoculated leaves the AO activity slowly, but steadily and significantly, increased from 12 hpi to 96 hpi.

3.2.10. Ascorbate content

Ascorbate is a redox component in apoplast involved in defense against oxidative stress. There was a continuous decrease in ascorbate content in *M. grisea* and *A. alternata* f. sp. *lycopersici* -inoculated leaves. *M. grisea*-challenged tomato leaves had 1.7-fold decrease in ascorbic content at 72 hpi whereas 2-fold decrease was observed in *A. alternata* f. sp. *lycopersici*-inoculated leaves at 96 hpi (Fig 3.8B). A significant spurt in apopalstic ascorbate occurred at 96 hpi in *M. grisea*-inoculated leaves.

3.2.11. Changes in carbohydrate metabolism

3.2.11.1. Cw-Inv

Cw-Inv hydrolyses the sucrose in to glucose and fructose. Cw-Inv increased in both non-host/host pathogen-inoculated leaves compared to mock-inoculated (Fig 3.9A). Highest activity of cw-Inv detected at 24 hpi in both the interactions and then gradually decreased in both the interactions.

3.2.11.2. Total SS content

Carbohydrate content in the leaf tissue increased in tomato leaves inoculated with non-host and compatible pathogens. Accumulation of SS was detected at 96 hpi in *M. grisea*-challenged leaves and at 48 hpi in *A. alternata* f. sp. *lycopersici*-inoculated leaves compared to mock-inoculated leaves (Fig 3.9B).

3.2.11.3. SS composition in AF

To assess the changes in the soluble sugars in the apoplast during non-host and compatible interactions, time-course experiments were conducted. Sucrose, glucose and fructose were the main sugars present in the AF obtained from mock-inoculated tomato leaves (Fig 3.10). None of these sugars was identified in the AF of tomato leaves inoculated with non-host and compatible pathogen throughout the time-course i.e. from 12 hpi to 96 hpi. The chromatogram shows the absence of sucrose, glucose and fructose in the apoplast of tomato leaves inoculated with both the pathogens.

3.3. Apoplast proteome analysis in tomato during non-host and compatible interactions

The apoplast represents a highly dynamic compartment serving as a continuum from roots through the stem to leaves and is potentially important as a bridge that perceives and transduces signals from the environment to the symplast. Proteins secreted into apoplast might contribute to biotic and abiotic stress response as a first line of defense. To elucidate the role of these secreted proteins during NHR, proteomic analysis was done using 2-DE and identified using MALDI-TOF-MS.

3.3.1. Purity assessment of IBP and AF fractions

The AF and IBP fractions were extracted from pathogen- and mock-inoculated tomato leaves for proteomic analysis. Cytosolic contamination, if any, in the AF was evaluated by SDS-PAGE analyses and also measuring of MDH activity (described in section 3.2.1).

The cytosolic contamination in the IBP fraction was quantitatively evaluated by three different approaches. A quantitative marker for cytosolic contamination, catalase, was assayed in TSP and IBP extracts immediately after extraction. Cytosolic contamination was calculated as the percentage CAT activity in the IBP compared with the activity in the TSP. The IBP showing a value of ≤0.5% was accepted for further analyses (Fig 3.11A). The presence of the subunits of

Rubisco, the major intracellular protein in leaf tissues, was examined in IBP fraction by SDS-PAGE analysis. Representative TSP and IBP SDS-PAGE patterns were compared (Fig 3.11B). The band corresponding to the Rubisco large subunit (~50 kDa) was detected in TSP, but was absent in the IBP fraction.

Western blot analysis of fructose 1,6-bis phosphatase, a marker for cytosolic contamination, in TSP, AF, and IBP fractions was performed using polyclonal antibodies. Band corresponding to fructose 1,6-bis phosphatase was detected in TSP and absent in AF and IBP fractions after staining with NBT (Fig 3.11C). Thus, contamination of AF and IBP fractions with other cytosolic proteins was considered nil or negligible.

3.3.2. Comparative 2-DE analysis of AF proteins

Changes in abundance of the AF proteins in tomato leaves during non-host and compatible interactions were analyzed by 2-DE. The AF proteins displayed on 2-D gels were relatively small and mostly distributed in the range of pH 3-10 and 10-100 kDa range (Fig 3.12A). Approximately, 56 protein spots were detected on colloidal coomassie brilliant blue-stained 2-D gel. Comparative 2-D gel analysis identified 14 and 10 differentially expressed protein spots during non-host and compatible interactions of tomato, respectively, compared to mock-inoculated leaves (Fig 3.12B).

Of these protein spots, the spot no's 150, 110, 100, and 72 had no change in expression level in compatible interactions, whereas they were up-regulated in non-host interactions of tomato. Protein spot no. 48 was down-regulated in both the interactions. Four spots (98, 61, 130, and 206) were down-regulated in compatible interactions, whereas they were up-regulated in non-host interactions of tomato. The differentially expressed spots were marked on 2-D gels (Fig 3.12 A). Protein spot no. 97 was down-regulated at 72hpi in compatible interactions but up-regulated in non-host interactions. Protein spot no. 107 was up-regulated at 72hpi in compatible interactions but induced in non-host interactions.

3.3.3. Identification of differentially expressed AF proteins by MS

Differentially regulated protein spots were excised from the 2-D gels, digested with trypsin, and analyzed by MALDI-TOF-MS (Table 3.1). Among the differentially expressed proteins, four protein spots were identified as chitinases (spot no. 98, 107, 110, and 97). Remaining protein spots match with α-amylase/trypsin inhibitor (spot no. 150), lignin-forming anionic peroxidase (spot no. 61), 2-Cys peroxiredoxin BAS1-like (spot no. 130), probable RNA-dependent RNA polymerase 4 (spot no. 34), ACC oxidase homolog 1 (spot no. 48), hypothetical protein (spot no. 101), unknown (spot no. 100), ribulose-phosphate 3-epimerase (spot 206), ferredoxin-thioredoxin reductase (spot 73), and G-type lectin S-receptor-like ser/thr-protein kinase (spot 72). The differentially expressed proteins could be classified into different functional classes like defense (chitinases and α-amylase/trypsin inhibitor), oxidative reductases (lignin-forming anionic peroxidase, ferredoxin-thioredoxin reductase, and 2-Cys peroxiredoxin BAS1-like), signalling (G-type lectin S-receptor-like serine/threonine-protein kinase), proteins with unknown function (probable RNA-dependent RNA polymerase 4 and ACC oxidase homolog 1), and unknown category (hypothetical protein, and unknown protein) (Fig 3.13A and B).

3.3.4. Comparative 2-DE analysis of IBPs in apoplast

Changes in the abundance of IBPs in the pathogen-inoculated tomato leaves were compared with mock-inoculated leaves by 2-DE. The AF proteins detected on 2-D gels were mostly distributed in the range of pH 4–10 and 10–100 kDa (Fig 3.14A). Approximately, 62 protein spots were detected on colloidal coomassie brilliant blue-stained 2-D gel. Comparative 2-D gels analyses identified 12 and 10 differentially expressed protein spots during non-host and compatible-interactions, compared to mock-inoculated tomato leaves (Fig 3.14B).

Of these protein spots, three spots (788, 1050 and 856) were induced in non-host and compatible interactions, whereas three spots (624, 563, and 569) were suppressed in both the interactions of tomato. Four spots (1009, 788, 1050 and 816) were induced in non-host interaction but suppressed in compatible interactions of tomato. Two spots (1184 and 1588)

did not show change in compatible interactions but were differentially expressed in non-host interactions.

3.3.5. Identification of differentially expressed apoplastic IBPs by MS

Differentially expressed protein spots were excised from the 2-D gels, digested with trypsin, and analyzed by MALDI-TOF-MS for identification (Table 3.2). Among the differentially expressed proteins, two protein spots each were identified as glucanases (spot no. 856, and 788), and NAD(P)H-quinone oxidoreductase (spot no. 981 and 1588). Remaining protein spots were identified as endochitinase (spot no 972), ferredoxin--NADP reductase (spot no.624), lignin-forming anionic peroxidase (spot no. 816), putative callose synthase (spot no. 1009), formin-like protein 12 (spot no. 1184), Ca^{2+}/calmodulin-dependent ser/thr-protein kinase (spot no. 1050), binding / catalytic/ coenzyme binding (spot no. 563), and oxygen-evolving enhancer (OEE) protein 2 (spot no. 569). The differentially expressed apoplastic IBPs were classified into different functional classes like defense (endochitinase, glucanase, and callose synthase), oxidative reductase (lignin-forming anionic peroxidase, ferredoxin-thioredoxin reductase, ferredoxin-NADP reductase, and NAD(P)H-quinone oxidoreductase), signalling (formin-like protein 12, and Ca^{2+}/calmodulin-dependent ser/thr-protein kinase) and proteins with unknown function (binding / catalytic/ coenzyme binding, and oxygen-evolving enhancer protein 2) (Fig 3.15A and B).

3.4. Transcriptional changes in tomato during non-host and compatible interactions

To obtain more details of the mechanism underlying the NHR, we studied the transcriptome changes in tomato against inoculation with *M. grisea* and *A. alternata* f. sp. *lycopersici*. We used the GeneChip ® Tomato Genome Array (Agilent) to measure and compare the difference in transcript accumulation (44, 000 probes) between pathogen- and mock-inoculated tomato leaves at 6 and 24 hpi. Two independent replications of the experiment were conducted. All GeneChip data were analyzed using GeneSpring GX v11.5 software.

Tomato genes that were differentially regulated (more than one-fold change with a $P \leq 0.01$) upon inoculation with both the pathogens were identified at 6 and 24 hpi, using samples harvested at 0 hpi as reference samples, to which the samples from all other time points were compared. In the compatible interaction, the number of up-regulated genes were 1525, 2815 genes at 6 and 24 hpi, respectively, while the down-regulated genes were 1614, 3263 genes at 6 hpi and 24 hpi, respectively (Fig 3.16A). In non-host interactions of tomato, the number of genes up-regulated genes amounted to 1713 and 1709 at 6 and 24 hpi, respectively. The number of genes down-regulated was 1856 and 1620 genes at 6 and 24 hpi, respectively (Fig 3.16A).

Often, similar gene sets were induced during different plant-pathogen interactions like compatible, incompatible and non-host interactions albeit the regulation occurs with different rates and amplitudes. To account for temporal variation, differentially regulated genes, monitored at different time points, were pooled one gene set for the compatible and one set for non-host interactions at a single time point. Roughly two-thirds of the regulated genes overlapped between the interactions (Fig 3.16B).

Even though tomato genome sequence was available, information regarding gene annotations was limited, for many tomato genes the function is still unknown. Thus, obtaining insight into the underlying biology for microarray experiments is difficult. A starting point to explore poorly characterized genes and predict plant protein function is the use of Gene Ontology (GO) annotation (the gene ontology consortium), a controlled formal vocabulary that consists of general terms for gene and protein annotations in any organism, making comparisons of taxa possible. Therefore, we performed an orthology prediction (majorly with *Solanum tuberosum*) for all gene probe sets that can be probed by the GeneChip and were able to assign GO annotation to all genes identified as differentially regulated transcripts. These were used to identify the major differentially regulated biological processes, showing that most differentially regulated genes belong to the categories carbohydrate metabolism, photosynthesis, transport, transcription, defense, stress, cell wall, lipid metabolism and signal transduction (Fig 3.17). Among the nine functional categories, more number of transcripts belong to “transport” and

"transcription". Genes responding to infection and related to defense, signaling, secondary metabolism, cell wall, gene regulation, and oxidative stress will be further discussed as genes in these categories are of greater importance in determining the outcome of disease development, with special reference to NHR.

3.4.1. Carbohydrate metabolism

Carbohydrate metabolism-related genes were severely down-regulated in tomato leaves inoculated with non-host and compatible pathogens compared to mock-inoculated leaves. Transcripts encoding for enzymes involved in starch biosynthesis like starch synthase 1, starch synthase 2 and starch branching enzyme II were down-regulated in both the interactions (Table 3.3). Transcripts encoding for enzymes involved in starch degradation like starch-granule-bound R1 protein, α-amylase, and α-glucosidase were up-regulated in both the interactions. Transcripts encoding for enzymes involved in sucrose catabolism like sucrose-phosphate-synthase, apopalstic invertase, acid invertase and β-fructofuranosidase were up-regulated in both the interactions. Most of the genes involved in glycolysis pathway like glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, phosphoglycerate kinase and enolase were down-regulated. Transcripts encoding Calvin cycle enzymes were also down-regulated (Table 3.3). Photosynthesis-related genes were also severely down-regulated in both the interactions except respiratory burst oxidase protein (rbohF) (Table 3.4). Transcripts encoding for PsaA and psaB, which are main subunits of photosystem 1 were down-regulated in both the interactions. Transcripts encoding for light-harvesting complex (LHC) like chlorophyll a,b binding protein type I, chlorophyll a/b binding protein Lhcb1-4, and chlorophyll a/b binding protein were down regulated (Table 3.4).

3.4.2. Transporters

Transcripts encoding genes involved in transportation in regular cellular processes were differentially regulated in both the interactions. Transcripts annotated as hexose transporters (HXT) like HXT1 and HXT3 were up-regulated in both the interactions (Table 3.5). Sucrose transporter (SUT1), polyol transporter 6 and plastidic ATP/ADP-transporter were also up-

regulated in both the interactions (Table 3.5). Transcripts encoding glucose-6-phosphate/phosphate translocator were significantly down-regulated in compatible interactions (-10.00-fold) compared to non-host interactions (-7.66-fold) at 24 hpi. Transcripts encoding triose phosphate/phosphate translocator, and ammonium transporter were down-regulated in both the interactions (Table 3.5B). Transcripts encoding for transmembrane ATPase transporters like ATP synthase subunit a, H(+)-transporting ATPase, and ATPase-like were down-regulated in both the interactions (Table 3.5).

3.4.3. Defense

Differentially regulated transcripts in the defense category included several genes annotated as being related to fungal cell-wall degradation, PR proteins, and putative *R*-genes. Transcripts related to different classes of *PR*-genes increased in abundance in both the interactions (Table 3.6). Among these genes, the protein family PR-5 (osmotin or thaumatin-like proteins) was highly up-regulated (13-fold) in both the interactions at 6 hpi and maintained till 24 hpi. PR-1 was relatively in abundance during non-host interactions, compared to compatible interactions at 6 hpi. There were no significant differences in expression of other *PR*-genes in both the interactions. In addition, transcript annotated as 9-divinyl ether synthase, which is involved in the biosynthesis of the anti-fungal and anti-bacterial toxins colneleic acid and colnelenic acid, was up-regulated (8-fold) in both the interactions.

Transcripts of wound-induced proteins like WIN1 and WIN2 were up-regulated in both the interactions. WIN2 was highly up-regulated in compatible interactions (7.20-fold) compared to non-host interactions (4.43-fold) at 6 hpi but expression levels were same at 24 hpi in both the interactions. Transcript encoding xyloglucan specific endoglucanase inhibitor 3 was up-regulated from 6 hpi (2.28-fold) to 24 hpi (3.44-fold) in non-host interactions, and down-regulated in compatible interactions from 6 hpi (2.31-fold) to 24 hpi (2-fold). Avr9/Cf-9 rapidly elicited protein 75 and MLO1 protein were also up-regulated in both the interactions (Table 3.6). Transcripts annotated as possible *R*-genes were differentially regulated during non-host and compatible interactions. *R*-genes belonging to coiled coil nucleotide binding site-leucine

rich repeat (CC-NBS-LRR) (two transcripts), as well as mildew resistance locus O 1(MLO1) were up-regulated (Table 3.6). Transcript encoding ss-galactosidase was significantly up-regulated in compatible interactions (6.55-fold) compared to non-host interactions (4.63-fold).

Transcript encoding cystein protease was up-regulated significantly at 6 hpi (5.25-fold) and decreased at 24 hpi (3.21-fold) in non-host interactions. But, in compatible interactions expression levels increased from 6 hpi (4.31-fold) to 24 hpi (5.07-fold). Transcript encoding multicystatin (putative cystein protease inhibitor) was down-regulated significantly at 6 hpi in non-host interactions (-8.40-fold) compared to compatible interactions (-6.48-fold). Transcript encoding metallocarboxypeptidase inhibitor was highly down-regulated from 6 hpi (-6.20-fold) to 24 hpi (-8.48-fold) in compatible interactions, but in non-host interactions, this transcript was up-regulated from 6 hpi (-5.90-fold) to 24 hpi (-4.89-fold). Other protease inhibitors like aspartic protease inhibitor, proteinase inhibitor type-2, and chymotrypsin inhibitor were also down-regulated in both the interactions.

Transcript encoding endo-β-1,4-glucanase was highly down-regulated in compatible interactions from 6 hpi (-3.51-fold) to 24 hpi (-2.15-fold) compared to non-host interactions, where no change was observed from 6 hpi (-2.16-fold) to 24 hpi (-2.15-fold). *R*- gene-like transcript was down-regulated significantly high in compatible interactions (-8.00-fold) compared to non-host interactions (-5.63-fold).

3.4.4. Transcription factors

Several transcripts corresponding to transcription were differentially regulated in both the interactions. The transcriptional factors belonging to different families, like WRKY, MYB and NAM/NAC factors were differentially regulated. WRKY transcription factor 71, WRKY protein, MYBAS1, NAC2 and NAC domain protein were up-regulated in both the interactions (Table 3.7A). Transcript annotated as CUP-SHAPED COTYLEDON3 (CUC3) which encodes a putative NAC-domain transcription factor was highly up-regulated in both the interactions. We also found that the transcripts encoding ethylene response factors (ERF), EIL3, APETALA2-like

protein MADS transcriptional factor, C2H2-type zinc finger protein were up-regulated in both the interactions.

Aux/IAA (IAA) proteins are short-lived transcriptional factors that function as repressors of early auxin response genes at low auxin concentrations and involved in auxin-mediated signaling pathway. Different Aux/IAA proteins like IAA11, IAA7 and IAA2 were down-regulated in both the interactions. Along with these, MADS-box proteins 5, AGAMOUS-like MADS-box protein and Dof zinc finger protein were down-regulated in both the interactions (Table 3.7B).

3.4.5. Stress

Transcripts encoding different peroxidase proteins were up-regulated in both the interactions. Transcripts encoding glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase were also up-regulated in both the interactions (Table 3.8). Transcript encoding ascorbate oxidase was highly up-regulated in both the interactions at 6 hpi. Transcripts encoding genes involved in abiotic stress like trehalose-6-phosphate synthase and proline dehydrogenase were up-regulated in both the interactions. Other transcripts annotated as genes in stress conditions like polyphenol oxidase D, catalase and stress-associated protein 3 were up-regulated in both the interactions. Transcripts encoding different heat shock proteins (HSP) like HSP 90, HSP70, HSP 40, ethylene-responsive hsp and 60 kDa chaperonin were down-regulated in both the interactions (Table 3.8).

3.4.6. Cell wall

Among cell wall-modifying enzymes, transcripts for proteins like cellulose synthase (CesA2 and CesA4), putative xyloglucan endotransglycosylase, xyloglucan endotransglucosylase-hydrolase (XTH3, XTH6), and pectin methylesterase 3 were up-regulated (Table 3.9). Seven transcripts encoding for expansins, which are involved in loosening of cell wall, CesA1, CesA3, XTH4 and XTH6 were down-regulated in both the interactions. Along with these genes, glycine-rich protein and β-mannosidase enzyme were also up-regulated in both the interactions.

3.4.7. Signal transduction

Several genes involved in signal transduction events were differentially expressed in both the interactions such as receptor kinases, protein kinases and calcium-mediated signal transduction proteins. A transcript encoding a wall-associated kinase-like protein (WLK) was up-regulated in both the interactions. Genes encoding mitogen-activated protein kinase (MAPK) pathway like MAP3K-like protein kinase, MAPKK, MKK4 and MKK4 were up-regulated in both the interactions (Table 3.10). The SNF1 kinase complex anchoring protein was up-regulated in both the interactions and is well characterized as one of the major components in sugar signaling. In our current study, Ca^{2+}-dependent protein kinases like CDPK4, CDPK and calmodulin 5/6/7/8-like protein were up-regulated in both the interactions (Table 3.10).

An increase in transcript accumulation following inoculation with both the pathogens was observed for many genes associated with hormone metabolism, such as genes encoding enzymes involved in the JA biosynthesis pathway, including a lipoxygenase (LOX), and an allene oxide synthase (AOS and AOS2). Other transcripts encoding the 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) and ACC synthase that are involved in the ethylene biosynthesis pathway were induced in both the interactions. Several other plant hormone-related genes also responded to infection. Transcript encoding a gene related to ABA synthesis was down-regulated. Gibberellic acid synthesis-related genes like gibberellin 20-oxidase-1, gibberellin 7-oxidase and gibberellin 2-oxidase 1 were down-regulated in both the interactions (Table 3.10). Transcripts encoding for enzymes involved in lipid signaling pathways like phospholipases (PLDa2, PLC3 and PLC2) were up-regulated at early hours of interactions (Table 3.10).

3.4.8. Fatty acid metabolism

Transcripts encoding genes involved in oxylipin biosynthesis were differentially regulated in both the interactions. Transcripts encoding linoleate 9S-lipoxygenase 2 and linoleate 13S-lipoxygenase 2-1 and alpha-DOX1 were up-regulated in both the interactions. Linoleate 13S-lipoxygenase 3-1 and probable linoleate 9S-lipoxygenase 5 were down-regulated in both the

interactions. Transcripts encoding 3-ketoacyl-CoA synthase fatty acid hydroperoxide lyase were down-regulated in both the interactions (Table 3.11).

3.4.9. Secondary metabolism

The phenylpropanoid biosynthetic pathway is the most important pathway which is involved in biosynthesis of secondary metabolites like isoflavinoid, flavonoid and anthocyanins. Phenylalanine ammonia-lyase (PAL) is a key biosynthetic catalyst in phenyl propanoid pathway. PAL catalyses the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid. The transcript related PAL1 was down-regulated in both the interactions (Table 3.12).

Transcripts belonging to the flavonoid synthesis were differentially expressed in abundance. For example, the transcripts of flavonoid 3',5'-hydroxylase, flavanone 3 β-hydroxylase and flavonol synthase increased in abundance in both the interactions. But, gene that belongs to isoflavanoid biosynthesis like isoflavone reductase (IFR) was down-regulated. This study revealed that genes related to lignin metabolism were differentially expressed. Cinnamoyl CoA reductase, the enzyme responsible for diverting the phenylpropanoid pathway into the branch of lignin synthesis was down-regulated. The cinnamoyl CoA reductase gene decreased in both the interactions. In addition, other genes involved in the lignin synthesis pathway increased clearly in abundance in both the interactions such as caffeoyl-CoA-O-methyltransferas, 4-coumarate-CoA ligase 1, and HMG coenzyme A synthase.

Genes involved in alkaloid biosynthesis (tropinone reductase II, tropinone reductase I and monoterpene synthase 1) and carotenoid biosynthesis (lycopene β-cyclase, lycopene epsilon-cyclase, and phytoene synthase) were down-regulated in both the interactions (Table 3.12). Transcripts encoding for N-hydroxycinnamoyl-CoA:tyramineN-hydroxycinnamoyl transferase (THT1-3, THT7-1 and THT7-8) were up-regulated in tomato leaves inoculated with non-host and comaptible pathogens compared to mock-inoculated leaves. These three enzymes are known to be involved in synthesis of antimicobial compounds like *P*-coumaroyl tyramine (CT) and feruloyl-CoA-tyramine (FT).

3.4.10. Amino acid metabolism

Transcripts encoding aromatic amino acid decarboxylase (AADC1A, 1B, and 2), which mediates the biosynthetic pathway of volatile compounds like 2-phenylethanol, were up-regulated in both the interactions (Table 3.13). Transcripts encoding asparagine synthetase and arginase 2 were up-regulated in tomato during non-host and compatible interactions. These enzymes are reported to catalyze conversion of phenylalanine to phenethylamine and tyrosine to tyramine, respectively. Transcripts encoding glutamine synthetase, glycine dehydrogenase and arginine decarboxylase were down-regulated in both the interactions (Table 3.13).

3.4.11. Transcripts differentially regulated either in non-host or in compatible interactions

Some of the transcripts were differentially regulated in non-host interactions of tomato. For example, transcript encoding WRKY-type DNA binding protein was up-regulated (2.5-fold) at 24 hpi in non-host interactions (Table 3.14) but this transcript was suppressed (-2.38-fold) at 24 hpi in compatible interactions (Table 3.15). Transcripts encoding for branched chain α-keto acid dehydrogenase E1-α subunit (-2.48-fold) and dehydrin-like protein (-2.00-fold) were down-regulated in non-host interactions at 6 hpi. Transcripts like cytochrome c oxidase subunit 5C and S-adenosyl methionine decarboxylase were marginally induced i.e. less than 2-fold. Transcripts encoding 3-isopropylmalate dehydrogenase and CC-NBS-LRR protein were down-regulated more than 2-fold in non-host interactions. Transcripts encoding ABA 8'-hydroxylase CYPA3 variant 1 and phosphoglycerate kinase 4 protein were marginally down-regulated i.e. less than 2-fold in non-host interactions (Table 3.14).

Transcripts encoding peroxisomal acyl-CoA oxidase 1A (2.13-fold) and proline transporter 2 (1.60-fold) were up-regulated at 24 hpi in compatible interactions. Transcript encoding putative disease resistance protein was down-regulated (-3.40-fold) in compatible interactions at 24 hpi. Transcripts encoding auxin response factor 4 and TSW12 (non-specific lipid-transfer protein 1) (-2.72-fold) protein were down-regulated in compatible interactions.

Discussion

4.1. Plants are not a feast to pathogens

Plants are constantly exposed to several pathogenic micro-organisms, but, disease rarely develops from these contacts. In other words, resistance appears to be the rule in plants, and susceptibility to infection is an exception. The natural and innate resistance present in all plants against a majority of the pathogens is called as NHR. To understand the mechanisms involved in NHR, we selected a non-host pathosystem, a dicot model crop plant (tomato) and a strict monocot pathogen (*M. grisea*). For a comparative study, we selected a dicot pathogen (*A. alternata* f. sp. *lycopersici*) that infects tomato.

Apoplast is an extracellular space in plant cell and a connecting information center between cytosol and the external environment. To explore the role of apoplast in NHR, we conducted some of the biochemical and molecular (proteomic and transcriptome) analyses. This thesis majorly emphasizes on apoplastic antioxidative defenses, proteome analysis and changes in transcriptome during non-host and compatible interactions.

4.2. Histological characterization of non-host system

We performed histological characterization of non-host pathosystem involving the model crop plant tomato and the economically important fungal pathogen *M. grisea*. The epidermal cells challenged by fungal attack begin to synthesize callose and form papillae. The formation of papillae represents a physical defense response, and it is the fastest response before the integrity of host cells is threatened (Narusaka et al 2005). These defense responses are non-specific. Therefore, the defense response to *M. grisea* is non-host penetration resistance. Apart from callose deposition, induced defense responses, including elevation of ROS and exhibition of hypersensitive cell death were observed during non-host interactions of tomato. Such defense response is often induced in response to a pathogen stimulus, due to recognition and perception of structural and chemical feature of the pathogen or damage to the plant cell wall and plant plasma membrane is associated with the invasion.

4.3. Antioxidative responses of apoplast

One of the earliest responses of the plants to an attempted infection is the production of ROS. Increased production of ROS during incompatible and non-host interactions induce cell death and restrict further growth of pathogen in plants. But, in plant-necrotroph interactions, accumulation of ROS induces cell death and favours growth of pathogen in leaf tissues, suggesting the contrasting effects of ROS in different plant-pathogen interactions and differential effects of individual ROS (Barna et al 2012). We made an attempt to understand the dynamics of generation of ROS and antioxidant enzyme activities to understand their role(s) in non-host (*M. grisea*) and compatible (*A. alternata* f. sp. *lycopersici*) interactions of tomato. Patykowski and Urbanek (2003) reported the enzymatic activities involved in generation and metabolism of H_2O_2 in tomato apoplast against *Botrytis cinerea*.

4.3.1. Differential role of ROS in host- and non-host interactions

Extracellular accumulation of ROS such as $O_2^{\cdot-}$, •OH and H_2O_2 occurs during biotic and abiotic stress responses of plants. Accumulation of ROS and increase in the activitiy of antioxidant enzymes was observed in tomato leaves challenged with *M. grisea* and *A. alternata* f. sp. *lycopersici*. Extracellular $O_2^{\cdot-}$ levels increased rapidly at early hours of inoculation with *A. alternata* f. sp. *lycopersici*, and late hours of inoculation with *M. grisea* in tomato plants, as reported in tomato cotyledons treated with *Cladosporium fulvum* race specific elicitors (May et al 1996). Accumulation of extracellular H_2O_2 was observed in both non-host and host pathogen-challenged leaves. Non-host defenses of lettuce to *Pseudomonas syringae* pv. *phaseolicola* were associated with increased peroxidase, H_2O_2 accumulation and cell death (Bestwick et al 1998). High accumulation of H_2O_2 during non-host interactions of cereals against inappropriate powdery mildew induced cell death and penetration failure of fungal spores. In contrast, accumulation of $O_2^{\cdot-}$ was associated with successful penetration of fungal spores in infected cells (Trujillo et al 2004), suggesting the complex role of ROS in execution of NHR. •OH accumulated at early hours of inoculation during non-host interactions, whereas in compatible interactions accumulation of •OH occurred at late hours of inoculation. The results of our study

corroborate with the observations of Patykowski and Urbanek (2003) who reported accumulation of ROS during tomato-*Botrytis cinerea* interactions. Together, the results support the notion that timing of accumulation of ROS is often important than the quantity of ROS during plant-pathogen interactions.

Bolwell et al (1999) and Bindschedler et al (2006) reported that H_2O_2 produced in French bean/ *Colletotrichum lindemuthianum* interactions and *Arabidopsis* in response to a *Fusarium oxysporum* cell wall preparation was derived directly from cell wall peroxidases. Further, Martinez et al (1998) reported the involvement of apoplastic NADH-peroxidase in cotton-*Xanthomonas campestris* pv. *malvacearum* incompatible interactions in production of $O_2^{\cdot-}$. In *M. grisea*-challenged leaves, NADH-peroxidase was high compared to *A. alternata* f. sp. *lycopersici*-challenged leaves in early hours of inoculation. But, at late hours NADH-peroxidase activity increased in tomato-*A. alternata* f. sp. *lycopersici* interactions also. Thus, the accumulation of extracellular H_2O_2 correlates to increase in oxidation of NADH in apoplast. Increased NADH oxidation in the apoplast of *Prunus* species against plum pox virus was reported to be due to NADH-peroxidase and not NAD(P)H oxidase (Diaz-Vivancos et al 2006), and in the apoplast of tomato against *B. cinerea* (Patykowski and Urbanek 2003).

Oxidative stress caused by high accumulation of ROS during plant-pathogen interactions results in oxidative degradation of membrane lipids and carbonylation of proteins which finally lead to cell death. Increased $O_2^{\cdot-}$ during compatible interactions resulted in high lipid peroxidation and protein carbonylation compared to non-host interactions of tomato. Apparantly the ROS levels were regulated by strong antioxidant system in non-host interactions, but not in compatible interactions of tomato. ROS induced cell death restricts the growth of *M. grisea* but favors the growth of *A. alternata* f. sp. *lycopersici* on tomato. Hernandez et al (2001) reported that carbonylation of apoplast proteins during salt stress was more in salinity stress-sensitive pea cultivar compared to tolerant cultivar. The effect of oxidative stress during biotic stress was reported in plant-pathogen interactions (Hakmaoui et al 2012; Mittler et al 1999).

4.3.2. Active antioxidant system in non-host interactions of tomato

Antioxidant enzymes like SOD, PRX and APX are involved in regulation of ROS levels in plant tissues. SOD was reported in the apoplast from pine needles (Streller et al 1994), spinach (Ogawa et al 1994), tomato (Patykowski and Urbanek 2003), *Prunus* (Diaz-Vivancos et al 2006), and in monocots like oats and barley (Vanacker et al 1998). Hernandez et al (2001) reported that only SOD was present in the apoplastic space of pea leaves, whereas APX and PRX seemed to be absent. In the present study, SOD activity increased in both non-host and compatible interactions. SOD activity was significantly high in *M. grisea*-challenged leaves compared to *A. alternata* f. sp. *lycopersici*-challenged leaves. Consequently, the capacity to eliminate $O_2^{\cdot-}$ in the apoplast of tomato leaves decreased during compatible interactions compared with non-host interactions. SOD is an H_2O_2-generating enzyme that might contribute to the observed increase in the apoplastic H_2O_2 during compatible interactions. The low activity of SOD in tomato-*A. alternata* f. sp. *lycopersici*-interactions allowed high accumulation of superoxide radicals which lead to more damage to membranes and resulted in susceptibility. In contrast, high SOD activity in *M. grisea* inoculated leaves showed high concentration of H_2O_2 and conferred resistance to plants. In pathogen inoculated barley leaves increased SOD contributed to the synthesis of H_2O_2 during the oxidative burst to prevent accumulation of $O_2^{\cdot-}$ (Vanacker et al 1998). A role for apoplastic SOD was also described in the regulation of H_2O_2 required for the development of the secondary cell wall (Karlsson et al 2005).

Both APX and PRX use H_2O_2 as electron acceptor, and therefore compete for the same substrate. APX scavenges H_2O_2 using ascorbate as reductant and has high affinity to H_2O_2 than PRX. APX could be responsible for H_2O_2 detoxification, preventing its accumulation in the apoplastic space when produced in excess (De Pinto and de Gara 2004). Ros Barcelo´ et al (2006) reported APX and PRX activities in the apoplast of *Populus alba* and *Citrus aurantium*, respectively. But, both these enzymes were not found in the apoplast of *Zinnia elegans* or *Eucalyptus camaldulensis*. In the same way, absence of APX activity in apoplast of Norway spruce needles was reported by Polle et al (1990). PRX was involved in plant defense responses like lignification, stiffening of the cell wall, detoxification of ROS and defense against pathogen

penetration. In the present study, PRX activity consistently increased in non-host interactions compared to compatible interactions. High PRX activity in non-host interactions resulted in reduction of H_2O_2 concentration. Increase in APX activity at early hours was observed in compatible interactions of tomato than in non-host interactions of tomato. APX activity increased at late hours (96 hpi) of inoculations in non-host interactions. Similar results were observed in apoplast of tomato during the interactions with *Botrytis cinerea*, where APX activity increased at 48 and 72 hpi (Patykowski and Urbanek 2003). To avoid oxidative damage, apoplastic H_2O_2 must be under the control by the antioxidant mechanisms present in the apoplast, including APX and PRX (Polle et al 1990; Hernandez et al 2001). Therefore, increased antioxidant capacity of tomato in apoplast during non-host interactions might regulate ROS levels.

The quinones produced by oxidation of phenolics can undergo one-electron reduction, forming semiquinone radicals that can produce $O_2^{\cdot-}$ and H_2O_2 (Appel et al 1993). PPO is also one of the enzymes that catalyze the oxygen-dependent oxidation of phenols to quinones. Increased PPO activity at late hours (96 hpi) and concomitant decrease in total phenolic content compared to non-host interactions could correlate to constant high level of H_2O_2 in compatible interactions. The rapid synthesis and early accumulation of phenolics during non-host interactions, compared to compatible interactions, could correlate to defense responses of tomato against *M. grisea*. Increase in PPO activity was observed in both susceptible and resistant cultivar of apricot but increase in PPO activity was to a lesser extent than in the susceptible cultivar against plum pox virus (Hernandez et al 2001). An increase in extracellular proteolytic activity was reported in wheat leaves inoculated with *Septoria tritici* (Segarra et al 2002). In the present study, although there was no change in proteases activity, during initial hours of interactions in both non-host and compatible interactions, there was an increase in protease activity in *M. grisea*-inoculated leaves at 96 hpi compared to *A. alternate* f. sp. *lycopersici* challenged leaves.

4.4. Changes in carbohydrate metabolism

Sugars are involved in many metabolic and signalling pathways in plants. Sugar signals may also contribute to immune responses against pathogens and probably function as priming molecules leading to PAMP-triggered immunity and effector-triggered immunity in plants. Pathogen modulates host carbohydrate metabolism and affects source to sink status. This change is often accompanied with an increase in cw-Inv activity. In our present study, increased cw-Inv activity was detected in tomato leaves challenged with non-host and compatible pathogens. As per reports, cw-Inv activity also increases in compatible interactions (Fotopoulos et al 2003; Chou et al 2000) but quantitatively more rapid in the incompatible interaction. The increase in cw-Inv activity functions to cleave sucrose into glucose and fructose in apoplast. So, we measured the concentrations of hexoses (sucrose, glucose and fructose) in the tomato leaf apoplast, using HPLC, after inoculation with non-host and compatible pathogens. None of these soluble sugars was detected in pathogen-inoculated tomato leaves but in mock-inoculated tomato leaves, soluble sugars were detected till late hours of post inoculation. The content of SS in leaf tissue increased in non-host and compatible interactions of tomato. The reason for this, hexoses in apoplast transported into the cell by hexose transporters where they are believed to fulfill the energy and carbon requirements for the resistance response (Truernit et al 1996). This transport also reduces hexose concentration in the apoplast, thereby reducing potential nutrients for apoplast-colonizing pathogens (Fotopoulos et al 2003).

4.5. Apoplastic protein analysis

A growing number of extracellular proteins are believed to play a crucial role in plant defense against microbes. These proteins include several of the well known PR proteins that directly interact with pathogens, such as chitinases and endo-β-1,3-glucanases. However, plants also deploy a repertoire of proteins in the wall that act as a surveillance system to allow the early detection of an impending pathogen assault. It is important to note that protein numbers and composition of cell wall proteomes depend, among others, on the extraction procedure. Here, AF and IBP fractions were extracted from tomato leaves yielding protein patterns partially

overlapping with those of previous studies applying infiltration and exudation techniques (Haslam et al 200).

4.5.1. Apoplastic proteins in tomato leaves

In our study, 14 differentially expressed proteins were identified during non-host and compatible interactions. These 14 proteins were functionally annotated and classified into as proteins involved in defense responses, oxidoreductases, proteins with unknown function in apoplast and unknown. Defense-related proteins are more abundant and more diverse in the apoplast proteome (van Loon et al 2006). In our study, four of the up-regulated protein spots were identified as endochitinases (both basic and acidic). Ndimba et al (2003) reported that basic endochitinase was phosphorylated in *Arabidopsis* cell suspension cultures treated with fungal elicitors and suggested that signal transduction cascade events are present in apoplast. Endochitinases belong to glycosyl hydrolase 19 (GH19), chitinase class 1 family and have been shown to possess antifungal activity. Cell walls of various fungi contain chitin and β-1,3 or β-1,6-glucan. Chitinases and β-1,3-glucases produced by plants can be secreted into apopalst and inhibit fungal growth by degrading their cell wall. More rapidly, induction of various classes of chitinases is also reported during an incompatible *Cladosporium fulvum*-tomato interaction than during a compatible interaction (Wubben et al 1996).

In the present study, lignin-forming anionic peroxidase was up-regulated in apoplast of tomato during non-host interactions of tomato. Plant peroxidases are well-studied PR proteins that belong to the PR-9 family (Swapan and Muthukrishnan 1999). During pathogen invasion, extracellular matrix peroxidases are known to catalyse extensin/polysaccharide cross-linking, lignifications and suberisation of the cell wall. These events lead to the formation of a formidable physical barrier that prevents pathogen penetration and spread. Ndimba et al (2003) also identified induction of peroxidase in *Arabidopsis* cell suspension cultures treated with fungal elicitors. Another peroxidase identified in the present study, 2-Cys peroxiredoxin BAS1-like, is also up-regulated in apoplast of tomato during non-host interactions. This is an

antioxidant enzyme involved in the detoxification of alkyl hydroperoxides with reducing equivalents provided through the thioredoxin system.

We detected that G-type lectin S-receptor-like ser/thr-protein kinase, which belongs to ser/thr protein kinase family, is up-regulated in non-host interactions of tomato but no change in compatible interactions compared to mock-inoculated leaves. This is a receptor-like ser/thr-protein kinase that represses the disease resistance signaling pathway triggered in response to bacterial pathogen such as *Pseudomonas syringae* pv. *tomato* in *Arabidopsis.* Knockout of this gene in *Arabidopsis* resulted in enhanced resistance to the virulent bacterial pathogen *P. syringae* pv. *tomato* accompanied by an increase in PR1 expression (Kim et al 2009). Another secreted protein identified in tomato AF was α-amylase/trypsin inhibitor which belongs to protease inhibitor I6. It could be involved in both insect and fungal defense mechanisms.

4.5.2. IBPs of cell wall from tomato leaves

In the IBps isolated from the tomato cell wall, we identified two glucanases which have anti-fungal activity. These two glucanases were up-regulated in tomato inoculated with non-host and compatible pathogens. It is of interest that glucanases were identified only in IB fraction but not in AF fraction whereas Watson et al (2004) identified chitinases and β-1,3 glucanases ionically attached to the wall, we find chitnases in the IF and glucanases in IBPs. Callose synthase, which is involved in synthesis of callose during plant growth metabolism and defense responses, was up-regulated in non-host interactions of tomato. It is a multi-pass membrane protein and glycosyltransferase 48 family. Induction of callose synthesis is one of the important defense responses in NHR.

In our IBP proteome analysis, we identified formin-like protein which plays a central part in the control of actin organization and dynamics during normal growth. Formin-like protein was up-regulated in non-host interactions of tomato but no change in protein level in compatible interactions. Another signaling related protein, calcium/calmodulin-dependent ser/thr-protein kinase, was up-regulated in non-host and compatible interactions. This protein can mediate

calcium-dependent regulation of gene expression through the phosphorylation of transcriptional activation of proteins.

In our proteomic analysis of IBP fraction, two spots were identified as glucanase which are up-regulated in tomato leaves inoculated with non-host and compatible interactions. In AF fraction, we could not find any glucanases. Some of the IBPs showed partial homology with oxygen-evolving enhancer (OEE) subunits (1 and 3), which have been previously detected in the *Medicago* apoplast (Abbasi et al 2004; Soares et al 2007). In our study, we identified one spot as OEE 2, which is known to be phosphorylated by wall-associated kinase 1 in a manner regulated by the extracellular and glycine-rich protein AtGRP-3. It was suggested earlier that WAK1/AtGRP-3 phosphorylation of OEE2 leads to the induction of defense genes (Yang et al 2003).

4.5.3. Non-canonical proteins in apoplast

Non-canonical proteins are the known intracellular proteins that are present in the apoplast. High proportions, up to 50%, unexpected proteins was reported in many recent cell wall proteomic studies (Chivasa et al 2002; Slabas et al 2004; Watson et al 2004; Soares et al 2007), which cannot easily be explained as contamination. The presence of these proteins (e.g. enzymes of the glycolytic pathway, transcription factors and ribosomal proteins) is puzzling because they do not contain a predicted signal peptide, which is necessary for targeting to the secretory pathway, and do not have an understandable function in the wall. The existance of an unknown export mechanism should not be excluded (Slabas et al 2004). In our present study, we also identified some of the non-canonical proteins like binding / catalytic/ coenzyme binding, ACC oxidase homolog 1, and probable RNA-dependent RNA polymerase 4. Although the purity of cell wall preparations can be checked using marker enzymes or antibodies against known proteins, the analysis tool (MS) is more sensitive than classical biochemical and immunological tests. However, the negatively charged plant cell wall (Shomer et al 2003) is potentially susceptible to additional contamination from charged, symplastic proteins during wall isolation. When non-destructive techniques were used to isolate cell wall proteins (Boudart

et al 2005), only a few non-canonical proteins were found, supporting the idea that they are likely to be contaminants.

4.6. Transcriptome analysis in tomato during host and non-host interactions

We used microarrays to monitor total transcriptional responses of tomato upon inoculation with the non-host (*M. grisea*) and compatible (*A. alternata* f. sp. *lycopersici*) pathogens. Tomato transcriptional responses to compatible and non-host inoculations overlapped substantially. Tao et al (2003) also reported that the mRNA expression patterns associated with the non-host and host defense reactions of *Arabidopsis* are similar. NHR confers robust protection against pathogenic invaders and bears many similarities to host resistance (Heath 2000; Thordal-Christensen 2003). These observations are similar to other transcriptomics studies that compared compatible and incompatible plant-pathogen interactions (Eulgem et al 2004; Navarro et al 2004; Tao et al 2003; Thilmony et al 2006). Two-thirds of the genes that are differentially regulated in the non-host interaction are also differentially regulated in the compatible interaction. This complies with the currently held hypothesis that non-host or incompatible resistance can largely be seen as an accelerated and amplified basal defense response. Several studies have revealed that a substantial number of genes are commonly regulated by different defense/stress signals, including infection with a fungal pathogen, wounding, cold, drought or high salinity (Reymond et al 2000; Schenk et al 2000; Cheong et al 2003).

4.6.1. Regulation of defense-related genes

The type of resistance that operates even in susceptible plants to limit pathogen growth is called as basal resistance. The hallmark of triggering basal resistance defenses is transcriptional accumulation of *PR*-genes. Our microarray data analysis revealed that there are no significant quantitative changes in expression levels of *PR*-genes in tomato leaves inoculated with compatible and non-host pathogens (Tao et al 2003). Galactosidase, which releases the galactose from wall polymers containing β-1,4-D-galactan was highly up-regulated in compatible interactions compared to non-host interactions.

Cysteine proteases are as key enzymes in the regulation of cell death and cysteine protease inhibitors also exists as counterpart to these enzymes to control cell death. In our present study, we reported that cysteine protease was highly up-regulated at early hours of interactions (6 hpi) in non-host interactions compared to compatible interactions. Putative cystein protease inhibitor was down-regulated highly at early hours of inoculation in interactions (6 hpi) in non-host interactions compared to compatible interactions. Solomon et al (1999) reported that plant cell death can be regulated by activity poised between the cysteine proteases and the cysteine protease inhibitors.

4.6.2. Regulation of primary metabolism related genes during defense responses

A majority of the down-regulated genes in tomato, during non-host and compatible interactions, belonged to the growth i.e. photosynthesis and basic metabolism. The repression of photosynthesis-related genes has also been observed in many incompatible host-pathogen interactions (Matsumura et al 2003). Transcripts related to primary metabolism like photosynthesis, glycolysis, Calvin cycle and Krebs cycle were down-regulated in both host and non-host interactions (Fig 4.1), indicating that activation of NHR poses a significant metabolic cost to the plant. This inverse relationship between growth and defense responses has been observed in many cases (Berger et al 2004).

Up-regulation of different sucrose hydrolyzing enzymes like the cw-Inv, acid invertase and vacuolar invertase during both the interactions increases hexoses concentration. These hexoses were transported to interior of the cell through HXT. In the present study, we reported that transcripts encoding HXT1, HXT3, and SUT1 were up-regulated at high levels in tomato. The elevated monosaccharide levels, in turn, signal repression of genes encoding the photosynthetic machinery and activation of defense-related genes (Ehness et al 1997).

Soluble sugars are emerging as signalling molecules in plant resistance responses and induce *PR* gene expression (Gomez-Ariza et al 2007). Collectively, these observations indicate co-ordination of defense responses, including non-host resistance responses, and growth in plants, with metabolic resources shunted to defense responses and away from general metabolism

during pathogen attack. This leads to inhibition of starch synthesis and induces starch degradation which yields glucose to supply carbon skeleton for defense responses. The expression of transcripts required for starch degradation (α-amylase, and α-glucosidase) is significantly elevated in tomato plants inoculated with the pathogens. Increase in starch degradation was also reported in potato-*Erwinia* infection (Stewart et al 1994). Along with starch degradation, sucrose synthesis will increase during stress conditions. The expression of transcripts required for sucrose synthesis (sucrose synthase4, sucrose synthase and sucrose-phosphate synthase) was up-regulated in tomato during non-host and compatible interactions.

Trehalose is another well-known non-reducing sugar that has been shown to partially induce resistance against powdery mildew (*Blumeria graminis* f. sp. *tritici*) in wheat by activation of PAL and peroxidase genes (Reignault et al 2001). In our microarray data, we also detected the induction of transcript encoding treahalose-6-phosphate synthase at high level in tomato leaves inoculated with non-host and compatible interactions. The expression of a putative trehalose 6-phosphate synthase/phosphatase 11 (TPS11) gene increased in *Arabidopsis* plants infected with tobacco mosaic virus (Golem and Culver 2003).

Apart from carbohydrates, fatty acid metabolism will also affect during pathogen attack. During defense responses, LOX involved in synthesis of a number of different compounds with signaling functions (Creelman and Mullet 1997; Parchmann et al 1997), antimicrobial activity (Croft et al 1993; Weber et al 1999), or to the development of the HR (Rusterucci et al 1999). Our microarray data revealed that transcripts encoding 9-LOX and 13-LOX were up-regulated significantly in both the interactions. Rance et al (1998) reported that 9-LOX activity was up-regulated during tobacco-*Phytophtora parasitica* var. *nicotianae* interactions. Two 9-LOX-derived compounds with antimicrobial activity, colneleic and colnelenic acids are synthesized upon pathogen infection in the potato-*P. infestans* interaction (Weber et al 1999). We also reported that divinyl ether synthase gene involved in synthesis of colnelenic acid was up-regulated highly (8-fold) in tomato leaves inoculated with non-host and compatible pathogens.

Fatty acid peroxidation by 9-LOX leads to membrane damage and eventual cell death (Rusterucci et al 1999). In our studies, we have shown that allene oxide synthase (AOS1 and

AOS2) along with 13-LOX which involves in JA synthesis was up-regualted in tomato during non-host and compatible interactions. Activation of phospholipases contributes to production of a potent second messenger phosphatidic acid, which has been shown to modulate the activity of a variety of proteins involved in defense signaling (Legendre et al 1993). In our study, we identified that transcript encoding PLC2 and PCL3 were up-regulated in tomato during non-host and compatible interactions.

4.6.3. Defense signaling pathways

The JA/ET hormones are positive regulators of resistance to necrotrophic fungi (Glazebrook 2001) and may be part of the basal defense response. In our study, we have shown that ET/JA pathway related genes were up-regulated in tomato challenged with non-host and compatible pathogens. The SA signalling does not appear to contribute significantly to NHR of tomato against to *M. grisea*. However, Huitema et al (2003) and Zimmerli et al (2004) suggested that the JA/ET pathway is also activated during non-host interactions. Recent reports revealed that ET involved not only in necrotroph plan-pathogen interactions but also against to biotrophic pathogens like *Plasmodiophora brassicae* which causes clubroot disease in *Arabidopsis* (Knaust and Ludwig-Müller 2013).

The genes involved in degradation of gibberellins, in to non-active gibberellins, like gibberellin 2-oxidase, gibberellin 7-oxidase, and gibberellin 20-oxidase were up-regulated in both interactions of tomato. Lee et al (2004) also reported that gibberellin 2-oxidase was up-regulated during non-host interactions of hot-pepper against *Xag*. This suggests that the deactivation of gibberellin may also be a part of basal defense response.

Negative regulators of auxin signaling like IAA 11, 7, and 2 were highly up-regulated during non-host and compatible interactions of tomato. With respect to the role of auxin in plant defenses, repressing auxin signaling in *Arabidopsis* contributes to resistance against *P. syringae* (Navarro et al 2006), and SA was found to inhibit the growth of pathogens via repression of the auxin-signaling pathway (Wang et al 2007). Auxin produced by *P. syringae pv. savastanoi* appears to be required for the inhibition of plant defenses (Robinette and Matthysse 1990). Our

microarray data suggested that the transcripts encoding cytokinin catabolism (CKX3 and CKX5) were up-regulated in tomato during non-host and compatible interactions. Choi et al (2010) also reported that overexpression of *CKX4* gene in *Arabidopsis* favors fast growth of *Pseudomonas syringae* pv. *tomato* DC3000.

Components of MAP kinase cascades were also differentially regulated in line with the evidence that MAP kinase modules play important roles in plant immunity (Pedley and Martin 2004). In our microarray study, we identified that many transcripts encoding for MAP kinase siganalling cascade (MPK3, 4, 1, MAP7K, WIPK, and MKK4) were up-regulated in tomato during non-host and compatible interactions. The MAP kinase, LeMPK3 was implicated in resistance to *Pseudomonas* and *Xanthomonas* bacterial strains (Ekengren et al 2003; Mayrose et al 2004). The orthologues of tobacco SIPK and WIPK, tomato MPK2 and MPK3, are activated in the AvrPto-Pto system tomato (Pedley and Martin 2005). Tomato MKK4, a member of the D group MAPKKs, for which no function is yet known in any other species, also induced cell death and, like the tomato orthologue of the *N. benthamiana* MAPKKKa, can activate MPK2 and MPK3.

4.6.4. Regulation of transcriptional factors

The WRKY superfamily plays a central role in defense regulation by acting as positive or negative regulator (Eulgem and Somssich 2007; Pandey and Somssich 2009). Our micro array data revealed that WRKY transcription factors 2, and 71 were up-regulated in non-host and compatible interactions of tomato. Mohr et al (2010) also reported that WRKY transcription factors regulate expression of surveillance genes at the top of the defense-signaling cascade, including the positive regulation of an *R* gene by one or more WRKY proteins. Different transcripts of NAC/NAM were induced in tomato during non-host and compatible interactions. The NAC are a family of genes specific to plants and play a role in defense and abiotic stress responses as well as in a diverse set of developmental processes. CUP-SHAPED COTYLEDON (CUC) is a part of a larger NAC (for NAM, ATAF, and CUC) protein family of transcription factors was also up-regulated in tomato during non-host and compatible interactions (van Esse et al 2009). Some NAC genes from *Arabidopsis* and the StNAC gene from potato, are induced by

pathogen attack and wounding (Aida et al 1997; Collinge and Boller 2001). The barley NAC gene HvNAC6 was implicated in basal defense against the barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (Jensen et al 2007).

4.6.5. Differential activation of secondary metabolism-related genes

The most striking effects of the activation of the phenylpropanoid pathway are that it produces many secondary metabolites, such as lignins, flavonoids and isoflavonoids (Whitbred and Schuler 2000). In addition, the accumulation of SA appeared to depend on the activity of PAL in tomatoes (Schaller et al 2000). In our study, we have identified that transcript encoding PAL1 was down-regulated in tomato during non-host and compatible interactions. Our microarray data also revealed that the transcripts encoding enzymes involved in the biosynthetic pathway of alkaloids and terpenoids were highly down-regulated during the early and late time points after inoculation with non-host and compatible pathogen. But, transcripts encoding enzymes involved in flavones biosynthesis were up-regulated in tomato during non-host and compatible interactions.

Conjugation of hydroxyl cinnamic acids with tyramine forms hydroxyl cinnamoyl tyramine and further FT and *p*-CT (Fig 4.2). Such conjugates, incorporated into the plant cell wall, strengthen the cell wall, against microbial degradation, or potentially act as direct antimicrobial agents. Synthesis of cinnamic acid conjugates was activated in response to fungal elicitors as well as in response to attempted infection by fungi, viruses, and bacteria and, in some cases, wounding. In the present study, we observed that transcripts encoding enzymes involved in synthesis of FT and CT were up-regulated in tomato leaves challenged with non-host and compatible pathogens. Increased synthesis of FT and CT is also associated with the resistance of potato plants to *Phytophthora infestans* (keller et al 1996) and with both the non-host- and gene-for-gene-determined resistance reactions of pepper to *Xanthomonas campestris* (Newman et al 2001). Biosynthesis of FT is induced in tobacco by tobacco mosaic virus infection (Negrel and Jeandet 1987). In tomato, potato, and tobacco, accumulation of FT occurs after wounding although in wounded pepper and nightshade no FT can be detected (Pearce et al 1998). The

increased synthesis and accumulation of CT and FT in tomato is associated with elevated mRNA levels of the genes encoding 4-coumarate-CoA ligase (4CL), and THT. But PAL, which involved in synthesis hydroxyl cinnamic acids from phenylalanine, was down-regulated. The available hydroxyl cinnamic acids, produced during regular plant metabolism, might be used by 4CL and THT for the synthesis of CT and FT. Among the three THT isozymes, *THT1-3* was highly expressed in tomato during non-host and compatible interactions. Same results were observed in tomato against pathogens by Roepenack-Lahaye et al (2003). THT enzymes are involved in incorporation of FT and CT and reorganizations of cell wall upon pathogen attack.

In tomato, a small family of decarboxylases (LeAADC1A, LeAADC1B, and LeAADC2) is involved in conversion of phenylalanine to phenethylamine and tyrosine to tyramine (Tieman et al 2006) (Fig 4.2). Although tyrosine is the preferred substrate *in vitro*, phenylalanine levels in tomato fruits far exceed those of tyrosine, indicating that phenylalanine is a physiological substrate. All three genes are expressed at comparable levels in fruits, whereas LeAADC1B and LeAADC2 are also expressed in leaves. Phenethylamine is further converted to 2-phenylacetaldehyde and involved in synthesis of volatile compounds like 2-phenylethanol which gives special flavor to tomato. Tyramine involved in synthesis of antimicrobial compounds like FT and CT. In our study, we noticed that these three AADC isozymes were up-regulated highly in tomato during non-host and compatible interactions. Till now no report was available on the role AADC and 2-phenylethanol in defense responses.

4.6.6. Regulation of cell wall related genes

A consequence of plant basal defense against pathogens is the modification of the plant cell wall, which provides an important barrier against pathogen penetration (Huckelhoven 2007). Our microarray data revealed that transcripts encoding expansin were down-regulated and transcripts encoding for xyloglucanglycosylases (XET7, 6 and 4) were down-regulated in tomato during non-host and compatible interactions. Cellulose is the main load-bearing component of the cell wall, changes in cellulose content often lead to obvious consequences in either compensatory or integrity responses. For example, reductions in cellulose are generally

associated with long-term compensatory changes in cell wall components, indicating that the cell wall can not only perceive, but also adjust for physical changes in its structure. Loss-of-function or treatment with inhibitors of CESA3, which leads to decrease in the cellulose content of the wall, cause constitutive expression of genes of JA/ET siganlling or result in production of lignin in response to pathogen attack or wounding (Caño-Delgado et al 2003; Ellis et al 2002). In our microarray data also, the transcript coding for *CESA3* was down-regulated in both non-host and compatible interactions of tomato.

In this study, the transcripts encoding for antioxidative enzymes like catalase (CAT1), AO, and several peroxidases were up-regulated but CAT2 were down-regulated in tomato challenged with non-host and compatible pathogens. Several transcripts of cell death marker gene, *hsr203j* were also up-regulated, suggesting the possible initiation of PCD (Pontier et al 1998). *A. alternata* f. sp. *lycopersici* is a necrotrophic fungus, which feeds on dead cells. Expression of HR would most likely enhance pathogen growth and colonization (Govrin and Levine 2000). But, in case of *M. grisea*, cell death restricts the further growth on non-host plant, tomato.

4.6.7. Transcripts that are differentially regulated either in non-host or in compatible interactions

Our microarray data analysis revealed that some of the transcripts were differentially regulated in any one of the interaction i.e. either in non-host or in compatible interactions. The transcript encoding WRKY-type DNA binding protein was up-regulated in non-host interactions but down-regulated in compatible interactions, which is a well known transcription factor expressed during plant-pathogen interactions. Branched chain α-keto acid dehydrogenase E1-α subunit, involved in amino acid degradation to generate precursor molecules and energy to cells, was up-regulated only in non-host interactions.

Peroxisomal acyl-CoA oxidase 1A, which is involved in fatty oxidation in peroxisomes, was up-regulated only in compatible interactions. TSW12 (non-specific lipid-transfer protein 1) was down-regulated only in compatible interactions. Torres-Schumann et al (1992) reported that

TSW12 was induced in tomato during seed germination and its level increases after NaCl treatment or heat shock.

4.7. Overview of the study

Histological characterization of non-host pathosystem revealed that callose and cell death at early hours of post inoculation restricting the growth of *M. grisea* on tomato plants. High accumulation of ROS at early hours of post inoculation might be involved in cell death and restriction of progression of disease by non-host pathogen. Cell death induced by accumulation of ROS during compatible interactions favors the growth of pathogen because *A. alternata* f. sp. *lycopersici* is a necrotroph pathogen which feeds on dead tissue. Strong antioxidative system in non-host interactions, involved in regulation of ROS, decreases the detrimental effects of ROS on cells. Along with high accumulation of ROS, weak antioxidative system in compatible interactions led to high lipid peroxidation and carbonylation of apoplast proteins and finally resulted into cell death. In our present study, we have shown the apoplast responsiveness to pathogen inoculation, by describing the differentially expression of apoplast proteins during non-host and compatible interactions. The proteins we have identified in tomato leaf apoplast by a proteomic approach are implicated in diverse physiological and defense processes and while some are probably part of a general downstream signaling pathway. Transcriptome analysis revealed that compatible and non-host interactions didn't show largely distinct molecular mechanisms; rather, they shared many of the same molecular mechanisms. All these defense mechanisms are a major part of basal resistance which presents in every plant universally against every pathogen. Proteomic and transcriptome analyses revealed that PR-proteins like β-1, 3-glucanase and endochitinase were up-regulated in both the interactions.

Summary & Conclusions

5. Summary and Conclusions

Plants are continually exposed to a vast number of potential pathogens and, as a result, they have evolved intricate defense mechanisms to recognize and defend themselves against a wide array of pathogens. The resistance strategies are either constitutively present, or acquired during or upon recognition of pathogen infection. These responses include hyper sensitive response (HR) and increased expression of defense-related genes. Often, the plant disease resistance described as cultivar or accession specific and is referred to as host resistance. A second resistance, operating under less-understood mechanisms, provides resistance against pathogens throughout all members of a plant species called as non-host resistance (NHR). A pathogen that cannot cause disease on a non-host plant is referred to as a non-host pathogen.

NHR to pathogens can be defined as strong and broad resistance of all members of a given plant species against all known isolates of a given pathogen species. A better understanding of the genetics and molecular mechanisms underlying plant NHR bears the potential for targeted employment of this valuable trait to control host pathogens. To study this wonderful phenomenon, we selected tomato (*Lycopersicon esculentum* cv. money maker), as a model crop plant and *Magnaporthe grisea* which causes disease only in monocots like cereals, as non-host pathogen. For comparative study, we selected *Alternaria alternata* f. sp. *lycopersici* as a host pathogen which causes stem canker to tomato.

5.1. Overview of the study

This thesis provides a comprehensive study on the defense responses of tomato upon treatment with non-host (*M. grisea*) and compatible (*A. alternata* f. sp. *lycopersici*) pathogens. To understand the stage at which *M. grisea* was restricted by tomato, we performed histological characterization using staining methods like aniline blue and trypan blue. As apoplast is the first site attacked by fungal pathogens, we studied the antioxidant defense responses and changes in proteome in apoplast during non-host interactions of tomato. We also studied the transcriptional regulation during the non-host and host interactions of tomato.

5.2. Histochemical changes

Callose is an effective barrier that is induced at the sites of attack during the early stages of pathogen invasion. Histological characterization of non-host pathosystem revealed that callose deposition occurred at 24 hours post inoculation (hpi). So, the growth of *M. grisea* on tomato leaf was restricted at penetration stage. Germination of *M. grisea* spores and formation of appressorium was occurred at 5 and 12 hpi, respectively. As a consequence of further defense response, cell death was occurred at early hours of post inoculation i.e. also at 24 hpi restrict the further growth of *M. grisea* on tomato plants. These cellular responses contributed to NHR of tomato against *M. grisea.*

5.3. Biochemical analyses

Accumulation of reactive oxygen species (ROS) like $O_2^{\cdot-}$, •OH and H_2O_2 at apoplast is called as oxidative burst and this is one of the earliest event occurred in plants against pathogen attack. High H_2O_2 and •OH accumulation at early hours of post inoculation might be involved in cell death and restriction of progression of disease by non-host pathogen. Accumulation of $O_2^{\cdot-}$ in compatible interactions was high compared to non-host interactions. This led to high lipid peroxidation and carbonylation of apoplast proteins in compatible interactions and therefore induces cell death. Increased superoxide dismutase (SOD) could be responsible for dismutaion and maintenance of low concentrations of $O_2^{\cdot-}$ during non-host interactions. In non-host interactions, strong antioxidative system i.e. high activity of extracellular peroxidase (PRX) and ascorbate peroxidase (APX), involved in regulation of ROS and decreased the negative effects of oxidative stress to the cells. Early production and rapid accumulation of phenolics in non-host interactions compared to compatible interactions also involved in restriction of growth of *M. grisea* on tomato cells. High PPO activity might be responsible for high accumulation of H_2O_2 in compatible interactions. The biochemical changes related to oxidative defenses in the apoplast of tomato, during non-host interactions with *M. grisea*, therefore appear to be important in the multilayered NHR.

5.4. Proteomic approach

The apoplast protein composition is influenced by several kinds of stress. To get the comprehensive data about proteins in apoplast, we analyze apoplast proteome in two parts i.e. apoplast fluid (AF) fraction and ionically cell wall bound protein (IBP) fraction. In AF and IBP fractions, 14 and 12 differentially expressed proteins were identified during non-host and compatible interactions, respectively. These proteins were characterized by using MALDI-TOF-MS. Identified proteins were classified based on function as defense, oxidoreductases, proteins with unknown function in apoplast and unknown proteins. We detected the changes in abundance of protein levels in apoplast of tomato after inoculation with non-host and host pathogens by describing the decreased levels of some proteins and the induction of others. We identified different sets of proteins in two different fractions i.e. chitinases only in AF fraction and glucanases only in IBP fraction. Some of the proteins which are not actually belonging to apoplast were also identified in both fractions like probable RNA-dependent RNA polymerase 4, ACCoxidase homolog 1, and oxygen-evolving enhancer protein 2. The proteins identified in tomato leaf apoplast both in AF and IBP by a proteomic approach are implicated in defense (chitinases and glucanases), oxidative burst (peroxidases) and while some are probably part of a general downstream signaling pathway (G-type lectin S-receptor-like ser/thr-protein kinase, and Ca^{2+}/calmodulin-dependent ser/thr-protein kinase). From this study, we can conclude that apoplast is rich with pathogenesis related (PR)-proteins and actively induces to external cues like pathogen attack.

5.5. Microarray-based analysis

The sensitivity, accuracy, and broad spectrum of large-scale mRNA expression profiling revealed the quantitative nature of plant responses to pathogens. We demonstrated that apparently specific biological phenomena, such as compatible and non-host interactions, may not necessarily be attributable to largely distinct molecular mechanisms; rather, they may share many of the same molecular mechanisms. The transcripts that are differentially regulated

during both the non-host and host interactions majorly belonged to basal disease response, which is a common defense responses induced by all pathogens in plants.

Transcripts related to the pathways, which are involved in production of energy or growth like photosynthesis, Calvin cycle, Krebs cycle, and glycolysis were down-regulated. Basal disease response like accumulation of *PR*-genes, secondary metabolite synthesis and strengthening of cell wall related transcripts were up-regulated. Catabolic processes like starch and sucrose hydrolysis were up-regulated and led to accumulation of soluble sugars (fructose and glucose). These soluble sugars involves in synthesis of secondary metabolites and polysaccharides which in turn, incorporated in to cell wall. From these results, we can conclude that the induction of the wide array of defense mechanisms involves a massive redistribution of energy toward the defense response. Some of the transcripts were down-regulated (branched chain α-keto acid dehydrogenase E1-alpha subunit, and dehydrin-like protein) and up-regulated (WRKY-type DNA binding protein, and cytochrome c oxidase subunit 5C) only during non-host interactions of tomato. During compatible interactions of tomato also some of the transcripts were up-regulated (peroxisomal acyl-CoA oxidase A1 and proline transporter 2) and down-regulated (putative disease resistance protein and TSW12). Transcripts encoding WRKY-type DNA binding protein was up-regulated in non-host interactions and down-regulated in compatible interactions. Further analysis of these transcripts may give more information about non-host interactions of tomato against *M. grisea*.

5.6. Major conclusions

The role of apoplast was studied for the first time in non-host interactions of tomato against *M. grisea*. In this thesis, we reported that the apoplastic antioxidative defenses play an important role in execution of NHR in tomato against *M. grisea*. Apoplast proteome also influenced and abundance of many PR-proteins has changed during non-host and compatible interactions. Transcriptome analysis revealed that genes involved in the synthesis of volatile compounds like 2-phenylethanol was highly up-regulated in both the non-host and compatible interactions. The significance of these volatile compounds during defense responses is not yet to be proved.

Bibliography

Abbasi FM, Komatsu S. A proteomic approach to analyze salt-responsive proteins in rice leaf sheath. Proteomics 2004;4:2072-81.

Able AJ, Sutherland MW, Guest DI. Production of reactive oxygen species during non-specific elicitation non-host resistance and field resistance expression in cultured tobacco cells. Funct Plant Biol 2003;30:91-9.

Aebi H, Lester P. Catalase in vitro. Methods Enzymol. 1984;105:121-6.

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. Genes involved in organ separation in *Arabidopsis*: An analysis of the cup shaped cotyledon mutant. Plant Cell 1997;9:841-57.

Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nature Protocols 2007;2:875-7.

Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 2004;55:373-99.

Appel HM. Phenolics in ecological interaction: the importance of oxidation. J Chem Ecol 1993; 19:1521-52.

Assaad FF, Qiu JL, Youngs H, Ehrhardt D, Zimmerli L, Somerville CR, Thordal-Christensen H. The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Mol Biol Cell 2004;15:5118-29.

Baker CJ, Orlandi EW. Active oxygen in plant pathogenesis. Annu Rev Phytopathol 1995;33:299-321.

Barna B, Fodor J, Harrach BD, Pogány M, Király Z. The Janus face of reactive oxygen species in resistance and susceptibility of plants to necrotrophic and biotrophic pathogens. Plant Physiol Biochem 2012;59:37-43.

Bayer EM, Bottrill AR, Walshaw J, Vigouroux M, Naldrett MJ, Thomas CL, Maule AJ. *Arabidopsis* cell wall proteome defined using multi-dimensional protein identification technology. Proteomics 2006;6:301-11.

Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971;44:276-87.

Berger S, Papadopoulos M, Schreiber U, Kaiser W, Roitsch T. Complex regulation of gene expression, photosynthesis and sugar levels by pathogen infection in tomato. Physiol Plant 2004;122:419-28.

Berger S, Sinha AK, Roitsch T. Plant physiology meets phytopathology: plant primary metabolism and plant pathogen interactions. J Exp Bot 2007;58:4019-26.

Bestwick CS, Brown IR, Mansfield JW. Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol 1998;118:1067-78.

Biemelt S, Sonnewald U. Plant-microbe interactions to probe regulation of plant carbon metabolism. J Plant Physiol 2006;163:307-18.

Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, et al. Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. Plant J 2006;47:851-63.

Blume B, Nurnberger T, Nass N, Scheel D. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 2000;12:1425-40.

Bolton MD. Primary metabolism and plant defense-fuel for the fire. Mol Plant-Microbe Inter 2009;22:487-97.

Bolwell GP, Blee KA, Butt VS, Davies DR, Gardner SL, Gerrish C, et al. Recent advances in understanding the origin of the apoplastic oxidative burst in plant cells. Free Rad Res 1999;31: 137-45.

Boudart G, Jamet E, Rossignol M, Lafitte C, Borderies G, Jauneau A, et al. Cell wall proteins in apoplastic fluids of *Arabidopsis thaliana* rosettes: Identification by mass spectrometry and bioinformatics. Proteomics 2005;5:212-21.

Caño-Delgado A, Penfield S, Smith C, Catley M, Bevan M. Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. Plant J 2003;34:351-62.

Chance B, Maehly M, in: S.P. Colowick, N.P. Kaplan (Eds.), Methods in enzymology, Academic Press, New York, 1955, p. 764.

Cheong YH, Chang HS, Gupta R, Wang X, Zhu X, Luan S. Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in *Arabidopsis*. Plant Physiol 2003;129:661-77.

Chivasa S, Ndimba BK, Simon WJ, Robertson D, Yu XL, Knox JP, et al. Proteomic analysis of the *Arabidopsis thaliana* cell wall. Electrophoresis 2002;23:1754-65.

Chivasa S, Simon WJ, Yu XL, Yalpani N, Slabas AR. Pathogen elicitor-induced changes in the maize extracellular matrix proteome. Proteomics 2005;5:4894-904.

Choi J, Huh SU, Kojima M, Sakakibara H, Paek KH, Hwang I. The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR-1-dependent salicylic acid signalling in *Arabidopsis*. Dev Cell 2010;19:284-95.

Chou HM, Bundock N, Rolfe SA, Scholes JD. Infection of *Arabidopsis thaliana* leaves with *Albugo candida* (white blister rust) causes a reprogramming of host metabolism. Mol Plant Pathol 2000;1:99-113.

Collinge M, Boller T. Differential induction of two potato genes, Stprx2 and StNAC, in response to infection by *Phytophthora infestans* and to wounding. Plant Mol Biol 2001;46:521-9.

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, et al. SNARE-protein-mediated disease resistance at the plant cell wall. Nature 2003;425:973-7.

Creelman RA, Mullet JE. Biosynthesis and action of jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol 1997;48:355-81.

Croft KPC, Juttner F, Slusarenko AJ. Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola*. Plant Physiol 1993;101:13-24.

Curto M, Camafeita E, Lopez JA, Maldonado AM, Rubiales D, Jorrín JV. A proteomic approach to study pea (*Pisum sativum*) responses to powdery mildew (*Erysiphe pisi*). Proteomics 2006;6:163-74.

Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP. The apoplastic oxidative burst peroxidase in *Arabidopsis* is a major component of pattern-triggered immunity. Plant Cell 2012;24:275-87.

De Pinto MC, de Gara L. Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associated with cell differentiation. J Exp Bot 2004;55:2559-69.

Diaz-Vivancos P, Rubio M, Mesonero V, Periago PM, Ros Barcelo A, Martı´nez-Go´mez P, Herna´ndez JA. The apoplastic antioxidant system in *Prunus*: response to long-term plum pox virus infection. J Exp Bot 2006;57:3813-24.

Doke N. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol Plant Pathol 1983;23:345-57.

Dubois M, Gilles KA, Hamilton JK, Rebus PA, Smith F. A colorimetric method for the determination of sugars and related substances. Anal Chem 1956;28:350-6.

Ehness R, Ecker M, Godt DE, Roitsch T. Glucose and stress independently regulate source and sink metabolism and defense mechanisms via signal transduction pathways involving protein phosphorylation. Plant Cell 1997;9:1825-41.

Eichmann R, Biemelt S, Schafer P, Scholz U, Jansen C, Felk A, et al. Macroarray expression analysis of barley susceptibility and nonhost resistance to *Blumeria graminis*. Plant Physiol 2006;163:657-70.

Ekengren SK, Liu Y, Schiff M, Dinesh Kumar SP, Martin GB. Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. Plant J 2003;36:905-17.

Ellis C, Karafyllidis I, Wasternack C, Turner JG. The *Arabidopsis* mutant cev1 links cell wall signalling to jasmonate and ethylene responses. Plant Cell 2002;14:1557-66.

Essmann J, Schmitz-Thom I, Schon H, Sonnewald S, Weis E, Scharte J. RNA interference-mediated repression of cell wall invertase impairs defence in source leaves of tobacco. Plant Physiol 2008;147:1288-99.

Eulgem T, Somssich IE. Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol 2007;10:366-71.

Eulgem T, Weigman VJ, Chang HS, McDowell JM, Holub EB, Glazebrook J, et al. Gene expression signatures from three genetically separable resistance gene signaling pathways for downy mildew resistance. Plant Physiol 2004;135:1129-44.

Floerl S, Druebert C, Majcherczyk A, Karlovsky P, Kües U, Polle A. Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. *napus*) attenuate *Verticillium longisporum* growth but not disease symptoms. BMC Plant Biol 2008;8:1-15.

Floerl S, Majcherczyk A, Possienke M, Feussner K, Tappe H, Gatz C, et al. *Verticillium longisporum* infection affects the leaf apoplastic proteome, metabolome, and cell wall properties in *Arabidopsis thaliana*. PLoS ONE 2012;7:31435.

Fotopoulos V, Gilbert MJ, Pittman JK, Marvier AC, Buchanan AJ, Sauer N, et al. The monosaccharide transporter gene, AtSTP4, and the cell-wall invertase, Atβ fruct1, are induced in *Arabidopsis* during infection with the fungal biotroph *Erysiphe cichoracearum*. Plant Physiol 2003;132:821-9.

Gay C, Gebicki JM. A critical evaluation of the effect of sorbitol on the ferric-xylenol orange hydroperoxide assay. Anal Biochem 2000;284:217-20.

Gillespie KM, Ainsworth EA. Measurement of reduced, oxidized and total ascorbate content in plants. Nature Protocols 2007;2:871-4.

Glazebrook J. Genes controlling expression of defence responses in *Arabidopsis*: 2001 status. Curr Opin Plant Biol 2001;4:301-8.

Golem S, Culver J. Tobacco mosaic virus induced alterations in the gene expression profile of *Arabidopsis thaliana*. Mol Plant-Microbe Inter 2003;16:681-8.

Gomez-Ariza J, Campo S, Rufat M, Estopa M, Messeguer J, San Segundo B, Coca M. Sucrose-mediated priming of plant defence responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. Mol Plant-Microbe Inter 2007;20:832-42.

Gomez-Gomez L, Boller T. Flagellin perception: a paradigm for innate immunity. Trends Plant Sci 2002;7:251-6.

Govrin EM, Levine A. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biol 2000;10:751-7.

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J. The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J 2000;23:441-50.

Grogan RG, Kimble KA, Misaghi I. A stem canker disease of tomato caused by *Alternaria alternata* f.sp. *lycopersici*. Phytopathol 1975;65:880-6.

Hakmaoui A, Pérez-Bueno ML, García-Fontana B, Camejo D, Jiménez A, Sevilla F, Barón M. Analysis of the antioxidant response of *Nicotiana benthamiana* to infection with two strains of pepper mild mottle virus. J Exp Bot 2012;63:5487-96.

Halliwell B, Gutteridge JMC. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. FEBS Lett 1981;128:347-52.

Harris GP, Jaffcoat B. Effects of temperature on the distribution C^{14}-labelled assimilates in the flowering shoot of Carnation. Ann Bot 1974;38:77-83.

Haslam RP, Downie AL, Raveton M, Gallardo K, Job D, Pallett KE, et al. The assessment of enriched apoplastic extracts using proteomic approaches. Ann Appl Biol 2003;143:81-91.

Hathout Y. Approaches to the study of the cell secretome. Exp Rev Proteomics 2007;2:239-48.

Heath MC. Non-host resistance and nonspecific plant defenses. Curr Opin Plant Biol 2000;3:315-9.

Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: I.Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 1968;125:189-98.

Herbers K, Meuwly P, Frommer WB, Metraux JP, Sonnewald U. Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. Plant Cell 1996;8:793-803.

Herbers K, Takahata Y, Melzer M, Mock HP, Hajirezaei M, Sonnewald U. Regulation of carbohydrate partitioning during the interaction of potato virus Y with tobacco. Mol Plant Pathol 2000;1:51-9.

Hernandez JA, Ferrer MA, Jimenez A, Ros-Barcelo A, Sevilla F. Antioxidant systems and $O_2^{\cdot-}$ /H_2O_2 production in the apoplast of *Pisum sativum* L. leaves: its relation with NaCl-induced necrotic lesions in minor veins. Plant Physiol 2001;127:817-31.

Hernandez JA, Talaveraa JM, Martınez-Gomez P, Dicentab F, Sevillaa F. Response of antioxidative enzymes to plum pox virus in two apricot cultivars. Physiol Plant 2001;111:313-21.

Hood ME, Shew HD. Applications of KOH-aniline blue fluorescence in the study of plant-fungal interactions. Phytopathology 1996;86:704-8.

Horsfall J, Dimond A. Interaction of tissue sugar, growth substances and disease susceptibility. Phytopath Z 1957;64:415-21.

Huckelhoven R, Dechert C, Kogel KH. Non-host resistance of barley is associated with a hydrogen peroxide burst at sites of attempted penetration by wheat powdery mildew fungus. Mol Plant Pathol 2001;2:199-205.

Huckelhoven R, Fodor J, Trujillo M, Kogel KH. Barley Mla and Rar mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f. sp. *hordei* are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion. Planta 2000;212:16-24.

Huckelhoven R. Cell wall-associated mechanisms of disease resistance and susceptibility. Annu Rev Phytopathol 2007;45:101-27.

Huitema E, Vleeshouwers GAA, Francis DM, Kamoun S. Active defence responses associated with nonhost resistance of *Arabidopsis thaliana* to the oomycete pathogen *Phytothphora infestans*. Mol Plant Pathol 2003;4:487-500.

Jackson P, Galinha C, Pereira C, Fortunato A, Soares N, Amâncio S, Ricardo CPP. Rapid deposition of extensin during the elicitation of grapevine callus cultures is specifically catalysed by a 40-kilodalton peroxidase. Plant Physiol 2001;127:1065-76.

Jarosch B, Jansen MM, Schaffrath U. Acquired resistance functions in mlo barley, which is hyper susceptible to *Magnaporthe grisea*. Mol Plant-Microbe Inter 2003;16:107-14.

Jensen MK, Rung JH, Gregersen PL, Gjetting T, Fuglsang AT, Hansen M, et al. The HvNAC6 transcription factor: a positive regulator of penetration resistance in barley and *Arabidopsis*. Plant Mol Biol 2007;65:137-50.

Jones JDG, Dangl JL. The plant immune system. Nature 2006;444:323-9.

Jones AME, Thomas V, Bennett MH, Mansfield J, Grant M. Modifications to the *Arabidopsis* defense proteome occur prior to significant transcriptional change in response to inoculation with *Pseudomonas syringae*. Plant physiol 2006;142:1603-20.

Joosten MHAJ, Hendrickx LJM, De Wit PJGM. Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of *Cladosporium fulvum* (syn. *Fulvia fulva*). Neth J Plant Pathol 1990;96:103-12.

Jung YH, Jeong SH, Kim SH, Singh R, Lee J, Cho YS, et al. Systematic secretome analyses of rice leaf and seed callus suspension-cultured cells: workflow development and establishment of high-density two-dimensional gel reference maps. J Proteome Res 2008;7:5187-210.

Kaffarnik FA, Jones AM, Rathjen JP, Peck SC. Effector proteins of the bacterial pathogen *Pseudomonas syringae* alter the extracellular proteome of the host plant, *Arabidopsis thaliana.* Mol Cell Proteomics 2009;8:145-56.

Kang L, Li J, Zhao T, Xiao F, Tang X, Thilmony R, et al. Interplay of the *Arabidopsis* non-host resistance gene *NHO1* with bacterial virulence. Proc Natl Acad Sci USA 2003;100:3519-24.

Kanzaki H, Saitohet H, Ito A, Fujisawa S, Kamoun S, Katou S, et al. Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana*. Mol Plant Pathol 2003;4:383-91.

Kar M, Mishra D. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiol 1976;57:315-9.

Karlsson M, Melzer M, Prokhorenko I, Johansson T, Wingsle G. Hydrogen peroxide and expression of hipI-superoxide dismutase are associated with the development of secondary cell walls in *Zinnia elegans*. J Exp Bot 2005;418:2085-93.

Keller H, Hohlfield H, Wray V, Hahlbrock K, Scheel D, Strack D. Changes in the accumulation of soluble and cell-wall bound phenolics in elicitor-treated cell suspension cultures and fungus-infected leaves of *Solanum tuberosum*. Phytochemistry 1996;42:389-96.

Kim HS, Jung MS, Lee SM, Kim KE, Byun H, Choi MS, et al. An S-locus receptor-like kinase plays a role as a negative regulator in plant defense responses. Biochem Biophys Res Commun 2009;381:424-8.

Kim ST, Cho KS, Yu S, Kim SG, Hong JC, Han CD, et al. Proteomic analysis of differentially expressed proteins induced by rice blast fungus and elicitor in suspension-cultured rice cells. Proteomics 2003;3:2368-78.

Kim ST, Kim SG, Hwang H, Kang SY, Kim HJ, Lee BH, et al. Proteomic analysis of pathogen-responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. Proteomics 2004;4:3569-78.

Knoester M, Van Loon LC, Heuvel JVD, Hennig J, Bol JF, Linthorst HJM. Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proc Natl Acad Sci USA 1998;95: 1933-7.

Kobayashi Y, Yamada M, Kobayashi I, Hitoshi K. Actin microfilaments are required for the expression of non-host resistance in higher plants. Plant Cell Physiol 1997;38:725-33.

Kocal N, Sonnewald U, Sonnewald S. Cell wall-bound invertase limits sucrose export and is involved in symptom development and inhibition of photosynthesis during compatible interaction between tomato and *Xanthomonas campestris* pv. *vesicatoria*. Plant Physiol 2008;148:1523-36.

Koch E, Slusarenko A. *Arabidopsis* is susceptible to infection by a downy mildew fungus. Plant Cell 1990;2:437-45.

Knaust A, Ludwig-Müller J. The ethylene signaling pathway is needed to restrict root gall growth in *Arabidopsis* after infection with the obligate biotrophic protist *Plasmodiophora brassicae*. J Plant Growth Reg 2013;32:9-21.

Kwon C, Neu C, Pajonk S, Yun HS, Lipka U, Humphry M, et al. Co-option of a default secretory pathway for plant immune responses. Nature 2008;451:835-40.

Lee S, Kim SY, Chung E, Joung YH, Pai HS, Hur CG, Choi D. EST and microarray analyses of pathogen responsive genes in hot pepper (*Capsicum annuum L*.) non-host resistance against soybean pustule pathogen (*Xanthomonas axonopodis* pv. *glycines*). Funct Integr Genomics 2004;4:196-205.

Legendre L, Yueh YG, Crain R, Haddock N, Heinstein PF, Low PS. Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J Biol Chem 1993;268:24559-63.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, et al. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464-78.

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, et al. Pre- and post invasion defenses both contribute to non-host resistance in *Arabidopsis*. Science 2005;310:1180-3.

Lipka V, Panstruga R. Dynamic cellular responses in plant–microbe interactions. Curr Opin Plant Biol 2005;8:625-31.

Lo´pez-Milla´n AF, Morales F, Andaluz S, Gogorcena Y, Abadı´a A, Rivas JDL, Abadı´a J. Responses of sugar beet roots to iron deficiency: Changes in carbon assimilation and oxygen use. Plant Physiol 2000;124:885-97.

Martinez C, Montillet JL, Bresson E, Agnel JP, Dai GH, Daniel JF, et al. Apoplastic peroxidase generates superoxide anions in cells of cotton cotyledons undergoing the hypersensitive reaction to *Xanthomonas campestris* pv. *malvacearum* race 18. Mol Plant-Microbe Inter 1998;11:1038-47.

Matsumura H, Reich S, Ito A, Saitoh H, Kamoun S, Winter P, et al. Gene expression analysis of plant host-pathogen interactions by SuperSAGE. Proc Natl Acad Sci USA 2003;100:15718-23.

May MJ, Hammond-Kosack KE, Jones JDG. Involvement of reactive oxygen species, glutathione metabolism, and lipid peroxidation in the *Cf*-gene-dependent defense response of tomato cotyledons induced by race-specific elicitors of *Cladosporium fulvum*. Plant Physiol 1996;110:1367-79.

Mayrose M, Bonshtien A, Sessa G. LeMPK3 is a mitogen-activated protein kinase with dual specificity induced during tomato defense and wounding responses. J Biol Chem 2004;279:14819-27.

Mellersh DG, Heath MC. Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell 2001;13:413-24.

Misra HP, Fridovich I. The univalent reduction of oxygen by reduced flavins and quinines. J Biol Chem 1972;247:188-92.

Mittler R, Herr EH, Orvar BL, Van Camp W, Willekens H, Inze D, Ellis BE. Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyper responsive to pathogen infection. Proc Natl Acad Sci USA 1999;96:14165-70.

Mohr TJ, Mammarella ND, Hoff T, Woffenden BJ, Jelesko JG, McDowell JM. The *Arabidopsis* downy mildew resistance gene RPP8 is induced by pathogens and salicylic acid and is regulated by W box cis elements. Mol Plant-Microbe Inter 2010;23:1303-15.

Montesano M, Brader G, Palva T. Pathogen-derived elicitors: searching for receptors in plants. Mol Plant Pathol 2003;4:73-9.

Moser O, Kanellis AK. Ascorbate oxidase of *Cucumis melo* L. var. *reticulatus*: purification, characterisation and antibody production. J Exp Bot 1994;45:717-24.

Mysore KS, Ryu CM. Non-host resistance: how much do we know? Trends Plant Sci 2004;9:97-104.

Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 2001;22:867-80.

Narusaka Y, Narusaka M, Seki M, Ishida J, Shinozaki K, Nan Y, et al. Cytological and molecular analyses of non-host resistance of *Arabidopsis thaliana* to *Alternaria alternata*. Mol Plant Pathol 2005;6:615-27.

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, et al. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 2006;312:436-9

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JD. The transcriptional innate immune response to flg22: Interplay and overlap with *Avr* gene-dependent defense responses and bacterial pathogenesis. Plant Physiol 2004;135:1113-28.

Ndimba BK, Chivasa S, Hamilton JM, Simon WJ, Slabas WJAR. Proteomic analysis of changes in the extracellularmatrix of *Arabidopsis* cell suspension cultures induced by fungal elicitors. Proteomics 2003;3:1047-59.

Negrel J, Jeandet P. Metabolism of tyramine and feruloyl tyramine in TMV inoculated leaves of *Nicotiana tabacum*. Phytochemistry 1987;26:2185-90.

Newman MA, Roepenack-Lahaye E, Parr A, Daniels MJ, Maxwell Dow JM. Induction of hydroxycinnamoyl-tyramine conjugates in pepper by *Xanthomonas campestris*, a plant defense response activated by *hrp* gene-dependent and *hrp* gene-independent mechanisms. Mol Plant-Microbe Inter 2001;14:785-92.

Nurnberger T, Scheel D. Signal transmission in the plant immune response. Trends Plant Sci 2001;6:372-9.

O'Brien JA, Daudi A, Finch P, Butt VS, Whitelegge JP, Souda P, et al. A peroxidase-dependent apoplastic oxidative burst in cultured *Arabidopsis* cells functions in MAMP-elicited defence. Plant Physiol 2012;158:2013-27.

Ogawa K, Kanematsu S, Asada K. Intra- and extra-cellular localization of "cytosolic" Cu,Zn-superoxide dismutase in spinach leaf and hypocotyl. Plant Cell Physiol 1996;37:790-9.

Oh IS, Park AR, Bae MS, Kwon SJ, Kim YS, Lee JE, et al. Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola.* Plant Cell 2005;17:2832-47.

Okushima Y, Koizumi N, Kusano T, Sano H. Secreted proteins of tobacco cultured BY2 cells: identification of a new member of pathogenesis-related proteins. Plant Mol Biol 2000;42:479-88.

Pandey SP, Somssich IE. The role of WRKY transcription factors in plant immunity. Plant Physiol 2009;150:1648-55.

Parchmann S, Gundlach H, Mueller MJ. Induction of 12-oxo-phytodienoic acid in wounded plants and elicited plant cell cultures. Plant Physiol 1997;115:1057-64.

Patykowski J, Urbanek H. Activity of enzymes related to H_2O_2 generation and metabolism in leaf apoplastic fraction of tomato leaves infected with *Botrytis cinerea*. J Phytopathol 2003;151:153-61.

Pearce G, Marchand PA, Griswold J, Lewis NG, Ryan CA. Accumulation of feruloyltyramine and *p*-coumaroyltyramine in tomato leaves in response to wounding. Phytochemistry 1998;47:659-64.

Peart JR, Lu R, Sadanandom A, Malcuit I, Moffett P, Brice DC, et al. Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc Natl Acad Sci USA 2002;99:10865-9.

Pedley KF, Martin GB. Identification of MAPKs and their possible MAPK kinase activators involved in the Pto-mediated defense response of tomato. J Biol Chem 2004;279:49229-35.

Pirovani CP, Carvalho HA, Machado RC, Gomes DS, Alvim FC, Pomella AW, et al. Protein extraction for proteome analysis from cacao leaves and meristems, organs infected by *Moniliophthora perniciosa*, the causal agent of the witches broom disease. Electrophoresis 2008;29:2391-401.

Polle A, Chakrabarti K, Schumann W, Rennenbreg H. Composition and properties of hydrogen peroxide decomposing systems in extracellular and total extracts from needles of Norway Spruce (*Picea abies* L., Karst.). Plant Physiol 1990;94:312-9.

Pontier D, Tronchet M, Rogowski P, Lam E, Roby D. Activation of hsr203, a plant gene expressed during incompatible plant-pathogen interactions, is correlated with programmed cell death. Mol Plant-Microbe Inter 1998;11:544-54.

Ramagli L. Quantifying protein in 2-D PAGE solubilization buffers. Methods Mol Biol 1999;112: 99-103.

Rampitsch C, Bykova NV, McCallum B, Beimcik E, Ens W. Analysis of the wheat and *Puccinia triticina* (leaf rust) proteomes during a susceptible host-pathogen interaction. Proteomics 2006;6:1897-907.

Rance I, Fournier J, Esquerre-Tugaye MT. The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. Proc Natl Acad Sci USA 1998;95:6554-9.

Reignault P, Cojan A, Muchembled J, Sahouri AL, Durand R, Sancholle M. Trehalose induces resistance to powdery mildew in wheat. New Phytol 2001;149:519-29.

Reymond P, Weber H, Damond M, Farmer EE. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 2000;12:707-20.

Robinette D, Matthysse AG. Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of development of the hypersensitive response elicited by *Pseudomonas syringae* pv. *phaseolicola*. J Bacteriol 1990;172:5742-9.

Roepenack-Lahaye E, Newman MA, Schornack S, Hammond-Kosack KE, Lahaye T, Jones JDG, et al. *p*-Coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense against pathogens. J Biol Chem 2003;278:43373-83.

Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK. Extracellular invertase: key metabolic enzyme and PR protein. J Exp Bot 2003;54:513-24.

Rojo E, Sharma VK, Kovaleva V, Raikhel NV, Fletcher JC. CLV3 is localized to the extracellular space, where it activates the *Arabidopsis* CLAVATA stem cell signaling pathway. Plant Cell 2002;14:969-77.

Ros Barcelo A, Gomez-Ros LV, Ferrer MA, Hernandez JA. The apoplastic antioxidant enzymatic system in the wood-forming tissues of trees. Trees–Structure and Function 2006;20:145-56.

Rustérucci C, Montillet JL, Agnel JP, Battesti C, Alonso B, Knoll A, et al. Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein of tobacco leaves. J Biol Chem 1999;274:36446-55.

Sarma AD, Oehrle NW, Emerich DW. Plant protein isolation and stabilization for enhanced resolution of two-dimensional polyacrylamide gel electrophoresis. Anal Biochem 2008;379: 192-5.

Schaller A, Roy P, Amrhein N. Salicylic acid-independent induction of pathogenesis-related gene expression by fusicoccin. Planta 2000;210:599-606.

Scharte J, Schon H, Weis E. Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with *Phytophthora nicotianae*. Plant Cell Environ 2005;28: 1421-35.

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proc Natl Acad Sci USA 2000;97:11655-60.

Schmelzer E. Cell polarization, a crucial process in fungal defence. Trends Plant Sci 2002;7:411-5.

Scholes JD, Lee PJ, Horton P, Lewis DH. Invertase-understanding changes in the photosynthetic and carbohydrate-metabolism of barley leaves infected with powdery mildew. New Phytol 1994;126:213-22.

Schweizer P. Nonhost resistance of plants to powdery mildew-New opportunities to unravel the mystery. Physiol Mol Plant Pathol 2007;70:3-7.

Segarra CI, Casalongue CA, Pinedo ML, Cordo CA, Conde RD. Changes in wheat leaf extracellular proteolytic activity after infection with *Septoria tritici*. J Phytopathol 2002;150:105-11.

Seo YS, Cho JI, Lee SK, Ryu HS, Han M, Hahn TR, et al. Current insights into the primary carbon flux that occurs in plants undergoing a defense response. Plant Stress 2007;1:42-9.

Sharma PC, Ito A, Shimizu T, Terauchi R, Kamoun S, Saitoh H. Virus-induced silencing of WIPK and SIPK genes reduces resistance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in *Nicotiana benthamiana*. Mol Genet Genomics 2003;269:583-91.

Shevchenko A, Wilm A, Vorm O, Mann M. Mass spectrometric sequencing of protein from silver-stained polyacrylamide gels. Anal Chem 1996;68:850-8.

Shimada C, Lipka V, O'Connell R, Okuno T, Schulze-Lefert P, Takano Y. Nonhost resistance in *Arabidopsis-Colletotrichum* interactions acts at the cell periphery and requires actin filament function. Mol Plant-Microbe Inter 2006;19:270-9.

Shomer I, Novacky AJ, Pike SM, Yermiyahu U, Kinraide TB. Electrical potentials of plant cell walls in response to the ionic environment. Plant Physiol 2003;133:411-22.

Slabas AR, Ndimba B, Simon WJ, Chivasa S. Proteomic analysis of the *Arabidopsis* cell wall reveals unexpected proteins with new cellular locations. Biochem Soc Trans 2004;32:524-8.

Soares NC, Francisco R, Ricardo CP, Jackson PA. Proteomics of ionically bound and soluble extracellular proteins in *Medicago truncatula* leaves. Proteomics 2007;7:2070-82.

Sonderby IE, Geu-Flores F, Halkier B. Biosynthesis of glucosinolates-gene discovery and beyond. Trends Plant Sci 2010;15:283-90.

Solomon M, Belenghi**a** B, Delledonne**b** M, Menachem**a** E, Levine**a** A. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 1999;11:431-43.

Stein M, Dittgen J, Sánchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, et al. *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to non-host resistance to inappropriate pathogens that enter plants by direct penetration. Plant Cell 2006;18:731-46.

Stewart D, Lyon GD, Tucker EJB. A Fourier-transform infrared spectroscopic and microscopic study of the infection of potato tubers by *Erwinia carotovora* ssp *carotovora* in aerobic and anaerobic conditions. J Sci Food Agric 1994;66:145-54.

Streller S, Wingsle G. *Pinus sylvestris* L. needles contain extracellular CuZn-superoxide dismutase. Planta 1994;192:195-201.

Swapan KD, Muthukrishnan S. Pathogenesis-related proteins in plants, CRC Press, New York 1999, pp. 1–278.

Swarbrick PJ, Schulze-Lefert P, Scholes JD. Metabolic consequences of susceptibility and resistance (race-specific and broad-spectrum) in barley leaves challenged with powdery mildew. Plant Cell Environ 2006;29:1061-76.

Takahashi Y, Nasir KHB, Ito A, Kanzaki H, Matsumura H, Saitoh H, et al. A high-throughput screen of cell-death-inducing factors in *Nicotiana benthamiana* identifies a novel MAPKK that mediates INF1-induced cell death signaling and non-host resistance to *Pseudomonas cichorii*. Plant J 2007;49:1030-40.

Takemoto D, Yoshioka H, Doke N, Kawakita K. Disease stress-inducible genes of tobacco: expression profile of elicitor-responsive genes isolated by subtractive hybridization. Physiol Plant 2003;118:545-53.

Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, Han B, et al. Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. Plant Cell 2003;15:317-30.

Thilmony R, Underwood W, He SY. Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. Plant J 2006;46:34-53.

Thordal-Christensen H, Zhang Z, Wie Y, Collinge DB. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 1997;11:1187-94.

Thordal-Christensen H. Fresh insights into processes of nonhost resistance. Curr Opin Plant Biol 2003;6:351-7.

Torres-Schumann S, Godoy JA, Pintor-Toro JA. A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. Plant Mol Biol 1992;18:749-57.

Tieman D, Taylor M, Schauer N, Fernie AR, Hanson AD, Klee HJ. Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. Proc Natl Acad Sci USA 2006;103:8287-92.

Truernit E, Schmid J, Epple P, Illig J, Sauer N. The sink specific and stress-regulated *Arabidopsis* STP4 gene: Enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. Plant Cell 1996;8:2169-82.

Trujillo M, Kogel KH, Hückelhoven R. Superoxide and hydrogen peroxide play different roles in the nonhost interaction of barley and wheat with inappropriate formae speciales of *Blumeria graminis*. Mol Plant-Microbe Inter 2004;3:304-12.

Tsuchida O, Yamagota Y, Ishizuka J, Arai J, Yamada J, Ta-keuchi M, Ichishima E. An alkaline proteinase of an alkalophilic *Bacillus* sp. Current Microbiol 1986;14:7-12.

Uma B, Rani TS, Podile AR. Warriors at the gate that never sleep: Non-host resistance in plants. J Plant Physiol 2011;168:2141-52.

Van Esse HP, Fradin EF, de Groot PJ, de Wit PJ, Thomma BP. Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct. Mol Plant-Microbe Inter 2009;22:245-58.

Vanacker H, Carver TLW, Foyer CH. Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. Plant Physiol 1998:117:1103-14.

Van Loon LC, Rep M, Pieterse CMJ. Significance of inducible defence-related proteins in infected plants. Annu Rev Phytopathol 2006;44:135-62.

Wang D, Pajerowska-Mukhtar K, Culler AH, Dong X. Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr Biol 2007;17:1784-90.

Watson BS, Lei ZT, Dixon RA, Sumner LW. Proteomics of *Medicago sativa* cell walls. Phytochemistry 2004;65:1709-20.

Weber H, Chetelat A, Caldelari D, Farmer EE. Divinyl ether fatty acid synthesis in late blight-diseased potato leaves. Plant Cell 1999;11:485-93.

Weisiger R, Fridovich I. Superoxide dismutase. Organelle specificity. J Biol Chem 1973;248: 3582-92.

Whitbred JM, Schuler MA. Molecular characterization of *CYP73A9* and *CYP82A1* P450 genes involved in plant defense in Pea. Plant Physiol 2000;124:47-58.

Wojtaszek P. Oxidative burst: an early plant response to pathogen infection. Biochem J 1997; 322:681-692.

Wubben JP, Lawrence CB, DeWit PJGM. Differential induction of chitinase and 1, 3-β-glucanase gene expression in tomato by *Cladosporium fulvum* and its race-specific elicitors. Physiol Mol Plant Pathol 1996;48:105-16.

Yamahara T, Shiono T, Suzuki T, Tanaka K, Takio S, Sato K, et al. Isolation of a germin-like protein with manganese superoxide dismutase activity from cells of a moss *Barbula unguiculata*. J Biol Chem 1999;274:33274-8.

Yang EJ, Oh YA, Lee ES, Park AR, Cho SK, Yoo YJ, Park OK. Oxygen-evolving enhancer protein 2 is phosphorylated by glycine-rich protein 3/wall-associated kinase 1 in *Arabidopsis.* Biochem Biophys Res Commun 2003;305:862-8.

Yoda H, Fujimura K, Takahashi H, Munemura I, Uchimiya H, Sano HH. Polyamines as a common source of hydrogen peroxide in host- and nonhost hypersensitive response during pathogen infection. Plant Mol Biol 2009;70:103-12.

Yun BW, Atkinson HA, Gaborit C, Greenland A, Read ND, Pallas JA, Loake GJ. Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises nonhost resistance in *Arabidopsis* against wheat powdery mildew. Plant J 2003;34:768-77.

Zellerhoff N, Himmelbach A, Dong W, Bieri S, Schaffrath U, Schweizer P. Nonhost resistance of barley to different fungal pathogens is associated with largely distinct, quantitative transcriptional responses. Plant Physiol 2010;152:2053-66.

Zhang S, Klessig DF. MAPK cascades in plant defense signalling. Trends Plant Sci 2001;6:520-7.

Zhang Z, Feechan A, Pedersen C, Newman MA, Qiu JL, Olesen KL, Thordal-Christensen H. A SNARE-protein has opposing functions in penetration resistance and defense signaling pathways. Plant J 2007;49:302-12.

Zimmerli L, Stein M, Lipka V, Schulze-Lefert P, Somerville S. Host and nonhost pathogens elicit different jasmonate/ethylene responses in *Arabidopsis*. Plant J 2004;40:633-46.

Journal of Plant Physiology 168 (2011) 2141–2152

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Review

Warriors at the gate that never sleep: Non-host resistance in plants

Battepati Uma, T. Swaroopa Rani, Appa Rao Podile*

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

ARTICLE INFO

Article history:
Received 16 July 2011
Received in revised form
19 September 2011
Accepted 20 September 2011

Keywords:
Extracellular matrix
Non-host resistance
Oxidative burst
Plasma membrane
Vesicle trafficking

ABSTRACT

The native resistance of most plant species against a wide variety of pathogens is known as non-host resistance (NHR), which confers durable protection to plant species. Only a few pathogens or parasites can successfully cause diseases. NHR is polygenic and appears to be linked with basal plant resistance, a form of elicited protection. Sensing of pathogens by plants is brought about through the recognition of invariant pathogen-associated molecular patterns (PAMPs) that trigger downstream defense signaling pathways. Race-specific resistance, (*R*)-gene mediated resistance, has been extensively studied and reviewed, while our knowledge of NHR has advanced only recently due to the improved access to excellent model systems. The continuum of the cell wall (CW) and the CW–plasma membrane (PM)-cytoskeleton plays a crucial role in perceiving external cues and activating defense signaling cascades during NHR. Based on the type of hypersensitive reaction (HR) triggered, NHR was classified into two types, namely type-I and type-II. Genetic analysis of *Arabidopsis* mutants has revealed important roles for a number of specific molecules in NHR, including the role of SNARE-complex mediated exocytosis, lipid rafts and vesicle trafficking. As might be expected, *R*-gene mediated resistance is found to overlap with NHR, but the extent to which the genes/pathways are common between these two forms of disease resistance is unknown. The present review focuses on the various components involved in the known mechanisms of NHR in plants with special reference to the role of CW–PM components.

Contents

Abbreviations: AGP, arabinogalactose protein; AVR, avirulence; CW, cell wall; CWA, cell wall apposition; eATP, extracellular adenosine triphosphate; ECM, extracellular matrix; GPI, glycosyl phosphatidyal inositol; HR, hypersensitive reaction; LR, lipid rafts; MAPK, mitogen-activated protein kinase; NHR, non-host resistance; PAMP, pathogen associated protein kinase; PCD, programmed cell death; PEN, penetration; PM, plasma membrane; PRR, pattern recognition receptor; R, resistance; ROS, reactive oxygen species; SNARE, soluble N-ethylmalemide-sensitive factor protein receptor; WAK, wall-associated kinase.

* Corresponding author. Tel.: +91 40 23134503; fax: +91 40 23010120.
E-mail address: arpsl@uohyd.ernet.in (A.R. Podile).

0176-1617/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2011.09.005

Non-host resistance is pathogen non-specific and is at least of two types

Plants encounter a variety of pathogenic microbes. If every pathogen were to infect every plant, plants would have faced an uphill task to survive. During the co-evolution of plants and pathogenic microbes, plants have learnt ways and means to keep most pathogens away by their ever vigilant surface recognition features (Burdon and Thrall, 2009; Dodds and Rathjen, 2010). It would, therefore, be interesting to look into the details of plants' recognition features and subsequent responses to the attempted non-specific infection by pathogens. Resistance against a majority of potential pathogens by most plant species is known as non-host resistance (NHR), which is the most common form of disease resistance that confers durable protection (Heath, 2000). Reviews on NHR have focused mainly on the following aspects: different types of NHR (Mysore and Ryu, 2004), pathogen-associated molecular patterns (PAMPs)-based recognition of non-adapted pathogen (Nurnberger and Lipka, 2005), powdery mildews in *Arabidopsis* (Ellis, 2006), molecular evolutionary concepts (Schulze-Lefert and Panstruga, 2011) and different obstacles during execution of NHR (Thordal-Christensen, 2003). This review discusses the possible mechanisms/components contributing to NHR with special reference to cell wall (CW)–plasma membrane (PM) continuum.

NHR is pathogen nonspecific general resistance that is classified into two types based on the execution of hypersensitive reaction (HR) (Mysore and Ryu, 2004). Type-I NHR is the most common form and does not produce visible symptoms. In this type, non-adapted pathogens fail to overcome the preformed and general elicitor-induced plant defense responses such as CW thickening, phytoalexin accumulation, other plant secondary metabolites and papilla formation. On the other hand, type II NHR is always associated with rapid localized necrotic HR. In this type, non-adapted pathogens are overcome by the preformed and general elicitor-induced plant defense responses, e.g. by producing detoxifying enzymes. Specific pathogen elicitors are recognized by the plant surveillance system and trigger plant defense leading to HR. The same plant species can exhibit both types of NHR (Peart et al., 2002), and the same pathogen can trigger different NHR types in different plant species (Lu et al., 2001).

During non-host interactions, a potential pathogen is recognized by invariant PAMP that activates signaling pathways and inducible plant defense responses. Inducible structural barriers like papillae restrict further growth of the pathogen and the antimicrobial compounds are mobilized to the papillae by vesicle trafficking and cytoskeleton reorganization. A variety of CW proteins like arabinogalactan proteins (AGPs), cellulose synthase and wall-associated kinase (WAK), have the potential to mediate CW–PM interactions during a pathogen invasion. This suggests that the molecules at CW–PM interface not only have a structural role through their extracellular domains, but also play a crucial role in signaling through their cytoplasmic domains, thereby establishing communication between apoplasm and cytoplasm.

The first part of this review presents a comprehensive description of available information on NHR, while the second part presents a focused discussion on the importance of the CW–PM continuum.

Insights into non-host disease resistance in plants

Pathogen recognition of by non-host plants

Recognition of non-self is the key to the activation of innate defense mechanisms in plants in response to microbial attacks. Plants resist pathogen invasion by deploying various defense responses that are activated by two main branches of their immune system (Jones and Dangl, 2006). The first branch consists of transmembrane pattern recognition receptors (PRRs) that activate basal defense responses by recognizing extracellular PAMPs common to many classes of microbes. A successful pathogen defeats this first line of defense with effectors that enhance their virulence. Pathogen effectors (virulence factors) are specifically recognized by corresponding resistance (*R*) genes in the second branch of the plant immune system.

PAMPs trigger receptor-mediated defense responses

When a potential pathogen overcomes the constitutive defense layers of a non-host plant, it is recognized at the PM of plant cells (Fig. 1). Pathogen recognition in non-host plants is brought about by PAMPs, also known as general or exogenous elicitors (Gomez-Gomez and Boller, 2002; Montesano et al., 2003; Nurnberger et al., 2004). Recognition of PAMPs by cognate PRRs induces multiple defense responses referred to as PAMP-triggered immunity (PTI) leading to basal resistance or NHR in plants. Basal disease resistance at the first glance is PTI plus weak effector-triggered immunity (ETI), minus effector-triggered susceptibility (ETS) (Jones and Dangl, 2006; Hoefle and Huckelhoven, 2008; Zhou and Chai, 2008; Nicaise et al., 2009). Pep-13 (Nurnberger et al., 1994), *Phytophthora* CW transglutaminase (TGase), lipopolysaccharide (LPS), N-terminal 22-mer fragment of eubacterial flagellin (flg22), a cold-shock-inducible RNA-binding protein (RNP-1) (Felix and Boller, 2003), and breakdown products of the plant CW (endogenous elicitors) that are probably released by CW degrading enzymes are some of the general elicitors. The dichotomy between PTI and ETI at present is blurred, where different molecules activate different defense signaling pathways, depending on the trigger, the receptor, and environmental conditions (Thomma and Nurnberger, 2011). PTI is yet to be clearly understood but novel PRRs are known to provide useful tools for genetic engineering that can provide sustainable quantitative broad spectrum disease resistance (Zipfel and Robatzek, 2010).

The links between NHR and gene-for-gene interactions

Multiple *R*-genes present in non-host plants may simultaneously recognize their corresponding *avr* gene-encoded products to activate the plant surveillance system. Cloned *avr*-genes from bacterial pathogens are recognized by previously unidentified *R*-genes

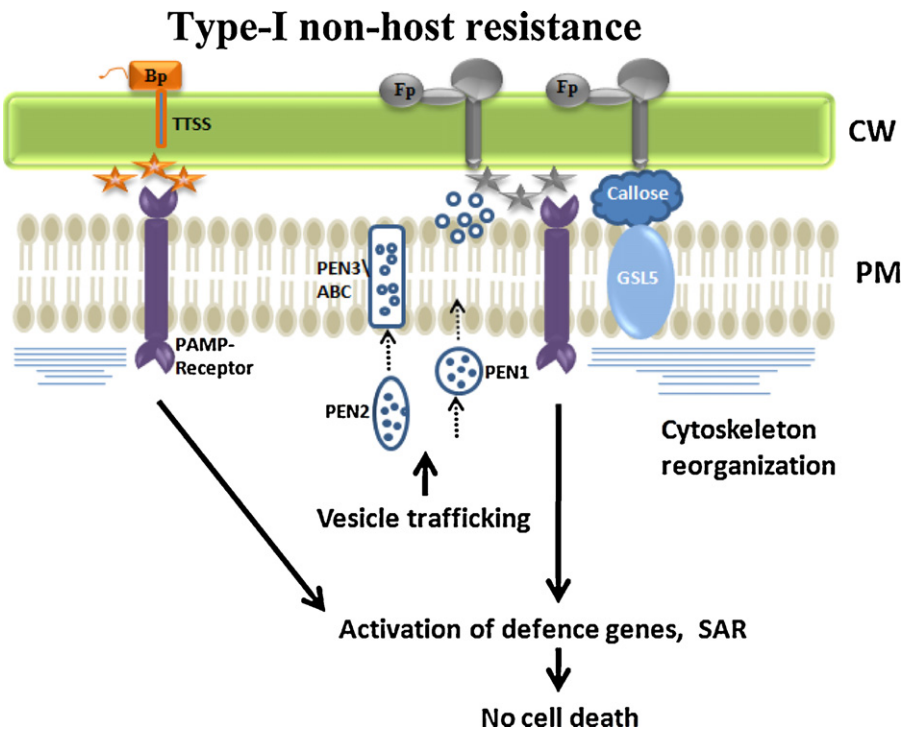


Fig. 1. Schematic representation showing type I non-host resistance. Plant cells possess a variety of membrane receptors that perceive exogenous signals (e.g. PAMP, represented as stars), which can be either peptides or oligosaccharides. A given ligand activates a specific receptor that initiates downstream signaling events. Penetration of a non-host pathogen is restricted at the cell wall by callose deposits, cytoskeleton reorganization and polarization of secretory components. Actin microfilaments (horizontal lines below the PM) become focused at the penetration site. A PEN1-mediated and vesicle-based secretion system delivers cargo molecules to the penetration site. PEN3-encoded ABC transporters deposit antimicrobial compounds at the pathogen invasion site, synthesized in peroxisomes in a PEN2-mediated pathway. Defense gene expression is induced by general elicitors of the non-host pathogen leading to type I non-host resistance without any associated cell death.

in non-host plants (Whalen et al., 1988; Kobayashi et al., 1989). This has given rise to the hypothesis that NHR is also determined by gene-for-gene interactions. Bacterial pathogens like *Pseudomonas phaseolicola* (*Psp*) are capable of sensing non-host plants and delivering effectors to the plant cell *via* the type III secretion system (Arnold et al., 2001). The absence of known *R*-genes in plants does not result in susceptibility to non-host pathogens. Although *R*-gene mediated resistance certainly contributes to resistance in non-host interactions, other mechanisms, in addition to gene-for-gene recognition, are likely to be involved in NHR. The extent to which PAMP and/or gene-for-gene recognition systems are involved in execution of NHR is still to be investigated. Increasing evidence has established a strong relationship between basal resistance mechanisms like NHR, and the *R*-gene mediated PCD (Wang et al., 2011).

Signal transduction during defense response

PAMP perception triggers activation of signaling cascade and transcriptional changes. Early events in the cascade involve ion fluxes, protein phosphorylation, enhanced cytosolic Ca^{2+} concentrations, Ca^{2+}-dependent protein kinases, mitogen-activated protein kinase (MAPK), extracellular adenosine triphosphate (eATP) and reactive oxygen species (ROS) accumulation (Huckelhoven, 2007). Ca^{2+} influx into the cytoplasm results in the decrease of apoplastic Ca^{2+} concentration which might influence CW rigidity as calcium plays a role in non-covalent cross-linking. This Ca^{2+} influx is essential for alkalization of apoplast and initial ROS accumulation (Blume et al., 2000; Grant et al., 2000). MAPKs are important in cross-talk during stress signaling that results in protection against microbial invasion (Nurnberger and Scheel, 2001; Zhang and Klessig, 2001; Gomez-Gomez and Boller, 2002; Jonak et al., 2002). In *Nicotiana benthamiana*, virus-induced gene silencing of NbSIPK and NbWIPK (orthologues of AtMPK6 and AtMPK3, respectively) allows multiplication of non-host bacterium *Pseudomonas cichorii* revealing the importance of MAPK in NHR (Sharma et al., 2003). High-throughput over expression and NbMKK1-silencing in *N. benthamiana* has further confirmed the role of MAPKs in controlling NHR (Takahashi et al., 2007).

Plant hormones like salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) appear to be involved in the later signaling events of induced immune responses. The genetic evidence about the role played by hormones in non-host interactions came from the mutant analysis of *Arabidopsis*, defective in SA (sid2), JA (jar1) and ET-mediated defense signaling pathways (Knoester et al., 1998; Zimmerli et al., 2004). Different non-host *Uromyces* sp. displayed different penetration frequencies on these *Arabidopsis* mutants. Sunflower rust fungus (*Puccinia helianthi*) failed to form haustoria on any of the *Arabidopsis* mutants lacking the production of SA and JA/ET. This was attributed to strong expression of wall-associated defense responses which are not induced by *Uromyces* spp. (Mellersh and Heath, 2003). Mysore and Ryu (2004) showed the crucial importance of JA, ET and SA not only in cultivar specific resistance but also in the maintenance of NHR in specific plant–non-host microbe interactions. The differential response of *Arabidopsis* mutants to different pathogen in NHR suggests the need to further dissect the role of hormones in this form of resistance.

Pre- and post-haustorial phase of NHR

NHR during plant–pathogen interactions operates *via* two phases. An attempted penetration of the CW by appressoria is aborted within the infection-induced papillae at a majority of the infection sites (Collins et al., 2003; Assaad et al., 2004). In type I NHR, only the pre-haustorial phase operates, while in the post-haustorial phase, haustoria formed by the appressoria are encased by callose to undergo HR (Fig. 1). In type II NHR, post-haustorial phase plays

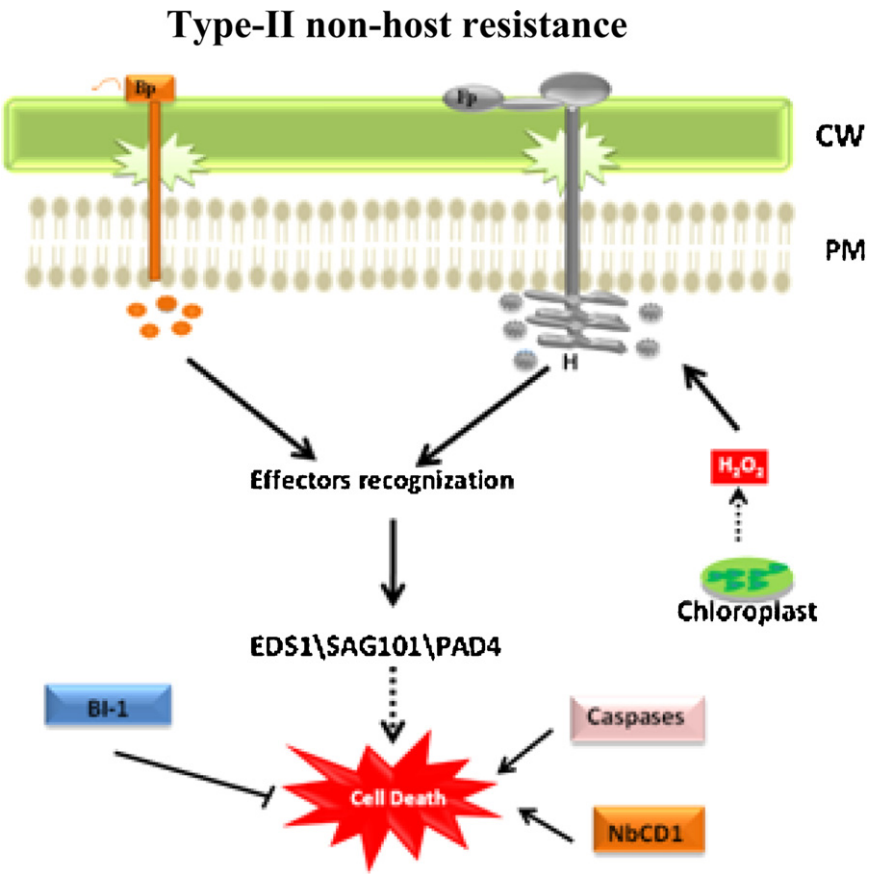


Fig. 2. Schematic representation showing type II non-host resistance. In type II non-host resistance, the non-host pathogen is able to overcome preformed and general elicitor induced plant defense responses, probably by producing detoxifying enzymes. Specific pathogen elicitors are then recognized by the plant's surveillance system, and this triggers plant defense reactions leading to cell death. EDS1, SAG101 and PAD4 genes, which are involved in R-gene mediated resistance, can also function in perceiving effector molecules (represented as stars below the PM) secreted by the non-host pathogen. H_2O_2 produced during electron transport in chloroplasts has been shown to be involved in the restriction of haustoria. Cell death regulators like BI-1 (Bax inhibitor 1), caspases and NbCD1 also play a role in triggering cell death during type II non-host resistance.

a major role (Fig. 2). A major difference between *R*-gene mediated resistance and the type I NHR is that in the former resistance occurs in the post-haustorial phase, whereas in the latter, it occurs mainly in pre-haustorial phase which is not likely to be associated with HR (Ellis, 2006).

Pre-haustorial resistance is effective against penetration by non-host pathogens

CW-associated defense mechanisms play a major role in pre-haustorial penetration resistance phase of NHR. Most of the non-host pathogens are unable to breach the plant CW as the central feature of penetration resistance is the formation of a cell wall apposition (CWA), or papillae, which constitute a physical and chemical barrier to cell penetration (Schmelzer, 2002) (Fig. 1). One of the earliest responses of plants to penetration by pathogens is the rapid translocation of cytosolic and subcellular components to the infection site (Takemoto and Hardham, 2004; Lipka et al., 2005). At least two vesicle-associated soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE)-mediated exocytosis pathways appear to drive focal or non-directional secretion of antimicrobial cocktails into the apoplastic space (Fig. 1). The antimicrobial secretions include proteins, small molecules and CW building blocks. Pre-haustorial phase of NHR involves multi-component secretory pathways, cytoskeletal elements and NAC transcription factors (Jensen et al., 2007).

Involvement of R-gene mediated resistance in post-haustorial resistance

Post-haustorial resistance in non-host plants may also involve a small but significant portion of *R*-gene mediated resistance. During non-host pathogen interaction, some of the spores escape the pre-haustorial resistance and form haustoria which are mopped up by the HR-mediated cell death (Fig. 2). This form of resistance is dependent on several genes linked to *R*-gene-mediated resistance that stabilizes R proteins (Peart et al., 2002; Kanzaki et al., 2003). Genes like *EDS1*, *PAD4*, *SAG101*, and *NHO1* are likely to participate in various non-host responses after penetration resistance has been breached (Table 1). Compromising of NHR is evident in *pen2 pad4 sag101* triple and *pen3eds1* double mutants, but not in *pad4sag101* double mutant (Zimmerli et al., 2004; Lipka et al., 2005; Stein et al., 2006). NHR is severely compromised in *Arabidopsis* against *Bgt* when the actin cytoskeleton functions in combination with *eds1* (Yun et al., 2003). The silencing of SGT1 (ubiquitin ligase-associated protein) also compromised NHR against *Pseudomonas syringae* pv. *maculicola* and *Xanthomonas axonopodis* pv. *vesicatoria* (Peart et al., 2002). Silencing of heat shock proteins (Hsp70 and Hsp90) results in the reduction of NHR against non-host pathogen *P. cichorii* due to the reduction in expression and HR (Kanzaki et al., 2003). Another mutational analysis of *Arabidopsis* indicates the involvement of specific gene NHO1 (glycerol kinase) in NHR against bacteria (Kang et al., 2003). Precise role of SGT1 and the Hsps in NHR remains to be investigated.

Table 1
List of genes/proteins that are involved in the execution of non-host resistance.

Gene/protein	Function in non-host resistance (NHR)	References
PAMP involved in NHR		
Pep-13	Induces defense responses in non-host plants like parsley and potato.	Nurnberger et al. (1994
Harpin (Hrp Z)	Elicits HR-like cell death and defense responses in various plants.	He et al. (1993)
Avirulennce (*avr*) genes	These genes from bacterial pathogens are recognized by previously unidentified *R*-genes in non-host plants.	Kobayashi et al. (1989) and Arnold et al., 2001
Genes involved in NHR		
PEN1(*PENETRATION 1*)/*ROR2*	This gene is involved in timely deposition of papillae during non-host interactions.	Collins et al. (2003)
PEN2	Encodes myrosinase involved in hydrolysis of indole glucosinolates to release potential antimicrobial components at the site of non-host interaction.	Lipka et al. (2005) and Bednarek et al. (2009)
PEN3/PDR8	May be involved in exporting toxic materials to the site of non-host pathogen interaction and intracellular accumulation of toxins.	Stein et al. (2006)
ETR1-1	Ethylene insensitive (*etr1-1*) tobacco plants lost resistance against many non-host pathogens; but *N*-gene-mediated gene-for-gene resistance against TMV was not compromised.	Knoester et al. (1998)
NHO1	Required for NHR of *Arabidopsis* against *Pseudomonas syringae* pv. *phaseolicola*.	Kang et al. (2003)
R-gene mediated genes involved in NHR		
EDS1	This gene is necessary for *R*-gene-mediated resistance to many pathogens in *Arabidopsis* and also involved in execution of NHR against isolates of *Peronospora parasitica* and *Albugo candida*.	Parker et al. (1996)
SGT1	Silencing of *SGT1* in *N. benthamiana* compromises NHR against *P. syringae* pv. *maculicola* and *Xanthomonas axonopodis* pv. *vesicatoria*	Peart et al. (2002)
Heat-shock proteins (Hsps)	Silencing of Hsp90 and Hsp70 in *N. benthamiana* individually compromised NHR against *P. cichorii*.	Kanzaki et al. (2003)
WIPK and *SIPK*	In *N. benthamiana* virus-induced gene silencing of *NbSIPK* and *NbWIPK* allowed multiplication of non-host bacterium *P. cichorii*.	Sharma et al. (2003)
PAD4/SAG101	*Pad4* and *sag101* single mutation have little effect on the frequency of *Bgh* haustoria formation in *Arabidopsis*. But along with *pen* mutation (*pen2 sag101pad4*) NHR was compromised.	Lipka et al. (2005) and Stein et al. (2006)

The pathways leading to NHR and gene-for-gene resistance show similarities as well as significant differences (Fig. 3). The major difference between NHR and incompatible interactions are to be located at the first barrier of the plant during pathogen recognition. The initial contact of the pathogen with ECM triggers a series of downstream signaling cascades. This contact point appears as the most crucial interacting platform for NHR. Therefore, the next part of this review closely examines this part of the interaction and the involvement of related components in NHR, in the light of the above discussion.

Proposed involvement of CW–PM continuum and its various components in NHR

Extracellular matrix as a fence/barrier against attack by non-host pathogen

The plant CW or extra cellular matrix (ECM) is a complex arrangement of carbohydrates and proteins (Showalter, 1993), whose composition has been difficult to study in part due to its limited solubility. The ECM plays a variety of roles in the growth, development, and defense of the cell against environmental stresses besides providing structural support to it (Ringli, 2010). It serves as a channel for signals, as a depository for important signaling molecules, and also as a depository of extracellular domains of the PM constituents. The first contact of a pathogen with a plant must include some form of interaction with the CW. Increasing evidence suggests that there is continuous cross-talk between ECM and the cytoskeletal network (Kobayashi et al., 1997) (Fig. 3). The nature and mechanism(s) by which these signals are perceived by the ECM and conveyed to the cell interior are poorly understood. Several molecules like mechanosensors, Arg-Gly-Asp motifs (RGD-motifs), AGP, callose synthase, cellulose synthase, formins etc., act as links between elements of the cytoskeleton and the ECM components (Kohorn, 2000; Baluska et al., 2003) (Fig. 3). The extent of involvement of these links in transferring signals from exterior to interior environment during non-host interactions is yet to be deciphered. Host and non-host pathogens are recognized at the CW and further downstream signaling takes place through the CW–PM continuum. Apoplastic defenses like CW strengthening, CWAs, apoplastic oxidative burst, antimicrobial proteins and phytoalexins are induced upon pathogen recognition. As a second layer of defense, HR-mediated cell death restricts the growth of non-pathogens due to accumulation of ROS.

Sensing of pathogens by plant mechanosensors

The mechano-sensing network is typically located within the CW–PM–cytoplasm continuum (Jaffe et al., 2002; Braam, 2005). Modifications of CW cellulose and contact of pathogen with the CW also induce defense response. Mutation in constitutive expression of VSP1 (CEV1), which encodes the cellulose synthase isoform CesA3, leads to constitutive production of JA/ET resulting in enhanced resistance to broad range of pathogens (Ellis and Turner, 2001; Ellis et al., 2002). Cellulose binding effector lectin (CBEL) of *Phytophthora parasitica* has no hydrolase activity and is useful for the attachment of the pathogen to the CW. However, such interaction induces local HR in the host plant (e.g. tobacco), as well as in the non-host plant *Arabidopsis*, supporting the possibility of cellulase synthase acting as a mechanosensor (Gaulin et al., 2006). Theseus1 (The1) is also involved in sensing cellulose alterations in the CW. Apart from cellulose synthase, other reported mechanosensors like callose synthase, PM glucan synthase (gls5) and calcium

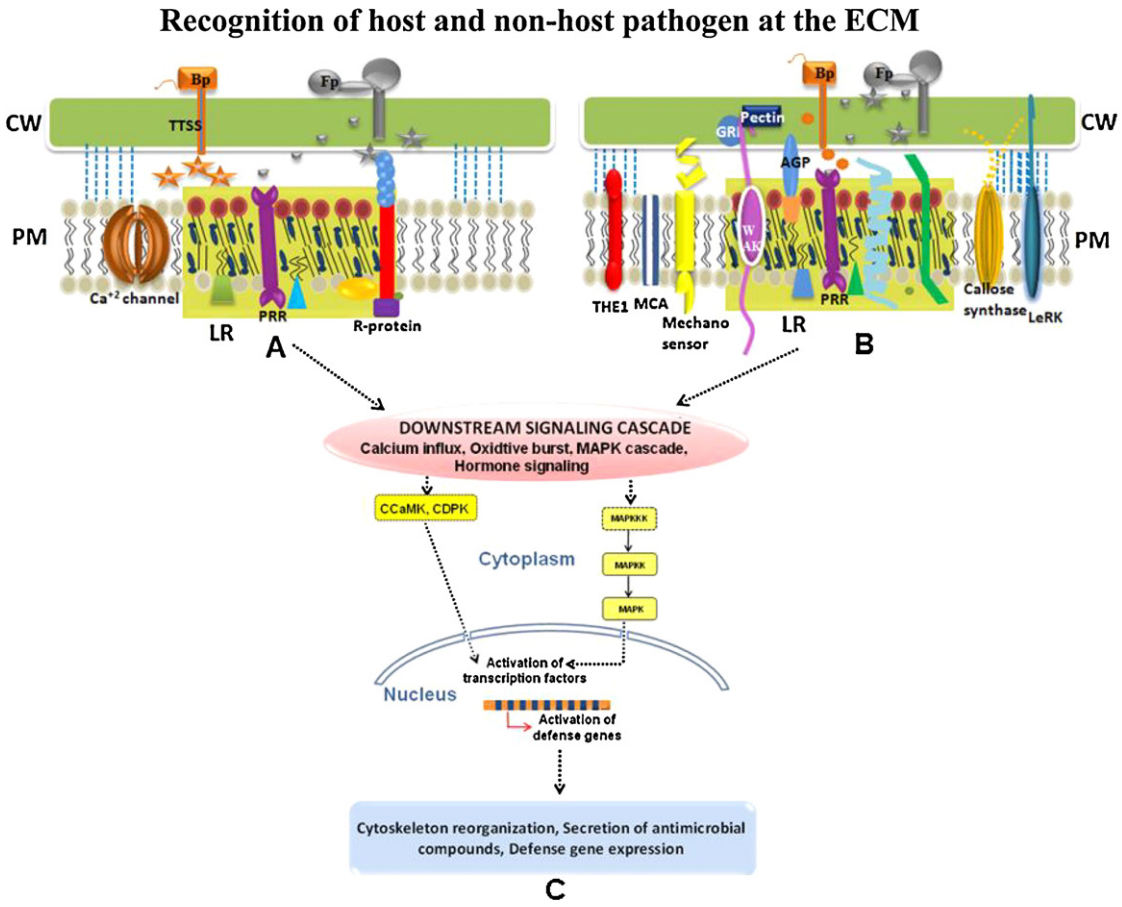


Fig. 3. Schematic representation of the sensing of (A) host, (B) non-host pathogen and (C) downstream signaling at the ECM. (A) Plant cells sense host pathogens, either bacterial (Bp) or fungal (Fp), through recognition of elicitor molecules (indicated as stars) at the ECM by membrane receptors like pattern recognition receptors (PRR) and R-proteins. These molecules along with R-protein adapter molecules and some integral PM proteins may associate with lipid rafts (LR-boxed area of the plasma membrane) forming a signallosome complex and process downstream signaling. (B) During non-host pathogen interaction, plant cell walls may sense the pathogens by monitoring the integrity of the cell wall through mechanosensors like THESEUS1 (THE1), cellulose synthase (CESA) and Ca^{2+} channels like MCA1. CW–PM linkers like wall-associated kinases (WAKs), glycosylphosphatidylinositol (GPI), anchored arabinogalactan proteins (AGPs) and Hechtian strands (dotted lines between the CW and PM) can sense the disturbance caused by non-host pathogens by spanning the PM and ECM. CW–PM linker molecules along with PRR and other putative peripheral and integral PM proteins may also form during R-gene mediated resistance and further signals downstream. (C) Though the sensing of pathogens at the plant's CW–PM interface appears to be different in R-gene mediated and non-host resistance, both processes converge for downstream signaling and induce rapid defense responses such as the activation of the MAPK cascade, hormone signaling, protein phosphorylation, cytoskeletal reorganization, and induction of defense genes to produce defense-related compounds like phytoalexins, protease inhibitors and proteases at the CW.

channel (MCAI) in *Arabidopsis* are localized in PM which activate upon mechanical stimulation (Nishimura et al., 2003; Nakagawa et al., 2007).

Callose synthase a CW–PM linker

Callose synthase, encoded by the gene PMR4, synthesizes callose and is rapidly up-regulated upon diverse stress situations, including mechanical stress (Sivaguru et al., 2000; Jaffe et al., 2002). Callose synthase mutant plants (*gsl5*) are resistant to pathogens, rather than being susceptible. Double-mutant analysis indicated that blocking of SA defense signaling pathway was sufficient to restore susceptibility to *pmr4* mutants (Jacobs et al., 2003; Nishimura et al., 2003). This proves that callose or callose synthase negatively regulates the SA pathway. Unconventional plant specific myosin VIII binds directly or *via* some further adaptor proteins to the callose synthase complex. This has revealed that callose synthases along with plant specific myosin VIII act as one class of cytoskeleton–CW linkers of plant cells (Fig. 3) (Verma and Hong, 2001; Ostergaard et al., 2003).

WAKs involvement in CW–PM continuum

CW-associated receptor kinases (WAK) physically link the plant ECM to the PM and are potential signaling molecules (He et al., 1999). At least five WAK are known in *Arabidopsis*. WAKs have a cytoplasmic Ser/Thr protein kinase domain, spanning the PM and extending a domain into the CW (He et al., 1999). WAKs link the PM to the carbohydrate matrix and have the potential to convert the signal into cellular events through their kinase domain. Mutations in WAKs demonstrated their centrality in plant development and also in plants' response to pathogens (He et al., 1998, 1999; Wagner et al., 1999). Induced expression of *WAK* is required for the survival of the plant during the response to a pathogen (He et al., 1998; Brutus et al., 2010). Implication of *WAK1* as a pathogenesis related (PR) gene was confirmed by its SA-mediated induction in an *NPR1*-dependent manner (He et al., 1998). Moreover, *WAK1* is up-regulated during systemic acquired resistance and is also induced by the fungal pathogen *Alternaria brassicicola* and by the defense related signaling molecules methyl jasmonate and ET (Maleck et al., 2000; Schenk et al., 2000). Kohorn (2001) showed that pectin along with secreted Gly-rich proteins (GRPs) (Mangeon et al., 2010) is a ligand of WAKs. Pectins are already known as major signaling

components of the CW involved in a series of physiological responses including morphogenesis, cell differentiation and host–pathogen interactions (Ridley et al., 2001). The WAKs are good candidates for receptors that monitor pectin integrity during pathogen attack and trigger defense response. Therefore, studies on the involvement of WAKs in signaling from the pectin ECM, in coordination with GRPs, are a key to understand the role of CW–PM continuum in cell growth and NHR.

Role for extracellular ATP and ADP in cell signaling

Extracellular ATP (eATP) and ADP along with other nucleoside triphosphates (NTP) and diphosphates (NDP) function as both autocrine and paracrine signaling molecules in the animal ECM by activating downstream signal transduction cascades. In plants, ATP (NTP/NDP) exits the cytosol and enters the ECM by passive means, resulting from the loss of PM integrity, or by wound-induced cell damage. eATP initiates signaling across the plant PM through various mechanisms like depolarizing the PM (Lew and Dearnaley, 2000; Demidchik et al., 2003; Jeter et al., 2004), triggering Ca^{2+} influx (Jeter et al., 2004), stimulating the accumulation of superoxide and nitric oxide (Song et al., 2006; Foresi et al., 2007; Wu and Wu, 2008), and inducing expression of MAPK and ethylene biosynthesis genes (Jeter et al., 2004; Song et al., 2006). eATP-mediated Ca^{2+} influx and transcriptional activation of MAPK in *Arabidopsis* roots are driven by PM NADPH oxidase (AtRBOHC)-dependent production of ROS (Demidchik et al., 2009). The decrease of eATP in the ECM, either upon pathogen inoculation or by enzymatic depletion, leads to over expression of PR genes against tobacco mosaic virus and *P. syringae* pv. *tabaci* in tobacco (Chivasa et al., 2009). The eATP present in ECM appears to play a pivotal role in NHR.

AGP signaling molecules in the ECM

AGPs and other glycosyl phosphatidyl inositol (GPI) anchored proteins (Fig. 3) identified in *Arabidopsis* play a key role in cell surface processes such as signaling, adhesion, matrix remodeling and pathogen response (Kohorn, 2000; Borner et al., 2002). AGPs are heavily glycosylated proteins which contain signals for the addition of a carboxy-terminal GPI anchor. Upon secretion, AGPs remain on the PM exposed to the CW, and have strong potential to bind to components of the CWAs (Oxley and Bacic, 1999; Celio et al., 2004). On binding with the CW pectins, AGPs can efficiently act as integrity sensors and affect downstream signal transduction (Majewska-Sawka and Nothnagel, 2000). We need to obtain compelling evidence to assign a specific role to the AGPs and their signaling in NHR.

RGD motifs in CW–PM attachments

In mammalian cells, the extracellular proteins that interact with integrins contain a characteristic RGD motif and serve as transmembrane mechanical links. RGD-containing peptides directly activate integrins (Hynes, 2002). The observation that plant PM proteins exhibit high affinity for the RGD sequence led to the identification of RGD receptors (integrin-like) (Canut et al., 1998) projecting the role of RGD motifs in CW–PM attachments. Further, the presence of integrin-like domains (Laval et al., 1999) in plants suggests some conservation between proteins involved in ECM–PM interactions in plant and animal systems. Interestingly, one of the responses to RGD peptides is the disruption of Hechtian strands, which link the CW to the PM and which appear during plasmolysis (Lee-Stadelmann and Stadelmann, 1989; Canut et al., 1998), substantiating the role of RGD motifs in CW–PM attachments.

CW–PM adhesion increases in host interactions such as powdery mildew-barley/cowpea and also in non-host interactions such as powdery mildew (*Erysiphe polygoni*)–non-host cowpea plants. The importance of CW–PM adhesion was revealed by Mellersh and Heath (2001), who show that rust fungi inoculated on cowpea cells pre-treated with powdery mildew fungi do not induce localized decrease in CW–PM adhesion below the penetration site, while also reducing the penetration frequency. A variety of RGD binding proteins may exist in plants, and they may differ in their specificity profiles. In non-host plants, the elimination of CW-associated responses by the application of RGD peptides was correlated with a significant increase in penetration efficiency by both the cowpea rust fungus and the plantain powdery mildew fungus, and with a significant increase in the intracellular growth of the rust fungus (Christopher-Kozjan and Heath, 2003). Thus, the role of RGD motifs in CW–PM adhesion is important for the expression of CW-associated responses.

Lectin receptor kinases

Lectins are proteins that bind to, but do not alter, carbohydrates (Jiang et al., 2010). A large family of receptor kinases has been identified which contain an extracellular lectin-like domain (Herve et al., 1999). Synthetic RGD peptides (RGD binding protein) allowed identification of lectin receptor kinases (LecRKs) in *Arabidopsis*. LecRK is involved in protein–protein interactions with RGD-containing proteins and plays an important role in structural and signaling pathways. *AtELP*s, which share homology with mammalian integrins, are up-regulated after bacterial infection (He et al., 1998; Laval et al., 1999). The CrRLK1 family was also identified as containing an RGD-binding motif in its extracellular domain and was thus similar to the LecRKs, implicated in mediating CW–PM contacts (Gouget et al., 2006). LecRKs are expected to bind polysaccharides in the plant CW and sense changes in host CWs upon pathogen intrusion.

We have highlighted AGPs, WAKs, cellulose synthase, and RGD as these are proteins known to directly contact the lipid bilayer and the carbohydrate complex. It appears that the AGPs and, perhaps WAKs, signal the CW architecture upon pathogen invasion. The specific and regulated association of cellulose synthase with the carbohydrate matrix possibly specifies cell position and signaling during pathogen attack. We know of only a few CW–PM contacts, as described here, but more are likely to be discovered. Of the defined ones, such as WAKs and AGPs, it remains to be determined how these molecules, among others, achieve an interaction that induces downward signal transduction pathways during host and NHR. In this sense, the proteins at the CW–PM interface might serve at least two functions, communication and structural (perhaps less specific) roles. Molecular interactions between the receptor kinases of PM and their corresponding ligands in the ECM are likely to initiate diverse signaling pathways that play key roles in NHR.

Lipid rafts/DRMs as signaling platforms

Lipids and proteins of the PM are not homogeneously distributed within membranes but rather form domains known as lipid rafts (LRs) (Pike, 2006). The LRs were implicated in signal transduction (Simons and Toomre, 2000), regulation of exocytosis (Salaun et al., 2004), endocytosis (Parton and Richards, 2003), apoptosis (Garcia et al., 2003), and actin cytoskeleton organization (Wickstrom et al., 2003; Falk et al., 2004). Subversion of the LR function was found to be important for pathogen entry (Rosenberger et al., 2000; Lafont et al., 2004). LRs are also called as detergent-resistant membranes (DRM) because of their insolubility in Triton-X 100. The DRMs may represent "signaling platforms" in which various components of signal transduction cascades are locally condensed (Hoessli et al., 2000). Upon stimulation, the rafts may cluster to form a larger platform where functionally related proteins can interact (Simons and Toomre, 2000; Garcia et al., 2003). An NADPH oxidase isoform, whose expression is specifically triggered upon treatment with pathogen elicitors, is recruited to the tobacco DRMs upon elicitation. Fluorescently tagged

versions of three proteins *At*PEN1 as well as *Hv*ROR2 and *Hv*MLO are evenly distributed in the PM of unchallenged (healthy) leaf epidermal cells but, focally accumulate at the attempted fungal pathogen entry sites, thereby defining the recruitment of proteins to lipid microdomains during particular biotic and/or abiotic stresses (Assaad et al., 2004; Bhat et al., 2005).

It is to be seen that most GPI-anchored proteins may be associated with presumptive DRMs (Brown and Rose, 1992; Schroeder et al., 1994; Varma and Mayor, 1998). Quantitative proteomic study by Stanislas et al. (2009) described the dynamic association of proteins with DRMs upon challenge of tobacco cells with an elicitor of defense reactions. In *Arabidopsis*, a novel membrane-bound RING motif protein, displaying E3 ubiquitin ligase activity, was upregulated by fumonisin B1 treatment and the pathogen infection. GFP fusion protein localization and cell fractionation experiments showed E3 ligase association with the DRM of PMs (Lin et al., 2008). Localization of Rac/Rop GTPases to DRMs is important for their activation in various signaling pathways (Bloch et al., 2005; Sorek et al., 2007; Yalovsky et al., 2008). In rice, *Os*Rac1 and *OsrbohB* localize to the DRM and interact with each other to activate ROS production and induce innate immunity (Ono et al., 2001; Wong et al., 2007; Fujiwara et al., 2009). The DRM at the PM plays a major role in perceiving signals from the cell periphery, and transferring the signal to the interior domain by interacting with both CW proteins and proteins residing in the cytosol. The exact role of all these CW–PM linkers in transduction of signals inside of the cell is not known. The signals transduced into the intracellular environment are yet to be identified.

Cytoskeleton and vesicle trafficking in the formation of CWA

During non-host plant–pathogen interactions, polarized accumulation of defense compounds at the pathogen intruder site depends on cytoskeletal rearrangements and secretory processes (Schmelzer, 2002; Lipka and Panstruga, 2005). It is thought that the actin cytoskeleton plays a pivotal role for cell polarization process by providing tracks for organelle and vesicle traffic during compatible, incompatible and non-host interactions (Takemoto et al., 2003). By the inhibition of actin polymerization using cytochalasins, allowed *Erysiphe pisi* penetrate and form haustoria in the non-host plant barley but there was no disease development because of HR (Kobayashi et al., 1997).

Molecular genetic analyses of *Arabidopsis*, showing altered pre-haustorial responses, led to the identification of genes encoding secretion-associated and efflux-associated proteins like PM-resident PEN1 syntaxin, PEN2 myrosinase, and PM-resident PEN3 ABC transporter (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006) (Table 1). In *Arabidopsis*, SNARE domain-containing PEN1 syntaxin, SNAP33 and VAMP721/722 assemble into a ternary SNARE complex which is thought to bind secretory vesicles containing unknown cargo for the plant PM (Kwon et al., 2008). This mobilization of the secretory pathway was precisely localized at sites of attempted penetration by non-adapted (*Bgh*) as well as adapted (*Golovinomyces orontii* and *Golovinomyces cichoracearum*) powdery mildew pathogens (Assaad et al., 2004; Bhat et al., 2005; Kwon et al., 2008). Depletion of VAMP721/722 enhances susceptibility to the adapted fungus *G. orontii*, the oomycete *Hyaloperonospora parasitica*, as well as to non-adapted *E. pisi*, implicating the involvement of additional syntaxins in vesicle-mediated defense (Kwon et al., 2008). ADP ribosylation factor1 (ARF1), whose primary function in plants is in vesicle trafficking (Lee et al., 2002; Pimpl et al., 2003; Contreras et al., 2004; Memon, 2004; Molendijk et al., 2004), exhibited a strong increase in the transcript levels upon inoculation with the non-host pathogen *P. cichorii*, but not with the virulent pathogen *Pst* in *N. benthamiana* (Coemans, 2008). *At*SYP122, a homolog of PEN1/*At*SYP121, is not involved in penetration resistance to *Bgh* as is evident by mutant analysis of fucose synthesis mutant *mur1* and *Atsyp122*, which were more strongly affected in CW composition than *pen1* (Assaad et al., 2004). This indicates a specialized role of PEN1 in penetration resistance, whereas SYP122 might have a general role in the secretion of CW material. SYP122, together with PEN1, controls SA, JA and ET-dependent defense as well as HR-mediated cell death indicating that the CW-associated defenses control other defense mechanisms to avoid inappropriate defense reactions (Zhang et al., 2007).

Secretory vesicles are involved in the deliberate poisoning of the apoplast by transporting secondary metabolites acting as toxic components to sites of incipient pathogen ingress. A striking example supporting this hypothesis comes from studies of non-host interaction between sorghum (*Sorghum bicolor*) and *Colletotrichum graminicola*, where accumulation of red-colored 3-deoxyanthocyanidin phytoalexins takes place at pathogen ingression areas (Snyder and Nicholson, 1990; Snyder et al., 1991). The polarized transport correlates with a reorganization of the actin cytoskeleton and results in a chemical CW reinforcement that involves binding by ether linkages of vesicle-transported hydroxycinnamic acid amides (McLusky et al., 1999). All three PEN genes are also involved in secretion and accumulation of anti-microbial components to the papillae (Fig. 1). In *pen1* mutants, the delay in papillae formation resulted in decreased penetration resistance but without disease development indicating that PEN1/*Hv*ROR2 is not the only component of complex NHR mechanisms (Assaad et al., 2004).

PEN2 accumulated underneath *Bgh* contact sites in *Arabidopsis*, where the enzyme intiates the metabolism of group of tryptophan-derived compounds known as indole glucosinotes (IGs), to release potential antimicrobial compounds. Glucosinolates together with their hydrolytic enzymes myrosinases are known to be involved in plant defense (Sonderby et al., 2010). Compared to the single mutant *pad3*, the double mutant *pen2pad3* was not only impaired in penetration resistance, but also supported hyphal growth of the non-adapted fungus *E. pisi* in *Arabidopsis*. These data indicate the importance of accumulation and sequential action of PEN-2 derived IGs, hydrolysis products and camelexin in NHR (Bednarek et al., 2009; Sanchez-Vallet et al., 2010). Pathogen entry rates of pathogen were twice as high in the double mutant *pen1pen2*, when compared to single mutants of either *pen1* or *pen2* (Lipka et al., 2005), suggesting that *PEN1* and *PEN2* act in separate defense pathways. PEN3/PDR8 may be involved in exporting toxic materials to attempted invasion sites, and intracellular accumulation of these toxins in *pen3* mutants may secondarily activate the SA pathway (Stein et al., 2006). Vesicle trafficking-mediated extracellular accumulation of toxic antimicrobial compounds, though, could be contained within the papillary CW scaffold, and might form a fine-tuned chemical and structural barrier against microbial intruders together with chemical cross-linking of the newly synthesized CW polymers (Kwon et al., 2008).

Oxidative burst at apoplast restricts the entry and growth of non-host pathogens

Inhibition of secretory system by inhibitors like monensin, brefeldin A and NEM leads to differential inhibition of ROS production, suggesting the involvement of secretory components generation of apoplastic H_2O_2 (Bolwell et al., 2002; Davies et al., 2006). Both CW and ECM can respond to environmental clues like biotic and abiotic stress through the release of signaling molecules like H_2O_2. The production of ROS such as O_2^-, H_2O_2 and $HO^\bullet$ at the cell surface, well known as the "oxidative burst", is one of the earliest events detectable during the incompatible and non-host plant–pathogen interactions (Yoshioka et al., 2008). ROS production in the CW is to modify CW components by the

process of CW softening/stiffing, to control plant growth, and to restrict the growth of pathogens. The major sources of ROS are PM-localized NADPH/NADH-dependent generation of superoxide or CW-localised peroxidase-dependent generation of H_2O_2 (Bolwell, 1999). During the non-host interaction of tobacco–*P. cichorii*, accumulation of polyamines in the apoplast and increase in the activity of poly(di)amine oxidase were observed (Yoda et al., 2009). Several lines of evidence from various plant species, now suggest that the sources of ROS are different in NHR and in incompatible interaction, but the pathways may interact with each other. The apoplastic oxidative burst during basal resistance is likely to be peroxidase-dependent, as is evidenced in *Arabidopsis* (Soylu et al., 2005; Bindschedler et al., 2006). Different plants exhibit different oxidative burst phases with an early production of ROS in both compatible and incompatible interactions (Grant et al., 2000; Apel and Hirt, 2004). In plant–fungal interactions, oxidative burst occurs in two or three phases, whereas biphasic accumulation of ROS occurs in plant–bacterial interaction (Baker and Orlandi, 1995; Thordal-Christensen et al., 1997; Huckelhoven et al., 2000). Increase in extracellular peroxidase activity and H_2O_2 accumulation in lettuce-*Psp* non-host interaction (Bestwick et al., 1998) has been known for a long time. Subcellular production of ROS also plays a role in NHR. Non-host interactions between tobacco and *Xanthomonas campestris* pv. *vesicatoria*, resulted in ROS accumulation in chloroplasts, followed by the appearance of localized lesions typical of the HR (Zurbriggen et al., 2009). Despite the similarities to host resistance, the involvement of ROS in non-host interactions may vary in terms of the intensity and timing of ROS bursts (Huckelhoven et al., 2001; Able et al., 2003). A mechanical transducer detecting CW–PM perturbations has been proposed to be involved in the initiation of responses, such as extracellular ROS production. The existence of a physical link between sensors in CW and receptors in PM with pathogen ligands might be involved in controlling recognition of the challenging pathogen and activation of ROS production (Kacperska, 2004).

Involvement of conventional cell death regulators in execution of NHR

Cell death regulators play a crucial role in the regulation of HR-mediated cell death in NHR. So cell death regulators identified so far as being involved in NHR are NbCD1, BAX inhibitor-1 (BI-1) and caspases. NbCD1, a novel class II ethylene-responsive element binding factor (ERF), is a potent inducer of the HR-like cell death and is expressed upon treatment with non-host pathogen *P. cichorii* (Nasir et al., 2005). Over expression of HvBI-1, suppressors of PCD, in barley epidermal cells weakened CW-associated H_2O_2 formation, penetration resistance to appropriate and inappropriate *Blumeria graminis*, and *MLA12*-mediated post penetration resistance (Eichmann et al., 2004, 2006). Inhibition of caspases, the key executers of PCD, delayed HR in non-host plants like cowpea or French bean and in the tobacco–*Psp* non-host system, but not in cowpea–rust fungus interaction. Cell death during non-host interactions requires caspase-like activity in several non-host combinations, but not in incompatible host resistance (Christopher-Kozjan and Heath, 2003), indicating the involvement of conventional cell death regulators in incompatible as well as non-host interactions. Whether this suicide response is also controlled by key regulators of cell death associated with *R*-gene mediated resistance is being investigated.

Conclusion

Plant pathogenic microorganisms need to breach the CW (the gate) barrier to gain access to the host. Plants have guarding molecules (warriors) built-in to the CWs to sense the danger signals of pathogens. Mechanosensors like THESEUS1 (THE1), enzymes like cellulose synthase, Ca^{2+} channels like MCA1, CW–PM linkers like WAKs, etc., located in the CW–PM continuum are some of the guarding molecules involved in activation of NHR in plants. Activation of innate immunity is due to recognition of danger signals that are directly derived from microbe or microbial invasion-derived damage or from other molecules not known to be present in the healthy plants. The progress made towards understanding NHR compared to *R*-gene-mediated resistance, has been limited due to the complexity of NHR. Recently, host components associated with NHR have been identified, including a few involved in both forms of resistance. NHR is widely thought to be the result of multiple barriers that must be overcome by the pathogens (Thordal-Christensen, 2003). Non-host and *R*-gene-mediated resistance have separate signal transduction pathways with a significant amount of cross-talk between them and a possible convergence of the two pathways at a later stage (Mysore and Ryu, 2004). The existence of a structural continuum connecting the cellular interior through the PM with the ECM is well documented in animal cells. In contrast, the existence of an analogous continuum between CW–PM and the cytoskeleton in plant cells has been postulated but only a few potential linkers have been actually identified. Nevertheless, CW–PM connections proved to be essential for signal transduction during pathogen attacks as is evident in the roles of RGD motifs, DRMs and LecRKs. Whether these signals are transmitted directly to the cytoskeleton or *via* cell surface receptors is unknown, and further details of the NHR mechanisms that effectively prepare plants to withstand microbial attacks have to be uncovered. It should be possible to further dissect NHR using appropriate mutants as well as genomic and proteomic studies of CW, apoplast, DRMs and genes and proteins involved in signal transduction pathways of NHR. Such studies should contribute to a better understanding of the dynamics of plant disease resistance against a broad range of pathogens.

Acknowledgements

We thank DBT-CREBB and UGC-CAS programs of the School of Life Sciences and DST-FIST Level II support to the Department of Plant Sciences, University of Hyderabad, for the infrastructure support and Prof. B.M. Moerschbacher, University of Münster, Germany, for critical reading and Dr. V. Vamsicharan, University of Hyderabad for language editing of the article. BU and TSR thank DBT-CREBB for the doctoral fellowship.

References

Able AJ, Sutherland MW, Guest DI. Production of reactive oxygen species during non-specific elicitation non-host resistance and field resistance expression in cultured tobacco cells. Funct Plant Biol 2003;30:91–9.

Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 2004;55:373–99.

Arnold DL, Gibbon MJ, Jackson RW, Wood JR, Brown J, Mansfeld JW, et al. Molecular characterization of avrPphD, a widely-distributed gene from *Pseudomonas syringae* pv. *phaseolicola* involved in non-host recognition by pea (*Pisum sativum*). Physiol Mol Plant Pathol 2001;58:55–62.

Assaad FF, Qiu JL, Youngs H, Ehrhardt D, Zimmerli L, Somerville CR, et al. The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Mol Biol Cell 2004;15:5118–29.

Baker CJ, Orlandi EW. Active oxygen in plant pathogenesis. Annu Rev Phytopathol 1995;33:299–321.

Baluska F, Samaj J, Wojtaszek P, Volkmann D, Menzel D. Cytoskeleton-plasma membrane-cell wall continuum in plants: emerging links revisited. Plant Physiol 2003;133:482–91.

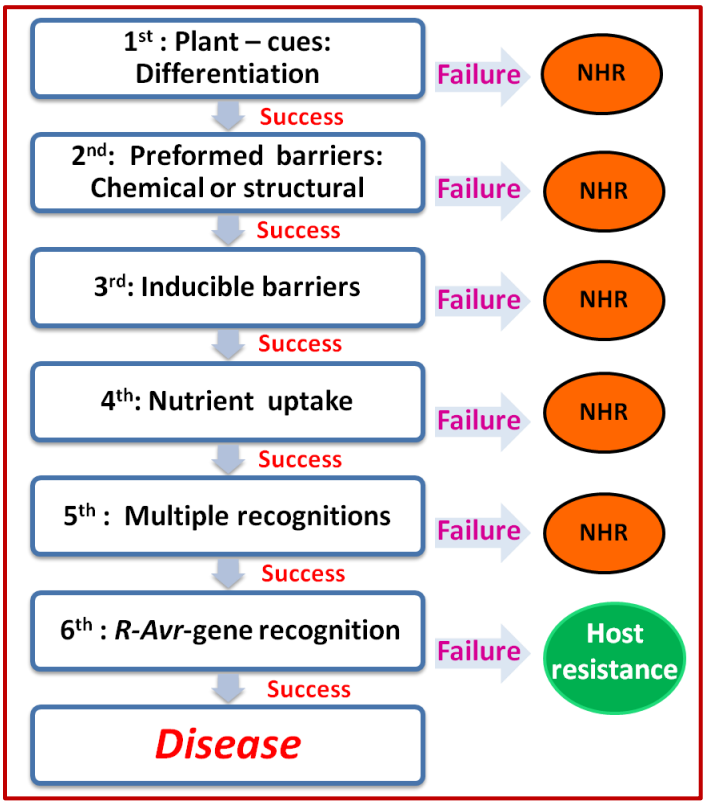
Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsk J, Mansurova M, et al. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 2009;323:101–6.

Bestwick CS, Brown IR, Mansfield JW. Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol 1998;118:1067–78.

Bhat RA, Miklis M, Schmelzer E, Schulze-Lefert P, Panstruga R. Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. Proc Natl Acad Sci USA 2005;102:3135–40.
Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, et al. Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. Plant J 2006;47:851–63.
Bloch D, Lavy M, Efrat Y, Efroni I, Bracha-Drori K, Abu-Abied M, et al. Ectopic expression of an activated RAC in *Arabidopsis* disrupts membrane cycling. Mol Biol Cell 2005;16:1913–27.
Blume B, Nurnberger T, Nass N, Scheel D. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 2000;12:1425–40.
Bolwell GP. Role of reactive oxygen species and NO in plant defense responses. Curr Opin Plant Biol 1999;64:163–76.
Bolwell GP, Bindschedler LV, Blee KA, Butt VS, Davies DR, Gardner SL, et al. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. J Exp Bot 2002;372:1367–76.
Borner GHH, Sherrier DJ, Stevens TJ, Arkin IT, Dupree P. Prediction of glycosylphosphatidyl inositol-anchored proteins in *Arabidopsis*: a genomic analysis. Plant Physiol 2002;129:486–99.
Braam J. In touch: plant responses to mechanical stimuli. New Phytol 2005;165:373–89.
Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 1992;68:533–44.
Brutus A, Siciliaa F, Maconeb A, Cervonea F, De Lorenzoa G. A domain swap approach reveals a role of the plan wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. Proc Natl Acad Sci USA 2010;107:9452–7.
Burdon JJ, Thrall PH. Coevolution of plants and their pathogens in natural habitats. Science 2009;324:755–6.
Canut H, Carrasco A, Galaud JP, Cassan C, Bouyssou H, Vita N. High affinity RGD-binding sites at the plasma membrane of *Arabidopsis thaliana* links the cell wall. Plant J 1998;16:63–7.
Celio GJ, Mims CW, Richardson EA. Ultrastructure and immunocytochemistry of the host–pathogen interface in *Poinsettia* leaves infected with powdery mildew. Can J Bot 2004;82:421–9.
Chivasa S, Murphy AM, Hamilton JM, Lindsey K, Carr JP, Slabas AR. Extracellular ATP is a regulator of pathogen defence in plants. Plant J 2009;60:436–48.
Christopher-Kozjan R, Heath MC. Cytological and pharmacological evidence that biotrophic fungi trigger different cell death execution processes in host and nonhost cells during the hypersensitive response. Physiol Mol Plant Pathol 2003;62:265–75.
Coemans B. High-throughput *in planta* expression screening identifies an ADP-ribosylation factor (ARF1) involved in non-host resistance and R gene-mediated resistance. Mol Plant Pathol 2008;9:25–36.
Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, et al. SNARE-protein-mediated disease resistance at the plant cell wall. Nature 2003;425:973–7.
Contreras I, Ortiz-Zapater E, Aniento F. Sorting signals in the cytosolic tail of membrane proteins involved in the interaction with plant ARF1 and coatomer. Plant J 2004;38:685–98.
Davies DR, Bindschedler LV, Strickland TS, Bolwell GP. Production of reactive oxygen species in *Arabidopsis thaliana* cell suspension cultures in response to an elicitor from *Fusarium oxysporum*: implications for basal resistance. Mol Plant Microbe Interact 2006;4:477–88.
Demidchik V, Nichols C, Oliynyk M, Dark A, Glover B, Davies J. Is ATP a signalling agent in plants? Plant Physiol 2003;133:456–61.
Demidchik V, Shang Z, Shin R, Thompson E, Rubio L, Laohavisit A, et al. Plant extracellular ATP signaling by plasma membrane NADPH oxidase and $Ca^{(2+)}$ channels. Plant J 2009;58:903–13.
Dodds PN, Rathjen JP. Plant immunity: towards an integrated view of plant–pathogen interactions. Nat Genet 2010;11:539–48.
Eichmann R, Biemelt S, Schafer P, Scholz U, Jansen C, Felk A, et al. Macroarray expression analysis of barley susceptibility and nonhost resistance to *Blumeria graminis*. Plant Physiol 2006;163:657–70.
Eichmann R, Schultheiss H, Kogel KH, Huckelhoven R. The barley apoptosis suppressor homologue BAX inhibitor-1 compromises nonhost penetration resistance of barley to the inappropriate pathogen *Blumeria graminis* f. sp. *tritici*. Mol Plant Microbe Interact 2004;17:484–90.
Ellis J. Insights into nonhost disease resistance: can they assist disease control in agriculture? Plant Cell 2006;18:523–8.
Ellis C, Turner JG. The *Arabidopsis* mutant *cev*1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell 2001;13:1025–33.
Ellis C, Karafyllidis I, Wasternack C, Turner JG. The *Arabidopsis* mutant *cev*1 links cell wall signalling to jasmonate and ethylene responses. Plant Cell 2002;14:1557–66.
Falk J, Thoumine O, Dequidt C, Choquet D, Faivre-Sarrailh C. NrCAM coupling to the cytoskeleton depends on multiple protein domains and partitioning into lipid rafts. Mol Biol Cell 2004;15:4695–709.
Felix G, Boller T. Molecular sensing of bacteria in plants. The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. J Biol Chem 2003;278:6201–8.
Foresi N, Laxalt A, Tonón C, Casalongué C, Lamattina L. Extracellular ATP induces nitric oxide production in tomato cell suspensions. Plant Physiol 2007;145:589–92.
Fujiwara M, Hamada S, Hiratsuka M, Fukao Y, Kawasaki T, Shimamoto K. Proteome analysis of detergent-resistant membranes (DRMs) associated with OsRac1-mediated innate immunity in rice. Plant Cell Physiol 2009;50: 1191–200.
Garcia A, Cayla X, Fleischer A, Guergnon J, Cañas FA, Rebollo MP, et al. Rafts: a simple way to control apoptosis by subcellular redistribution. Biochimie 2003;85:727–31.
Gaulin E, Drame N, Lafitte C, Torto-Alalibo T, Yves Martinez Y, Ameline-Torregrosa C, et al. Cellulose binding domains of a *Phytophthora* cell wall protein are novel pathogen-associated molecular patterns. Plant Cell 2006;18:1766–77.
Gomez-Gomez L, Boller T. Flagellin perception: a paradigm for innate immunity. Trends Plant Sci 2002;7:251–6.
Gouget A, Senchou V, Govers F, Sanson A, Barre A, Rougé P, et al. Lectin receptor kinases participate in protein–protein interactions to mediate plasma membrane–cell wall adhesions in *Arabidopsis*. Plant Physiol 2006;140: 81–90.
Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J. The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J 2000;23:441–50.
He SY, Huang HC, Collmer A. *Pseudomonas syringae* pv. *syringae* $harpin_{pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 1993;73:1255–66.
He ZH, He D, Kohorn BD. Requirement for the induced expression of a cell wall associated receptor kinase for survival during pathogen response. Plant J 1998;14:55–63.
He ZH, Cheeseman I, He D, Kohorn BD. A cluster of five cell wall-associated kinase genes, Wak1-5, are expressed in specific organs of *Arabidopsis*. Plant Mol Biol 1999;39:1189–96.
Heath MC. Non-host resistance and nonspecific plant defenses. Curr Opin Plant Biol 2000;3:315–9.
Herve C, Serres J, Dabos P, Canut H, Barre A, Rouge P, et al. Characterization of the *Arabidopsis* LecRK-a genes: members of a superfamily encoding putative receptors with an extracellular domain homologous to legume lectins. Plant Mol Biol 1999;39:671–82.
Hoefle C, Huckelhoven R. Enemy at the gates – traffic at the plant cell pathogen interface. Cell Microbiol 2008;10:2400–7.
Hoessli DC, Ilangumaran S, Soltermann A, Robinson PJ, Borisch B, Nasir-Ud-Din. Signaling through sphingolipid microdomains of the plasma membrane: the concept of signaling platform. Glycoconjug J 2000;17:191–7.
Huckelhoven R. Cell wall-associated mechanisms of disease resistance and susceptibility. Annu Rev Phytopathol 2007;45:101–27.
Huckelhoven R, Fodor J, Trujillo M, Kogel KH. Barley *Mla* and *Rar* mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f. sp. *hordei* are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion. Planta 2000;212:16–24.
Huckelhoven R, Dechert C, Kogel KH. Non-host resistance of barley is associated with a hydrogen peroxide burst at sites of attempted penetration by wheat powdery mildew fungus. Mol Plant Pathol 2001;2:199–205.
Hynes R. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673–87.
Jacobs AK, Lipka V, Burton RA, Panstruga R, Schulze-Lefert P, Fincher GB, et al. An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell 2003;15:2503–13.
Jaffe MJ, Leopold AC, Staples RC. Thigmo responses in plants and fungi. Am J Bot 2002;89:375–82.
Jensen MK, Rung JH, Gregersen PL, Gjetting T, Fuglsang AT, Hansen M, et al. The HvNAC6 transcription factor: a positive regulator of penetration resistance in barley and *Arabidopsis*. Plant Mol Biol 2007;65:137–50.
Jeter C, Tang W, Henaff E, Butterfield T, Roux SJ. Evidence of a novel cell signalling role for extracellular adenosine triphosphates and diphosphates in *Arabidopsis*. Plant Cell 2004;16:2652–64.
Jiang SY, Ma Z, Ramachandran S. Evolutionary history and stress regulation of the lectin superfamily in higher plants. BMC Evol Biol 2010;10:79–103.
Jonak C, Okresz L, Bogre L, Hirt H. Complexity, cross talk and integration of plant MAP kinase signalling. Curr Opin Plant Biol 2002;5:415–24.
Jones JDG, Dangl JL. The plant immune system. Nature 2006;444:323–9.
Kacperska A. Sensor types in signal transduction pathways in plant cells responding to abiotic stressors: do they depend on stress intensity? Physiol Plant 2004;122:159–68.
Kang L, Li J, Zhao T, Xiao F, Tang X, Thilmony R, et al. Interplay of the *Arabidopsis* non-host resistance gene *NHO1* with bacterial virulence. Proc Natl Acad Sci USA 2003;100:3519–24.
Kanzaki H, Saitohet H, Ito A, Fujisawa S, Kamoun S, Katou S, et al. Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana*. Mol Plant Pathol 2003;4:383–91.
Knoester M, Van Loon LC, Heuvel JVD, Hennig J, Bol JF, Linthorst HJM. Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proc Natl Acad Sci USA 1998;95:1933–7.
Kobayashi DY, Tamaki SJ, Keen NT. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. Proc Natl Acad Sci USA 1989;86:157–61.
Kobayashi Y, Yamada M, Kobayashi I, Hitoshi K. Actin microfilaments are required for the expression of non-host resistance in higher plants. Plant Cell Physiol 1997;38:725–33.

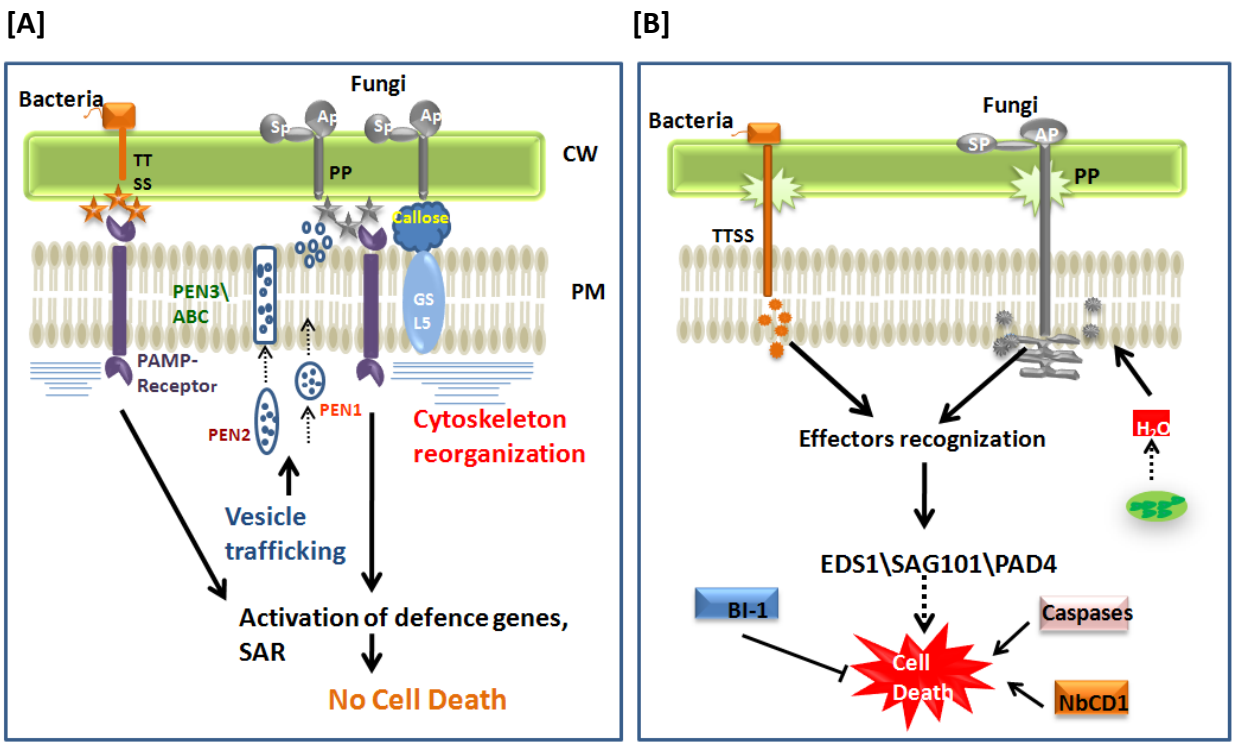
Kohorn BD. Plasma membrane–cell wall contacts. Plant Physiol 2000;124:31–5.
Kohorn BD. Cell wall associated kinases. Curr Opin Cell Biol 2001;13: 529–33.
Kwon C, Neu C, Pajonk S, Yun HS, Lipka U, Humphry M, et al. Co-option of a default secretory pathway for plant immune responses. Nature 2008;451:835–40.
Lafont F, Abrami L, van der Goot FG. Bacterial subversion of lipid rafts. Curr Opin Microbiol 2004;7:4–10.
Laval V, Chabannes M, Carriere M, Canut H, Barre A, Rouge P, et al. A family of *Arabidopsis* plasma membrane receptors presenting animal β-integrin domains. Biochim Biophys Acta 1999;1435:61–70.
Lee MH, Min MK, Lee YJ, Jin JB, Shin DH, Kim DH. ADP-ribosylation factor 1 of *Arabidopsis* plays a critical role in intracellular trafficking and maintenance of endoplasmic reticulum morphology in *Arabidopsis*. Plant Physiol 2002;129:1507–20.
Lee-Stadelmann OY, Stadelmann EJ. Plasmolysis and deplasmolysis. Methods Enzymol 1989;174:225–46.
Lew R, Dearnaley J. Extracellular nucleotide effects on the electrical properties of growing *Arabidopsis thaliana* root hairs. Plant Sci 2000;153:1–6.
Lin SS, Martin R, Mongrand S, Vandenabeele S, Chen KC, Jang IC, et al. RING1 E3 ligase localizes to plasma membrane lipid raft to trigger FB1-induced programmed cell death in *Arabidopsis*. Plant J 2008;56:550–61.
Lipka V, Panstruga R. Dynamic cellular responses in plant–microbe interactions. Curr Opin Plant Biol 2005;8:625–31.
Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Schulze-Lefert P, et al. Pre- and post invasion defenses both contribute to non-host resistance in *Arabidopsis*. Science 2005;310:1180–3.
Lu M, Tang X, Zhou JM. *Arabidopsis NHO1* is required for general resistance against *Pseudomonas* bacteria. Plant Cell 2001;13:437–47.
Majewska-Sawka A, Nothnagel EA. The multiple roles of arabinogalactan proteins in plant development. Plant Physiol 2000;122:3–9.
Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, Lawton KA, et al. The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. Nat Genet 2000;26:403–10.
Mangeon A, Junqueira RM, Sachetto-Martins G. Functional diversity of the plant glycine-rich proteins superfamily. Plant Signal Behav 2010;5:99–104.
McLusky SR, Bennett MH, Beale MH, Lewis MJ, Gaskin P, Mansfield JW. Cell wall alterations and localized accumulation of feruloyl 3′-tyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarization, peroxidase activity and suppression of flavonoid biosynthesis. Plant J 1999;17:523–34.
Mellersh DG, Heath MC. Plasma membrane–cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell 2001;13:413–24.
Mellersh DG, Heath MC. An investigation into the involvement of defense signaling pathways in components of the non-host resistance of *Arabidopsis thaliana* to rust fungi also reveals a model system for studying rust fungal compatibility. Mol Plant Microbe Interact 2003;16:398–404.
Memon AR. The role of ADP-ribosylation factor and SAR1 in vesicular trafficking in plants. Biochim Biophys Acta 2004;1664:9–30.
Molendijk AJ, Ruperti B, Palme K. Small GTPases in vesicle trafficking. Curr Opin Plant Biol 2004;7:694–700.
Montesano M, Brader G, Palva T. Pathogen-derived elicitors: searching for receptors in plants. Mol Plant Pathol 2003;4:73–9.
Mysore KS, Ryu CM. Non-host resistance: how much do we know? Trends Plant Sci 2004;9:97–104.
Nakagawa Y, Katagiric T, Shinozaki K, Qie Z, Tatsumi H, Furuichie T, et al. *Arabidopsis* plasma membrane protein crucial for Ca^{2+} influx and touch sensing in roots. Proc Natl Acad Sci USA 2007;104:3639–44.
Nasir KH, Takahashi Y, Ito A, Saitoh H, Matsumura H, Kanzaki H, et al. High-throughput in planta expression screening identifies a class II ethylene-responsive element binding factor-like protein that regulates plant cell death and non-host resistance. Plant J 2005;43:491–505.
Nicaise V, Roux M, Zipfel C. Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. Plant Physiol 2009;150:1638–47.
Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC. Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science 2003;301:969–72.
Nurnberger T, Lipka V. Non-host resistance in plants: new insights into an old phenomenon. Mol Plant Pathol 2005;6:335–45.
Nurnberger T, Scheel D. Signal transmission in the plant immune response. Trends Plant Sci 2001;6:372–9.
Nurnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K, Scheel D. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell 1994;78:449–60.
Nurnberger T, Brunner F, Kemmerling B, Piater L. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev 2004;198:249–66.
Ono E, Wong HL, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K. Essential role of the small GTPase Rac in disease resistance of rice. Proc Natl Acad Sci USA 2001;98:759–64.
Ostergaard L, Petersen M, Mattsson O, Mundy J. An *Arabidopsis* callose synthase. Plant Mol Biol 2003;49:559–66.
Oxley D, Bacic A. Structure of the glycosylphosphatidylinositol anchor of an arabinogalactan protein from *Pyrus communis* suspension-cultured cells. Proc Natl Acad Sci USA 1999;25:14246–51.
Parker JE, Holub EB, Frost LN, Falk A, Gunn ND, Daniels MJ. Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. Plant Cell 1996;8:2033–46.
Parton RG, Richards AA. Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic 2003;4:724–38.
Peart JR, Lu R, Sadanandom A, Malcuit I, Moffett P, Brice DC, et al. Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc Natl Acad Sci USA 2002;99:10865–9.
Pike LJ. Rafts defined: a report on the keystone symposium on lipid rafts and cell function. J Lipid Res 2006;47:1597–8.
Pimpl P, Hanton SL, Taylor JP, Pinto-daSilva LL, Denecke J. The GTPase ARF1p controls the sequence-specific vacuolar sorting route to the lytic vacuole. Plant Cell 2003;15:1242–56.
Ridley BL, O'Neill MA, Mohnen D, Pectins:. structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 2001;57:929–67.
Ringli C. Monitoring the outside: cell wall-sensing mechanisms. Plant Physiol 2010;153:1445–52.
Rosenberger CM, Brumell JH, Finlay BB. Microbial pathogenesis: lipid rafts as pathogen portals. Curr Biol 2000;10:823–5.
Salaun C, James DJ, Chamberlain LH. Lipid rafts and the regulation of exocytosis. Traffic 2004;5:255–64.
Sanchez-Vallet A, Ramos B, Bednarek P, Lopez G, Bednarek MP, Schulze-Lefert, et al. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. Plant J 2010;63:115–27.
Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, et al. Co-ordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proc Natl Acad Sci USA 2000;97:11655–60.
Schmelzer E. Cell polarization, a crucial process in fungal defence. Trends Plant Sci 2002;7:411–5.
Schroeder R, London E, Brown D. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc Natl Acad Sci USA 1994;9:12130–4.
Schulze-Lefert P, Panstruga R. A molecular evolutionary concept connecting non-host resistance, pathogens host range, and pathogen speciation. Trends Plant Sci 2011;16:117–25.
Sharma PC, Ito A, Shimizu T, Terauchi R, Kamoun S, Saitoh H. Virus-induced silencing of WIPK and SIPK genes reduces resistance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in *Nicotiana benthamiana*. Mol Genet Genomics 2003;269:583–91.
Showalter AM. Structure and function of plant cell wall proteins. Plant Cell 1993;5:9–23.
Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 2000;1:31–9.
Sivaguru M, Fujiwara T, Yang Z, Osawa H, Samaj J, Baluska F. Aluminum-induced 1-3-β-glucan inhibits cell-to-cell trafficking of molecules through plasmodesmata: a new mechanism of Al toxicity in plants. Plant Physiol 2000;124: 991–1008.
Snyder BA, Nicholson RL. Synthesis of phytoalexins in Sorghum as a site-specific response to fungal ingress. Science 1990;248:1637–9.
Snyder BA, Leite B, Hipskind J, Butler LG, Nicholson RL. Accumulation of sorghum phytoalexins induced by *Colletotrichum graminicola* at the infection site. Physiol Mol Plant Pathol 1991;39:463–70.
Sonderby IE, Geu-Flores F, Halkier B. Biosynthesis of glucosinolates-gene discovery and beyond. Trends Plant Sci 2010;15:283–90.
Song C, Steinebrunner I, Wang X, Stout S, Roux SJ. Extracellular ATP induces the accumulation of superoxide via NADPH oxidases in *Arabidopsis*. Plant Physiol 2006;140:1222–32.
Sorek N, Poraty L, Sternberg H, Bar E, Lewinsohn E, Yalovsky S. Activation status-coupled transient acylation determines membrane partitioning of a plant Rho-related GTPase. Mol Cell Biol 2007;27:2144–54.
Soylu S, Brown I, Mansfield JW. Cellular reactions in *Arabidopsis* following challenge by strains of *Pseudomonas syringae*: from basal resistance to compatibility. Physiol Mol Plant Pathol 2005;66:232–43.
Stanislas T, Bouyssie D, Rossignol M, Vesa S, Fromentin J, Morel J, et al. Quantitative proteomics reveals a dynamic association of proteins to detergent-resistant membranes upon elicitor signaling in tobacco. Mol Cell Proteomics 2009;8:2186–98.
Stein M, Dittgen J, Sánchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, et al. *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to non-host resistance to inappropriate pathogens that enter plants by direct penetration. Plant Cell 2006;18:731–46.
Takahashi Y, Nasir KHB, Ito A, Kanzaki H, Matsumura H, Saitoh H, et al. A Novel MAPKK involved in cell death and defense signaling. Plant Signal Behav 2007;25:396–8.
Takemoto D, Hardham AR. The cytoskeleton as a regulator and target of biotic interactions in plants. Plant Physiol 2004;136:3864–76.
Takemoto D, Yoshioka H, Doke N, Kawakita K. Disease stress-inducible genes of tobacco: expression profile of elicitor-responsive genes isolated by subtractive hybridization. Physiol Plant 2003;118:545–53.
Thomma BPHJ, Nurnberger T, Joosten MHAJ. Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell 2011;23:4–15.
Thordal-Christensen H. Fresh insights into processes of nonhost resistance. Curr Opin Plant Biol 2003;6:351–7.
Thordal-Christensen H, Zhang Z, Wie Y, Collinge DB. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive

response during the barley-powdery mildew interaction. Plant J 1997;11: 1187–94.
Varma R, Mayor S. GPI-anchored proteins are organized in submicron domains at the cell surface. Nature 1998;20:798–801.
Verma DPS, Hong Z. Plant callose synthase complexes. Plant Mol Biol 2001;47:693–701.
Wagner T, Anderson C, Kohorn BD. Wall-associated receptor kinases: the dynamics of the plant ECM. Inst Juan March de Estudios e Invest 1999;101: 17–8.
Wang W, Barnaby JY, Tada Y, Li H, Tor M, Caldelari D, et al. Timing of plant immune responses by a central circadian regulator. Nature 2011;470: 110–5.
Whalen MC, Stall RE, Staskawicz BJ. Characterization of a gene from a tomato pathogen determining hypersensitive resistance in non-host species and genetic analysis of this resistance in bean. Proc Natl Acad Sci USA 1988;85:6743–7.
Wickstrom SA, Alitalo K, Keski-Oja J. Endostatin associates with lipid rafts and induces reorganization of the actin cytoskeleton via down-regulation of RhoA activity. J Biol Chem 2003;278:37895–901.
Wong HL, Pinontoan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, et al. Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell 2007;19:4022–34.
Wu SJ, Wu JY. Extracellular ATP-induced NO production and its dependence on membrane Ca^{2+} flux in *Salvia miltiorrhiza* hairy roots. J Exp Bot 2008;59:4007–16.
Yalovsky S, Bloch D, Sorek N, Kost B. Regulation of membrane trafficking, cytoskeleton dynamics, and cell polarity by ROP/RAC GTPases. Plant Physiol 2008;147:1527–43.
Yoda H, Fujimura K, Takahashi H, Munemura I, Uchimiya H, Sano HH. Polyamines as a common source of hydrogen peroxide in host- and nonhost hypersensitive response during pathogen infection. Plant Mol Biol 2009;70:103–12.
Yoshioka H, Bouteau F, Kawano T. Discovery of oxidative burst in the field of plant immunity: looking back at the early pioneering works and towards the future development. Plant Signal Behav 2008;3:153–5.
Yun BW, Atkinson HA, Gaborit C, Greenland A, Read ND, Pallas JA, et al. Loss of actin cytoskeletal function and *EDS1* activity, in combination, severely compromises nonhost resistance in *Arabidopsis* against wheat powdery mildew. Plant J 2003;34:768–77.
Zhang Z, Feechan A, Pedersen C, Newman MA, Qiu JL, Olesen KL, et al. A SNARE-protein has opposing functions in penetration resistance and defence signaling pathways. Plant J 2007;49:302–12.
Zhang S, Klessig DF. MAPK cascades in plant defense signalling. Trends Plant Sci 2001;6:520–7.
Zhou JM, Chai J. Plant pathogenic bacterial type III effectors subdue host responses. Curr Opin Microbiol 2008;11:179–85.
Zimmerli L, Stein M, Lipka V, Schulze-Lefert P, Somerville S. Host and nonhost pathogens elicit different jasmonate/ethylene responses in *Arabidopsis*. Plant J 2004;40:633–46.
Zipfel C, Robatzek S. Pathogen- associated molecular pattern-triggered immunity: veni, vidi. . .? Plant Physiol 2010;154:551–4.
Zurbriggen MD, Carrillo N, Tognetti VB, Melzer M, Peisker M, Hause B, et al. Chloroplast-generated reactive oxygen species play a major role in localized cell death during the non-host interaction between tobacco and *Xanthomonas campestris* pv. *vesicatoria*. Plant J 2009;60:962–73.



Redrawn from Thordal-Christensen, Curr. Opin.Plant Biol. (2003)

Fig 1.1: A simplified view of the challenges encountered by a pathogen as it attempts to cause disease. Stages at which non-host and host resistance can be manifested are indicated on the right.

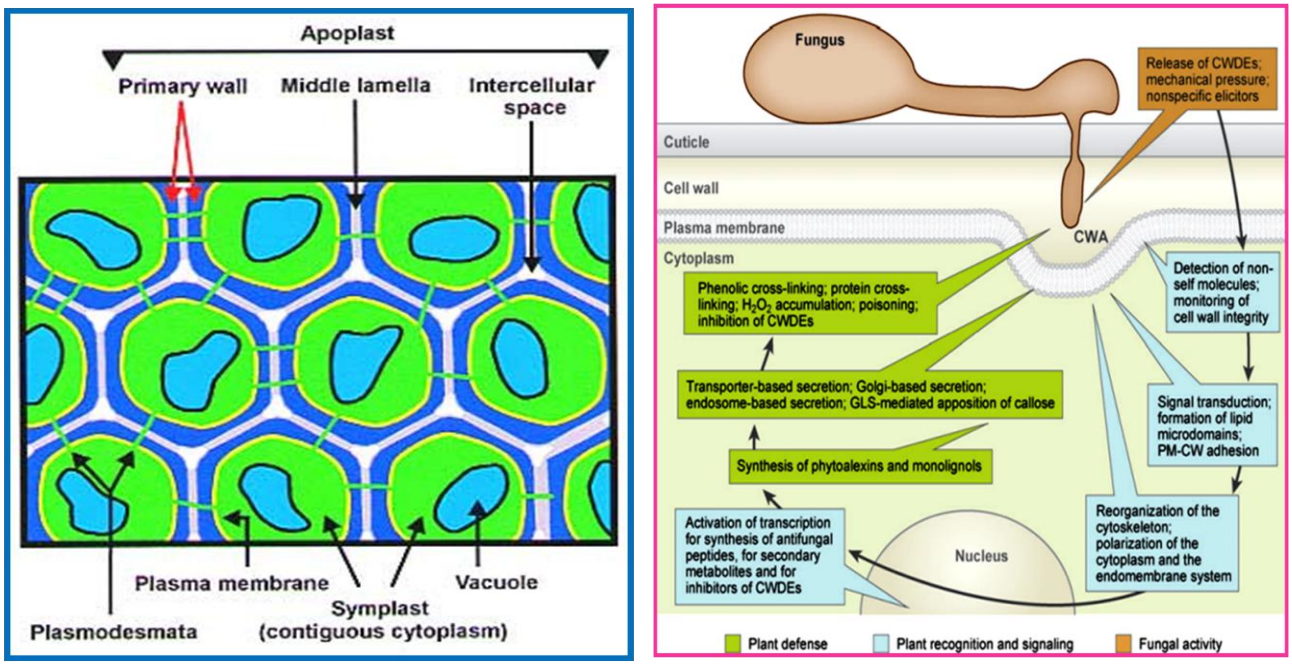


Uma et al, J. Plant Physiol. (2011)

Fig 1.2: Schematic representation of pre-and post-penetration resistance.

(A) During pre-penetration resistance, pathogen is not able to overcome the preformed barriers and general elicitor-induced plant defense responses such as callose deposition and accumulation of antimicrobial compounds like phytoalexins. PEN1, PEN2 and PEN3-mediated vesicle-based secretion system delivers antimicrobial compounds molecules to the penetration site. *Pathogenesis-related* (*PR*) gene expression as a component of systemic-acquired resistance (SAR) can be induced by general elicitors of the non-host pathogen.
(B) Pathogens that overcome pre-penetration resistance are recognized by the plant surveillance system and this triggers plant defense leading to a hypersensitive response (HR) called as post-penetration resistance. EDS1, SAG101 and PAD4 genes, which are involved in *R*-gene mediated resistance, also plays important role in execution of post-penetration resistance. Cell death regulators like BI-1 (Bax inhibitor 1), caspases and NbCD1 also play a role in triggering cell death during type II non-host resistance.

[A] **[B]**



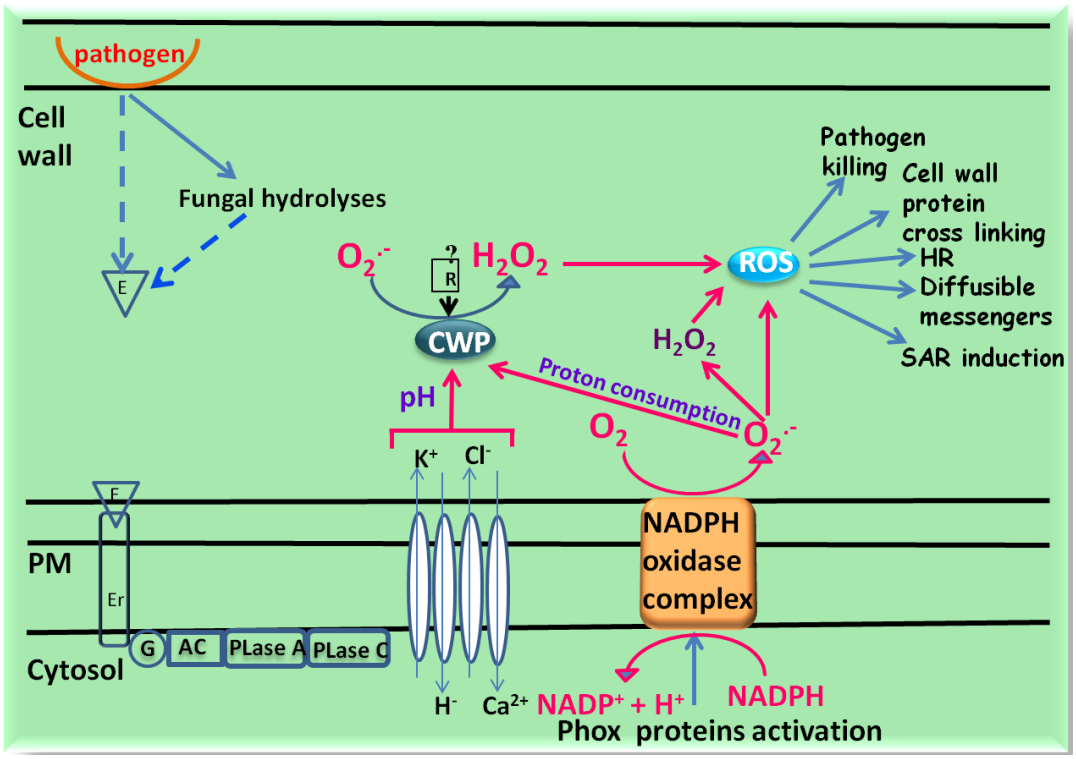
http://www.ccrc.uga.edu/~mao/intro/Apoplast.jpg

Adopted from Huckelhoven, Ann. Rev. Plant Pathol. (2007)

Fig 1.3: Schematic representation of apoplast.

(A) Apoplast is the free diffusional space outside the plasma membrane which includes primary wall, middle lamella and intercellular space. Structurally, the apoplast is formed by the continuum of cell walls of adjacent cells as well as the extracellular spaces, forming a tissue level compartment comparable to the symplast.

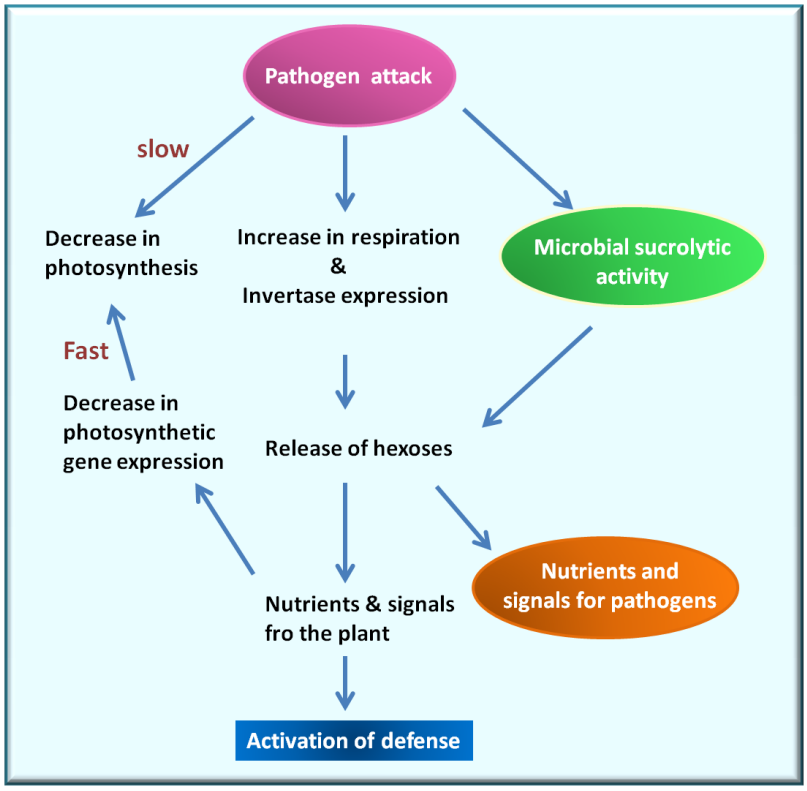
(B) Schematic representation of biochemical and molecular mechanisms for cell wall-associated defense. CW, cell wall; CWA, cell wall apposition; CWDE, cell wall-degrading enzyme; GLS, glucan synthase; PM, plasma membrane.



Adopted from Wojtaszek, Biochem. J. (1997)

1.4: Schematic representation of major hypotheses describing the possible origin of ROS.

During plant-pathogen interactions, pathogens are recognized by elicitors at apoplast. These elicitors when bound to respective receptors, located at the plasma membrane, initiate the cascade(s) of signalling events leading to the activation of either NADPH oxidase and/or cell-wall-bound peroxidases (marked in blue) generating ROS (pink) used further in various aspects of the plant defense response. Abbreviations used : AC, adenylate cyclase ; CWP, cell-wall-bound peroxidase ; E, elicitor ; Er, receptor ; G, GTP-binding protein(s) ; PLase A and PLase C, phopholipases A and C ; R, reductant.



Adopted from Berger et al., J. Exp. Bot. (2007)

Fig 1.5: Model of changes in carbohydrate metabolism in response to pathogen attack

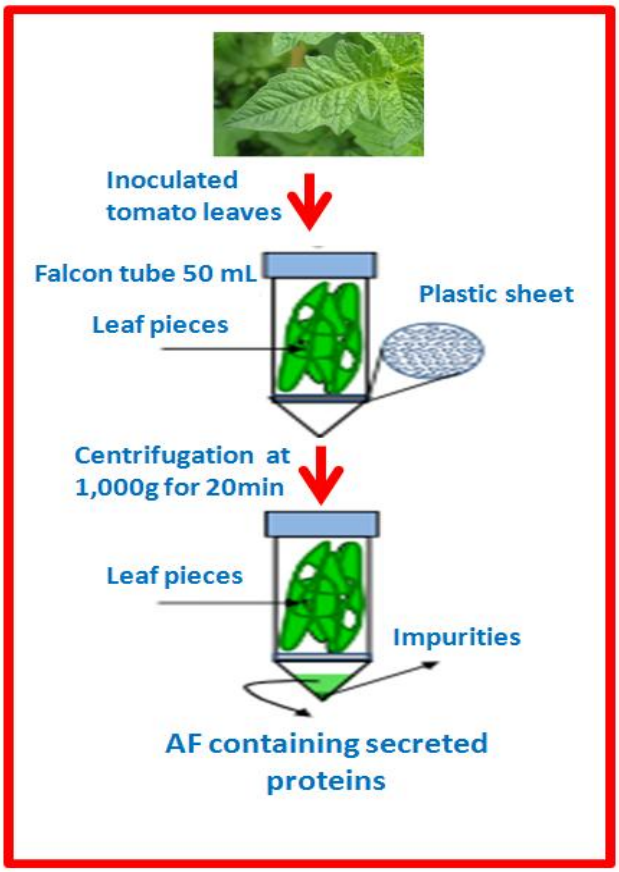


Fig 2.1: Collection of apoplastic fluid (AF) from tomato leaves by gravity extraction.

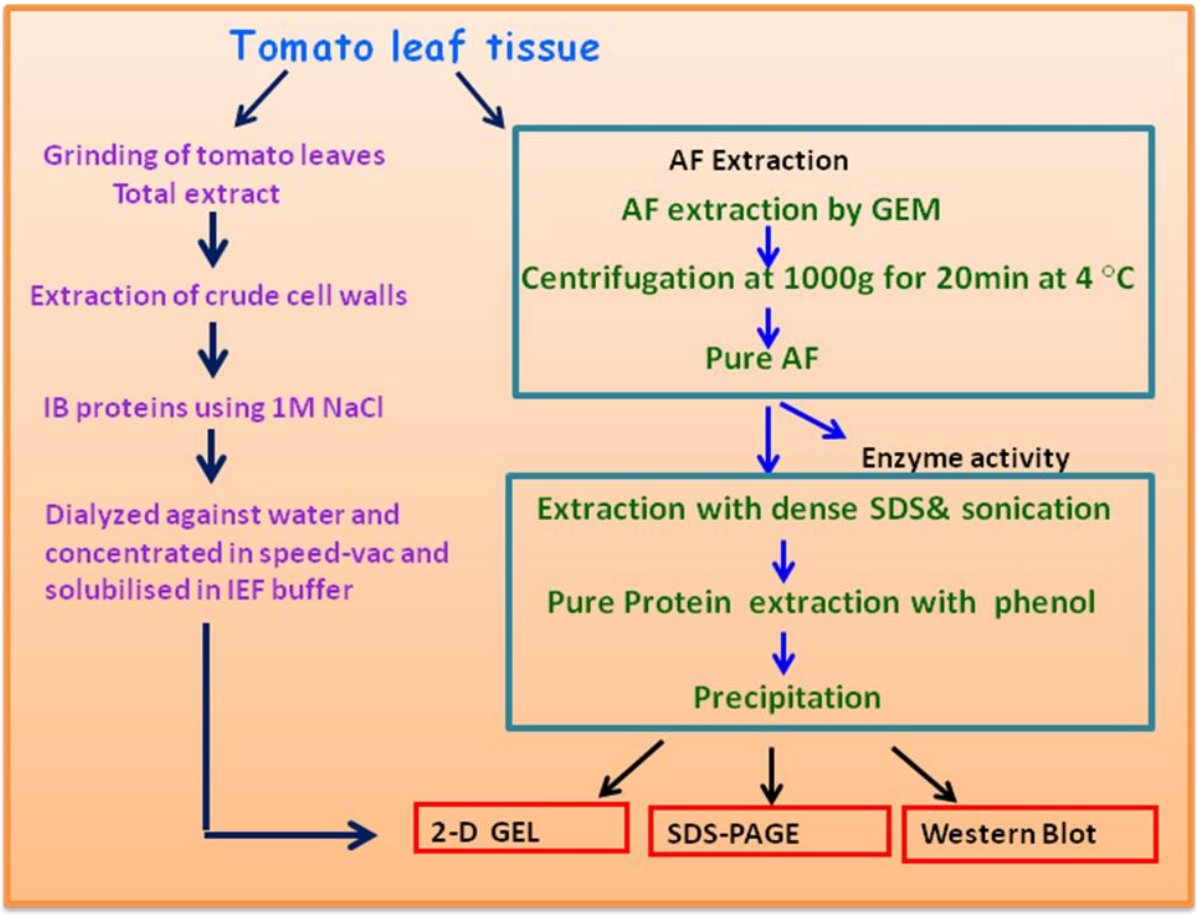


Fig 2.2: Experimental design to study the apoplast proteome of tomato

Schematic representation of the steps involved in extraction of apoplastic fluid (AF) and ionically bound protein (IBP) from tomato leaves for 2D-electrophoresis.

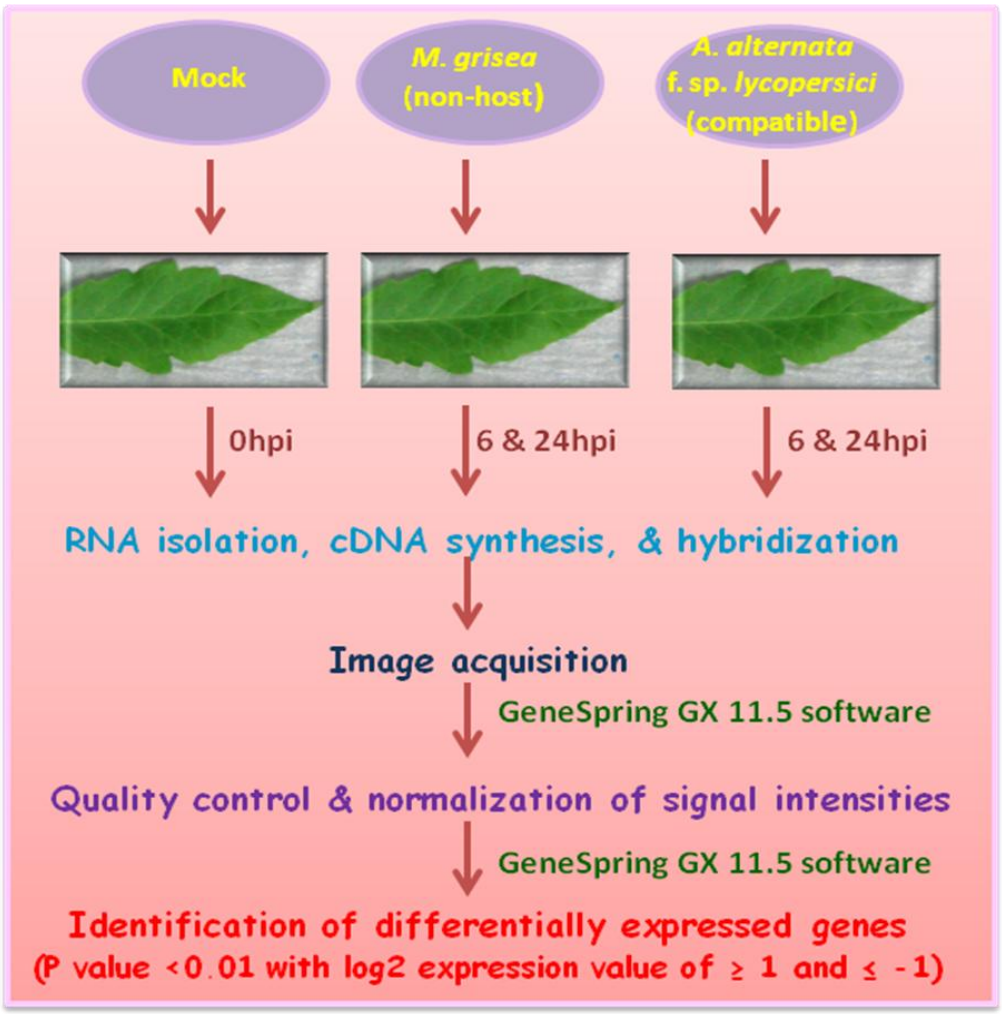


Fig 2.3: Experimental design for the transcriptome analysis of tomato

Six different sets of tomato plants, consisting of three plants per set, were treated with *A. alternaria* f. sp. *lycopersici* or *M. grisea*. Leaf samples were collected from all three plants of each set (each set is one biological replicate) and total RNA was isolated and pooled to minimize the background noise. Mock-treated set served as control.

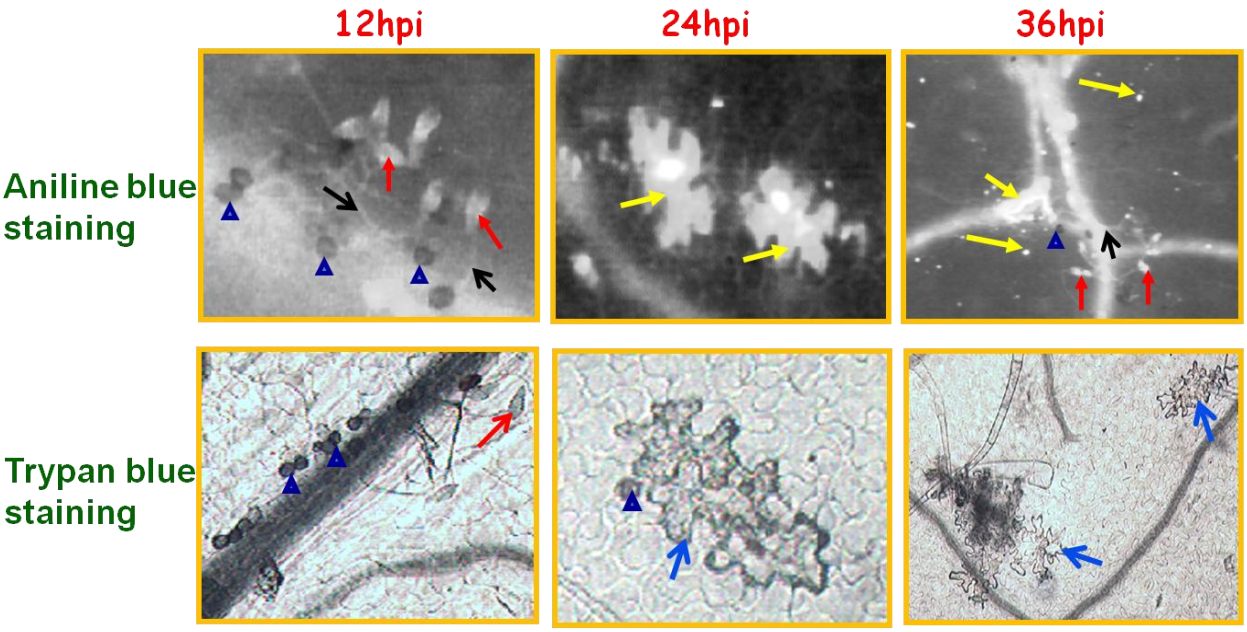


Fig 3.1: Histochemical observations of callose deposition and cell death.

Callose deposition and cell death detected using aniline blue and trypan blue staining, respectively in tomato leaves inoculated with *M. grisea* spores. Red arrows indicate the spores, yellow arrows indicate the callose deposition, blue arrows indicate the cell death, black arrows indicate the germ tube, and ▲- indicate the appressorium.

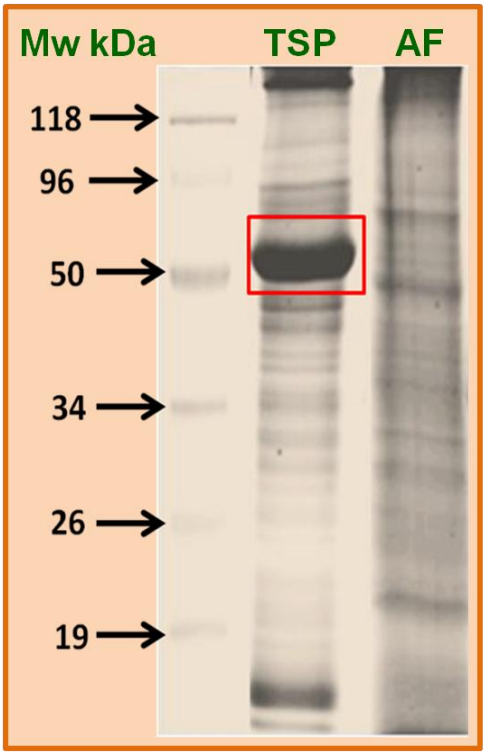


Fig 3.2: Assessment of purity of apoplastic fluid (AF) extracted by gravity extraction.

SDS-PAGE analysis of the total soluble protein (TSP) and AF from tomato leaves. Gels were stained with coomassie brilliant blue G-250 stain solution. Rubicso large subunit (~50 kDa) is marked with red box. Lane 1: pre-stained protein marker, Lane 2: 10µg of TSP, and Lane 3: 10µg of AF.

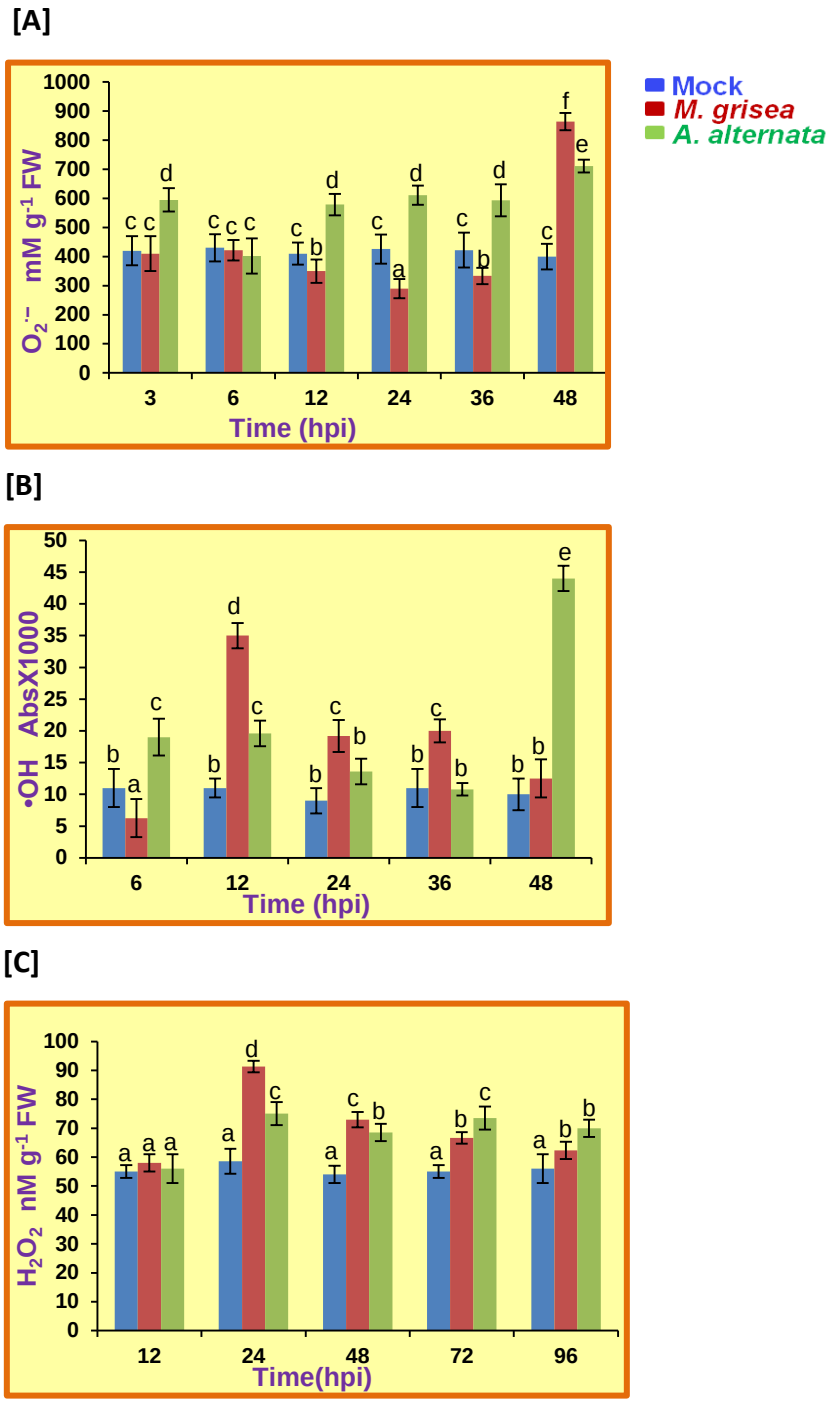


Fig 3.3: Measuring $O_2^{\cdot-}$, •OH, and extracellular H_2O_2 in tomato leaves.

Time course of formation of $O_2^{\cdot-}$ (A), •OH (B), and extracellular H_2O_2 (nM g^{-1} fresh weight) (C) in tomato leaves inoculated with *M. grisea* and *A. alternata* f. sp. *lycopersici* compared with mock-inoculation. Error bars represent the standard deviation of the mean from three independent experiments. Bars of the histogram with same letter on the top do not differ significantly ($P \leq 0.05$). hpi- hours post inoculation.

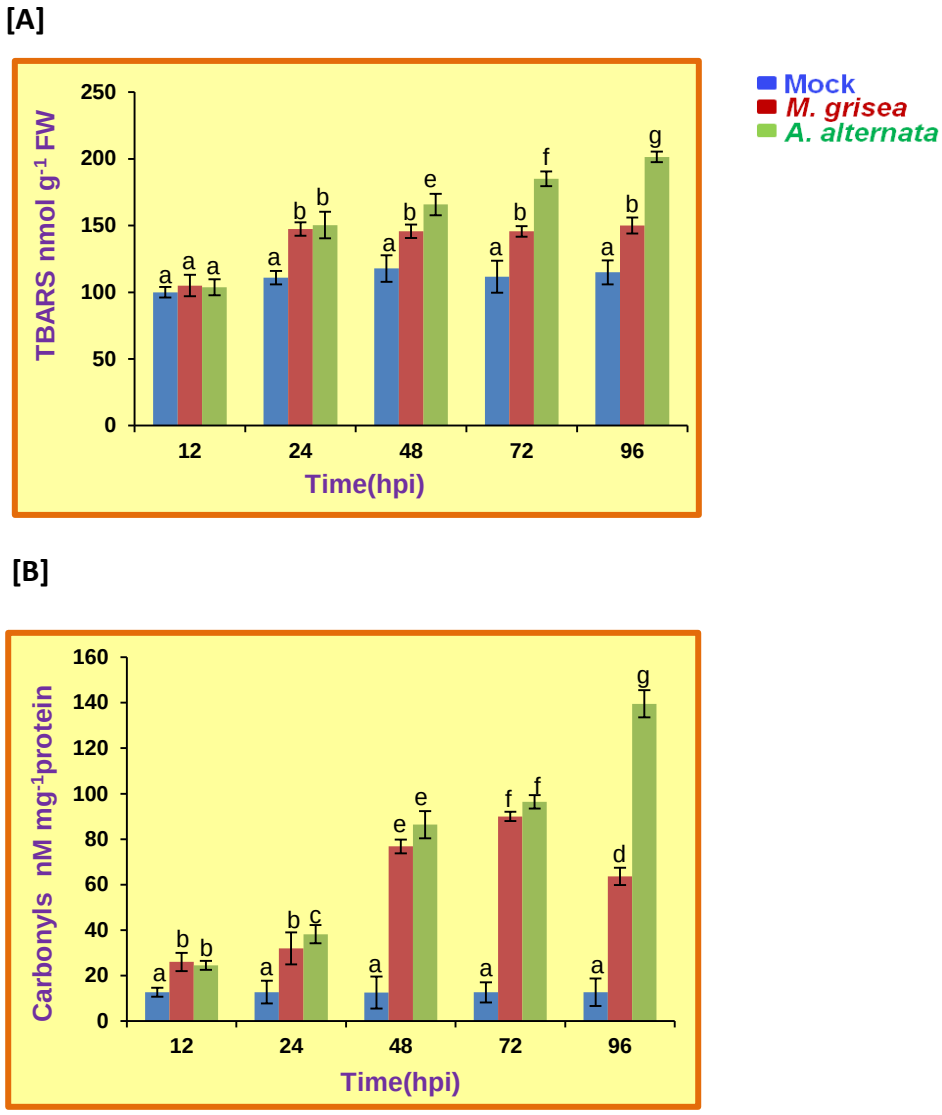


Fig 3.4: Measuring lipid peroxidation and carbonyl content in apoplast of tomato leaves.

Levels of lipid peroxidation (nM g^{-1} fresh weight) (A), and carbonyl content in apoplast (nM mg^{-1} protein) (B) in tomato leaves inoculated with *M. grisea* and *A. alternata* f. sp. *lycopersici* compared with mock-inoculated leaves. Error bars represent the standard deviation of the mean from three independent experiments. Bars of the histogram with same letter on the top do not differ significantly ($P \leq 0.05$). hpi- hours post inoculation.

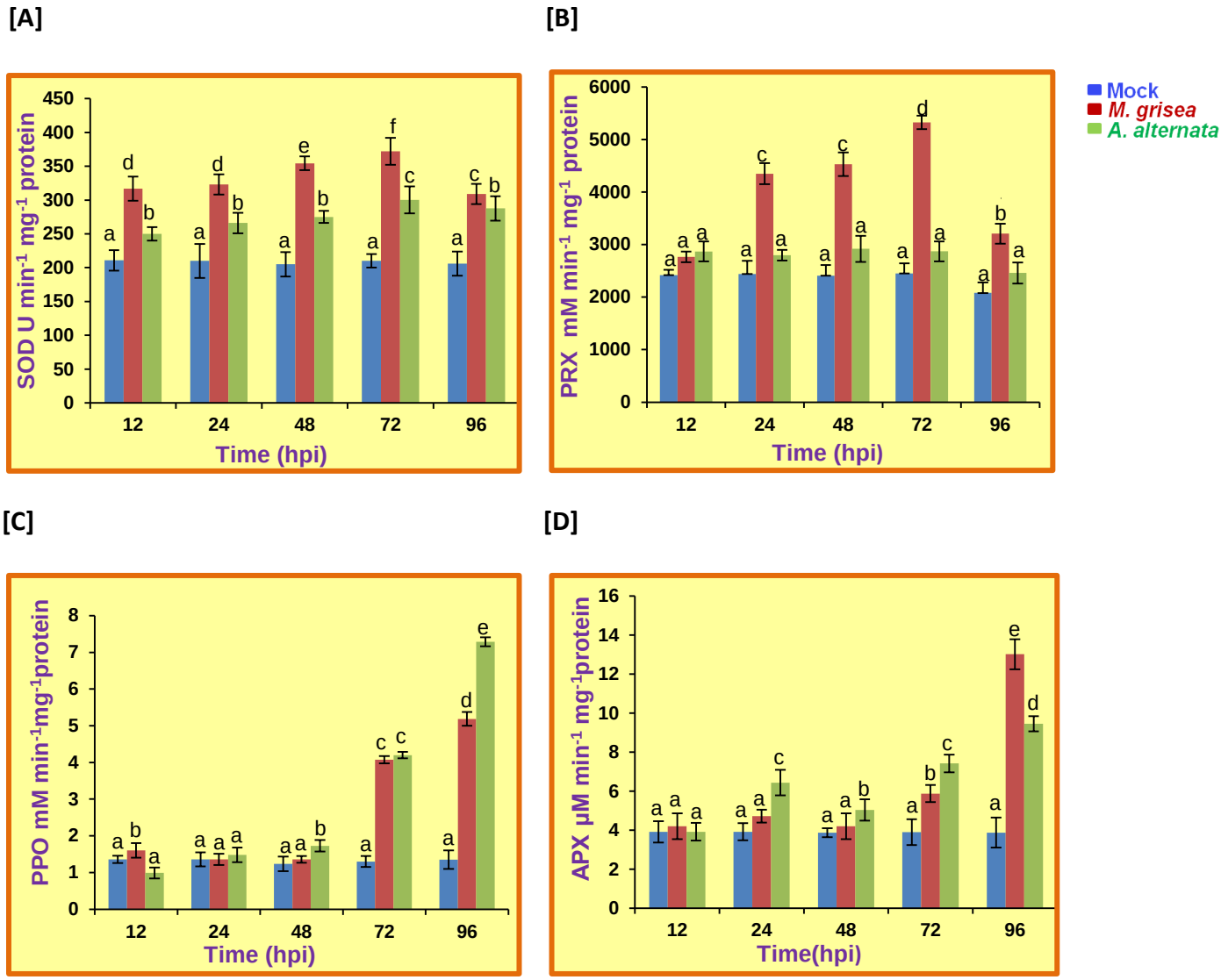


Fig 3.5: Profile of activity of antioxidative enzymes in apoplast of tomato leaf apoplast.

Superoxide dismutase (SOD) (A), extracellular peroxidase (PRX) (B), polyphenol oxidase (PPO) (C) and ascorbate peroxidase (APX) (C), and in tomato leaves inoculated with *M. grisea* and *A. alternata* f. sp. *lycopersici* compared to mock-inoculated leaves. All enzyme activities were expressed as mM $min^{-1}mg^{-1}$ protein. Error bars represent the standard deviation of the mean from three independent experiments. Bars of the histogram with same letter on the top do not differ significantly ($P \leq 0.05$). hpi-hours post inoculation.

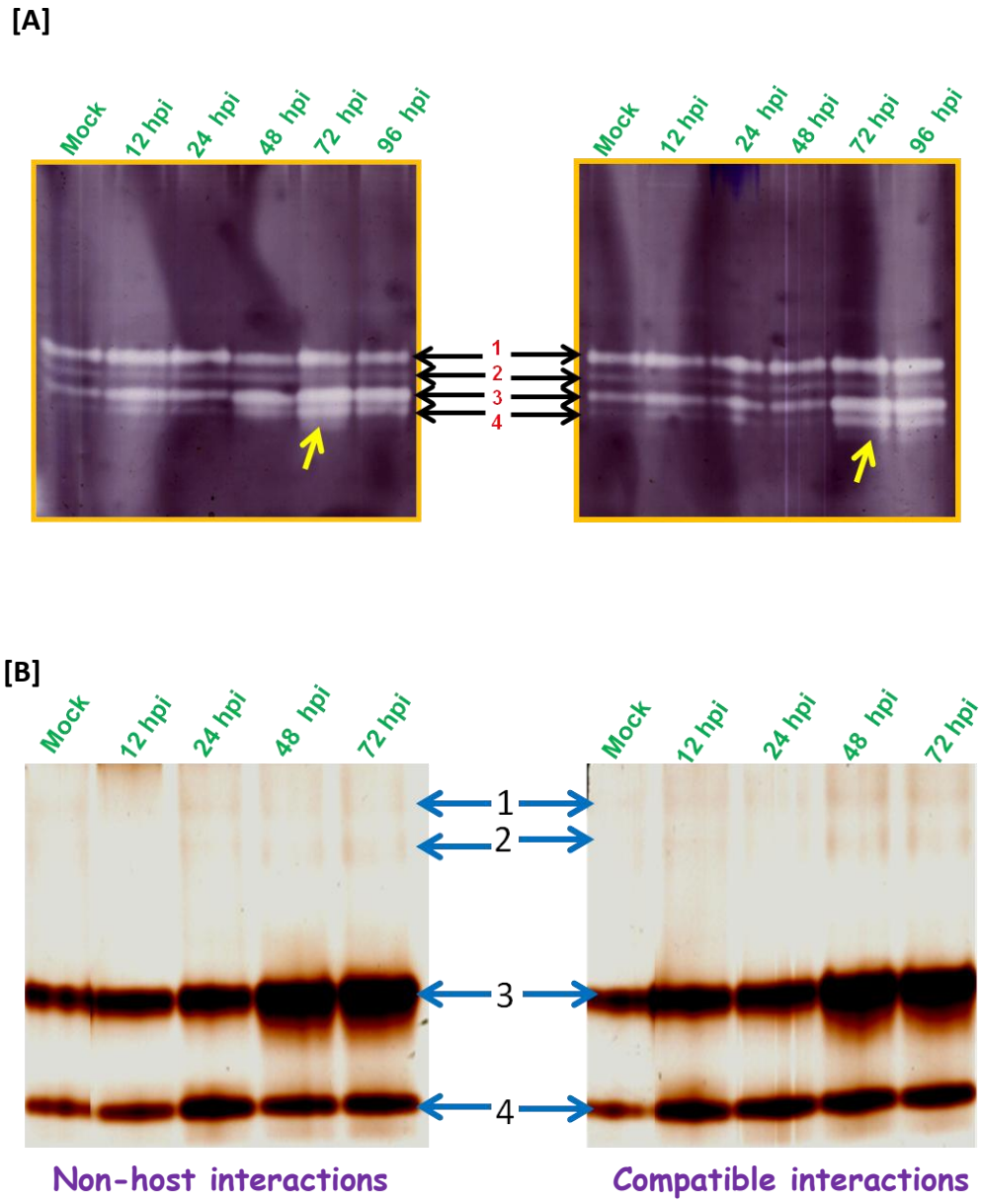


Fig 3.6: Analysis of superoxide dismutase (SOD) and extracellular peroxidase (PRX) isozymes in apoplast of tomato leaves.

Separation of SOD (A), and PRX (B) isozymes by native PAGE from the apoplast of tomato leaves inoculated with *M. grisea* (left) and *A. alternata* f. sp. *lycopersici* (right) compared to mock-inoculation. Yellow arrow indicates the transient induction of new band at 72 hpi in both non-host and compatible interactions. Black arrows indicate the 4 SOD isozymes (A) and blue arrows indicate the 4 PRX isozymes (B).

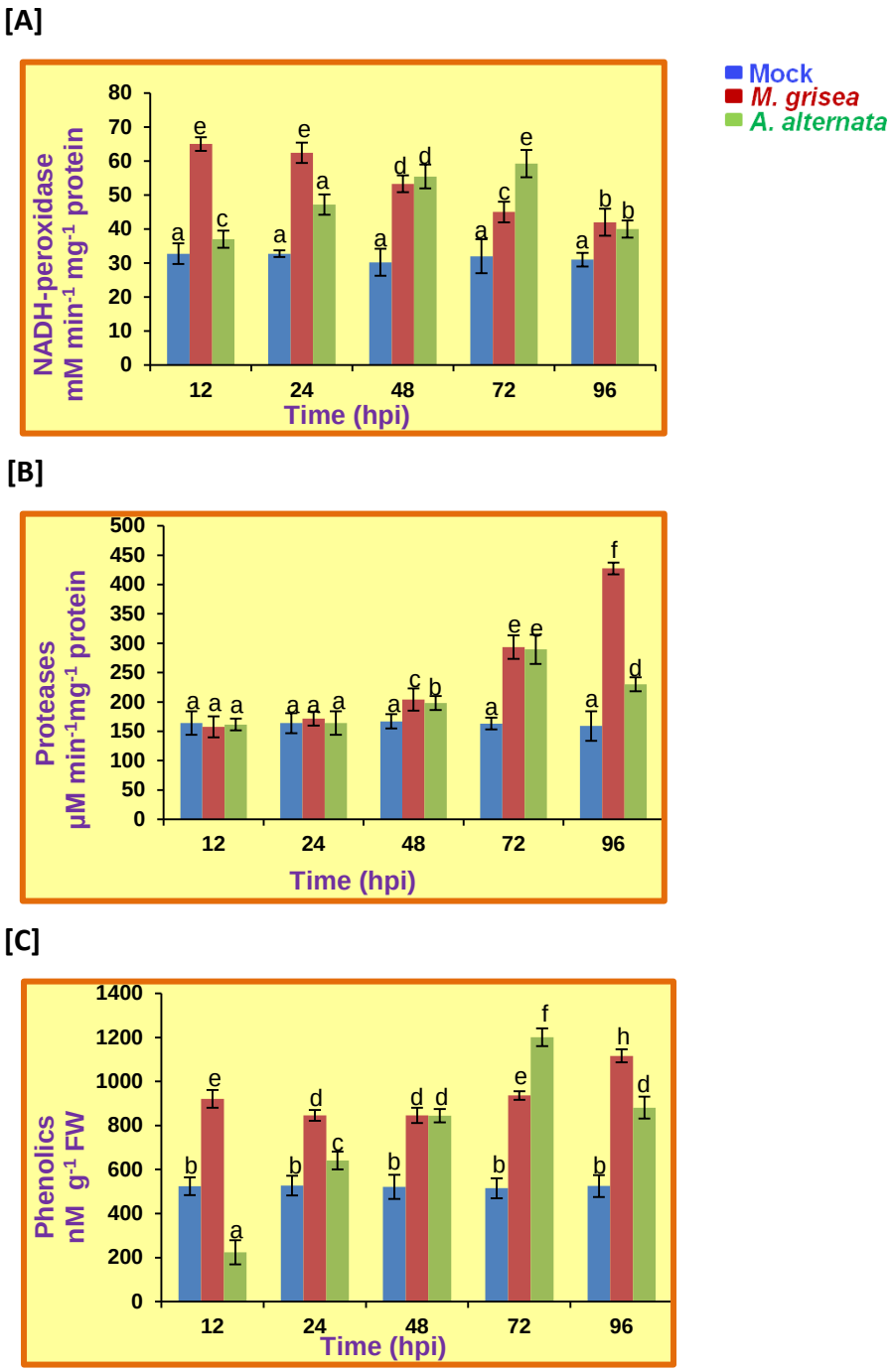
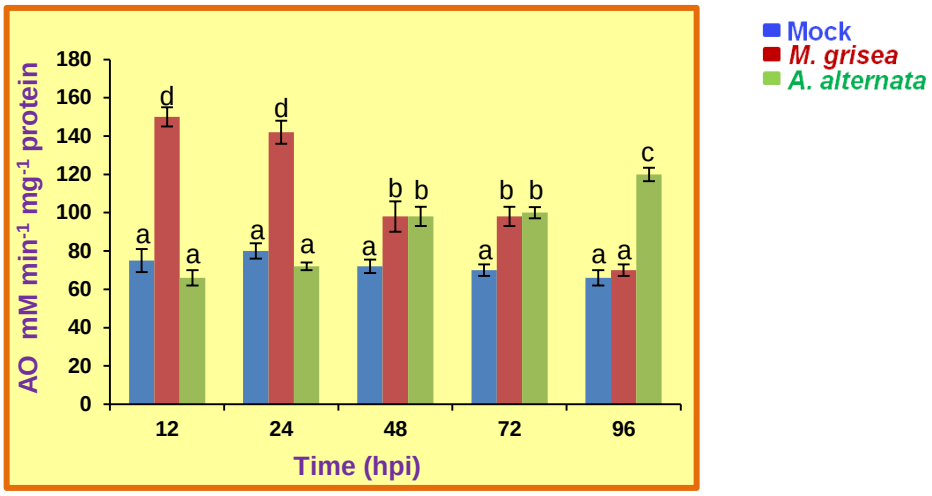


Fig 3.7: Measuring NADH-peroxidase, protease activity, and phenolic content in apoplast of tomato leaves.

NADH-peroxidase activity (mM min^{-1} mg^{-1} protein) (A), proteases activity (µM mg^{-1} protein) (B) and phenolic content (nM gallic acid equivalent g^{-1} fresh weight) (C) in tomato leaves inoculated with *M. grisea* and *A. alternata* f. sp. *lycopersici* compared with mock-inoculation. Error bars represent the standard deviation of the mean from three independent experiments. Bars of the histogram with same letter on the top do not differ significantly ($P \leq 0.05$). hpi-hours post inoculation.

[A]



[B]

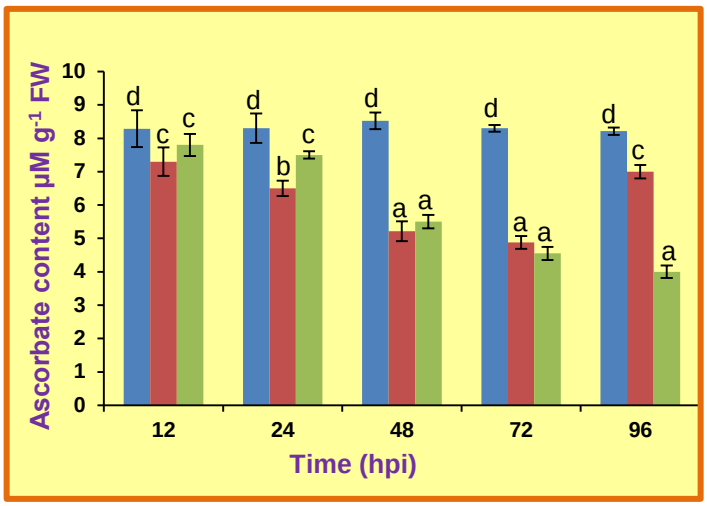
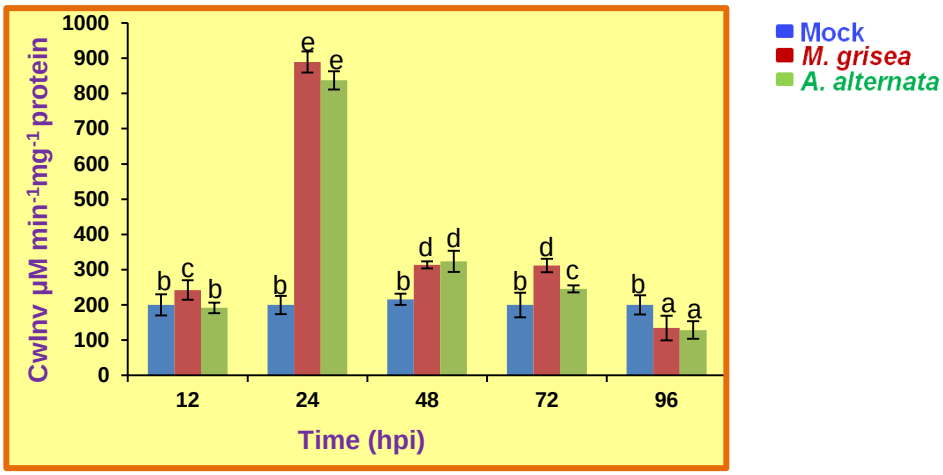


Fig 3.8: Measuring ascorbate oxidase activity and ascorbic acid content in apoplast of tomato leaves.

Ascorbate oxidase (AO) activity (mM min^{-1} mg^{-1} protein) (A) and ascorbic acid content (B) in tomato leaves inoculated with *M. grisea* and *A. alternata* f. sp. *lycopersici* compared to mock-inoculation. Error bars represent the standard deviation of the mean from three independent experiments. Bars of the histogram with same letter on the top do not differ significantly ($P \leq 0.05$). hpi-hours post inoculation.

[A]



[B]

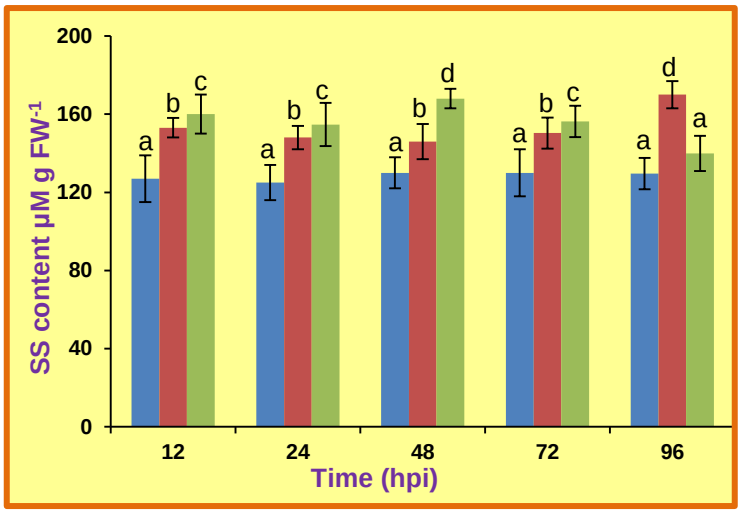


Fig 3.9: Quantification of cell wall invertase (Cw-Inv) and soluble sugar (SS) content in tomato leaves.

Activity of Cw-Inv (A), and SS content in whole-leaf tissue (B) in the tomato leaves inoculated with *M. grisea* and *A. alternata* f. sp. *lycopersici* compared to mock-inoculation. Error bars represent the standard deviation of the mean from three independent experiments. Bars of the histogram with same letter on the top do not differ significantly ($P \leq 0.05$). hpi-hours post inoculation.

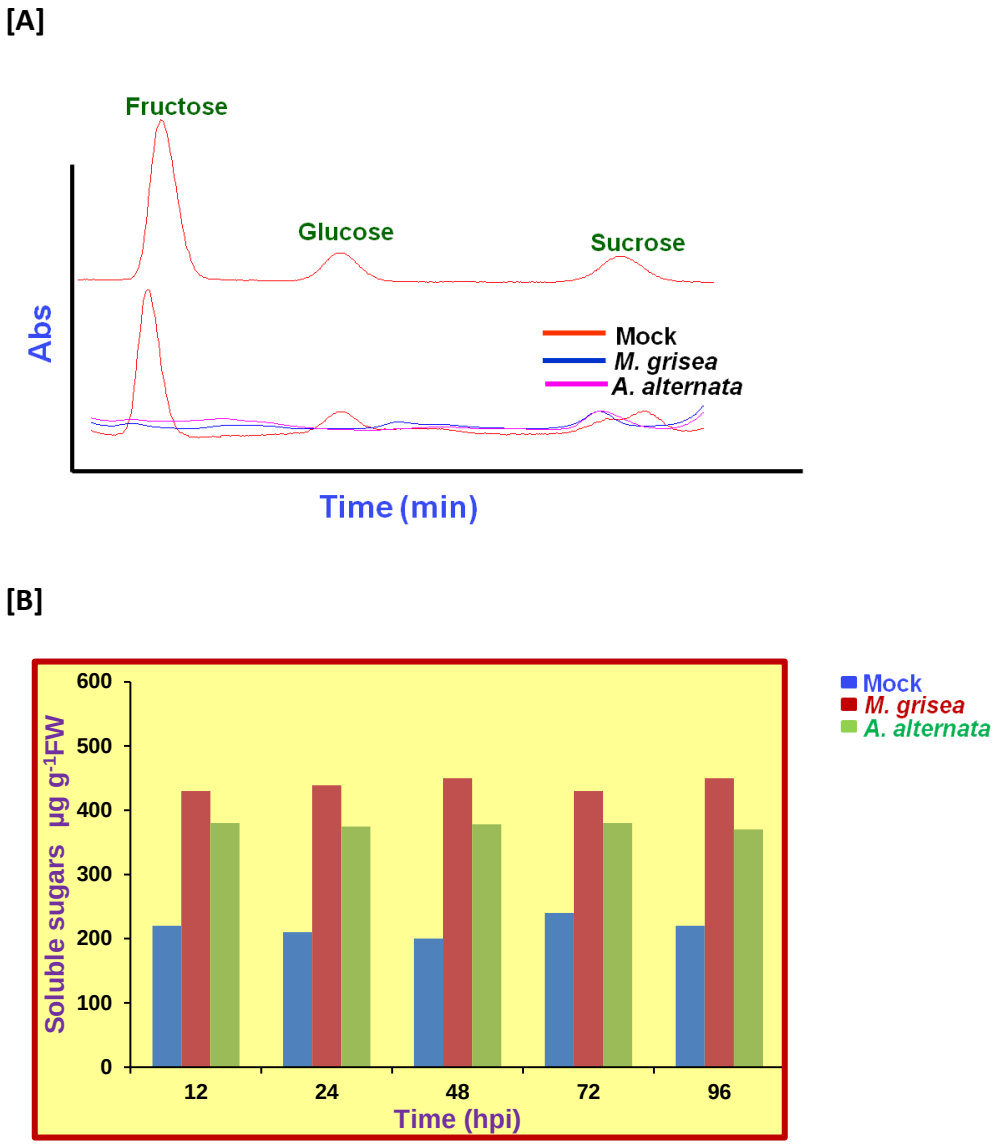


Fig 3.10: Quantification of soluble sugars in tomato apoplast using HPLC.

A) Time-course estimation of sucrose, glucose, and fructose concentration using HPLC in AF of tomato during non-host (blue line) and compatible interactions (pink line) compared to mock-inoculated (red line) at different time points. B) Concentration of sucrose, glucose, and fructose in AF extracted from mock-inoculated tomato leaves up to 96 hours post inoculation (hpi).

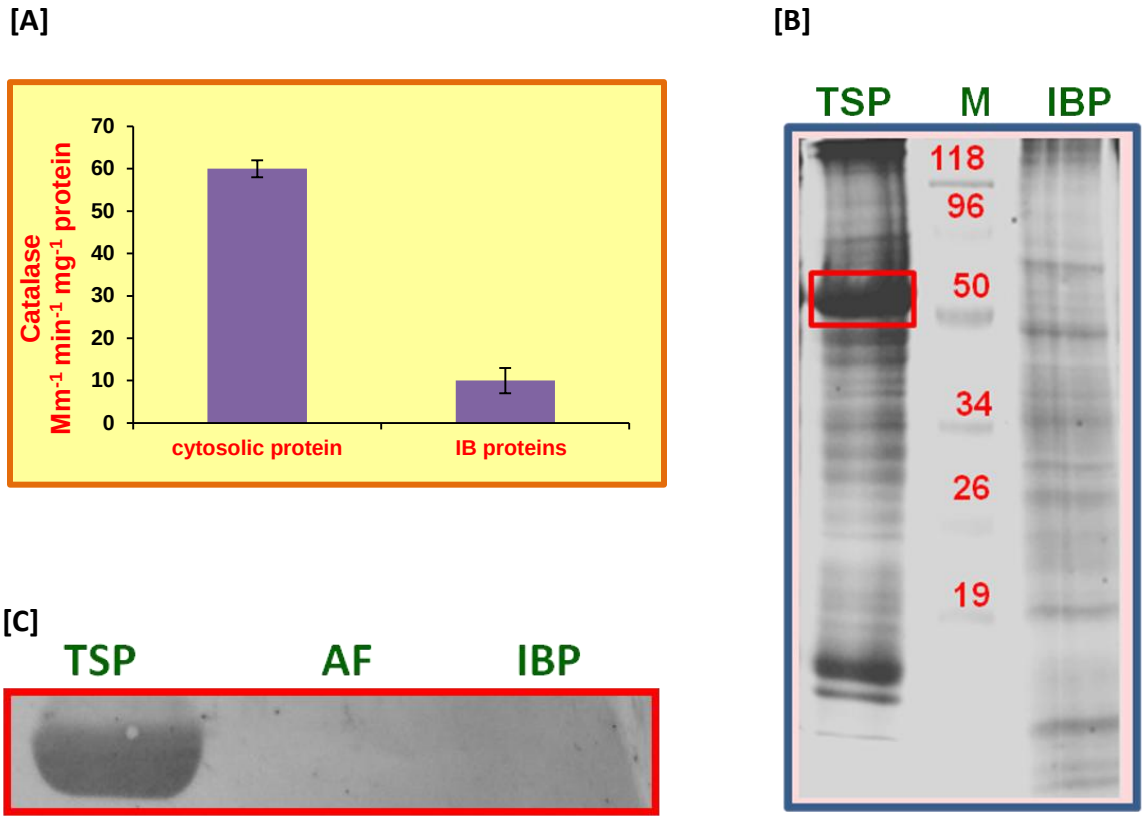


Fig 3.11: Assessment of purity of ionically bound protein (IBP) fraction.

A) Cytosolic marker enzyme, catalase activity (µM min^{-1} mg^{-1} protein) in total soluble protein (TSP) and IBP fraction.

B) SDS-PAGE analyses of TSP and IBP from tomato leaves. Rubicso large subunit (~50 kDa) is marked with red colour box. Lane 1: 10µg TSP, Lane 2: pre-stained protein marker, and Lane 3: 10µg IBP

C) Western blot analysis of fructose 1,6-bis phosphatase (a cytosolic marker enzyme) from TSP, AF and IBP factions.

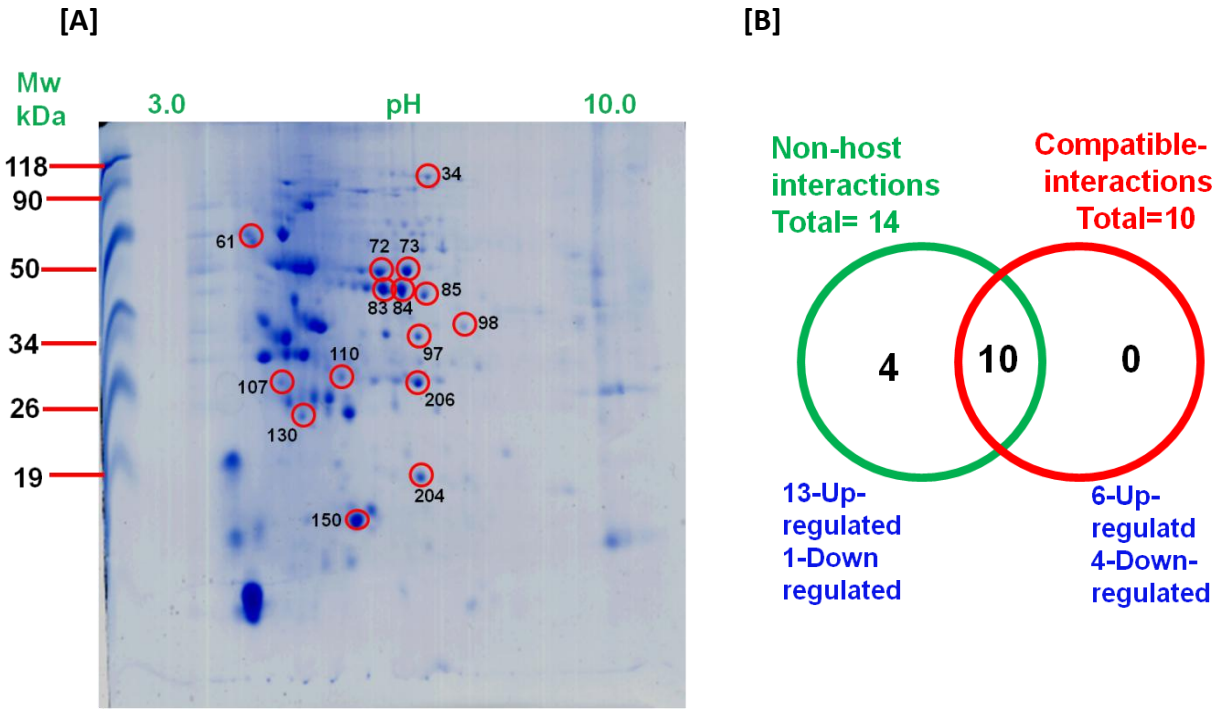


Fig 3.12: Reference 2-D electrophoretic pattern and venn diagrams of differential expressed proteins in the apoplastic fluid.

A) Reference 2-D electrophoretic patterns of proteins in the apoplastic fluid. Proteins were resolved using a linear gradient pH 3-10 in the first dimension and a 12% SDS-PAGE in the second dimension. Differentially expressed protein spots in non-host and compatible interactions that are identified by MALDI-TOF-MS were shown in circles. All gels were loaded with 350 µg total protein

B) Venn diagrams showing the number of proteins that are differentially expressed during non-host and compatible interactions of tomato.

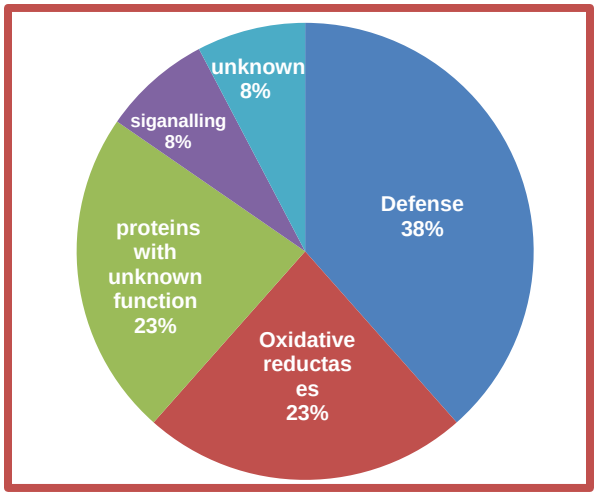
Spot ID*	Protein identification	Accession number	Estimated pI	Theor-itical pI	Estimated Mr(kDa)	Theor-itical Mr(kDa)	Interaction** Non-host	Host
150	α-amylase/trypsin inhibitor	P16850	5.5	7.49	16	16	↑	=
98	Basic 30 kDa endochitinase	Q05538	7	6.19	34	16	↑	↓
107	Acidic 27 kDa endochitinase	Q05540	4	4.68	30	27	↑	↑
110	Acidic endochitinase pcht28	Q40114	5.5	8.37	30	40	↑	=
61	Lignin-forming anionic peroxidase	P11965	4	4.69	50	35	↑	↓
130	2-Cys peroxiredoxin BAS1-like	Q9C5R8	5	5.55	26	29	↑	↓
34	Probable RNA-dependent RNA polymerase 4	Q5QMN4	6.5	6.65	95	133	↑	↑
48	ACCoxidase homolog 1	Q84MB3	5	5.17	70	40	↓	↓
101	Hypothetical protein	002271029	6	6.07	34	52	↑	↑
100	Unknown	BK22551	6	6.02	26	27	↑	=
206	Ribulose-phosphate 3-epimerase	Q9ZTP5	6.5	8.65	28	29	↑	↓
73	Ferredoxin-thioredoxin reductase	Q6LBH9	6	5.91	40	13	↑	↑
97	Basic 30 kDa endochitinase	Q05538	6.5	6.19	30	16	↑	↑
72	G-type lectin S-receptor-like ser/thr-protein kinase	Q9SXB8	6	8.27	50	95	↑	=

Apoplastic fluid (AF) fraction from *M. grisea*, *A. alternata* f. sp. *lycopersici* and mock-inoculated tomato leaves were separated by 2-D-gel electrophoresis, picked, trypsinated and used for determination fragment composition by MALDI-TOF-MS. Identification was achieved by data bank queries as outlined under materials and methods. Spot numbers refer to those shown in Fig. 3.12.

*As shown in fig 3.12, ↑up regulated, ↓ down-regulated, = no change, ** Interaction with M. *grisea* (non-host) and *A. alternata* f. sp. *lycopersici* (host).

Table 3.1: Details of peptide matches for the tomato leaf apoplast proteins resolved by 2-D PAGE.

[A]



[B]

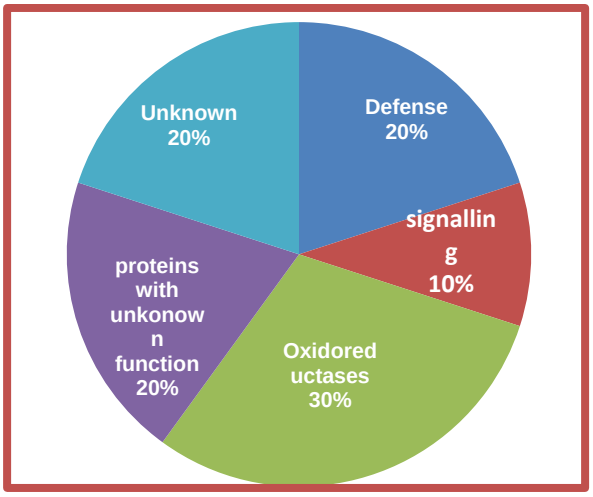
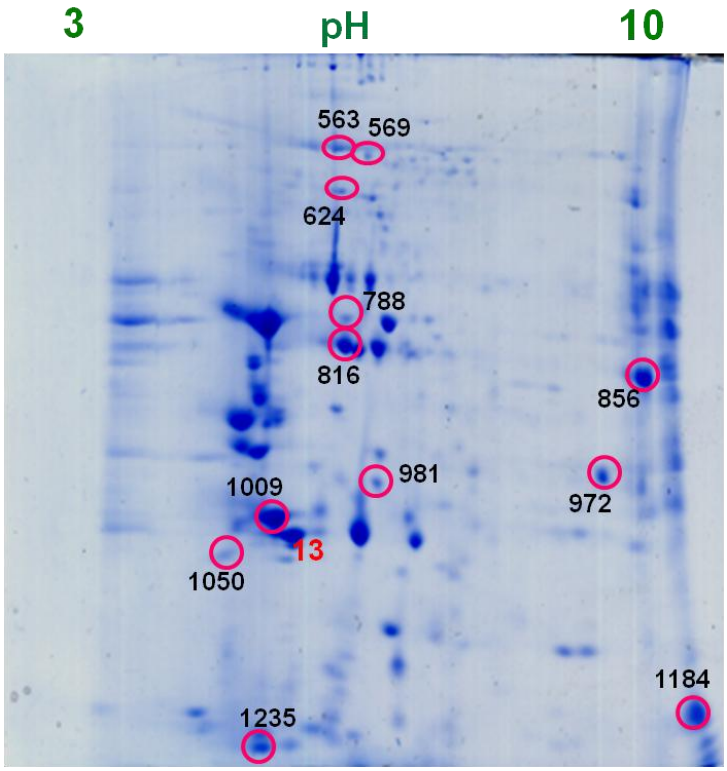


Fig 3.13: A functional classification of differentially expressed proteins in the apoplastic fluid of tomato during non-host (A) and compatible (B) interactions.

[A]



[B]

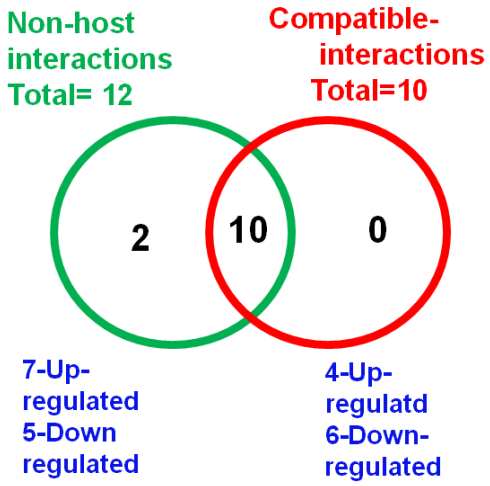
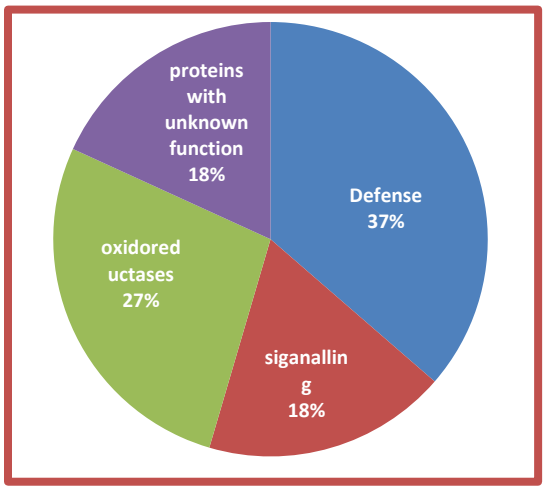


Fig 3.14: Reference 2-D electrophoretic pattern and venn diagrams of differential expressed ionically bound cell wall proteins (IBP) in the apoplast.

A) Reference 2-D electrophoretic patterns of IBP fraction. Proteins were resolved using a linear gradient pH 3-10 in the first dimension and a 12% SDS-PAGE in the second dimension. Differentially expressed protein spots in non-host and compatible interactions that are identified by MALDI-TOF-MS were shown in circles. All gels were loaded with 350 µg total protein.
B) Venn diagrams showing the number of proteins that were differentially expressed during non-host and compatible interactions of tomato.

[A]



[B]

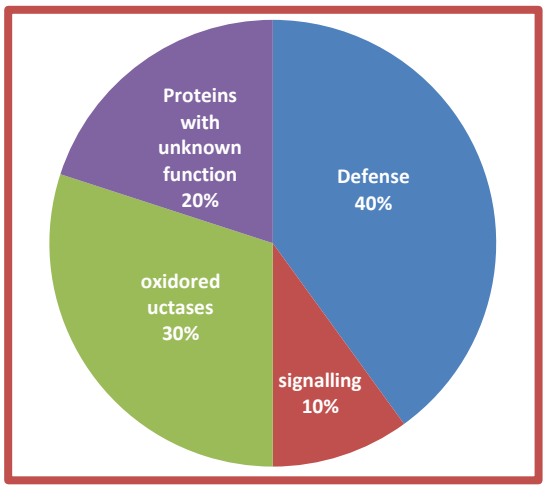


Fig 3.15: A functional classification of differentially expressed proteins in the ionically bound cell wall protein (IBP) fraction of tomato apoplast during non-host (A) and compatible (B) interactions.

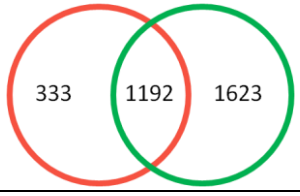
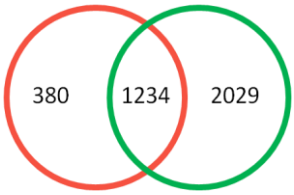
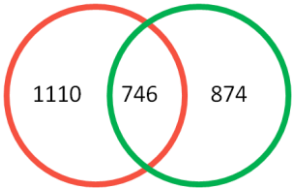
Spot ID*	Protein identification	Accession number	Estimated *pI*	Theor-Itical *pI*	Estimated Mr (kDa)	Theor-itical Mr(kDa)	Interactions** Non-host	Host
856	Basic β-1,3-glucanase	M80608	10	7.851	35	3975	↑	↑
624	Ferredoxin--NADP reductase	O04977	6	8.37	66	39751	↓	↓
1009	Putative callose synthase	Q9LYS6	5	8.76	20	223998	↑	↓
981	NAD(P)H-quinone oxidoreductase	Q6EW45	6.5	6.06	22	18727	↓	↓
1184	Formin-like protein 12	Q7XWS7	10	5.55	18	184674	↑	=
788	Glucan endo-1,3-β-glucosidase	Q01413	6.5	7.85	40	39751	↑	↑
1050	Ca^{2+}/calmodulin-dependent ser/thr-protein kinase	Q07250	4.5	9.11	20	46748	↑	↑
1588	NAD(P)H-quinone oxidoreductase		6		35		↓	=
816	Lignin-forming anionic peroxidase	P11965	6	4.68	35	35108	↑	↓
563	binding / catalytic/ coenzyme binding	NP_565868	5.5	8.37	110	34972	↓	↓
569	Oxygen-evolving enhancer protein 2	P29795	6.5	8.26	110	27.946	↓	↓
972	Endochitinase	P05315	9.5	8.64	25	36376	↑	↑

Ionically bound cell wall protein (IBP) fraction from *M. grisea*, *A. alternata* f. sp. *lycopersici* and mock-inoculated tomato leaves were separated by 2-D-gel electrophoresis, picked, trypsinated and used for determination fragment composition by MALDI-TOF-MS. Identification was achieved by data bank queries as outlined under materials and methods. Spot numbers refer to those shown in Fig. 3.14.

*As shown in fig 3.12, ↑up regulated, ↓ down-regulated, = no change, ** Interaction with M. *grisea* (non-host) and *A. alternata* f. sp. *lycopersici* (host)

Table 3.2: Details of peptide matches for ionically cell wall bound proteins the tomato leaf apoplast proteins resolved by 2-D PAGE.

[A]

	Compatible interactions		Non-host interactions	
	6 hpi	24 hpi	6 hpi	24 hpi
Up-regulated genes				
Down-regulated genes				

[B]

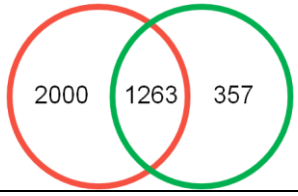
	6hpi		24hpi	
	Compatible	Non-host	Compatible	Non-host
Up-regulated genes				
Down-regulated genes				

Fig 3.16: The number of differentially expressed transcripts during non-host and compatible interactions.

Venn diagrams showing the number of transcripts that were differentially expressed in tomato leaves during non-host and compatible interactions at a level of $\log_2$ expression value of ≥1 and ≤-1 with *P*<0.01. A) Number of differentially regulated transcripts during compatible interactions *vs* mock inoculated (left) and non-host interactions *vs* mock-inoculated (right) at 6 and 24 hpi compared. B) Number of specific and overlapped transcripts between compatible and non-host interactions of tomato at 6 and 24 hpi.

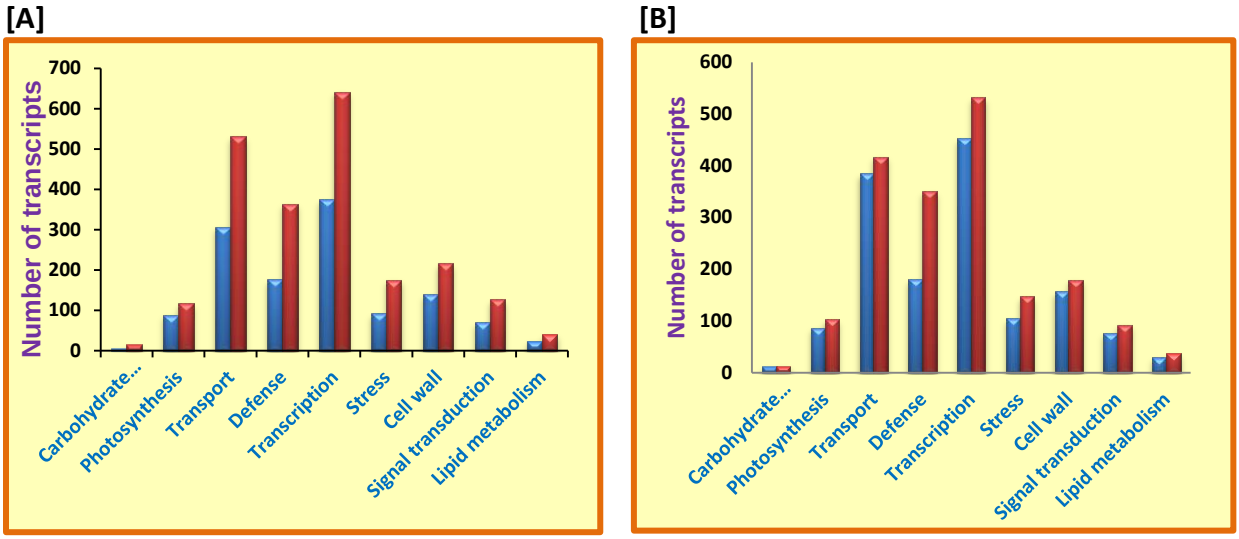


Fig 3.17: Functional categories of differentially expressed transcripts during non-host and compatible interactions.

Assigned functional categories of differentially expressed transcripts using cut-off statistical parameter $P<0.01$ with $\log_2$ expression value of ≥1 and ≤-1. Differentially expressed transcripts in *A. alternata* f. sp. *lycopersici*-challenged (A) and *M. grisea*-challenged (B) tomato leaves compared to mock-inoculated tomato leaves. The blue and red bars represent the differentially regulated transcripts at 6 and 24 hpi in each functional category, respectively.

Annonation	Fold change (P<0.01)			
	M. grisea		*A. alternata*	
Starch metabolism				
	6hpi	**24hpi**	**6hpi**	**24hpi**
Starch-granule-bound R1 protein	1.32	3.36	1.25	2.05
Alpha-amylase	2.72	5.05	2.91	6.03
Alpha-1,4 glucan phosphorylase L-2	5.62	1.24	5.04	2.30
Alpha-glucosidase	8.44	9.55	9.66	10.13
Starch phosphorylase L	-4.48	-4.23	-4.47	-3.87
Isoamylase isoform 2	-2.66	-2.64	-3.15	-3.27
Granule-bound starch synthase 1	-2.66	-2.64	-3.08	-3.97
Granule-bound starch synthase 2	-5.64	-8.17	-5.47	-9.18
Alpha-glucan water dikinase chloroplastic	-4.73	-5.15	-4.90	-4.90
Starch branching enzyme II	-4.83	-4.83	-5.32	-4.84
Soluble starch synthase	-3.11	-3.08	-2.94	-4.27
Sucrose metabolism				
β-fructofuranosidase	4.27	3.49	4.28	3.97
Sucrose synthase 4	3.19	3.13	3.76	4.17
sucrose synthase	1.76	2.02	2.26	2.80
Sucrose-phosphate-synthase	2.06	3.35	3.00	4.43
Apoplastic invertase	1.95	3.23	2.19	2.44
Acid invertase	1.55	1.39	1.81	1.70
Glycolysis pathway				
Hexokinase	4.21	4.17	4.10	4.22
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha	5.06	6.76	5.33	6.76
Fructose-bisphosphate aldolase	2.35	2.86		
Plastid enolase	1.81	1.65	2.05	1.49
Glyceraldehyde-3-phosphate dehydrogenase	-2.92	-5.06	-2.87	-5.41
Fructose-bisphosphate aldolase	-3.44	-2.82	-3.22	-5.04
Pyruvate kinase, cytosolic isozyme	-3.48	-3.50	-3.94	-3.34
Phosphoglycerate kinase	-2.44	-4.16	-2.29	-3.87
Calvin cycle				
Transaldolase 2	8.78	5.42	7.12	6.38
RUBISCO- large subunit	4.47	1.75	4.18	2.56
Ribulose-1,5-bisphosphate carboxylase, small subunit	-3.78	-4.46	-3.72	-6.58
Chloroplast Rubisco activase	-3.44	-3.57	-3.14	-4.46
Fructose-1,6-bisphosphatase class 1 2	-2.55	-5.04	-2.55	-4.47
Ribulose-phosphate 3-epimerase	-2.31	-4.17	-2.31	-4.47
Ribulose bisphosphate carboxylase small chain 3B	-2.85	-4.31	-1.93	-4.45
Carbonic anhydrase	-2.35	-5.98	-2.27	-5.33
Ribulose bisphosphate carboxylase/oxygenase activase	-2.49	-3.36	-2.72	-3.31
Transketolase, chloroplastic (TK)	-2.27	-3.10	-2.45	-3.78

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock-inoculated and *M. grisea* and *A. alternata* f. sp. *lycopersici* are given. hpi-hours post inoculation.

Table 3.3: Carbohydrate metabolism-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
Respiratory burst oxidase protein F	3.68	2.13	3.71	2.76
Chlorophyll a,b binding protein type I	-8.14	-8.55	-8.42	-8.29
Chlorophyll a/b binding protein Lhcb1-4	-4.19	-3.16	-4.31	-4.74
Chlorophyll a/b binding protein	-5.20	-8.15	-5.14	-8.58
PsaA 1	-2.13	-2.58	-2.50	-2.46
Phytochrome B	-2.43	-2.98	-2.59	-2.48
Chlorophyll a/b binding protein precursor CAB6A	-3.26	-5.20	-3.33	-5.59
Chlorophyll a/b-binding protein precursor	-4.25	-4.26	-4.57	-5.73
Cytochrome b6 1	-2.80	-2.63	-3.13	-2.78
Apocytochrome f 1	-2.67	-3.73	-3.12	-2.06
33kDa precursor protein of oxygen-evolving complex	-2.43	-3.15	-1.79	-2.55
Photosystem II oxygen-evolving complex protein 3	-1.43	-2.02	-1.55	-3.71
Pre-plastocyanin (AA -64 to 106)	-2.57	-3.61	-2.42	-4.42
PsaD	-1.23	-1.80	-1.35	-2.93
violaxanthin de-epoxidase	-2.54	-6.04	-2.53	-7.61
PSII polypeptide	-2.50	-1.65	-2.35	-3.33
NADH-quinone oxidoreductase subunit A 1	-1.13	-4.39	-1.36	-4.63
PsaB 1	-1.81	-1.55	-2.11	-1.52
Oxygen-evolving enhancer protein 2	-1.25	-1.89	-1.56	-1.87
NADPH oxidase RBOHD	-2.75	-4.51	-3.11	-5.77
Ferredoxin-I	-2.39	-1.95	-2.40	-2.63
Ferredoxin-NADP reductase	-2.22	-3.95	-2.08	-5.19
Subunit A of ferredoxin-thioredoxin-reductase	-1.09	-1.96	-1.15	-2.71
Cytochrome b6-f complex iron-sulfur subunit	-1.29	-1.11	-1.36	-1.28
Ferrochelatase	-1.62	-3.59	-1.48	-4.05

Fold changes in up-(with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.4: Photosynthesis-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
Voltage-dependent anion-selective channel protein	8.56	9.27	8.98	9.43
Probable polyol transporter 6	6.71	7.37	7.10	9.50
Transporter-related	5.88	3.76	6.43	5.30
Inorganic phosphate transporter	5.21	4.38	5.62	5.30
Alternative oxidase 1b	4.48	2.96	6.00	3.90
st3 protein	4.11	3.55	3.98	3.64
Plastid quinol oxidase	3.46	1.80	3.37	1.33
Amino acid permease	3.94	3.07	4.24	4.27
Vacuolar proton translocating ATPase 116 kDa subunit a isoform 4	3.13	4.98	3.29	4.45
HXK3	4.21	4.17	4.10	4.22
Plastidic ATP/ADP-transporter	2.94	1.87	3.00	1.70
Ferritin	2.63	3.19	2.67	2.56
Hexose transporter 1	2.45	2.70	2.70	4.12
Amino acid transporter	2.44	1.97	2.82	2.95
Hexose transporter 3	2.08	1.46	1.92	1.81
ADP,ATP carrier protein-like	2.22	2.51	2.00	2.13
Sucrose transport protein	1.25	1.76	1.43	1.34
Plastidic ATP/ADP-transporter	1.87	2.94	3.00	1.70
Sucrose transport protein-SUT1	1.85	1.09	1.42	1.75
Envelope membrane protein 1	1.74	1.89	1.60	1.90
Plasmalemma Na+/H+ antiporter-like	1.49	1.29	1.23	1.24
LeOPT1 protein	1.29	1.24	1.25	1.56
ABCG subfamily transporter	-8.86	-6.83	-8.74	-7.06
Glucose-6-phosphate/phosphate translocator 2	-7.66	-10.77	-10.06	-11.57
Neryl diphosphate synthase 1	-5.81	-4.77	-3.81	-5.14
Triose phosphate/phosphate translocator	-6.78	-5.85	-6.68	-7.23
ABC transporter-like	-6.06	-4.41	-6.43	-4.69
ATP synthase subunit a	-6.05	-6.13	-5.40	-6.90
Non-specific lipid-transfer protein	-3.88	-2.91	-1.83	-2.88
H(+)-transporting ATPase	-3.08	-5.07	-3.36	-4.26
Non-specific lipid-transfer protein	-3.34	-3.47	-3.25	-4.59
Ammonium transporter	-3.10	-6.56	-3.87	-6.76
X intrinsic protein	-3.14	-3.79	-2.96	-2.58
Non-specific lipid transfer protein	-3.87	-5.23	-4.30	-8.78
Potassium channel	-2.05	-1.87	-1.69	-2.06
Ca2+-ATPase	-2.26	-2.83	-2.26	-2.58
Membrane channel protein	-2.07	-1.26	-3.83	-3.76
ABCG subfamily transporter protein (PDR4)	-2.43	-2.89	-2.28	-4.50
ABCG subfamily transporter protein	-1.77	-1.90	-1.55	-2.65
P-glycoprotein	-1.25	-2.62	-1.71	-2.27
Putative inward rectifying potassium channel	-1.03	-2.70	-1.10	-3.59
Hexose transporter (Fragment)	-1.90	-4.04	-1.93	-4.12
Oxoglutarate/malate translocator	-1.15	-1.10	-1.49	-1.47
ATPase-like	-1.18	-1.16	-1.12	-1.67
Remorin (pp34)	-1.10	-2.84	-1.28	-3.28

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.5: Transport-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (p<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
PR5-like protein	13.03	13.32	13.42	12.72
Pathogenesis-related protein 1b	8.18	10.96	8.64	11.18
β-1,3-glucanase	8.78	8.78	8.88	10.36
Pathogenesis-related protein STH-2	7.95	10.08	8.58	12.03
Probable glutathione S-transferase	5.92	6.14	7.20	6.27
Pathogenesis-related protein PR P23	4.43	5.48	4.71	4.72
Pathogenesis-related protein 10	4.40	6.22	4.69	6.60
Subtilisin-like protease	4.00	2.56	4.18	4.56
Pathogenesis-related protein 1	2.78	4.68	3.41	5.37
Acidic endochitinase precursor	2.47	2.21	1.76	3.59
Putative thaumatin-like protein	2.87	2.63	3.03	1.54
Endochitinase 1	2.97	5.26	3.35	4.87
Pathogenesis related protein PR-1	2.68	4.92	1.24	5.18
Acidic class II 1,3-beta-glucanase	1.63	2.18	1.40	2.15
Proteinase inhibitor type-2	3.90	10.50	6.52	13.34
Cationic peroxidase precursor	8.53	8.11	8.63	9.79
9-divinyl ether synthase (StDES)	8.23	7.99	8.91	9.07
Xyloglucan-specificfungal endoglucanase inhibitor protein precursor	7.03	8.87	8.45	8.45
Wound-induced protein WIN2	4.43	6.19	7.20	6.27
Wound-induced protein WIN1	4.22	6.02	4.81	5.82
Subtilisin-like protease	4.00	2.56	4.18	4.56
WRKY transcription factor 5	4.06	4.53	3.90	5.38
Putative disease resistance protein	3.52	4.59	4.14	5.37
Chymotrypsin inhibitor I, A, B and C subunits	3.39	4.21	3.26	3.50
Avr9/Cf-9 rapidly elicited protein 75	2.74	4.37	3.62	4.60
CC-NBS-LRR protein	3.20	3.42	3.42	2.52
MLO1 protein	3.65	3.09	3.87	3.53
CC-NB-LRR protein	2.81	3.47	3.06	3.94
Xyloglucan specific endoglucanase inhibitor3	2.28	3.44	2.31	1.99
Putative disease resistance protein	2.89	2.24	2.50	2.97
Cysteine protease	5.25	3.61	4.31	5.07
Wounding-induced ribonuclease gene protein	1.74	3.05	1.95	3.38
ss-galactosidase	1.90	4.63	1.51	6.55
Putative cytochrome P450	1.81	1.83	1.90	2.89
Tm-2 ToMV resistance protein	1.19	1.60	1.22	1.84
Multicystatin	-8.40	-8.15	-6.48	-7.55
Metallocarboxypeptidase inhibitor	-5.90	-4.89	-6.20	-8.48
EDS1 protein	-3.37	-4.62	-3.25	-4.87
Aspartic protease inhibitor 1	-3.41	-2.44	-3.32	-2.62
RGC1 (Fragment)	-2.10	-3.02	-2.02	-2.53
Resistance gene-like	-2.53	-5.63	-2.23	-8.08
Endo-beta-1,4-D-glucanase	-2.16	-2.15	-3.51	-4.84
Ankyrin repeat-rich protein	-2.38	-2.24	-2.53	-2.23
Harpin binding protein 1	-2.77	-2.60	-3.02	-3.01
Polygalacturonase inhibitor protein	-2.27	-3.34	-3.08	-4.58
Snakin-1	-2.21	-3.20	-2.14	-2.27
Xyloglucan-specific endoglucanase inhibitor 4	-1.19	-1.73	-1.78	-3.32
UDP-apiose/xylose synthase	-1.35	-2.07	-1.85	-2.24

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.6: Defense-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P<0.01*)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
RNA1 polyprotein	7.14	7.90	7.72	9.24
NAC domain protein NAC2	5.53	6.79	5.69	8.37
LeCBF1 protein	4.95	3.60	4.48	3.05
Putative NAC domain protein	4.91	4.58	5.73	5.01
C2H2-type zinc finger protein	4.75	4.03	5.19	5.46
Cup-shaped cotyledon3	4.24	6.47	5.44	3.78
Probable WRKY transcription factor 71	4.44	4.86	5.50	6.37
Ripening regulated protein DDTFR10/A	4.09	2.48	3.60	3.83
Putative regulator of chromosome condensation	5.73	5.97	5.60	6.56
ERF transcription factor	4.66	5.52	5.36	7.34
Probable WRKY transcription factor 71	4.44	4.86	5.50	6.37
Putative arginine/serine-rich protein-like	2.22	3.51	2.24	2.91
Ethylene-responsive factor 1	3.62	5.04	4.10	4.29
Pti4	3.85	3.87	4.20	4.82
DREB3	2.30	2.20	2.26	2.16
Histone H2B	2.61	4.00	3.19	4.75
WRKY protein	2.54	2.55	2.83	2.47
Heat stress transcription factor A-5	2.34	3.79	2.42	5.07
Mitochondrial SBP40	3.78	3.44	4.61	3.66
Myb-related protein MYBAS1	3.10	2.73	2.06	2.36
APETALA2-like protein	1.88	1.30	2.19	2.23
myb-related transcription factor	1.88	2.19	1.99	1.38
GAGA-binding transcriptional activator	1.74	2.86	1.44	2.29
Nam-like protein 1	1.78	2.46	2.26	2.77
myb-related transcription factor	1.88	2.19	1.99	1.38
Anaerobic basic leucine zipper protein	1.66	5.70	2.77	6.42
Class II knotted-like homeodomain protein	1.67	1.90	1.43	1.64
EIL3 protein	1.44	2.31	1.72	2.28
Nam-like protein 4	1.55	2.28	1.65	3.07
PGPD14	1.58	3.13	1.59	3.62
Transcription factor JERF1	2.61	2.68	2.40	2.06
WRKY transcription factor 2	2.66	2.32	3.19	4.02
Transcription factor JERF1	2.61	2.68	2.40	2.06
Ribonuclease	2.86	3.19	3.14	4.53
Adenosine kinase isoform 1T-like protein	2.31	2.05	2.30	3.08
MADS transcriptional factor	2.10	2.18	2.78	2.58

Fold changes in up-regulated between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.7A: Transcription factors that are up-regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
MADS-box protein 5	-6.13	-5.35	-5.45	-6.18
Agamous-like MADS-box protein	-6.13	-5.35	-5.45	-6.18
Dof zinc finger protein	-5.85	-2.42	-6.55	-1.98
DREB	-4.04	-2.97	-4.15	-3.93
40S ribosomal protein S27	-3.79	-4.67	-3.73	-4.60
IAA11 protein	-2.10	-3.07	-3.00	-4.99
IAA7 protein	-3.20	-1.92	-3.30	-2.94
Self-pruning G-box protein	-3.00	-2.16	-2.95	-3.43
CRT binding factor 2A	-2.66	-3.14	-2.74	-3.89
vsf-1 protein	-2.09	-1.57	-2.14	-1.98
NO APICAL MERISTEM	-2.28	-2.13	-2.69	-2.29
Transcription factor	-2.54	-3.01	-2.95	-3.88
Bell-like homeodomain protein 4	-2.28	-5.14	-3.39	-5.82
VP1-ABI3-like protein	-2.00	-2.17	-2.26	-2.25
TDR3 protein	-2.75	-1.61	-2.23	-1.40
Translation initiation factor eIF(Iso)4E	-1.90	-2.55	-2.22	-2.85
IAA2 protein	-1.29	-1.68	-3.28	-2.58
RNA polymerase IV largest subunit	-1.88	-2.64	-2.39	-2.69
Ribonuclease E	-1.41	-2.45	-1.56	-2.50
Deoxyuridine triphosphatase	-1.35	-3.15	-1.61	-3.56
Transcription factor MYB48	-1.44	-1.28	-2.42	-2.58
Deficiens analogue	-1.40	-1.16	-2.64	-2.30

Fold changes in down-regulated between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.7B: Transcription factors that are down-regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annoatations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
Polyphenol oxidase D	9.57	9.08	10.38	7.47
Peroxidase	8.54	9.55	9.58	9.79
Putative peroxidase	7.56	8.31	7.91	9.26
Putative peroxidase	6.20	6.68	6.14	6.45
Leucine aminopeptidase	5.79	7.91	7.13	2.69
Ascorbate oxidase	5.50	5.34	5.99	6.05
Proline dehydrogenase	5.49	3.27	4.79	3.09
Catalase activity	2.94	3.17	2.76	4.53
Wound/stress protein	2.41	2.61	2.50	1.20
Peroxidase	2.81	4.06	2.11	3.65
ER6 protein	2.73	2.86	2.77	2.69
Catalase	2.72	3.75	2.65	4.29
Trehalose-6-phosphate synthase	2.27	4.25	2.93	6.28
Heat stress transcription factor A-5	2.16	4.78	2.40	4.23
Rapid alkalinization factor 1	2.06	3.75	2.61	4.48
Cold-stress inducible protein	2.02	1.75	1.81	1.71
Stress-associated protein 3	1.79	2.62	1.70	2.99
Glutathione peroxidase	1.28	1.67	1.95	2.62
Glutathione reductase	1.90	1.84	2.10	2.22
hsr203j	6.23	9.87	6.95	11.21
Ethylene-responsive catalase	-5.05	-4.49	-4.76	-2.85
Methyl jasmonate esterase	-5.15	-4.51	-4.37	-5.62
Zeaxanthin epoxidase	-5.84	-4.08	-4.57	-5.90
Chloroplast heat shock protein	-4.28	-3.10	-4.40	-1.69
Heat shock protein 90	-3.30	-4.85	-3.76	-5.85
Glutaredoxin	-3.48	-4.64	-2.41	-4.32
Cold-stress inducible protein	-3.27	-1.61	-3.18	-3.18
Small heat shock protein	-3.04	-3.21	-6.28	-3.06
Vacuolar sorting receptor protein PV72	-2.52	-4.59	-3.11	-3.97
Heat shock 70 kDa protein	-2.92	-4.08	-2.99	-2.40
Heat shock cognate protein	-2.62	-3.68	-2.59	-3.24
Molecular chaperone Hsp90-1	-2.30	-2.01	-2.34	-3.11
Chaperone protein DnaJ 5	-2.20	-4.27	-1.99	-5.08
Cathepsin D inhibitor	-2.85	-4.75	-2.84	-6.95
Prosystemin	-2.14	-1.92	-2.20	-1.54
Dehydroascorbate reductase(DHAR2)	-2.04	-2.08	-2.15	-1.53
Trehalose-phosphate phosphatase	-1.66	-2.09	-2.19	-1.87
Ascorbate peroxidase	-1.84	-5.65	-1.99	-5.47
Ethylene-responsive heat shock protein	-1.53	-1.76	-1.68	-2.22
60 kDa chaperonin (GroEL protein)	-1.74	-4.62	-2.37	-5.45
Drought-induced stress protein	-1.37	-3.96	-1.25	-3.82

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.8: Stress-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annonations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
Cellulose synthase (StCesA4)	7.36	7.38	7.78	7.00
Pectin methylesterase 3	6.85	7.39	6.59	7.44
Hydroxyproline-rich glycoprotein	6.59	5.86	6.81	6.47
Feruloyl transferase	4.41	5.71	5.05	8.19
Xyloglycan endo-transglycan	5.22	2.62	5.18	2.85
Xyloglucan ndotransglycosylase/hydrolase XTH-6	5.77	7.38	5.45	9.02
Xyloglucanendotransglucosylase-hydrolase XTH3	4.42	1.43	3.85	1.31
Expansin-like protein precursor	4.03	2.95	3.58	3.86
β-mannosidase enzyme	4.96	3.38	4.65	2.01
Pectin methylesterase	3.23	2.74	3.27	1.93
UDP-glucose:protein transglucosylase-like	2.72	2.09	2.71	1.78
Glycine-rich protein	3.65	4.57	3.85	3.97
Expansin	2.28	3.06	1.87	3.23
Expansin 8	2.11	3.04	2.31	3.51
Extensin (class I)	2.53	5.11	2.63	2.85
alpha-L-arabinofuranosidase	1.72	1.95	2.16	2.65
Expansin 4	-6.50	-4.50	-6.62	-7.21
Expansin2	-6.58	-6.00	-7.13	-7.75
xyloglucan endotransglycosylase	-4.80	-2.98	-3.19	-3.85
Xyloglucan endotransglycosylaseXTH4	-4.60	-3.99	-3.28	-3.67
UDP-GlcNac-dolichyl-phosphateN-acetylglucosaminephosphotransferase	-4.33	-5.17	-4.36	-6.18
Pectinesterase	-4.41	-5.09	-6.09	-5.22
Expansin11	-3.12	-3.01	-3.79	-4.93
Cellulose synthase (StCesA1)	-3.13	-7.39	-4.69	-9.02
Arabinogalactan	-3.04	-4.27	-2.95	-5.62
Polygalacturonase-like protein-like	-2.49	-6.14	-2.32	-4.44
Methionine rich arabinogalactan	-1.24	-3.40	-1.82	-4.33
Expansin12	-1.88	-4.51	-3.38	-6.20
Expansin10	-1.22	-2.06	-1.79	-2.93
Expansin A4	-1.47	-1.26	-1.64	-1.95
Xyloglucanendotransglucosylaseydrolase XTH6	-1.44	-2.27	-1.35	-3.51
Xyloglucanendotransglucosylase-hydrolase XTH7	-1.69	-3.93	-2.58	-5.09
Xyloglucan galactosyltransferase	-1.43	-2.32	-1.45	-2.12
Cellulose synthase (StCesA3)	-1.11	-4.15	-1.76	-3.98

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.9: Cell wall-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
MPK3	10.34	9.04	10.43	9.69
F-box domain-containing protein	7.31	7.73	7.41	7.53
EIX receptor 1	5.40	5.39	5.91	6.75
Calmodulin 5/6/7/8-like protein	5.27	6.98	6.32	8.43
Mitogen-activated protein kinase	4.37	5.21	3.75	5.21
Auxin-regulated protein	4.33	4.08	5.02	4.64
MAP3K-like protein kinase	3.06	4.26	3.46	3.98
Putativereceptor-likeser-thr protein kinase	3.15	1.83	3.13	2.83
Putative ethylene receptor protein	2.87	3.00	2.95	2.22
Phospholipase PLDa2	2.96	2.40	2.86	2.91
Nitric oxide synthase-associated protein I	2.92	4.40	3.55	5.30
Calcium-dependent protein kinase 4	2.49	3.20	2.17	2.83
SNF1 kinase complex anchoring protein	2.31	2.74	2.58	3.93
MAPKK	2.24	2.35	1.97	2.58
Calcium dependent protein kinase	2.17	3.23	2.22	3.18
PI-phospholipase C PLC3	1.75	2.36	1.80	2.28
EIN3-binding F-box protein 1	1.68	2.26	1.83	1.22
Serine/threonine protein kinase-like	1.36	2.43	1.39	3.15
LRR receptor-like kinase	1.40	4.62	1.81	5.81
PI-phospholipase C PLC2	1.40	2.11	1.90	2.59
ACC synthase	1.86	3.57	2.5	3.65
ACC oxidase	5.52	6.24	6.09	5.64
WIPK	1.79	1.22	2.36	1.82
MAPK7	1.94	1.59	2.12	2.61
MEK2	1.93	2.06	2.22	2.18
MKK4	2.08	2.61	2.03	2.17
MPK4	2.28	1.81	2.76	2.67
MKP1	1.42	1.89	2.27	2.06
SERK3B	1.81	1.74	1.79	1.78
SERK1	3.54	3.87	2.87	3.90
Fructokinase 2-like protein	-9.06	-6.57	-8.45	-6.69
NAM-like protein	-4.19	-3.49	-2.79	-2.25
LRR receptor-like kinase	-4.13	-4.77	-4.95	-5.58
Phototropin-2	-3.00	-3.23	-3.36	-5.31
Resistance gene-like	-2.45	-5.84	-2.20	-8.55
Putative ethylene receptor	-2.60	-3.13	-2.82	-3.15
Putative uncharacterized protein	-2.41	-2.53	-3.04	-4.13
Calcineurin B-like interacting protein kinase	-1.90	-2.87	-2.11	-0.95
Serine/threonine protein kinase-like	-1.62	-4.16	-2.99	-3.99
WD-40 repeat protein-like protein	-1.74	-3.35	-1.83	-3.47
14-3-3 protein	-1.46	-1.43	-1.54	-2.20
ADP-ribosylation factor 1	-1.36	-3.72	-1.25	-3.76
Zeaxanthin epoxidase	-5.84	-4.08	-4.57	-5.90
Gibberellin 20-oxidase-1	-6.22	-6.19	-7.09	-5.52
Gibberellin 7-oxidase	-7.26	-5.77	-7.16	-7.59
Gibberellin 2-oxidase 1	-9.46	-6.96	-9.18	-8.14
Neoxanthin synthase	-1.93	-2.51	-2.00	-2.04

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.10: Signal transduction-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
alpha-DOX1	7.51	8.25	8.63	9.08
Linoleate 9S-lipoxygenase 2	4.02	4.60	4.87	4.19
Linoleate 13S-lipoxygenase 2-1	4.36	1.06	4.16	1.93
Putative sterol desaturase	2.45	2.43	2.41	2.22
Phosphatidate cytidylyltransferase	1.76	2.49	1.64	2.51
Peroxisomal acetoacetyl-coenzyme A thiolase	1.26	2.28	1.38	3.28
Oxysterol-binding protein	1.81	1.33	1.97	1.98
Linoleate 13S-lipoxygenase 3-1	-2.75	-3.43	-2.81	-2.55
Fatty acid hydroperoxide lyase	-2.70	-2.73	-2.63	-2.17
Gamma-tocopherol methyltransferase	-5.09	-6.45	-6.28	-7.89
Probable linoleate 9S-lipoxygenase 5	-1.82	-2.87	-1.92	-3.02
Phosphoinositide-specific phospholipase C	-3.40	-3.35	-3.97	-4.80
Omega-3 fatty acid desaturase	-4.40	-6.62	-4.84	-3.72
3-ketoacyl-CoA synthase	-9.09	-8.15	-8.22	-9.48

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.11: Fatty acid metabolism-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P*<0.01)			
	M. grisea		*A. alternata*	
	6hpi	24hpi	6hpi	24hpi
Flavonol synthase	7.54	7.88	7.63	8.46
Flavanone 3 beta-hydroxylase	4.39	4.16	4.38	4.95
Flavonoid 3',5'-hydroxylase	4.17	4.26	4.33	4.92
Caffeoyl-CoAO-methyl transferase	2.77	2.94	3.23	3.03
Chalcone isomerase	1.41	1.54	1.60	1.78
N-hydroxycinnamoyl-CoA:tyramineN-hydroxycinnamoyl transferase THT1-3	3.93	3.76	4.80	4.28
N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase THT7-1	2.20	1.79	2.81	2.53
N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase THT7-8	1.67	1.72	2.48	3.32
Flavonoid3-glucosyl transferase	-8.73	-5.51	-6.21	-4.00
Chalcone synthase 2	-6.27	-5.86	-6.36	-6.33
Tropinone reductase II	-4.36	-2.66	-5.30	-2.68
Phytoene synthase	-4.59	-4.90	-4.55	-5.13
PAL 1	-4.04	-6.01	-4.37	-6.46
Lycopene beta-cyclase	-3.67	-3.89	-3.99	-4.47
Tropinone reductase I	-3.60	-3.96	-3.48	-5.40
Monoterpene synthase 1	-3.30	-2.35	-2.88	-3.81
Carotenoidcleavage oxygenase	-3.60	-3.72	-3.92	-2.98
Cinnamoyl CoA reductase 2	-3.02	-3.20	-2.80	-3.46
1-deoxy-D-xylulose-5-phosphate reductoisomerase activity	-2.59	-2.77	-2.18	-2.48
Putative tropinone reductase	-2.23	-2.24	-1.65	-4.48
HMG-CoA reductase	-2.46	-2.73	-3.35	-3.33
Lycopene epsilon-cyclase	-1.26	-4.37	-1.62	-5.30
Chalcone synthase	-2.22	-3.75	-2.48	-4.55
9-cis-epoxy-carotenoid dioxygenase 1	-3.83	-3.40	-3.97	-3.19
Cycloartenol synthase	-1.00	-3.27	-1.69	-4.08
Isoflavone reductase homolog	-1.79	-3.64	-1.71	-4.85

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.12: Secondary metabolism-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

Annotations	Fold change (*P*<0.01)			
	M. grisea		***A. alternata***	
	6hpi	**24hpi**	**6hpi**	**24hpi**
Aromatic amino acid decarboxylase 1B	5.02	3.72	5.93	4.09
Aromatic amino acid decarboxylase 1A	5.77	4.89	6.50	5.43
Aromatic amino acid decarboxylase 2	4.97	5.03	5.85	5.82
Asparagine synthetase	3.49	7.42	4.21	9.45
γ-aminobutyrate transaminase subunit precursor isozyme 1	3.19	3.13	3.76	4.17
Arginase 2	2.32	3.00	2.65	5.63
Glutamate--tRNA ligase-like protein	2.79	3.95	2.79	3.77
Glutamine synthetase	-3.41	-6.37	-3.24	-6.23
Aminomethyltransferase	-2.52	-5.08	-2.63	-4.41
Glutamate decarboxylase isoform3	-2.91	-5.73	-6.24	-9.59
Arginine decarboxylase	-2.17	-2.39	-1.64	-2.10
Glycine dehydrogenase	-2.64	-4.17	-3.02	-4.43
Polyprotein, putative	-1.78	-3.66	-1.91	-3.87

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and pathogen (*M. grisea* and *A. alternata* f. sp. *lycopersici)* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.13: Amino acid metabolism-related transcripts that are differentially regulated during compatible and non-host interactions compared to mock-inoculated tomato leaves.

M. grisea	
Annotation	**Fold change**
	6 hpi
Branched chain alpha-keto acid dehydrogenase E1-alpha subunit	-2.48
Dehydrin-like protein	-2.02
Alternative oxidase 1a	-1.4
ABA 8'-hydroxylase CYPA3 variant 1	-1.60
NAD(P)H-quinone oxidoreductase chain H	-1.40
Ripening regulated protein DDTFR19	-1.47
	24 hpi
WRKY-type DNA binding protein	2.49
Succinic semialdehyde dehydrogenase	1.21
S-adenosylmethioninedecarboxylase	1.36
Mitochondrial import receptor subunit TOM20	1.26
Photosystem II 10 kDa polypeptide	1.33
Cytochrome c oxidase subunit 5C	1.29
Copper chaperone	1.44
Cysteine protease inhibitor 1 (P340)	1.52
CC-NBS-LRR protein	-2.14
Root border cell-specific protein-like protein	-1.83
Knox-like protein	-1.48
Putative beta-subunit of K+ channels	-1.47
6-phosphofructokinase	-1.78
3-isopropylmalate dehydrogenase	-2.82
Phosphoglycerate kinase 4	-1.48
Peripheral-type benzodiazepine receptor	-1.70
DS2 protein response to stress	-1.45
Starch phosphorylase H	-1.85

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and *M. grisea* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.14: Transcripts that are differentially regulated only in non-host interactions of tomato.

A. alternata	
Annotation	**Fold range**
	24 hpi
Peroxisomal acyl-CoA oxidase 1A	2.13
Proline transporter 2	1.60
Putative glycosyltransferase	1.62
Galactinol synthase	1.49
Putative disease resistance protein	-3.40
TSW12 protein	-2.72
Auxin response factor 4	-2.58
WRKY-type DNA binding protein	-2.38
Steroid 5 alpha reductase DET2	-1.59
Spliceosomal protein	-2.03
Wound-induced proteinase inhibitor II prepeptide	-1.87
Fruit-ripening protein-like	-1.83
Ubiquitin-conjugating protein-like	-1.79
Prf interactor 30137	-1.48

Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and *A. alternata* f. sp. *lycopersici* inoculated tomato leaves are given. hpi-hours post inoculation.

Table 3.15: Transcripts that are differentially regulated only in compatible interactions of tomato.

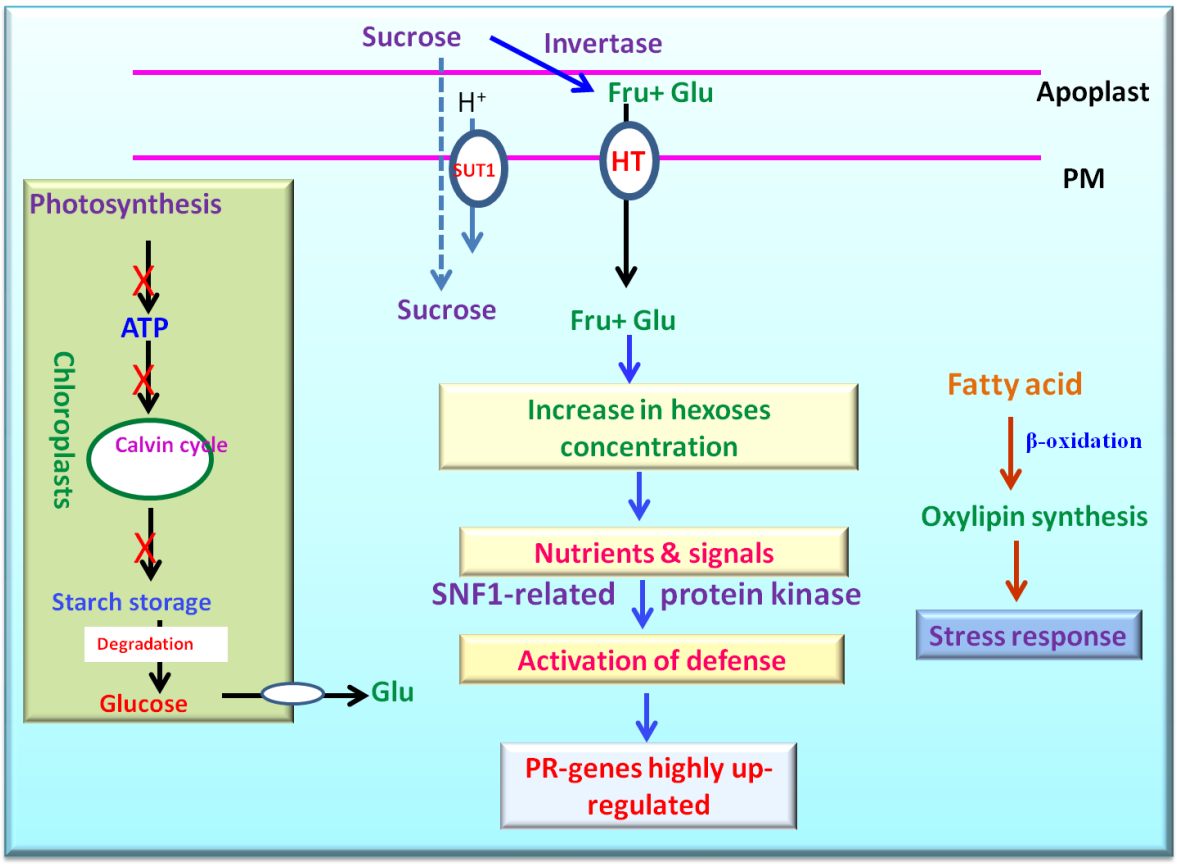


Fig 4.1: Overview of the seven metabolic pathways discussed in the text in detail.

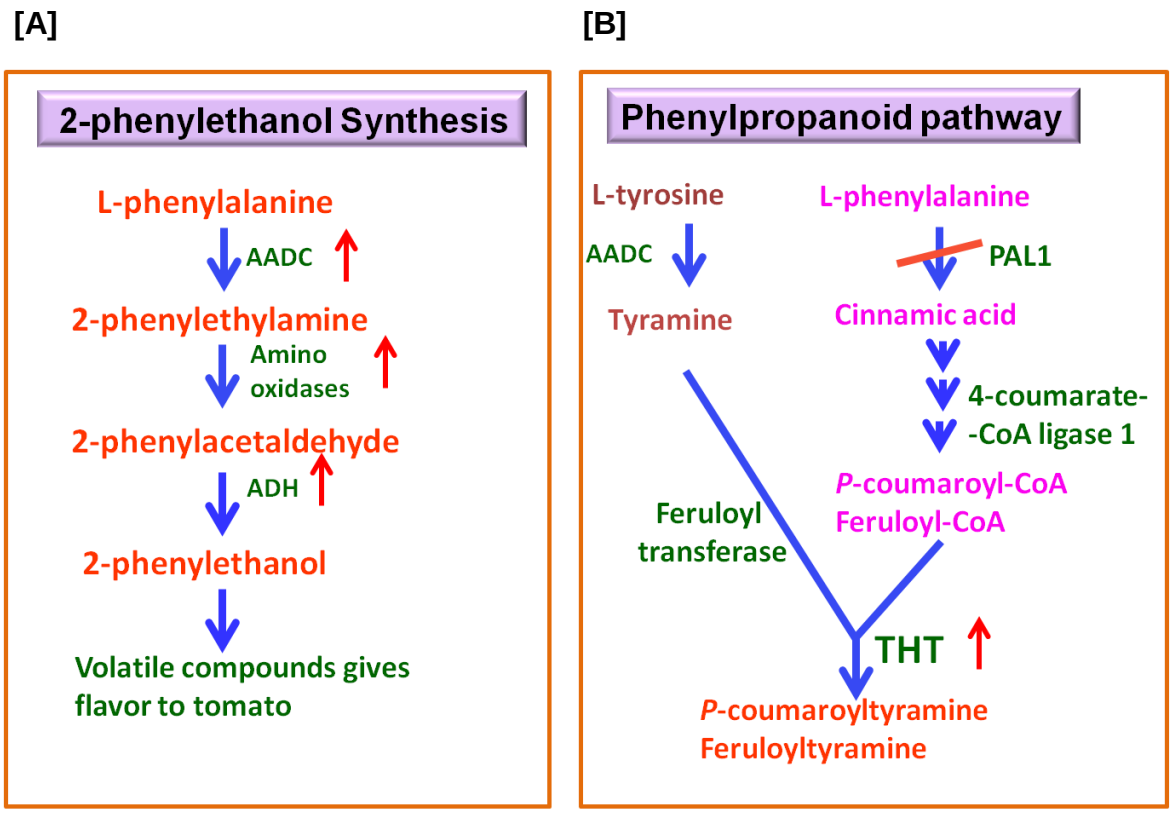


Fig 4.2: Schematic representation of changes in secondary metabolism

A) Pathway for production of the volatile compounds like 2-phenylethanol in plants. Phenylalanine is decarboxylated to phenethylamine by an aromatic amino acid decaeboxylase (AADC) enzyme. Phenylethylamine is converted to 2-phenylacetaldehyde by aminooxidasese followed by conversion to 2-phenylethanol by alcohol dehydrogenases.

B) Phenyl propanaoid pathway for production of antimicrobial compounds like *P*-coumaroyltyramine and feruloyltyramine. L-tyrasine is decarboxylated to tyramine by an AADC. N-hydroxycinnamoyl-CoA:tyramineN-hydroxycinnamoyl transferase (THT) catalyzes the synthesis of *P*-coumaroyltyramine from the *P*-coumaroyl-CoA and tyramnine. The enzymes PAL1 and 4-coumarate--CoA ligase 1 (4CL) of the phenylpropanoid pathway are involved in the synthesis of *p*-coumaroyl-CoA. Feruloyl transferase catalyzes the synthesis of feruloyltyramine from feruloyl-CoA and tyramine.