Archana Singh · Indrakant K. Singh
Editors

Molecular Aspects of Plant-Pathogen Interaction

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Preface

Pathogen attack has been one of the chief constrains that reduce crop productivity worldwide. Plants have established sophisticated mechanisms to counter and acclimatize over these invading pathogens at physiological, biochemical as well as molecular levels. Due to severe crop losses by pathogen outbreak, it is mandatory to completely understand the resistance/defense mechanisms against pathogen and develop advanced tactics to improve biotic stress tolerance in crop plants.

We present this book with an objective to realize the plant defense against different pathogens better and to document fundamentals as well as recent findings. This book has an amalgamation of basic information about disease resistance along with current insights into plant-pathogen interaction. The book has 15 chapters to disseminate the most updated information and detailed overviews on the present knowledge on molecular aspects of plant responses and adaptation to biotic stresses. This book is an essential reading for researchers and professionals in plant pathology, cell biology, molecular biology and genetics. This is highly recommended for the ones who are involved in plant disease resistance and crop improvement and to all plant scientists and undergraduates.

Depending on their modes of nutrition, phytopathogens have been categorized as necrotrophs, biotrophs and hemibiotrophs. These pathogens can be bacterial and fungal and cause various diseases in plants. In addition, viruses are another important class of pathogens and are causal agents for many common plant diseases. Plants counter to pathogens by activating a cascade of genes, encoding different receptors, signaling and protective molecules. During biotic stress, first of all effector molecules i.e. pathogen-associated molecular patterns (PAMPs) are perceived by plant recognition receptors (PRRs), after which PRRs interact with additional trans-membrane proteins that act as signaling adapters or amplifiers to achieve full functionality and PAMP triggered immunity (PTI). Defense response by receptor-like protein is a complex strategy, characterized by specific interaction between disease resistance (*R*) genes of plants and corresponding avirulence (*avr*) genes of pathogen that induce effector-triggered immunity (ETI) through hypersensitive response.

The NBS-LRR genes are important class of resistance gene families and their products recognize factors secreted by pathogens, which activates downstream signaling pathways leading to defense. Mitogen-activated protein kinases (MAPKs), which are cell-signaling enzymes that also show vital functions in transmitting extracellular signals to the nucleus during biotic stress. To achieve defense against

pathogen, transcription factors such as WRKY transcription factors bind to plant-specific *cis*-regulatory elements and activate gene expression thereby inducing transcriptional reprogramming and proteomic alterations to coordinate the perception and activation of pathways specific to the type of pathogen in question. Mainly phytohormones, small RNAs and other factors regulate this change at transcript level and protein level. Amongst all the targets, the induction and accumulation of pathogenesis-related (PR) proteins and biosynthesis of secondary metabolites are an integral component of innate immune responses in plants during pathogen attack.

Overall this volume will convey an overview of plant-pathogen interactions and it is a must read to understand this process for the genetic improvement of crops for disease resistance.

We are obliged to the authors of various chapters of this book for writing their chapters methodically and with great responsibility. We are extremely thankful to Dr. Rama, Principal, Hans Raj College, University of Delhi and Dr. Ajay Arora, Principal, Deshbandhu College, University of Delhi for providing overall support for our research and academic pursuits. We would also like to convey our gratitude to Dr. V. K. Kawatra, Mr. P. K. Singh and Dr. Vijay Rani Rajpal for always motivating us. We appreciate the beautiful ambiance created by our little angels Saumya and Kimaya, which allowed us to work tirelessly and gave us all emotional support. We are grateful to our parents for their constant support and blessings. Last but not the least, our sincere thanks to the handling editors and publisher.

We are optimistic that this book will be effective in broadcasting the latest knowledge about the plant-pathogen interaction.

New Delhi, India

Archana Singh
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Dr. Archana Singh is a Ph.D. in Plant Molecular Biology from the National Institute for Plant Genome Research (NIPGR). Since 2009, Dr. Archana has been working as an Assistant Professor in the Department of Botany, Hans Raj College, University of Delhi, where she teaches plant biotechnology, bioinformatics, genetics, cell biology and molecular biology. She has been awarded with Young Scientist Under Fast-Track Scheme (2013–2016) by Science and Engineering Research Board (SERB), Ministry of Science of Technology, Government of India. She has also been awarded with a prestigious Raman Post-Doctoral Fellowship by University Grant Commission, Ministry of Human Resources and Development, Government of India. Her quality research is evidenced by her publications in peer-reviewed journals of international repute like *Journal of Experimental Botany*, *Scientific Reports*, *Journal of Integrated Plant Biology*, *Journal of Asia-Pacific Entomology*, *Australasian Plant Pathology*, *Computational Biology and Chemistry* and *Plant Gene*. She has trained and supervised a few Junior Research Fellows and M.Sc. and B.Tech. students for their research projects. Over the years, she expanded her research interests from functional genomics of biotic stress to bioinformatics and proteomics.

Dr. Indrakant K. Singh is a Ph.D. in Zoology from the University of Delhi (DU). For last ten years, he has been working as an Assistant Professor in the Department of Zoology, Deshbandhu College, University of Delhi, where he teaches genetics, genomics, computation biology and immunology. He has been awarded with Young Scientist Award/Fellowship (2013–2016) by Science and Engineering Research Board (SERB), Ministry of Science of Technology, Government of India. He has also been awarded with a prestigious Raman Post-Doctoral Fellowship (2016–2017) by University Grant Commission, Ministry of Human Resources and Development, Government of India. He has published his quality research in peer-reviewed journals of international repute like *Journal of Experimental Botany*, *Scientific Reports*, *Journal of Biomedical Research*, *Journal of Asia-Pacific Entomology*, *Australasian Plant Pathology, Computational Biology and Chemistry* and *Plant Gene.* Presently, two Ph.D. scholars are working under his supervision. He has trained several B.Sc. and M.Sc. students for their research projects and now they are pursuing their doctoral research abroad. Over the years, he expanded his research interest from molecular basis of insect-plant interaction to functional genomics and proteomics.

Abbreviations

12-OPDA	12-oxophytodienoic acid
24-EpiBL	24-epibrassinolide
28-HomoBL	28-homobrassinolide
2D-DIGE	Two dimensional differential gel electrophoresis
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
5-HPT	5-hydroxytryptophan
AABL1	AB15 like1
ABA	Abscisic acid
ABC	ATP binding cassette
ACC	1-aminocyclopropane-1-carboxylic aid
ACMV	African cassava mosaic virus
ACS6	1-aminocyclopropane-1-carboxylic acid synthase
AGO	Argonaute
ALS	Amyotrophic lateral sclerosis
AMV	Alfaalfa mosaic virus
AP2	Apetala2
APAF	Apoptotic protease activating factor
APX	Ascorbate peroxidase
ARID	A/T rich interaction domain
as-1	Activation sequence 1
At	*Agrobacterium tumefaciens*
AtAGO1	*Arabidopsis* AGO1
AtEFR	*Arabidiopsis thaliana* EF-TU receptor
AtFSL2	*Arabidopsis thaliana* flagellin sensing 2
ATRM	*Arabidopsis* transcriptional regulatory map
ATX1	*Arabidopsis* homolog of trithorax
Aux	Auxin
Avr	Avirulence
BAK1	BRI1 associated receptor kinase 1
BAW	Beet armyworm
Bc	Botrytis cinerea
BECs	Blumeria effector candidates
BHNs	Broad host range necrotrophs
BIA	β-(Isoxazolin-5-on-2yl)-alanine

BIK1	BRI1 associated kinase
BIN2	Brassinosteroid insensitive 2
BL	Brassinolide
BMAA	β-methylamino-L-alanine
BPH	Brown plant hopper
BRI1	Brassinosteroid insensitive 1
BRs	Brassinosteroids
BWMK1	Blast and wounding activated map KINASE 1
bZIP	Basic domain leucine zipper
CaBD	Calmodulin binding domain
CaM	Calmodulin
CARD	Caspase recruitment domain
Cas	CRISPR associated
CAT	Catalase
CBB	Coomassie brilliant blue
CBD	Chitin binding domain
CC	Coiled coil
CD	C-terminal common docking
cDNA	Complementary DNA
CDPK	Calcium dependent protein kinase
CEBiP	Chitin elicitor binding protein
CED4	Cell death protein 4
CERK1	Chitin elicitor receptor kinase 1
CESA	Cellulose synthase cataylic subunit
CGs	Cyogenic glycosides
ChIP	Chromatin immunoprecipitation
CHS	Chalcone synthase
CKs	Cytokinins
CMV	Cucumber mosaic virus
CNR	Crinkler & necrosis
Col	Columbia
COR	Coronatine
COX5	Cytochrome oxidase subunit v
CP	Coat protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CSEPs	Candidate secreted effector protein
CT	P-coumaroyl tyramine
CWDE	Cell wall degrading enzymes
D	Aspartate
DAMPs	Damage associated molecular patterns
DCL	DICER-like
DGE	Differential gene expression
DHAR	Dehydroascorbate reductase
DMT	DNA methyl transferase
DORN1	Does not respond to nucleotide 1

DRBs	Double stranded RNA binding proteins
DRE	Dehydration responsive element
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded Ribonucleic acid
DTI	Danger triggered immunity
eATP	Extracellular ATP
EBL	Epibrassinolide
ECC	*Erwinia carotovora*
ECM	Extra cellular matrix
ECS	Endocytosis cell signaling
EDS 1	Enhanced disease susceptibility 1
EF	Elongation factor
EFR	EF-Tu receptor
EF-Tu	Elongation factor Tu
EHC	Encasement of the haustorial complex
EHM	Extra haustorial membrane
EIX	Ethylene inducing xylanase
ELISA	Enzyme-linked immunosorbent assay
eLRR	Extra cytoplasmic leucine rich repeat
EPD	Eukaryotic promoter domain
EPSs	Extracellular polysaccharides
ER	Endoplasmic reticulum
ERE	Ethylene responsive elements
EREBP	Ethylene responsive element binding protein
ERF	Ethylene response factor
ESI	Electrospray ionization
ESTs	Expressed sequence tags
ET	Ethylene
ETI	Effector triggered immunity
ETR2	Ethylene resistant 2
ETS	Effector-triggered susceptibility
FISH	Fluorescence in-situ hybridization
FLAK	Phenylalanine, leucine, alanine, lysine
Flg	Flagellin
FLS 2	Flagellin sensing 2
FMs	Functional markers
FT	Feruloyl tyramine
GA	Gibberellic acids
GC-MS	Gas chromatography mass spectrometry
GE	Glucan elicitor
GEBP	GE-binding protein
GM	Genetically modified
GNA	*Galanthus nivalis* agglutinin
GR	Glutathine reductase
GST	Glutathione s-transferase

HCN	Hydrogen cynide
HCRSV	Hibiscus chlorotic ringspot carmovirus
hc-siRNAs	Hetrochromatic small interfering RNAs
HDA19	Histone deacetylase 19
HeMV	Hempane mosaic virus
HEN1	HUA ENHANCER
HGA	Homogalactouronam
HMGR2	3-hydroxy-3-methylglutaryl CoA reductase 2
HPLC	High performance liquid chromatography
HR	Hypersensitive response
hrc	HR and conserved
hrp	Hypersensitive reaction and pathogenicity
HSN	Host specific necrotroph
HSPs	Heat shock proteins
HSTs	Host specific toxins
HVMPK4	*Hordeum vulgare* signaling protein MAP kinase 4
HYL1	Hyponastic leaves 1
IAA26	Indole-3-acetic acid transcription factor
ICAT	Isotope coded affinity tag
IEF	Isoelectric focusing
IP-ELISA	Immune virus particle-ELISA
IPG	Immobilized pH gradient
IPP	Isopentenyl diphosphate
IPT	Isopentyl transferase
ISR	Induced systemic resistance
ITRAQ	Isobarric tagged for relative and absolute quantitation
JA	Jasmonic acid
JARE	Jasmonic acid responsive element
JS	Justamembrane
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
L-DOPA	L-3,4-dihydoxyphenylalanine
LEA	Late embrogenesis abundant
LecRK	Lectin receptor kinase
Ler	Landsberg erecta
LGD1	Lagging growth development 1
LIR	Localised induced responses
LOS	Lipo-oligosaccharide
LPS	Lipopolysachharide
LRK	LRR kinase
LRR	Leucine rich repeat
lsiRNAs	Long small interfering RNAs
LTP	Lipid transfer protein
LYK3	LysM receptor like kinase 3
LZNBS-LRR	Leucine zipper nucleotide binding site leucine rich repeat

m/z	Mass to charge ratio
MALDI	Matrix assisted laser desorption/ionisation
MAMPs	Microbe associated molecular patterns
MAPK	Mitogen activated protein kinase
MCPs	Methyl accepting chemotaxis proteins
MDP	Muramyl dipeptide
MeJA	Methyl jasmonate
MIMPs	Microbe induced molecular patterns
miRNA	MicroRNA
MKK	MAP kinase kinase
MKS	MPK4 substrate
MLA 10	Mildew resistance locus A10
MoAGOs	M. oryzae genome encoded AGOs
MoDCL1	M. oryzae genome encoded DCL1
MoDCL2	M. oryzae genome encoded DCL2
MPs	Movement proteins
MPSS	Massively parallel signature sequencing
MS	Mass spectrometry
MSUD	Meiotic silencing of unpaired DNA
MTA	5′-methylthyoadenosine
MTI	MAMP triggered immunity
MTI	Microbial associated molecular pattern(MAMP) triggered immunity
MudPIT	Multidimensional protein identification technology
NAG	N-acetylglucosamine
NAM	N-acetyl muramic
NAMP	Nematode associated molecular pattern
NASBA	Nucleic acid sequence based amplification
nat-siRNAs	Natural antisense transcript-derived small interfering RNAs
NBS	Nucleotide binding site
NDPR	Non-pathogen derived resistance
NDR 1	Non race specific disease resistance 1
NEP1	Necrosis and ethylene inducing protein 1
NF	Nodulation factor
NFP	NOD factor perception
NGS	Next generation sequencing
NLPs	Necrosis and ethylene inducing peptide 1 like proteins
NLPs	Nep L like proteins
NLS	Nuclear localization signal
NO	Nitric oxide
NOD	Nucleotide binding oligomerization domain
NPAA	Nonprotein amino acids
NRG1	N-requirement gene
NtMKP1	Tobacco MAP kinase phosphatase 1
nTNL	Non TIR-NBS-LRR
O_2	Oxygen

OBF	Octopine synthase element binding factor
Obpv	Obuda pepper virus
OG	Oligogalacturonides
ORCAs	Octadecanoid responsive catharanthus APETALA2 domain proteins
Osa	*Oryza sativa*
osNramp6	osa-miRNA negative regulation of natural resistance associated macrophage protein 6
PAB	Plant associated bacteria
PAD3	Phytoalexin deficient 3
PAL	Phenylalanine ammonia lyase
PAMPs	Pathogen associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PD	Plasmodesmata
PDR	Pathogen derived resistance
Pel	Pectate lyase
PEN	Penetration genes
PepMV	Pepino mosaic virus
PEST	Pro-Glu-Ser-Thr
PG	Polygalactouronases
PGIP	Polygalactouronase inhibiting protein
PGN	Peptidoglycan
pI	Isoelectric point
PKs	Protein kinases
PLRV	Potato leafroll virus
PMEs	Pectin methyl esterases
PNPs	Plant natriuretic peptides
Pop	*Pseudomonas* outer protein
PPO	Poly phenol oxidase
PPP	Pentose phosphate pathway
PPV	Plum pox potyvirus
PR	Pathogenesis related
PR1	Pathogenesis related elements
pre-miRNA	Precursor miRNA
pri-miRNA	Primary-miRNA
PRLs	PR like proteins
PRRs	Pathogen recognition receptors
PRRs	Pattern recognition receptors
PRSV	Papaya ring spot virus
PS I	Photosystem I
PS II	Photosystem II
PSE1	Penetration specific effector 1
PstDC3000	Pathovar tomato strain DC3000
PTA-ELISA	Plate trapped antigen-ELISA
PTGS	Post transcriptional gene silencing

PTGS	Post translational gene silencing
PTI	PAMP triggered immunity
PTMs	Post translational modification
PVX	Potato virus X
PVY	Potato virus Y
PX	Peroxiredoxins
QAs	Quinolizidine alkaloids
qPCR	Real time quantitative PCR
QS	Quorum sensing
QTL	Quantitative trait loci
R	Resistance
RALF	Rapid alkalisation factor
ra-siRNAs	Repeat associated small interfering RNAs
RdDM	RNA-directedDNAmethylation
RDR	RNA dependent RNA polymerase
RDV	Rice dwarf virus
REL	Reticuloendotheliosis
REn	Replication enhancer
RenSeq	R gene enrichment and sequencing
RGC2	Resistance gene candidate 2
RIP	Repeat induced point
RISC	RNA induced silencing complex
RLCKs	Receptor like cytoplasmic kinases
RLK	Receptor like kinase
RLP	Receptor like protein
RMs	Random markers
RNAi	RNA interference
RNS	Reactive nitrogen species
ROMT	Resveratrol-o-methyltransferase
ROS	Reactive oxygen species
RP	Reverse phase
RPA	Reverse phase protein microarray
RPW8	Resistance to powdery mildew 8
Rsp1	Repetitive secreted protein 1
RSS	RNA silencing suppressors
RTD	Read through domain
RTP	Read through protein
RT-PCR	Reverse transcriptase-PCR
RUBISCO	Ribulose-1,5-biphosphate carboxylase oxygenase
RYMV	Rice yellow mottle virus
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SAM	S-adenosyl methionine
SAR	Systemic acquired resistance
SARE	Salicylic acid responsive elements

SARE	SA-responsive element
SCF	SKP1-cullin-f-box protein
SCX	Strong cation exchange
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SE	Sieve element
See1	Seedling efficient effector 1
SEL	Size exclusion limit
SERK3	Somatic embryo receptor kinase 3
SIB1	Sigma factor binding protein 1
SIPK	Salicylic acid protein kinase
SIS	Sex induced silencing
SMV	Soybean mosaic virus
SNARE	Soluble N-Ethylmaleimide-sensitive factor attachment protein receptor
SNPs	Single-nucleotide polymorphisms
SOD	Superoxide dismutase
sRNAs	Small RNAs
ssDNA	Single stranded deoxyribonucleic acid
SSEM	Serologically specific electron microscopy
SSH	Suppression subtractive hybridization
SSITL	*Sclerotinia sclerotiorum* integrin like
SSPs	Small secretory proteins
SSR	Simple sequence tags
ssRNA	Single stranded ribonucleic acid
SSRs	Simple sequence repeats
STAND	Signal transduction ATPases with numerous domains
STB	Septoria tritici blotch
STS	Stilbone synthase gene
SYMRK	Symbiosis receptor kinase
ta-siRNAs	Trans-acting small interfering RNAs
TBP	TATA-box -binding protein
TCNL	TIR-CC-NBS-LRR
TCV	Turnip crinkle virus
TFs	Transcription factors
TGB	Triple gene block
TGS	Transcriptional gene silencing
THI2.1	Thionin 2.1
TIPK	Trichoderma induced MAPK
TIR	Toll interleukin 1 receptor
TLR 4	Toll like receptor 4
TMV	Tobacco mosaic virus
TNL	TIR-NBS-LRR
TOF-MS	Time of flight mass spectrometry
TrD	Transmembrane domain
tRNA	Transfer RNA

TSWV	Tomato spotted wilt virus
TTSS	Type three secretion system
TuMP	Turnip mosaic virus
TVMV	Tobacco vein molting virus
TYLCD	Tomato yellow leaf curl disease
VIP1	VirE2 interacting protein 1
VOCs	Volatile organic compounds
VRC	Viral replication complex
vRNP	Viral ribonucleoprotein complex
W	Tryptophan
WAK1	Wall associated kinase 1
WIPK	Wound induced protein kinase
Xop	Xanthomonas outer protein

1 *Arabidopsis thaliana* as a Model Organism to Study Plant-Pathogen Interactions

Shachi Agrawal

Abstract

Arabidopsis thaliana (*a crucifer*) provides a model system in every discipline of plant sciences including plant pathology with a varied array of molecular and genetic resources and biological information. Members of crucifer are widely distributed geographically and are well adapted to various plant pathogens such as fungi, bacteria, viruses, and nematodes. Besides small plant size, short life cycle, small genome size, availability of whole genome sequence, and easy genetic and mutational analysis, its response to the pathogen attack in a similar fashion as other higher plant species and an extensive collection of mutants available to determine defense pathway are the characteristics, which identify this plant as an indispensable research model in plant-pathogen interaction studies. This chapter mainly focuses on various existing model pathosystems of *Arabidopsis* with viral, bacterial, and fungal pathogens including an outlook on how this knowledge can be translated from *Arabidopsis*-pathogen model system to other crop plants. A general and brief overview of plant-pathogen interactions and how *A. thaliana* recognize and respond to pathogens is also portrayed.

Keywords

Effector molecules · Hypersensitive response (HR) · Plant defense · Plant defensin gene · PR proteins · Resistance genes · Signal molecules · Systemic acquired resistance (SAR)

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1.1 Introduction

When a pathogen attacks a plant, either a pathogen can proliferate and can cause development of disease or the plant can resist the pathogen by means of active or passive form of resistance. During resistance, plants recognize a race-specific avirulence determinant produced by the pathogen (Keen 1990; Scofield et al. 1996; Tang et al. 1996); defense mechanisms are activated leading to hypersensitive response (HR) (Matthews 1991). At the same time, expressions of pathogenesis-related (PR) proteins as well as plant defensins are induced due to gene-for-gene interactions and rapid localized cell death (Narasimhan et al. 2001; Asano et al. 2012). Signaling molecules, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and reactive oxygen species (ROS) are directly involved in plant defense against pathogens (Clarke et al. 2000; Kunkel and Brooks 2002; Hossain et al. 2007; Asano et al. 2012). There is rich information on plant-pathogen interaction on many species. Advanced molecular tools are also accessible that can be used to study the function and evolution of genes that are important for plant defense such as those that control responses to wide range of pathogens. However, studies in molecular plant pathology require large initial investments in molecular technologies. It is cost-effective since these investments are shared among multiple laboratories by means of publications, bioinformatic tools, and databases such as TAIR. Moreover, researchers gain in-depth biological understanding when they compare and match previous studies from a research community that shares the tools and resources of model organisms. Although, it is imperative to study individual plant-pathogen interactions at species level to gain better knowledge. But, at the same time, *A. thaliana* serves as a model system to answer many basic questions related to plant-pathogen interaction due to availability of complete genome sequence and having a small genome size together with the extensive collection of new mutants and germplasm as well as the presence of specialized transformation techniques, its rapid growth, can be handled easily in the laboratory conditions, mutagenesis can also be done easily and the possibility of using microarrays for gene expression analysis. *Arabidopsis* is susceptible to only a limited number of pathogens including viruses, bacteria, fungi, nematodes, and insect pests, and it responds to the pathogen attack in a similar fashion to those of other higher plant species.

A. thaliana (L.) Heynh. is an annual flowering plant that belongs to mustard family (Cruciferae or Brassicaceae). It is a native of Eurasia, which has a broad natural distribution throughout Europe, Asia, and North America. Of late it has been introduced and naturalized worldwide. It is speculated that its spread was facilitated by the expansion of agriculture (Francois et al. 2008). *A. thaliana* is considered as a weed as it grows in open or recently disturbed habitats. *Arabidopsis* shows extensive natural variation for different developmental, abiotic, and biotic stress resistance traits (Koornneef et al. 2004; Alonso-Blanco et al. 2009; Atwell et al. 2010). Till date, over 750 different ecotypes (accessions) of *A. thaliana* have been collected from natural populations that are available for experimental analyses. The most commonly used ecotypes of *Arabidopsis* for genetic and molecular studies are Columbia (Col) and Landsberg erecta (L*er*). The entire life cycle of *A. thaliana* is completed in 6 weeks, which includes seed germination, rosette formation, bolting,

Fig. 1.1 An *Arabidopsis* plant grown under laboratory condition

flowering, and maturation of seeds. The plant is a small-sized herb with overall length of around 15–20 cm; leaves are 1.5–5 cm long and 2–10 mm broad. Flowers (2 mm length and 3 mm diameter) undergo self-pollination but can be crossed manually. The fruit is called a silique (5–20 mm long) that contains 20–30 seeds. On germination, the seed develops into a rosette plant (2–10 cm diameter), wherein the whorls of leaves are covered with trichomes (Fig. 1.1). Under laboratory conditions *Arabidopsis* can be grown easily in petri plates, pots, or hydroponics, either under fluorescent lights or in a greenhouse. Inflorescence is a corymb that appears as a result of bolting after 3 weeks of planting. Several hundred siliques are produced per plant, which account for more than 5000 total seeds. The plant has a single primary root that grows vertically downward and produces smaller lateral roots that are easy to study in culture.

1.2 Plant-Pathogen Interactions

An array of pathogens including fungi, bacteria, and viruses attack the plant kingdom. Different strategies have been devised by different pathogens to invade, feed on, and reproduce in the host plants. Pathogens can be broadly classified as biotrophs and necrotrophs based upon the strategy used by them to invade and infect a plant (Oliver and Ipcho 2004). Biotrophic pathogens are those that require a living host tissue for its growth and reproduction. In some cases wherein the tissue dies in the later stages of the infection, the pathogens are classified as hemibiotrophs. On the

contrary, necrotrophic pathogens kill the host tissue as soon as they infect it and then grow and feed on the dead tissue. Viruses are classified as biotrophic pathogens, whereas bacteria and fungi follow both biotrophic as well as necrotrophic strategies of invasion. Plants respond to different kinds of pathogens differently. Pathogens can further be classified as those with different primary target tissues encountering different environmental conditions. Those pathogens that target the green, photosynthesizing, and assimilate-producing source tissues like leaves will encounter different kinds of defense responses in comparison to pathogens infecting the assimilate-importing tissue such as roots, flowers, and sink leaves (Berger et al. 2007).

Plant defense mechanism against pathogens can be either preformed (primary) or induced (secondary). The first and foremost step required for the activation of defense response is to recognize the presence of microorganisms. Elicitors are molecules that at very low concentrations induce plant defense response (Thakur and Sohal 2013). Recognition of microorganism-derived elicitors initiates the basal resistance in plants. This defense response involves activation of ion fluxes, phosphorylation/dephosphorylation of proteins by protein kinases and phosphatases, and production of signaling molecules such as adapter proteins, salicylic acid, jasmonic acid, ethylene, reactive oxygen species, and nitric oxide. These steps further initiate an array of signaling that leads to the regulation of expression of defense-related genes and the induction of defense responses. These responses include cell wall strengthening, accumulation of phytoalexins and pathogenesis-related (PR) proteins, and localized programmed cell death (PCD) (McDowell and Dangl 2000; Dangl and Jones 2001; Garcia-Brugger et al. 2006).

Plants also possess an innate immune system that perceives the presence of pathogens by recognition of molecules known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively) or by sensing effector proteins secreted by the host during plant-pathogen interactions. Early interactions between PAMPs/MAMPs and cell surface receptors (pathogen recognition receptors or PRRs) lead to appropriate defenses by activating multicomponent and multilayered responses. The establishment of defense is triggered by several pathways that can involve Ca^{2+} influx, generation of reactive oxygen and nitrogen species (ROS and RNS, respectively), and synthesis of phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), which act as signal molecules (Pieterse et al. 2009). Plant immunity may be described at two levels (Jones and Dangl 2006). The first one involves cell surface pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) and initiate PAMP-triggered immunity (PTI). The second involves nucleotide-binding leucine-rich repeat (NB-LRR) proteins, encoded by resistance (*R*) genes, which sense pathogen effectors and elicit a potent immune response called effector-triggered immunity (ETI). ETI is faster, longer, and stronger than PTI and usually leads to a local cell death, the hypersensitive response (HR), which stops the spread of the pathogen (Jones and Dangl 2006). In some cases, pathogens can evade such recognitions also and suppress host immunity with effectors, causing effector-triggered susceptibility

(ETS). R proteins recognize some effectors that enable the pathogen to overcome PTI, and the effectors are thus termed an avirulence (Avr) protein (Jones and Dangl 2006).

As per our current understanding, virulence of the virulent pathogenesis is contributed by the production of effector molecules which thereby suppress plant defense, and thus the compatible interactions allow the spread of the pathogen in the susceptible plant (Jones and Dangl 2006). Herein the pathogen proliferates at a rate in which the plant defense could not keep pace with that subsequently leads to the development of disease and necrosis. On the other hand, in resistant plants, the specific resistance is governed by the recognition of the activity of pathogen effector molecules (race-specific avirulence determinant) by plant receptor proteins (Keen 1990; Scofield et al. 1996; Tang et al. 1996; Berger et al. 2007). Hence, these incompatible interactions prevent the pathogen from spreading and impart resistance to the plant. The disease resistance "R" genes encode the microbe recognizing plant receptors. Those pathogens that cannot establish themselves in the host plant are called as avirulent strains of plant pathogens, and their early recognition combined with fast activation of plant defense mechanisms results in the inducible defense system (Jones and Dangl 2006). Moreover, the recognition of the avirulent strain determinant activates a hypersensitive response (HR) that is characterized by localized PCD resulting in small necrotic lesions that efficiently restrict the spread of biotrophic pathogens (Heath 2000; Narasimhan et al. 2001). In addition, plant defensins (PDF1.1, PDF1.2) mRNAs are expressed in response to gene for gene interaction (Narasimhan et al. 2001). As discussed earlier, various signaling molecules like jasmonic acid, salicylic acid, ethylene, and reactive oxygen species (ROS) are directly involved in such inducible defense systems (Clarke et al. 2000; Kunkel and Brooks 2002; Hossain et al. 2007). The jasmonate/ethylene signaling pathway seems to be the most important mechanism in defending against necrotrophic pathogens. On the other hand, in order to combat against the biotrophic pathogens, plants recruit the salicylic acid-dependent responses (Thomma et al. 2001).

1.3 How *Arabidopsis thaliana* Recognize and Respond to Pathogens?

In nature, plants are exposed to a large number of pathogens, but somehow they are susceptible to only a few of them. This may be due to the presence of different defense mechanisms exhibited by the plants (Nimchuk et al. 2003; Jones and Takemoto 2004). The disease resistance (*R*) genes that are involved in pathogen recognition show excessive polymorphism. This polymorphism has been speculated as a cause for plant resistance. In monoculture, loss of R gene polymorphism results in reduced resistance and increased susceptibility (Stahl and Bishop 2000). *Arabidopsis* is prone to infection by pathogens that includes viruses, bacteria, fungi, nematodes, and insects. As the mode of response to the pathogen attack is highly

conserved in higher plant species, study of *Arabidopsis*-pathogen interactions have greatly helped the scientists to understand the molecular and cellular basis of host-pathogen interactions, disease resistance, and pathogen virulence (Andargie and Li 2016).

As stated earlier, R genes are important for parasite recognition and initiation of defense mechanism. A total of 150 different R genes have been identified in *Arabidopsis* genome that are located unevenly on chromosomes with 49, 2, 16, 28, and 55 R gene loci on chromosome number 1, 2, 3, 4, and 5, respectively (*Arabidopsis*-Genome-Initiative 2000). These R genes encode for proteins that contain nucleotide-binding (NB) domain(s) that binds to ATP or GTP along with a carboxy-terminal leucine-rich repeat (LRR) domain (S) that facilitate protein-protein interactions and ligand binding. They are further classified as those that contain toll interleukin 1 receptor domain (TIR) or coiled-coil (CC) domain at their amino terminal. Thus, broadly they can be classified as TIR-NB-LRR and CC-NB-LRR. *Arabidopsis* genome contains 85 TIR-NB-LRR resistance genes at 64 loci and 36 CC-NB-LRR resistance genes at 30 loci (*Arabidopsis*-Genome-Initiative 2000). Some of these R genes carry additional domains also, like WRKY transcription factor domain and protein kinase domain that have also been implicated in plant defense.

Studies were carried out to compare the defense mechanisms in plants and animals. Nitric oxide production seems to be a common response in both plants and mammals in conditions of biotic stress. But distinct homologue of nitric oxide synthase gene was not found in *Arabidopsis*. REL (reticuloendotheliosis) domain transcription factors or similar proteins that are involved in innate immunity in both *Drosophila* and mammals or their homologs were not detected in *Arabidopsis thaliana*. Moreover, no homologues were detected for genes like classical caspases, bcl2/ced9, and baculovirus p35 that are involved in apoptosis regulation in animal cells; however, eight homologues of metacaspase family protein and 36 cysteine proteases were found in *Arabidopsis* (*Arabidopsis*-Genome-Initiative 2000; Uren et al. 2000). The production of reactive oxygen intermediates is a primary response that is common to both plant and animal during pathogen recognition. This process involves transfer of electrons across the plasma membrane in mitochondria to make superoxide by a specialized respiratory burst oxidase. In mammals, gp91 is the subunit of NADH oxidase that catalyzes the final step of electron transfer to molecular oxygen (O_2), resulting in the generation of superoxide ion (O_2^-) (Yu et al. 1998). The *Arabidopsis* genome has eight functional homologues of *gp91*. These homologues are called *Atrboh* genes and have been implicated in plant defense response (Torres et al. 2002). In mammals, gp91 activity requires the action of Rac proteins, but no Rac or Ras proteins are found in *Arabidopsis*; however, a large family of rop genes that are related to G-proteins are present and may carry the same function. The various pathogens of *Arabidopsis thaliana*, gene associated with natural variation of response to those pathogens and their molecular functions, are summarized in Table 1.1.

Table 1.1 Various pathogens of *Arabidopsis thaliana* along with gene associated with natural variation of response to pathogen interactions and their molecular functions (Roux and Bergelson 2016)

Pathogens of *Arabidopsis*	Associated gene locus	Class of the associated gene
Viruses		
Turnip crinkle virus (TCV)	HRT	CC-NBS-LRR protein
Cucumber mosaic virus (CMV)	RCY1	CC-NBS-LRR protein
Tobacco ringspot virus (TRSV)	TTR1	TIR-NBS-LRR protein
Tobacco etch virus (TEV)	RTM1	Jacalin-like lectin protein
	RTM2	Small heat shock-like protein
	RTM3	MATH domain-containing protein
Plum pox virus (PPV)	RTM1	Jacalin-like lectin protein
	RTM2	Small heat shock-like protein
	RTM3, rwm1/rpv1	MATH domain-containing protein
		Nucleus-encoded chloroplast phosphoglycerate kinase
Lettuce mosaic virus (LMV)	RTM1	Jacalin-like lectin protein
	RTM2	Small heat shock-like protein
	RTM3	MATH domain-containing protein
Plantago asiatica mosaic virus (PAMV)	JAX1	Jacalin-like lectin protein
Watermelon mosaic virus (WMV)	rwm1/rpv1	Nucleus-encoded chloroplast phosphoglycerate kinase
Bacteria		
Pseudomonas syringae	RPM1/RPS3	CC-NBS-LRR protein
	RPS2	CC-NBS-LRR protein
	RPS5	CC-NBS-LRR protein
	RPS4	TIR-NBS-LRR protein
	RRS1	TIR-NBS-LRR WRKY protein
	ACD6	Ankyrin-repeat transmembrane protein
Xanthomonas campestris	RPS4	TIR-NBS-LRR protein
	RRS1	TIR-NBS-LRR WRKY protein
	RKS1	A typical kinase
	AT5G22540	Protein of unknown function
Ralstonia solanacearum	RPS4	TIR-NBS-LRR protein
	RRS1	TIR-NBS-LRR WRKY protein
	ERECTA	LRR receptor-like kinase
Fungi		
Elicitor from *Sclerotinia sclerotiorum*	RLP30	Receptor-like protein
Botrytis cinerea	RLP30	Receptor-like protein
	EGM1	Receptor-like kinase
	EGM2	Receptor-like kinase
	RLM3	TIR-NB protein

(continued)

Table 1.1 (continued)

Pathogens of *Arabidopsis*	Associated gene locus	Class of the associated gene
Fusarium oxysporum	RFO1	Wall-associated receptor-like kinase
	RFO2	Receptor-like protein
	RFO3	Receptor-like kinase
Alternaria brassicicola	RLM3	TIR-NB protein
Alternaria brassicae	RLM3	TIR-NB protein
Colletotrichum higginsianum	RPS4	TIR-NBS-LRR protein
	RRS1	TIR-NBS-LRR WRKY protein
Oomycetes		
Albugos candida	RAC1	TIR-NBS-LRR protein

1.4 *Arabidopsis thaliana*: An Important Model Host for Studying Plant-Pathogen Interactions

A. thaliana is an important model host for studying plant-pathogen interactions due to several reasons as described earlier. *Arabidopsis* is susceptible to only a limited number of pathogens including viruses, bacteria, fungi, nematodes, and insect pests. Diseases resulting from these pathogens have been reported in the wild (Holub et al. 1994, 1995; Tsuji and Somerville 1992) suggesting both the pathogen and the host share an ecological niche, and when the appropriate environmental conditions are present, disease can occur. Diseases have also been observed in a laboratory setting where the host is deliberately exposed to the pathogen. Regardless of the setting, nature, or the laboratory, *Arabidopsis* responds to the pathogen attack in a similar fashion as other higher plant species when exposed to viral, prokaryotic, or eukaryotic pathogens (Andargie and Li 2016). Since the 1990s till today, several plants have been recognized as model systems for plant-pathogen interactions such as tobacco, tomato, etc., but *A. thaliana* has been used extensively as a model plant to have an overview of the plant-pathogen interactions with a wide variety of pathogens. The *A. thaliana* genetic system is significantly more tractable than those of the other plant species, which were hampered by long generation times and large, polyploid, or repetitive genomes. Agriculturally important crucifers such as *Brassica napus*, *Brassica rapa* (oilseed rape, canola), *B. oleracea*, *Brassica* spp., European cabbage, cauliflower, Chinese cabbage, and radish (*Raphanus* spp.) are the closest relatives of *Arabidopsis*, so all the informations available can be useful for studying plant-pathogen interactions in these related spp. that are economically important crops. But molecular studies can be more complex in these spp. since they are mostly polyploids.

Besides, *A. thaliana* exhibits all of the major kinds of defense responses described in other plants. Furthermore, a large number of virulent and avirulent bacterial, fungal, and viral pathogens of *A. thaliana* have been deciphered (Glazebrook et al. 1997). Mutants defective in almost every aspect of plant growth and development have been identified and studied by the various research groups over the world. Novel insights into events subsequent to pathogen recognition in *A. thaliana* have

been obtained from mutants altered in defense (Buell 1998). Several mutant groups in *A. thaliana* exist today: lesion mimic mutants, phytoalexin mutants, as well as enhanced susceptibility and resistance mutants. With the variety of mutants available, it is possible to determine which defense pathways are activated during pathogen attack and what leads to the subsequent resistance or susceptibility. As research progresses, the different mutants will be linked to specific genes finally leading to a better understanding of the various genes involved in plant response pathways (Glazebrook et al. 1997).

1.5 *A. thaliana*-Pathogen Interactions

Arabidopsis has been reported as a susceptible host to a range of pathogens and resistant to other pathogens. The findings related to defense mechanism in *Arabidopsis* have been successfully implemented in many model systems, which have been developed to better understand interactions between plants and pathogens. The primary response of *Arabidopsis* includes the perception of pathogens by cell surface pattern recognition receptors (PRRs) and is referred to as PAMP-triggered immunity (PTI). Activation of FLS2 and EFR triggers MAPK signaling pathway that activates defense genes for synthesis of antimicrobial compounds. *Arabidopsis* possess specific intracellular surveillance proteins (R proteins) to monitor the presence of pathogen virulence proteins. This ETI occurs with localized programmed cell death to arrest pathogen growth, resulting in cultivar-specific disease resistance.

1.5.1 *Arabidopsis*-Virus Interactions

Viral infections and their spread throughout a plant require numerous interactions between the host and the virus. Systemic viral infections in plants are complex processes that require compatible virus-host interactions in multiple tissues. These interactions include viral genome replication in the cytoplasm of the initially infected cells, cell-to-cell movement toward neighboring tissues, long-distance movement through the vascular tissue, phloem unloading, and cell-to-cell movement in non-inoculated systemic tissues (Carrington et al. 1996). Incompatibilities between virus and host factors at any of these stages could therefore lead to restrictions and delay establishment of a systemic infection. The utility of *Arabidopsis* as a model system has not gone unnoticed, and several viruses previously found to be pathogenic on crucifers have also been found to infect *Arabidopsis*. This model organism has proven to be useful to understand the relationship between the host plant and the virus replication and movement processes (Kunkel 1996; Yoshii et al. 1998). Susceptible interactions between plants and viruses can result in a variety of visible symptoms ranging from mild stunting to overall necrosis.

Although plant viruses are among the least genetically complex pathogens, they use a variety of strategies to suppress or bypass host defense and infect susceptible

hosts. In plants, these strategies may be an enhancement of infection by manipulating host resources, such as the formation of replication complexes (Hills et al. 1987), enlargement of the plasmodesmata size-exclusion limit (Wolf et al. 1989; Waigmann et al. 1994), evolution of viral suppressors of RNA silencing to counteract antiviral silencing (Burgyan and Havelda 2011), interference with plant cell cycle regulation (Lai et al. 2009), and using host components for its own replication. In turn, plants have evolved intricate mechanisms to fight viral infection, such as pathways mediated by gene silencing, hormone-mediated signaling pathways, and regulation of metabolism (Gao et al. 2016).

A number of viruses such as *turnip crinkle virus*, *cucumber mosaic virus*, *tobacco ringspot virus*, *tobacco etch virus*, *plum pox virus*, *lettuce mosaic virus*, *plantago asiatica mosaic virus*, and *watermelon mosaic virus* are reported to infect *A. thaliana*. In *Arabidopsis-turnip crinkle virus* pathosystems, *turnip crinkle virus* (TCV) is the smallest and simplest plant RNA virus belonging to *Tombusviridae* family *Carmoviruses*. It infects many *Arabidopsis* ecotypes (Dempsey et al. 1993). The ecotype Dijon (Di-O) of *A. thaliana* has been reported to be resistant to TCV-M infection. This resistance is not expressed at the cellular level but rather at the level of whole plant. In *Arabidopsis*, resistance to most viral pathogens does not involve an HR (Ishikawa et al. 1991; Leisner et al. 1993; Lee et al. 1994; Callaway et al. 1996). However, inoculation of turnip crinkle virus (TCV) on plants from the resistant ecotype Dijon (Di-0 or Di-17) shows both an HR and the induction of PR gene expression (Simon et al. 1992; Dempsey et al. 1993, 1997; Uknes et al. 1993). Furthermore, it was found that the HR and resistance are dependent on SA but independent of NPR1, ethylene, and JA-mediated defense signaling (Kachroo et al. 2000). Genetic analyses revealed that HR development is conferred by a single dominant gene termed HRT (for HR to TCV) (Dempsey et al. 1997). HRT gene has been reported to trigger defense gene expression and SA accumulation (Cooley et al. 2000; Ren et al. 2000; Jeong et al. 2008). Thus, HRT also appears to be required for resistance to TCV infection along with a second locus, named RRT (which regulates resistance to TCV). In contrast, TCV-susceptible ecotypes, including Columbia (Col-0), fail to mount an HR, exhibit delayed and weak PR gene expression, and develop systemic disease symptoms (Li and Simon 1990; Dempsey et al. 1993). TCV infection triggers the production of ROS as well as alteration of cellular redox in *Arabidopsis* (Pu et al. 2016).

1.5.2 *Arabidopsis*-Bacterium Interactions

Perhaps, bacterial pathogens are the more facile pathogens to work with in a laboratory setting as bacteria have several advantages over the other classes of pathogens for pathological studies. They can be cultured in vitro and have relatively rapid generation times (only minutes not even days). In addition, most bacterial plant pathogens elicit rapid host responses (hours to days) and have pathogenicity and avirulence factors that have been documented in other plant species. Thus, they provide a foundation to begin work in *Arabidopsis*. Only a small number of

bacterial species are pathogenic on *Arabidopsis*. The predominant bacterial pathogen utilized in *Arabidopsis* studies is *Pseudomonas syringae* on which many scientists have reported various important findings. Additional bacterial pathogens utilized in *Arabidopsis* research include *Erwinia* species (causal agents of soft rots), *Ralstonia* species (causal agents of vascular wilts), and *Xanthomonas campestris* pathovars (causal agents of blights and rots). A brief review of *Arabidopsis-Pseudomonas* pathosystems is presented in this chapter.

The interaction between *A. thaliana* and various phytopathogenic *Pseudomonas* pathovars presents an outstanding model to genetically define plant and bacterial loci necessary for generation of a hypersensitive response (HR) (Dangl et al. 1991). Certain isolates of *Pseudomonas syringae pv. maculicola* are virulent on *Arabidopsis*, while others are not. *Pseudomonas syringae* is a Gram-negative, rod-shaped bacterium with polar flagella (Agrios 1997). Different strains of *P. syringae*, however, are known for their diverse and host-specific interactions with plants (Hirano and Upper 2000). Understanding the molecular basis of this high level of host specificity has been a driving force in using *P. syringae* as a model for the study of host-pathogen interactions. In crop fields, infected seeds are often an important source of primary inoculum in *P. syringae* diseases, and epiphytic bacterial growth on leaf surfaces often precedes disease development (Hirano and Upper 2000). *P. syringae* enters the host tissues (usually leaves) through wounds or natural openings such as stomata, and in a susceptible plant, it multiplies to high population levels in intercellular spaces. Infected leaves show water-soaked patches, which eventually become necrotic. Depending on *P. syringae* strains, necrotic lesions may be surrounded by diffuse chlorosis. Some strains of *P. syringae* also cause cankers and galls (Agrios 1997). In resistant plants, on the other hand, *P. syringae* triggers the hypersensitive response (HR), a rapid, defense-associated death of plant cells in contact with the pathogen (Klement et al. 1964). In this situation, *P. syringae* fails to multiply to high population levels and causes no disease symptoms. Several strains belonging to pathovars *tomato*, *maculicola*, *pisi*, and *atropurpurea* of *Pseudomonas syringae* may infect the model plant *A. thaliana* (Crute et al. 1994). The establishment of the *Arabidopsis-P. syringae* pathosystem triggered a period of highly productive research that has contributed to the elucidation of the fascinating mechanisms underlying plant recognition of pathogens, signal transduction pathways controlling plant defense responses, host susceptibility, and pathogen virulence and avirulence determinants.

Upon infection with *P. syringae*, PTI and the subsequent SA-dependent and independent defenses are activated in *Arabidopsis* (Tsuda et al. 2008). About 36 protein effectors (Cunnac et al. 2009; Lindeberg et al. 2009) are secreted into the host cell by a remarkable system conserved in Gram-negative bacteria called the type III secretion system (TTSS). These effectors suppress both SA-independent and SA-mediated basal defenses (Nomura et al. 2005; Kim et al. 2008). Furthermore, *P. syringae* pathovars secrete the phytotoxin coronatine (COR) involved in bacterial virulence (Kloek et al. 2001). COR has been reported to target JA, ET, AUX, and ABA pathways, which play important roles to antagonize the SA-mediated defenses (Thilmony et al. 2006). COR also suppresses PTI and induces the reopening of

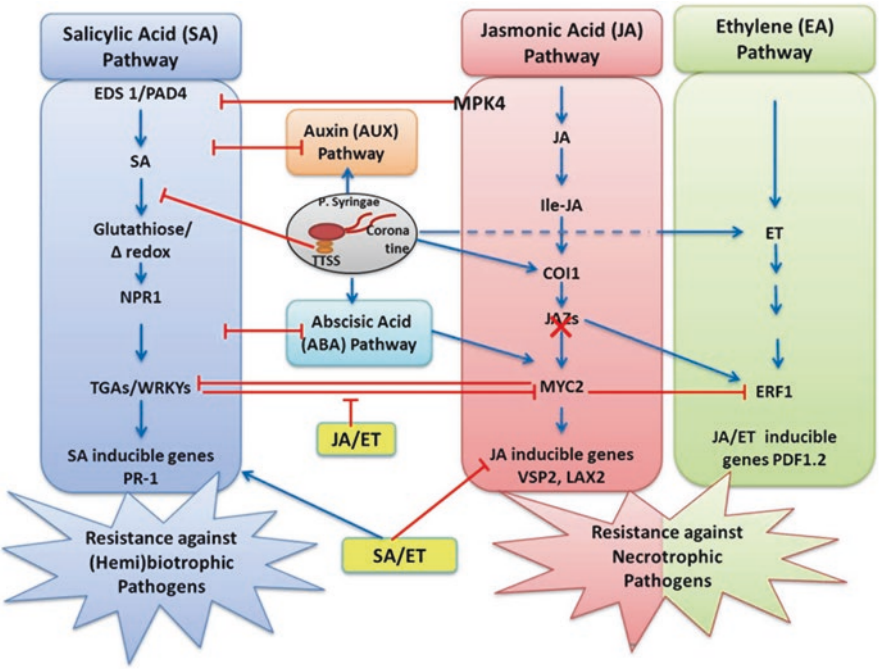

Fig. 1.2 Networking between the principal plant defense hormones during *Arabidopsis-Pseudomonas* interaction. The signaling cascades of the major plant defense pathways (salicylic acid (SA), jasmonic acid (JA), ethylene (ET), which are the backbone of defense signaling network) along with other hormonal signaling pathways feeding into it are shown here. The cross-communications between these signaling pathways that lead to protection against different pathogens may have synergistic (shown in blue color) as well as antagonistic (shown in red color) effects. In addition, the hemibiotrophic bacteria *Pseudomonas syringae* can suppress host defenses by manipulating plant hormone pathways through secretion of the phytotoxin coronatine and effectors delivered by the type III secretion system (TTSS) (Adapted from Pieterse et al. 2009)

stomata that have been closed upon recognition of the bacterial flagellin and lipopolysaccharides by *Arabidopsis* (Melotto et al. 2006). Both COR and TTSS effectors display common and distinct virulence effects in *Arabidopsis* such as targeting the JA pathway, thereby repressing the SA-inducible defenses (Zhao et al. 2003; He et al. 2004; Uppalapati et al. 2007). Thus, during *P. syringae* infections, TTSS effectors and COR plays important role in suppression of SA-inducible responses and promote full susceptibility (Laurie-Berry et al. 2006). Some TTSS effectors of *P. syringae* also interfere with the AUX (Navarro et al. 2006; Chen et al. 2007; Zhang et al. 2007) and the ABA pathways (de Torres-Zabala et al. 2007), both promoting the repression of SA-inducible defenses. These effectors interfere with the hormonal network of plant defenses, thereby leading to successful infection through the suppression of basal defenses including the SA pathway (Fig. 1.2).

Importantly, and in contrast to most fungal pathogens, *Pseudomonas syringae* is amenable to molecular genetic manipulations such as gene introduction, transposon

mutagenesis, and targeted gene replacement. Some *P. syringae* pv. tomato and pv. maculicola strains are virulent on some *Arabidopsis* ecotypes (Quirino and Bent 2003). Among its various strains, pathovar tomato strain DC3000 (PstDC3000) can usually infect the plant host *A. thaliana*. This *A. thaliana-Pseudomonas syringae* pv. tomato DC3000 (DC3000) pathosystem has been reported to be an ideal system to understand both microbial-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered susceptibility (ETS) processes at the transcriptional level (Xin and He 2013). DC3000 is highly virulent on *Arabidopsis* as it directly delivers 28 effector proteins (Cunnac et al. 2009) into the host cell through type III secretion system (TTSS) as well as small molecules such as coronatine (COR). These virulence factors collectively suppress MAMP-triggered immunity (MTI) and enhance nutrient availability, therefore allowing bacterial multiplication. A key structural component of the TTSS pilus is the HrpA protein. DC3000*hrpA*-mutants activate MTI but cannot form a TTSS to deliver the suite of effectors that can suppress MTI. They also produce minimal amounts of coronatine. Thus, DC3000*hrpA*-infection triggers MTI in the host (Lewis et al. 2015).

1.5.3 *Arabidopsis*-Fungus Interactions

A large number of fungal and oomycete pathogens have been reported to infect *A. thaliana*, either naturally or in the laboratory. These include *Ustilaginoidea*, *Botrytis*, *Fusarium*, *Colletotrichum*, obligate biotrophs (e.g., *Peronospora*, *Albugo*, and *Erysiphe*), hemibiotrophs (i.e., facultative biotrophs; e.g., *Phytophthora*), and necrotrophs (e.g., *Alternaria*, *Botrytis*, and *Rhizoctonia*). On infection, *Arabidopsis* respond to each pathogen in a specific manner. A brief review of *Arabidopsis-Ustilaginoidea* pathosystems is described here.

The biotrophic ascomycete fungus, *Ustilaginoidea virens* (Cooke) Tak (teleomorph: *Villosiclava virens*), causes one of the most severe false smut diseases in rice (*Oryza sativa*) (Ford et al. 1994; Talbot and Foster 2001). This pathogenic fungus has been reported to interact compatibly with the model plant *A. thaliana* also (Andargie and Li 2016). In rice, *U. virens* infection converts infected rice grains into smut balls, which results in sterility of the florets, whereas in *Arabidopsis* sterility of the flowers is evident without smut ball formation. A 39.4 Mb draft of *U. virens* genome that encodes 8426 predicted genes have been sequenced (Zhang et al. 2014). In addition to this, the pathogen has showed a decreased gene inventories for different metabolisms including nutrient uptake and polysaccharide degradation. This could arise possibly due to the adaptation of the pathogen to the specific floret infection and biotrophic lifestyles. *Once U. virens* colonize and attack the leaves, flowers, pods, and roots of a dicotyledonous plant, *A. thaliana*, different defense response proteins become induced in the infected plants as witnessed through gene expression analysis. *A. thaliana* has been reported to activate a hypersensitive response (HR) mechanism which precedes a slower systemic response that ultimately leads to systemic defense response. This mechanism reduces the damage and destruction caused by this biotrophic fungus. The progression of the fungal

hyphae on the surface of the leaves is through the attachment to the trichomes. Leaves, along with retention of fungal spores, also determine the survival, attachment, and penetration of the hyphae. The trichomes present on the leaf surface provide physical adhesion point for the hyphae in addition to retaining water on the plant surface and provide nutrients and protected environment for microbial growth (Lindow and Brandl 2003; Monier and Lindow 2003; Calo et al. 2006).

Basically both *Arabidopsis* and rice share similar defense response mechanisms. Chao et al. (2014) reported that during the first stage of *U. virens* infection in the infected rice spikelet, proteins that are involved in protein modification, protein degradation, and receptor phosphorylation become activated to a great extent. During this time, some receptor protein kinases can activate corresponding substrates to facilitate downstream signal transduction, and this is shown by MPK3 and MPK6, which phosphorylate WRKY33 to initiate phytoalexin biosynthesis in *Arabidopsis*. In addition, a protein kinase APK1B which is involved in stamen development and its repression can prevent pollen tube germination causing self-incompatibility in *Arabidopsis, is also involved in defense against U. virens causing rice spikelet infection*. The presence of protein kinase APK1B indicates that the pathogen manipulates host development signaling by prohibiting protein phosphorylation, hence allowing further infection of the plant with *U. virens* to occur.

Andargie and Li (2016) performed semiquantitative RT-PCR analysis with the RNA isolated from leaves, roots, flowers, and siliques of *Arabidopsis* plants following infection with the *U. virens* spores in order to test whether the pathogenesis-related genes or plant defensin genes were inducible by pathogen infection. Their findings indicate that *U. virens* isolates infect *Arabidopsis*, and the plant subsequently activates different defense response mechanisms, witnessed by the expression of pathogenesis-related genes, *PR-1*, *PR-2*, *PR-5*, *PDF1.1*, and *PDF1.2*. Thus *Arabidopsis* plants activate different defense strategies in order to limit the damage and destruction which are caused by this biotrophic fungus *U. virens* and may serve as a good model host species to study the interaction between infected plants and the rice false smut fungus *U. virens.*

Thus it is a novel pathosystem based on *U. virens* and *Arabidopsis as* rice pathogenic *U. virens* transformed colonies may infect and colonize endophytically on the different parts of the *Arabidopsis* plant. Since the processes that determine the outcome of an interaction between a microbial pathogen and a host plant are complex, understanding the molecular details of these interactions, like the pathogen genes required for infection, effective host defense responses, as well as mechanisms by which host and pathogen signaling networks are regulated, might be utilized to design new plant protection strategies. Generally, the established *A. thaliana-U. virens* pathosystem could be able to expand the model systems investigating fungi-plant interactions and will facilitate a full understanding of *U. virens* biology and pathology. It can advance our knowledge in order to describe the plant immune system. This pathosystem will now permit various follow-up molecular genetics and gene expression experiments to be performed to identify the defense signals and responses that restrict fungal hyphae colonization in plants and also provide initial evidence for tissue-adapted fungal infection strategies.

1.6 Conclusion

This chapter has focused on the well-suited model interactions of *A. thaliana with various plant pathogens (fungi, bacteria, and virus)*. Till now, various studies have been done pertaining to the interaction of *A. thaliana* with a large number of virulent and avirulent bacterial, fungal, and viral pathogens. This model species exhibits all of the major kinds of defense responses described in other plants. Regardless of the setting, nature, or the laboratory, *Arabidopsis* responds to the pathogen attack in a similar fashion as other higher plant species when exposed to pathogens. *A. thaliana* has been used extensively as a model plant not for any particular pathogen but to understand diverse range of plant-pathogen interactions with a wide variety of pathogens. The exhaustive analysis of *Arabidopsis* genes, whose expression is modulated during disease development, paves the way for dissecting plant networks activated by recognition of *pathogen* effectors in susceptible plants.

The lessons learned and information gathered from the model plant, *Arabidopsis*, can be applied to agronomically important crops. The study of this model plant interaction with various pathogens will contribute to our appreciation of how plants and pathogens have evolved to survive each other's attacks and counterattacks, which will in turn help us to develop sustained control measures by guided interception of pathogen virulence and/or by selective activation of plant defense. Thus it may address the key challenges of understanding how both plant defense and pathogen attacks are integrated and translating knowledge from *Arabidopsis* to crop plants. It would enable researchers to limit disease spread due to a better knowledge of the pathogen as well as provide them with a better understanding of the mechanisms involved in plant defense. Interfamily transfer of R genes from *Arabidopsis* can develop pathogen-resistant crops. It has been reported when RRS1/RPS4 pair of R genes from *Arabidopsis thaliana* was transferred to tomato, it caused resistance against *Ralstonia solanacearum*.

Glossary

Avirulence (Avr) protein Small proteins produced by pathogens and recognized by the host cell resistance proteins. These proteins trigger defense responses in plants. Avr proteins are often type III secretion system effectors, involved in pathogenicity.

Basal defense Plant defense that occurs early in the host-pathogen interaction in response to the perception by plant pattern recognition receptors of microbial-associated molecular patterns (MAMPs). Basal defense is MAMP-triggered immunity (MTI) plus weak effector-triggered immunity (ETI), minus effector-triggered susceptibility (ETS).

Biotroph A pathogen that colonizes living tissues for its growth and reproduction.

Effector molecules Pathogen-produced molecules that interfere with and suppress plant defense mechanisms, e.g., bacterial proteins, delivered by the bacterial type III secretion system (TTSS) to the plant cell interior.

Effector-triggered immunity (ETI) Immune responses triggered by recognition of specific pathogen effectors. The ETI response relies on R genes. Plant ETI often causes an apoptotic hypersensitive response.

Effector-triggered susceptibility (ETS) The state of a plant in which the plant's defense mechanism become suppressed by pathogen effector molecules.

Elicitor Any metabolite isolated from pathogens that at a very low concentration induces a hypersensitive response in host plants.

Hypersensitive response (HR) A complex defense response that is often associated with resistance (R) protein-mediated immunity. HR culminates in programmed cell death in cells in the vicinity of the pathogen, which may inhibit pathogen spreading.

MAMP-triggered immunity (MTI) Immunity raised after recognition of MAMPs by pattern recognition receptors (PRRs) localized on the surface of plant cells.

Microbial-associated molecular patterns (MAMPs) More recent term used for PAMPs. A series of essential and conserved molecular motifs of both pathogenic and nonpathogenic microbes that can be recognized by pattern recognition receptors in plants.

Necrotroph A pathogen that rapidly kills the host tissue and feeds on the dead tissue.

PAMP-triggered immunity (PTI) Immunity raised from the interaction of pattern recognition receptors (PRRs) in plant cells with elicitor molecules. It is a part of the first line of defense and results in a basal level of resistance.

Pathogen-associated molecular patterns (PAMPs) A set of molecular structures (epitopes) not shared with the host but shared by related pathogens, relatively invariant.

Pathogenesis-related (PR) genes Plant genes that activates after infection by pathogens.

Pathogenesis-related (PR) proteins Plant proteins that are synthesized in response to microbial attack and that serve to limit the growth pathogens. They are induced as a part of systemic acquired resistance.

Pattern recognition receptors (PRRs) Germ line-encoded proteins that can recognize microbe-associated molecular patterns and induce signaling cascade in innate immunity responses.

Plant defensin (PDF) Small, highly stable, cysteine-rich peptides that constitute a part of the innate immune system, mostly involved in defense against a broad range of fungal pathogens.

R genes and R proteins Plants have R genes (resistance genes) whose products mediate resistance to specific microbes, e.g., virus, bacteria, fungus, oomycete, nematode, etc. The product of R gene is R protein that allows recognition of specific pathogen effectors, either through direct binding or by recognition of the effector's alteration of a host protein.

Systemic acquired resistance (SAR) Inducible whole-body resistance. The development of a general immune capacity throughout the entire plant following an initial invasion by a pathogen.

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Fungal and Bacterial Biotrophy and Necrotrophy

2

Geeta and Reema Mishra

Abstract

Plant pathogens have been divided into two classes, namely, biotrophs and necrotrophs. These pathogens lead to significant economic losses by infecting various crops. Biotrophs complete their life cycle by using the living host cell machinery, while necrotrophs feed on the host cell after killing them. Hemibiotrophs, a third group, show both the forms for obtaining nutrition i.e., early biotrophic stage to later necrotrophic phase. After infecting the plants, both the groups of plant pathogens can trigger and suppress plant immune responses by synthesizing and secreting effector proteins. In case of biotrophic pathogens, effector proteins were found to be Avr proteins (identified by resistance proteins), *hrp* genes, and cell wall-degrading enzymes, while necrotrophic pathogen has additional effectors called as host-selective toxins. Significant differences have been observed between these two groups in the disease symptoms they cause, their host range, morphogenesis of the infection, production of secondary metabolites and hormones, and nature of plant resistance. Biotrophs possess a sophisticated way of infection, i.e., it enters the host cell using the haustoria, colonizes the intercellular space, and overpowers the host defenses. Necrotrophs have been further grouped into host-specific and broad host range necrotrophs depending on the toxins they secrete. In case of necrotrophic infections, host cell death has been shown to trigger production of hormones like ethylene, abscisic acid, salicylic acid, and jasmonic acid. Both bacterial and fungal plant pathogens belonging to the above mentioned category have been identified. In this chapter we are going to discuss the current state of knowledge about bacterial and fungal biotrophs and necrotrophs.

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2.1 Introduction

During the course of evolution, there has been an arms race between plant pathogens and their hosts or virulence and resistance. To date, many phytopathogens having varied infection strategies have been identified. These phytopathogens are broadly divided into two major categories on their modes of nutrition, namely, necrotrophs and biotrophs. These pathogens can be bacterial and fungal and cause various diseases in plants. Necrotrophic pathogens kill the host tissue rapidly and survive on the contents (Stone 2001), while biotrophic pathogens thrive on the nutrients of the living host. Biotrophs have a sophisticated or stealthy mechanism of invasion, and they develop specialized structures such as haustoria, appressoria, and hyphae for gaining entry into the host plant for absorbing nutrients and for blocking the immune responses of plants (Schulze-Lefert and Panstruga 2003; Mendgen and Hahn 2004). Biotroph sustains the host viability, causes comparatively little injury to the host, and suppresses hypersensitive response (HR; localized programmed cell death of host plant) as it restricts the nutrient supply. Necrotrophs are less sophisticated, rather are notorious and have various virulence strategies for killing and absorbing the nutrient from the host plant cells for their growth and reproduction. They macerate the tissues or can cause soft rots and stimulate HR-like host cell death. Based on the secretion of toxins, necrotrophs have been further divided into two subcategories, namely, host-specific necrotrophs (HSNs) and broad host range necrotrophs (BHNs) (Wolpert et al. 2002). As the name suggests, HSNs secrete host-specific toxins for the development of virulence. The fungal necrotrophs *Cochliobolus carbonum* and *Alternaria* spp. have been shown to secrete host-specific toxins for the pathogenicity (Walton 1996). The examples of necrotrophs belonging to BHN category include the bacterial pathogen *Erwinia carotovora* and fungal pathogens *Plectosphaerella cucumerina*, *Botrytis cinerea*, *Alternaria brassicicola*, and *Sclerotinia sclerotiorum* (Mengiste 2012). All these reports suggest the fact that there are significant differences between the two major classes of pathogens in terms of infection symptoms, host range, and effector proteins they secrete (Laluk and Mengiste 2010).

Another category of phytopathogens has been named as hemibiotrophs, which exhibit two phases of nutrient acquisition: an early biotrophic phase and later manifests necrotrophic phase. Hemibiotrophic plant pathogens include bacterium *Pseudomonas syringae*, fungi *Colletotrichum graminicola* and *Magnaporthe oryzae*, and oomycete *Phytophthora infestans* (Lee and Rose 2010). Irrespective of the category of pathogens, the basic events after the invasion include production of effector proteins by the pathogen for their colonization, while, in case of host plant, the defense mechanism is activated for limiting the spread of pathogen. In case of necrotrophic invasion, host cell death, and secretion of various secondary

metabolites and hormones named such as jasmonic acid, salicylic acid, abscisic acid, and ethylene, accumulation of reactive oxygen species and callose takes place (Mengiste 2012).

Plant fungal pathogens cause various diseases on economically important crops. They exhibit different infection strategies and develop specialized infection structures for deriving the nutrition. All the three modes of pathogenesis (necrotrophy, biotrophy, and hemibiotrophy) have been identified in fungi. Different fungi secrete different pathogen effector molecules during the infection process for their invasion and colonization. Pathogen effectors have been shown to be involved in pathogenicity, as silencing or disrupting pathogen effector coding genes showed reduced virulence (Stergiopoulos et al. 2013). Fungi also secrete certain toxins, cell wall-degrading enzymes, and toxic secondary metabolites to aid the infection process (Horbach et al. 2011). It is believed that infection strategies of biotrophic fungi are more complex when compared to necrotrophic ones.

Bacterial pathogens belonging to both the categories secrete virulence proteins, cell wall-degrading enzymes, toxins, and extracellular polysaccharides during the infection for their colonization, growth, and replication (Alfano and Collmer 1996). Bacteria possess a multi-protein secretory system for translocating the virulence factors to the host plant cell (Alfano and Collmer 2004) and also employ the quorum sensing to suppress the host immune system. The most well-characterized bacterial pathogens include *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia.* All the genera have three common features, i.e., they kill the cells, inhabit the intercellular spaces, and have *hrp* genes (hypersensitive response and pathogenicity; Alfano and Collmer 1997). The Hrp protein secretion system is very important for pathogenesis.

These pathogens cause various pre- and post-harvest diseases in economically important plants incurring significant economic losses. The plant responds to these pathogens by activating the immune system called as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), effector-triggered immunity (ETI), and phytohormone signaling (Bent and Mackey 2007; Willment and Brown 2007).

Whole genome sequencing and comparative genomics studies have helped in better understanding of the various effector proteins, strategies of pathogenesis, and resistance mechanism in plants. Different effector proteins have also been used as an imperative tool in disease resistance breeding (Vleeshouwers and Oliver 2014). This chapter provides an overview on the different lifestyles and infection strategies of fungal and bacterial phytopathogens characterized.

2.2 Fungal Biotrophy and Necrotrophy

Of the 1.5 million species of fungi projected to exist (Hawksworth 1991), very few of them have been functionally characterized. The lifestyle of pathogenic fungi can be saprophytic, biotrophic, or necrotrophic (Kahmann and Basse 2001; Mendgen and Hahn 2002; Glazebrook 2005; Howlett 2006; Ferreira et al. 2007). It has been suggested that most of the fungi are saprotrophic in nature, i.e., they derive their nutrition from decaying organic matter (Kahmann and Basse 2001; Ahmad et al.

2006). Fungal necrotrophs first infect the host cells, and this requires various stages of conidial attachment followed by germination, formation of lesion, and finally the softening of tissue and sporulation (Prins et al. 2000). After the initial phase of infection, the penetration is facilitated by production of toxins, appressoria, haustoria, hyphae formation, and secretion of cell wall-degrading enzymes (CWDEs) (Prell and Day 2001; Idnurm and Howlett 2001; Mendgen and Hahn 2002; Oliver and Ipcho 2004). All through the infection process, fungi also dynamically suppress the host cell defense by manipulating various processes, thereby aiding their own proliferation (Prins et al. 2000). Biotrophic pathogens obtain the nutrients from living cells by forming complex infection structures like hyphae or haustoria for exchanging nutrients from the host plant cell (Heath 1997, 2002; Schulze-Lefert 2004). The secretory activity of CWDEs is limited in case of fungal biotrophs (Idnurm and Howlett 2001; Tudzynski and Sharon 2003; Oliver and Ipcho 2004; Schulze-Lefert 2004). The definitions are categorical, but the relationship between necrotrophs and biotrophs is probably best represented as continuum with intermediates, i.e., hemibiotrophs, pathogens that initiate infection as biotrophs but later switch to necrotrophs (Glazebrook 2005). The economically important biotrophic, hemibiotrophic, and necrotrophic plant fungal pathogens have been recorded in Tables 2.1 and 2.2.

2.2.1 Effector Proteins Secreted by Fungal Plant Pathogens

Plants recognize fungal pathogens on the basis of pathogen-associated molecular patterns (PAMPs). PAMPs are conserved molecules important for pathogen and characterize a class of microbes (Bent and Mackey 2007; Willment and Brown 2007). Examples of PAMPs include fungal xylanase (Ron and Avni 2004) and chitin (Kaku et al. 2006; Miya et al. 2007). Detection of these PAMPs by pattern recognition receptors (PRRs) leads to a PAMP-triggered immune response (Bent and Mackey 2007; Hückelhoven 2007; Zipfel 2008, 2009). To counter the PTI, fungal pathogens secrete effector molecules that can directly suppress the host defense response and also alter the host cell physiology for their proliferation (Boller and He 2009; Göhre and Robatzek 2009; Stergiopoulos and de Wit 2009; Stergiopoulos et al. 2010; Koeck et al. 2011).

Chitin (the major structural component of filamentous fungus cell wall) is released by the action of plant chitinases and triggers the immune response of plants (Kaku et al. 2006; Miya et al. 2007). The secreted effector Ecp6 from *Cladosporium fulvum* competes with the plant receptors CEBiP, by sequestering chitin oligosaccharides, and suppresses chitin-triggered immunity (de Jonge et al. 2010).

As discussed earlier *Avr* genes are another category of pathogen effectors that are specifically recognized by plant resistance (R). R genes consist of leucine-rich repeat (LRR) domains and nucleotide-binding sites (NBS) (Jones and Dangl 2006). It is not clear how fungal pathogens translocate the effector proteins into the host cell and whether they have type III secretion system or not as it is present in bacterial pathogens (Jin et al. 2003; Büttner and Bonas 2006). Most of the fungal *Avr*

Table 2.1 List of important biotrophic, hemibiotrophic, and necrotrophic fungal plant pathogens

Name of the pathogen	Host plant of the pathogen	Name of the disease
Biotrophic pathogens		
Cladosporium fulvum	Tomato	Tomato leaf mold
Ustilago maydis	Maize	Maize smut
Blumeria graminis	Barley; wheat	Powdery mildew
Plasmopara halstedii	Sunflower	Downy mildew
Synchytrium endobioticum	Potato	Potato wart
Plasmopara viticola	Grapevine	Downy mildew
Hemibiotrophic pathogens		
Magnaporthe oryzae	Rice	Rice blast
Mycosphaerella graminicola	Wheat	*Septoria tritici* leaf blotch
Bipolaris sorokiniana	Barley; wheat	Spot blotch disease
Colletotrichum graminicola	Maize	Anthracnose stalk rot disease
Moniliophthora perniciosa	*Theobroma cacao*	Witches' broom disease
Necrotrophic pathogens		
Cochliobolus heterostrophus	Maize	Southern leaf blight
Ascochyta rabiei	Chickpea	Blight disease
Cochliobolus carbonum	Maize	Northern leaf spot and ear rot
Cochliobolus victoriae	Oat	Victoria blight
Alternaria alternata	Pear; strawberry; tangerine; apple; tomato; tobacco; citrus	Black/dark leaf spot
Alternaria solani	Tomato and potato	Tomato early blight; collar and fruit rot
Alternaria brassicicola	*Brassica* species (broccoli, cabbage, canola, mustard; cauliflower; turnip)	Black spot (leaf, stem, or pod spots)
Periconia circinata	Sorghum	Milo
Pyrenophora tritici-repentis (*Drechslera tritici-repentis*)	Wheat	Tan spot
Bipolaris sacchari	Sugarcane	Eyespot
Phyllosticta maydis (*Mycosphaerella zeae-maydis*)	Maize	Yellow corn leaf blight
Stagonospora nodorum (*Phaeosphaeria nodorum*)	Wheat	*Stagonospora nodorum* blotch
Stemphylium vesicarium	European pear	Brown spot
Botrytis fabae	Bell bean (*Vicia faba*)	Chocolate spot
Name of the pathogen	Host plant of the pathogen	Name of the disease
Botrytis elliptica	Lilly	Gray mold
Botrytis cinerea	Dicots; some monocots	Gray mold "*Botrytis* blight"

(continued)

Table 2.1 (continued)

Name of the pathogen	Host plant of the pathogen	Name of the disease
Sclerotinia sclerotiorum	Cabbage; bean; citrus; celery; coriander; melon; squash; soybean; tomato; lettuce; cucumber	White mold
Monilinia fructicola	*Prunus* species (apples; pears; and other pome fruits in Rosaceae)	Brown fruit rots
Fusarium graminearum/ Gibberella zeae	Cereals	*Fusarium* head blight
Cercospora zeae-maydis	Maize	Gray leaf spot
Exserohilum turcicum	Maize	Northern leaf blight
Leptosphaeria maculans	Oilseed rape (or canola) (*Brassica napus*)	Blackleg or stem canker disease
Diaporthe toxica	Lupin	*Phomopsis* stem blight
Phoma medicaginis	Pea	Leaf spot and spring black stem
Colletotrichum gloeosporioides	Lupin and mango	Anthracnose
Fusarium oxysporum	Tomato, banana, cotton, and many others	*Fusarium* wilt
Rhizoctonia solani	Lucerne, clovers, pasture grasses, grain legumes, cereals, and oilseed crops	Rhizoctonia canker/ root rot
Pythium spp.	Very broad	Seedling damping off
Leptosphaerulina trifolii	*Medicago* spp.	Lepto leaf spot
Pleiochaeta setosa	Lupin	Brown leaf spot
Stagonospora meliloti	Lucerne and medics	*Stagonospora* crown rot
Stemphylium botryosum	Tomato; alfalfa; lettuce	Leaf spot and foliage blight
Pseudopeziza medicaginis	Lucerne	Common leaf spot

Adapted from Laluk and Mengiste (2010) and Wang et al. (2014)

genes identified have been shown to contain two important motifs, namely, dEER (aspartate, glutamate, glutamate, arginine) and RxLR (arginine, any amino acid, leucine, arginine) (Dodds et al. 2009; Tyler 2009). It is believed that these two motifs are essential for the entry into host cell. Mutation in these two motifs prevented the delivery of Avr1b from *Phytophthora sojae* and Avr3a from *P. infestans* into the host cell (Whisson et al. 2007; Dou et al. 2008). N-terminal "RxLR-like" motifs have been identified in the *Avr* genes of *Fusarium oxysporum* f. sp. *lycopersici*, *Melampsora lini*, and *Leptosphaeria maculans* (Kale et al. 2010). Based on the observation that oomycete and fungal RxLR-like motifs interact with the phosphatidylinositol-3-phosphate (an abundant phospholipid present in the outer surface of plasma membrane of plants), Kale et al. (2010) proposed that lipid raft-mediated endocytosis allows the pathogen effectors to enter the plant cell. Approximately 1500 effectors with RxLR and dEER motifs have been identified in

Table 2.2 List of important bacterial plant pathogens

Name of the pathogen	Host plant of the pathogen	Name of the disease
Dickeya	Potato tubers, bulbs of vegetables, and ornamental crops	Necrosis, blight, and soft rot
Erwinia amylovora	Apple, pear	Fire blight
Erwinia carotovora	Carrots, potatoes, cucumbers, onions, tomatoes, lettuce	Soft rot
Erwinia chrysanthemi	*Chrysanthemums*, maize, *Dieffenbachia*, *Euphorbia pulcherrima*, bananas, *Philodendron*	Soft rots and wilts
Pectobacterium atrosepticum	Potato	Potato blackleg disease
Phytophthora infestans	Potato, tomato	Late blight of potato
		Late blight of tomato
Pseudomonas syringae pv. *tomato*	*Tomato, Arabidopsis thaliana*, *Brassica oleracea* var. *botrytis*	Bacterial speck
Pseudomonas viridiflava	Tomato, soybean, pepper	Bacterial leaf blight
Pseudomonas syringae pv. *syringae*	*Prunus* sp., tomato, cereals, citrus, and kiwi	Bacterial canker and blast
Ralstonia solanacearum	Potato, tomato, banana, groundnut, tobacco	Bacterial wilt
Xanthomonas campestris pv. *Vesicatoria*	Tomato, pepper	Bacterial leaf spot

Phytophthora species (Jiang et al. 2008; Tyler 2009). After entering the host cell, these effector molecules suppress the host defense and elicit cell death (Bos et al. 2006; Dou et al. 2008).

Another category of fungal effectors identified to suppress plant basal resistance and cause plant cell death is crinkler and necrosis (CRN) effectors (Torto et al. 2003; Win et al. 2007; Haas et al. 2009). The CRN effectors also contain a conserved FLAK (phenylalanine, leucine, alanine, lysine) domain followed by a signal peptide (Win et al. 2007; Haas et al. 2009). *Phytophthora* genome has been shown to contain 61–451 CRN genes (Haas et al. 2009: Liu et al. 2011). Transient expression of CRN C- terminal domain has been shown to elicit host cell death (Liu et al. 2011). CRN8 of *Phytophthora infestans* has also been shown to regulate host signaling during infection by kinase activity (van Damme et al. 2012). Stam et al. (2013) identified 84 CRN proteins in *P. capsici*.

In fairly recent reports, small RNAs (sRNA) produced by the fungal pathogens have been shown to suppress the host immunity and are termed as non-proteinaceous sRNA effectors (Weiberg et al. 2013, 2014; Wang et al. 2015). Fungal phytopathogens also produce various small secretory proteins (SSPs) important for virulence. A family of such SSPs implicated as effector proteins required for pathogen host association has been identified in different fungal species, and it is believed that biotrophs are likely to secrete more SSPs as compared to necrotrophs (Cheng et al. 2014; Kim et al. 2016).

2.2.2 Effectors Produced by Pathogens Disturb the Phytohormonal Signaling

Plant hormones like auxin, gibberellins (GAs), abscisic acid (ABA), jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) have been implicated in regulating plant defense responses. The three main players extensively characterized for the defensive roles till date are SA, JA, and ET. It has been reported that SA signaling is involved in eliciting resistance to biotrophic and hemibiotrophic phytopathogens, while ET and JA provide resistance to plants against necrotrophs (Glazebrook 2005; Di et al. 2016). Many fungal pathogens produce phytohormone and mimic during the infection for altering host cell response (Inomata et al. 2004). Many fungal pathogens themselves also produce plant hormones to disturb the host cell plant hormone signaling (Agrios 2005; Mobius and Hertweck 2009; Brodhun et al. 2013). Also many effector proteins synthesized by fungal pathogens have been shown to alter phytohormone biosynthesis and signaling pathways (Kazan and Lyons 2014).

In *Ustilago maydis* an increase in chorismate mutase 1 (Cmu1) that changes SA precursor chorismate into prephenate has been observed during infection process which alters SA biosynthesis pathway (Djamei et al. 2011). *Sclerotinia sclerotiorum* (a necrotrophic fungal pathogen) has also been shown to have SA-degrading effector protein (Penn and Daniel 2013). A secretory effector protein, SSITL (*Sclerotinia sclerotiorum* integrin-like), secreted by *S. sclerotiorum* has been shown to inhibit JA-regulated defense responses (Zhu et al. 2013). PSE1 (penetration-specific effector 1), an effector secreted from the pathogen *P. parasitica*, affected auxin efflux (Evangelisti et al. 2013). ABA confers susceptibility to fungal pathogens, namely, *B. cinerea* and *F. oxysporum* (Audenaert et al. 2002; Anderson et al. 2004).

2.2.3 Phytotoxins Released by Fungal Pathogens

Toxins released by fungal pathogens play an important role in pathogenicity (Mobius and Hertweck 2009; Stergiopoulos et al. 2013). These toxins damage the host cells thereby aiding in colonization of pathogen. These toxins are categorized into two classes: host-specific toxins (HSTs) and non-HSTs. HSTs are majorly synthesized by *Dothideomycetes* (Wolpert et al. 2002; Berestetskiy 2008). Various trichothecene phytotoxins (inhibits translation) are secreted by species of *Fusarium* for the proliferation inside the host plant (Desjardins et al. 2007). Fungal necrotrophs also synthesize phytotoxic proteins like NEP1 (necrosis and ethylene-inducing protein) and NEP1-like proteins involved in host cell death and ROS production (Pemberton and Salmond 2004). AAL (a toxin released by *Alternaria alternata*) and Fumonisin B1 toxins secreted by fungal pathogen inhibit the ceramide synthesis and result in buildup of free sphingoid bases which results in the production of ROS and host cell death (Abbas et al. 1994; Shi et al. 2007). Fumonisin B1, secreted by *Fusarium verticillioides*, lessens extracellular ATP and causes PCD.

2.2.4 Significant Effector Proteins Secreted by Few Biotrophic, Hemibiotrophic, and Necrotrophic Fungi

2.2.4.1 *Cladosporium fulvum*

C. fulvum is a fungal pathogen which is biotrophic in nature and causes leaf mold disease of tomato (Thomma et al. 2005). Avr9, a 28 amino acid cysteine-rich effector protein of this genus, elicits HR in tomatoes. At the time of infection, *C. fulvum* secretes Ecp6 effector protein containing LysM chitin-binding domain. This domain and another effector, Avr4, bind to the chitin and prevent the host recognition (van Esse et al. 2007; Sánchez-Vallet et al. 2013). It also produces Avr2 effector molecule which binds the cysteine proteases of plants and prevents the defense responses of plants (van Esse et al. 2008).

2.2.4.2 *Ustilago maydis*

U. maydis is a biotrophic fungus which is the causal agent of maize smut. It infects the aerial organs of maize and enters the cell wall with the help of special structures named as hyphae for deriving nutrition from the host plant (Brefort et al. 2009). During infection process 20% of its genes were found to be upregulated. Effector gene cluster deletion analysis was performed to delineate the function of different effector proteins in the infection process (Kamper et al. 2006). Pep1 effector of this genus codes for a 178 amino acid secretory protein and plays an important role during the invasion process. It also inhibits the activity of apoplastic plant peroxidases thereby suppressing the immune response of plants (Doehlemann et al. 2009; Hemetsberger et al. 2015). Hum3 (a hydrophobin domain and a repetitive repellent protein-like repetitive domain-containing protein) and Rsp1 (repetitive secreted protein 1, a protein with internal hydrophilic repeats) effector proteins from *U. maydis* have been shown to be significant for cell adhesion during the infection process (Muller et al. 2008). Another effector See1 (Seedling efficient effector 1) has been shown to be involved in reactivating DNA synthesis important for tumor proliferation in leaf cells (Redkar et al. 2015).

2.2.4.3 *Blumeria graminis*

It is an obligate biotrophic fungus and causes powdery mildew disease on barley and wheat. It forms filamentous hyphae for infecting the leaves and haustoria for deriving nutrition from its host. More than 500 candidate secreted effector proteins (CSEPs) also called as Blumeria effector candidates (BECs) have been identified in this fungus. Eight such BECs including BEC1054 (RNase-like effector) are required for the formation of feeding structure, i.e., haustoria (Pennington et al. 2016).

2.2.4.4 *Botrytis cinerea*

B. cinerea is a broad host range necrotrophic fungus and is the causal agent of gray mold disease. It penetrates the host epidermal cells by secreting various cell wall-degrading enzymes, cutinases, proteases, lipases, oxalic acid, and a nonhost-selective toxin named as botrydial (Prins et al. 2000; Kars et al. 2005).

2.2.4.5 *Sclerotinia sclerotiorum*

S. sclerotiorum is a necrotrophic fungus that infects more than 400 plant species. It secretes various enzymes like glucanases, pectinases, cellulases, and proteases for disrupting the tissue (Bolton et al. 2006). It also synthesizes oxalic acid during the infection process to suppress the host immune responses (Guimaraes and Stotz 2004). It causes necrosis of crucifers by the secretion of polyketides (Pedras and Ahiahonu 2004). A secretory protein of *S. sclerotiorum*, SSITL (SS1G_14133), has also been shown to suppress the host immune response (Zhu et al. 2013).

2.2.4.6 *Alternaria brassicicola*

A. brassicicola is a broad host range necrotrophic pathogen known to synthesize various toxins important for pathogenesis. Host-specific toxins are secreted by *Alternaria alternata*, another necrotrophic fungus, to suppress host resistance (Tsuge et al. 2013). Cell wall-degrading enzymes and lipases produced by this genus affect the various cellular process of the host (Cho 2015).

2.2.4.7 *Magnaporthe oryzae*

It is a hemibiotrophic fungus, which causes rice blast disease. It forms appressorial pegs for the penetration of leaf cuticle and epidermal cells of the host. The effector proteins of this genus accumulate in the biotrophic interfacial complex (BIC, a lobed structure formed at the tip of invasive hypha) (Khang et al. 2010). BAS1, PWL2, BAS2, BAS3, BAS4, and AvrPita1 are the different effector proteins found to be upregulated during the infection process (Mosquera et al. 2009; Khang et al. 2010). Another effector protein of this genus named as AVR-Pii has been shown to suppress the plant immune response by inhibiting the activity of host Os-NADP-ME (a rice NADP-malic enzyme2 protein), which is important for the production of ROS (Singh et al. 2016).

2.2.5 Bacterial Biotrophy and Necrotrophy

Phytopathogenic bacteria are accountable for the devastating damage to the agriculture. The interaction between bacteria and its plant host is very dynamic. Bacteria cause many diseases in higher plants by entering the intercellular spaces through the natural openings, stomata, and wounds. Fire blight, halo blights cankers, galls, and leaf spots are the various diseases elicited by the bacteria in higher plants. They have varied infection strategies and belong to both the biotrophic and necrotrophic categories of pathogens. Necrotrophic bacteria follows the brute force strategy in which the bacteria rapidly kill the parenchymatous tissues of the host plants, while biotrophic bacteria reproduces within the host cell and slowly kills the host (Collmer and Bauer 1994). Necrotrophic bacteria trigger hypersensitive response (HR), i.e., a localized programmed cell death of host plant that restricts the pathogen by cutting off the nutrient supply; cell death occurs that enhances the colonization of the bacteria and acts as a sign of successful infection (Van Kan 2006). For a successful infection to occur, phytopathogenic bacteria have many genes that are induced when

the contact between bacteria and plant is established. Using an in vivo expression technology, Boch et al. (2002) identified several genes that are induced when *P. syringae* pv. *tomato* attacks *Arabidopsis thaliana*. Few bacteria employ quorum-sensing mechanism to counter the host defense (Alfano and Collmer 1996).

Sometimes it becomes difficult to place the bacterial pathogens in a particular class as we have examples like *Pseudomonas syringae* which can be placed in biotrophic, hemibiotrophic, and partly in necrotrophic category; *Ralstonia solanacearum* is both a necrotrophic and biotrophic bacteria, *Xanthomonas* species are biotrophic, and *Erwinia amylovora* is often mentioned under necrotrophic category (Kraepiel and Barny 2016).

2.2.6 Basis of Bacterial Pathogenicity: The HRP System

The necrotrophic bacteria elicit the HR response using *hrp* genes (hypersensitive response and pathogenicity). These genes were first identified in *Pseudomonas syringae* pv. *syringae* (brown spot of bean) and were found to be present in clusters and are essential for the transport of the *Avr* gene-derived signal from the bacteria into host plant cells (Niepold et al. 1985; Lindgren et al. 1986). *hrp* genes have also been identified in Gram-negative bacterial pathogens, namely, *Xanthomonas campestris* pv. *vesicatoria*, *Erwinia amylovora*, and *Ralstonia solanacearum*. Mutation in any one of the *hrp* genes leads to failure of pathogen invasion (Van Gijsegem et al. 1993). *Hrp* and *Hrc* (hypersensitive response and conserved) genes encode the type III secretion system (TTSS) that helps in the injection of bacterial virulence "effector" proteins into host cells (Alfano and Collmer 2004; Cornelis and Van Gijsegem 2000). The HRP system delivers various effector proteins which promote the induction of disease by suppressing the basal defense mechanism of host plant and also triggers HR response (Chang et al. 2005; Collmer et al. 2002; Zwiesler-Vollick et al. 2002). These effector proteins are designated as hairpins, which are cysteine-lacking, glycine-rich proteins (Bauer et al. 1995), for example, Avr proteins (Keen 1990), Hop (Hrp outer protein, Alfano and Collmer 1997) of *Pseudomonas*, Xop (*Xanthomonas* outer protein; Noel et al. 2001), or Pop (*Pseudomonas* outer protein; Arlat et al. 1994) of *Ralstonia*. NLPs or Nep1-like proteins are a new family of proteins identified in pathogenic bacteria that induce HR response in plants (Pemberton and Salmond 2004). Various reports on the localization of effector proteins suggested nucleus and plasma membrane to be their target site (Yang and Gabriel 1995; Deslandes et al. 2003).

2.2.7 Effector Proteins Synthesized by the Bacteria

Phytopathogenic bacteria synthesize effector proteins also named as double-edged swords during the course of infection. These proteins enter the host cell cytoplasm and helps in suppressing the plant immune system, thereby playing an important part in virulence and pathogen survival (Mattoo et al. 2007; Shames and Finlay

2012). These effector proteins target various processes of host cell like RNA metabolism, secretion of proteins, and activation of kinases (Kaffarnik et al. 2009). These effector proteins can be recognized by the plant R proteins (resistance proteins) leading to development of plant resistance against the pathogen. Such proteins are called as avirulence proteins (Avr). The pathogen has an *Avr* (avirulence) gene that interacts with the corresponding R (resistance) gene of the host plant leading to establishment of HR and avirulence preventing development of disease (Flor 1971). Reports also suggest that pathogens deploy the secretion system of host plant invasion (Kaffarnik et al. 2009). Pathogenic bacteria *Pseudomonas syringae* have been shown to secrete about 30 effector proteins into the cytosol of plant (Chang et al. 2005). One of the effector proteins of *Pseudomonas syringae* named as AvrPto has been shown to bind the receptor kinases of *Arabidopsis* and blocks the immune response (Zong et al. 2008; Xiang et al. 2008).

2.2.8 Extracellular Polysaccharide and Toxins Produced by Bacteria

Extracellular polysaccharides (EPSs) produced by the bacteria alter the defense-activating signal, block the xylem, and protect the bacteria from the various environmental stresses (Denny 1995). EPSs secreted by *P. syringae* lead to the development of chlorotic and necrotic symptoms (Corsaro et al. 2001). The toxins mainly the secondary metabolites or peptides produced by various necrogenic bacteria are important for pathogenesis or have an antimicrobial activity that reduces the microbial competition (Gross 1991). Coronatine, a toxin produced by *P. syringae*, causes chlorosis in plants and also aids the entry of bacteria by triggering the opening of stomata and by defeating the host defenses (Brooks et al. 2005; Uppalapati et al. 2007). Some pathovars of *P. syringae* produces antimetabolite toxins (mangotoxin, phaseolotoxin, and tabtoxin) that inhibit the biosynthesis of aromatic amino acids thereby interfering with the nitrogen metabolism of host plant (Snoeijers et al. 2000).

2.2.9 Plant Cell Wall-Degrading Enzymes (PCWDE)

Soft rot pectinolytic bacteria under necrotrophic category, namely, *Erwinia carotovora*, *E. chrysanthemi*, *Pseudomonas viridiflava*, *Pectobacterium*, and *Dickeya*, using type II secretion system secrete plant cell wall-degrading enzymes (pectic enzymes, cellulases, proteases, and hemicellulases) that soften the host tissue and kill the cells and also help in nutrient uptake (Perombelon and Kelman 1980; Barras et al. 1994). A single pectate lyase (Pel) is secreted by *P. viridiflava* (Liao et al. 1988), while *E. carotovora* and *E. chrysanthemi* produce complex of pectic enzymes (Barras et al. 1994). Investigation also suggests that PCWDE are produced in a population density-dependent mechanism called quorum sensing (QS). It plays a

very important role in regulating bacterial pathogenesis (Whitehead et al. 2001). In case of soft rot-causing bacteria *Pectobacterium atrosepticum*, QS control the production of cell wall-degrading enzymes (Liu et al. 2008).

2.3 Plant Defense Responses Against Pathogen Attack

Necrotrophs and biotrophs confront almost the same basal plant defenses, with the major component being the waxy cuticle layer and rigid cell walls (Heath 2000a, 2002). Plants recognize pathogens on the basis of pathogen-associated molecular patterns (PAMPs). PAMPs are conserved molecules important for pathogen and characterize a class of microbes (Bent and Mackey 2007; Willment and Brown 2007). Examples of PAMPs include bacterial flagellin (Zipfel et al. 2004), fungal xylanase (Ron and Avni 2004), and chitin (Kaku et al. 2006; Miya et al. 2007). Detection of these PAMPs by pattern recognition receptors (PRRs) leads to a PAMP-triggered immune response (PTI) (Bent and Mackey 2007; Hückelhoven 2007; Zipfel 2008, 2009). This comprises the activation of a mitogen-activated protein (MAP) kinases followed by WRKY transcription factor phosphorylation leading to induction of plant defense responses (Heath 2000a; Dodds and Rathjen 2010). Plant responses associated with PTI include callose deposition in the cell wall, production of reactive oxygen species (ROS), and induction of pathogenesis-related proteins and defensins (Silverstein et al. 2005; van Loon et al. 2006). In addition to PAMPs, plant cells can recognize degraded damaged host cells mainly polysaccharides called damage-associated molecular patterns (DAMPs) (Lotze et al. 2007; Matzinger 2007; Hückelhoven 2007). Effector-triggered immunity response happens when an *Avr* gene is recognized by a plant *R* gene (Heath 1998, 2000b; Tudzynski and Sharon 2003; Schulze-Lefert 2004; Ferreira et al. 2007). ETI is associated with the production of reactive oxygen species (ROS) which results in HR response at the point of infection (Heath 1998, 2000b; Lamb and Dixon 1997; Torres and Dangl 2005).

2.4 Use of Effector Proteins in Plant Breeding

Effector molecules are the pathogen-induced molecules that help the bacteria or fungi to invade plants. "Effectoromics" is a high-throughput functional genomics approach used to identify the R genes, and it has played a very important role in modern resistance breeding. For the first time, this strategy was deployed for *Phytophthora infestans* and potato, leading to identification of many *R* and *avr* genes (Vleeshouwers et al. 2008, 2011). Detailed characterization of the effector molecules will help in hastening the R gene cloning and determining the redundancy and can help in deploying R gene in agriculture (Vleeshouwers and Oliver 2014).

2.5 Prospects

Plant-pathogen interactions are highly coevolved and dynamic at molecular and cellular levels. The model illustrating the interaction between plants and fungal/bacterial phytopathogens (necrotrophs/biotrophs) has been depicted in Fig. 2.1. Despite this substantial progress in this field, a critical challenge still remains in understanding the mechanism by which fungal/bacterial pathogens cause the disease and how plants respond to the elicitors secreted by these pathogens during invasion. Also it is not known whether fungi also have their own transport machinery, analogous to the bacterial type III secretion system. Research focused on the identification of diverse virulence factors important for pathogenesis will provide information about the genes present in the pathogenicity islands, which will definitely catapult our understanding of fungal and bacterial pathogenesis. In the coming years, amalgamation of genomics, transcriptomics, proteomics, and metabolomics data will help us in clarifying the various genes encoding different proteins important in plant-pathogen (biotroph/necrotroph) interactions. The study of interplay between the effector proteins secreted by pathogen and defense-promoting signaling in host cells will help us in elucidating the mechanism of disease development. It is very important to develop new methods to manage the biotrophic and necrotrophic plant pathogens. Plant biologists studying these plant-pathogen interactions will undoubtedly help in saving our crops from the devastating attacks.

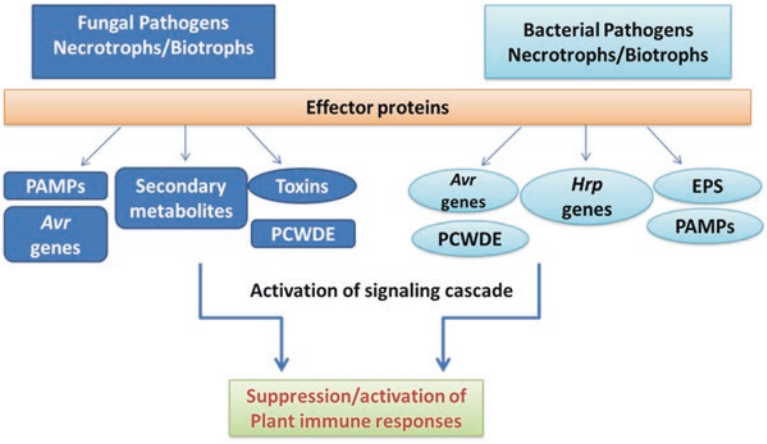

Fig. 2.1 Model for plant/pathogen (necrotroph/biotroph) interaction. *PAMPs* pathogen-associated molecular patterns, *PCWDE* (plant cell wall-degrading enzyme), *EPSs* extracellular polysaccharides

Glossary

Appressorial pegs It is a specialized cell characteristic of fungal plant pathogens and is used during infection process.

Effector proteins Proteins secreted by bacterial pathogens during the infection process and help in suppressing the immune system of host.

Extracellular polysaccharides High molecular weight sugar polymers synthesized by microorganisms. They play important roles in protecting the microorganism and also mediate their pathogenicity.

Hypersensitive response It is a defense mechanism evoked by pathogens and involves localized cell death to stop the spread of infection.

Phytohormone Chemicals or signal molecules synthesized by plants and play an important role in their growth and development.

Phytopathogens Pathogenic bacteria, viruses, or fungi which infect plants and cause many plant diseases.

Quorum sensing It is a phenomenon of cell-cell communication which helps bacteria to sense the cell density and coordinate their behavior accordingly.

Small RNAs Noncoding RNA molecules which are less than 200 nucleotide in length and have a role in RNA silencing and regulation of gene expression.

Virulence The extent of injury caused by pathogen to its host.

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Plant–Virus Interactions

3

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Abstract

Viruses are small pathogens not visible under light microscope and are causal agents for many common plant diseases. They lead to heavy economic losses in crop production and quality in different parts of the world. The simplest viruses are composed of nucleic acid and protein coat. Plant viruses mostly have single-stranded ribonucleic acid (ssRNA), but in few cases single- or double-stranded DNA may also be present. They are obligate parasites and require host machinery for their reproduction. They make their passive entry into plant cells through the wounds caused by either physical injuries, through environmental factors, or by the vectors which could be insects, nematodes, fungi, and even mites. Viral RNA disassembles, replicates, and converts its mRNA to proteins in the host cytoplasm using energy and proteins from the host cell. Once viruses enter the host, they move from infected cells to healthy neighboring cells locally. Long-distance transport via the vascular system for systemic infection is also the key feature of plant viruses. In response to the infection by viruses, plants also develop certain defense mechanisms. In this chapter the aspects related to movement of viruses in plant system, general response of plants to viruses, defense mechanisms developed by the plant like RNA silencing, virus-encoded suppressor proteins, development of disease-free tissues, and future aspects are considered.

Keywords

Pathogen · ssRNA · RNA silencing · Suppressor proteins

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3.1 Introduction

About 15% of the global crop production is lost due to plant diseases highlighting the fact that there is a continuous threat to the plants throughout the world. And more than one third of these plant diseases are thought to be caused by phytopathogenic viruses (Boualem et al. 2016). Viral infections pose serious threat to agricultural and horticultural crops and cause huge economic loss. Plant virus infections cannot be directly controlled by the use of various chemicals and insect vector management, although virus population and distribution can be restricted to some extent through the use of these chemical treatments. Thus the development of disease-resistant cultivars is a major challenge in plant breeding research because the usage of chemicals has more negative effect on humans and surrounding environment (Islam et al. 2017). Viruses being the most abundant life forms on earth can be found in all kingdoms of life. About one third of all the viruses are represented by plant viruses. Plant viruses are simple, obligate, intracellular parasites. One of the important characteristics shared by all viruses is their relatively small genome size, which has a very limited coding capacity. Viruses are nucleic acid-based pathogens and packed with a protein called capsid. They contain single-stranded (ss) or double-stranded (ds) RNA or DNA genome. For instance, a vast majority of known plant viruses are positive ssRNA viruses that typically encode not more than a dozen proteins. They lack necessary components for their independent survival, so they rely entirely on host machinery for their life cycle. They use protein and energy from the host cell to perform different processes inside/outside the host cytoplasm like entry into plant cell or uncoating of nucleic acid, translating viral proteins, viral nucleic acid replication, intracellular and systemic movement, encapsulation and suppression of host defenses on their accumulation, progeny virions assembly, and further transmission (Nelson and Citovsky 2005). They enter their host cells passively either through wounds caused by environmental physical injuries or by different vectors, which may be insects, nematodes, soil fungi, or mites. The obligate intracellular nature of viruses provides a platform for host–virus interaction (Pallas and Garcia 2011).

Viruses can replicate within the living cells of their respective hosts using their own enzymes like RNA-dependent RNA polymerase, DNA replicase, or reverse transcriptase, whereas subviral agents called viroids use RNA polymerase of their hosts for replication. Viroids till now are considered to be the smallest plant pathogens. They are nonprotein-encoding and highly structured, single-stranded RNA molecules (Navarro et al. 2012). The absence of a protein coat distinguishes viroids from viruses. Viroids completely have different ways of transmission as compared to plant viruses because they are not enveloped and do not encode any (movement) proteins. The most important transmission route is believed to be mechanical transmission through physical contact with insect parts and/or plant products (e.g., pollen).

During each stage of viral cycle, variant interactions are generated between the plant and the virus, which might lead to either a compatible or non-compatible relationship between virus and host plant. The development of disease in the plant is an exception and not an outcome of the viral infection as plants are capable of counteracting the harmful effects of viruses. The resistance is offered either through passive

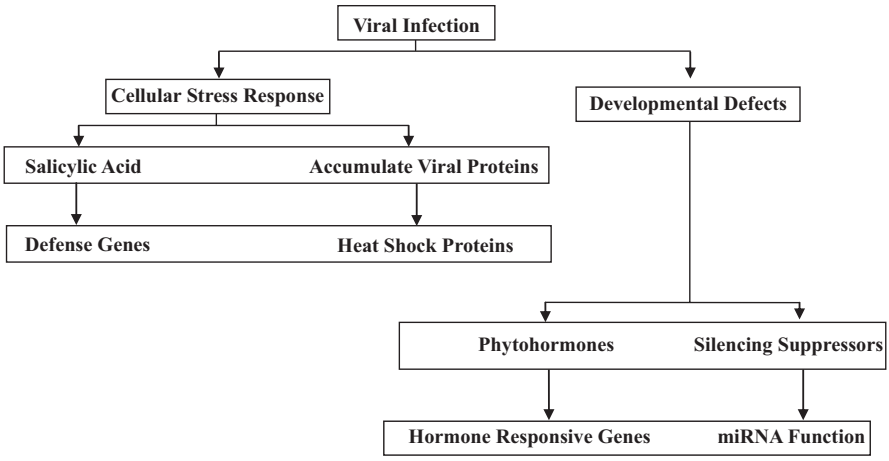

Fig. 3.1 Host plant and virus interactions leading to the expression of defense-related and heat shock genes under the control of signal transduction pathways. Viruses also cause developmental defects as they disrupt the phytohormone signaling and regulatory microRNAs

means where essential host susceptibility factors are absent or through the presence of defensive physical and chemical barriers which the virus has to cross. Once the virus has crossed these barriers, then it is exposed to certain nonspecific defense reactions. The virus causes the infection only if it produces virulence factors against these defense mechanisms, which may or may not be challenged by the plants depending on the defense response to virulence factors of virus (Jones and Dangl 2006).

Various plant proteins (like RNA polymerases, RNA helicases, Dicer-type dsRNA RNases, ssRNA RNases) induced by viral RNA participate in antiviral defense response via RNA silencing mechanism. Viruses have to escape this universal mechanism for its successful infection in the plant. The strategy that is generally adopted by the virus is the production of silencing suppressors (Valli et al. 2009). Viruses also have to complete their reproductive cycle apart from overcoming defense barriers. The positive polarity viruses uncoat and translate the genomic RNA once they enter the plant cell followed by their movement to neighboring cells and finally spread all over the plant (Fig. 3.1). Therefore undertaking plant–virus interaction mechanism is a better way to develop novel tools for plant protection against virus attack. In this chapter, we have highlighted the present state of knowledge about plant–virus interactions.

3.2 Environment and Evolution Modulate Plant Pathogenesis

Since the beginning of virology, a primary motivation for the study of plant viruses is that these are important pathogens of plants. And a major area of research has focused on viral plant pathogenesis (study of the capacity of viruses to cause diseases in

plants). Since the identification of first virus genes in mid-1980s that had the properties of avirulence (Avr) factors or that encoded functions associated with the development of specific symptoms, the knowledge on plant virus pathogenesis has increased (Baughman et al. 1988). An obvious antagonistic plant–virus interaction has focused on those viruses that cause disease in crops. However, recently, the concept of viruses being commensals or even mutualists has been emphasized, although based on limited experimental evidence. Still, plant pathogenesis is an ongoing subject of debate, and it is highly important to understand which factors favor the evolution of viruses toward pathogenicity and under which conditions viruses will act as mutualists or commensals. Although much studies on plant–virus interactions in wild ecosystems have not been undertaken (Roossinck and García-Arenal 2015), evidence point toward asymptomatic virus infection in wild plants (Stobbe and Roossinck 2014). For viruses that infect wild plants, the interactions affecting the composition and dynamics of wild plant ecosystems have been well documented (Prendeville et al. 2014).

However, the universal outcome of plant–virus interactions is not always a disease. Viruses that lead to manifestation of disease in some host plants can be asymptomatic in others emphasizing the role of plant–virus interactions in plant pathogenicity. The interaction between viral infection and environmental conditions may change the host–virus interaction from detrimental to beneficial to the host. For example, the effects of CMV (*Cucumber mosaic virus*) infection on *Arabidopsis thaliana* plants over a range of temperature and light intensity conditions varied according to plant genotype and environment, infection being detrimental, neutral, or beneficial to the host plant in terms of viable seed production (Roossinck and García-Arenal 2015).

Environmental modulation during pathogenesis does not involve any genetic change either in the virus or the host. However, it has been observed that change in the environment can result in virus evolution towards more severely pathogenic genotypes. For several plant–virus interactions, symptom expression has often been considered as an alternative to virulence (Doumayrou et al. 2013). Although evidences fail to show any correlation between virus multiplication and virulence, the trade-off hypothesis (there is evolution of highly virulent isolates of virus in environmental conditions that favor high rates of horizontal transmission) can be useful for the analyses of virulence/pathogenicity evolution in plant viruses (Stukenbrock and McDonald 2008). Diversity of habitat also modulates the evolution of pathogenesis. Viruses with a narrow host range have higher host availability in agricultural habitats than in wild resulting in higher rates of horizontal transmission. An increase in the transmission rate may result in the evolution toward high pathogenicity. Factors favoring or hindering the evolution of high pathogenicity in specific environments or across environments await future research.

3.3 Plant–Virus Relationships

The studies conducted on extremely complex plant–virus interactions for more than half a century have led to a partial elucidation of the mechanisms linked to the accumulation of viruses inside host cells, virus movement within the plant, and the plant

defense mechanisms. Most of the plant virus combinations do not necessarily lead to effective infections because virus must be able to find appropriate supportive host factors and should be able to escape the host defense responses. A virus may not be always pathogenic even though it is able to replicate. Numerous viral and host components interact within the context of viral needs, including host proteins, cell membranes, lipids, and metabolites (Stapleford and Miller 2010). Developmental abnormalities and other phenotypic manifestations described as disease symptoms appear only when virus infection disturbs host physiology. It is only at this stage that pathogenesis takes place (Pallas and García 2011). Numerous interactions between virus and host factors involved in the replication of plant viruses having either positive or negative effects result in an outcome of the infection. Both the factors, i.e., defense responses raised by the host against the infectious agent and effects of virus replication in the host, can lead to pathogenesis. Thus a single virus factor can even be the main responsible agent of the pathogenic process (Nagy and Pogany 2012).

Viruses do not normally cause the death of the host because they need living tissue for their multiplication, although there are exceptions. Plant viruses need to counter plant defense mechanisms and to usurp the functions of different host factors in order to complete their life cycle. Plants can defend themselves against various parasites and pathogens like insects, animals, other parasitic plants, viruses, and bacteria as they have well-developed recognition mechanisms which act as barriers preventing infection in the case of pathogens. Therefore, an interaction and/or interference is needed between viral and host components which, in some instances, would lead to symptom development through an alteration in the plant physiology. According to recent discoveries, plant–virus interactions are also known to be involved in plant development such as hormonal regulation, cell-cycle control, endogenous transport of macromolecules, etc. Nevertheless, the identities of all host factors involved in the viral cycle are still unknown (Hull 2009). However, the interactions differ widely in the mechanisms involved in the display of symptoms. When there is viral infection in the plant, both compatible and incompatible host–virus interactions exist.

3.3.1 Compatible Host–Virus Relationships

If the host is not able to recognize the virus, the two develop a compatible interaction, which may be favorable for the virus. In such a relationship, virus infects the host cell, and, depending on where the virus symptoms appear, local and systemic hosts are distinguished. Sometimes both the symptoms appear simultaneously. Local and systemic symptoms are called *external symptoms*.

A variety of disease symptoms can be observed due to viral infections in plants, like leaf rolling, wilting, yellowing, stunting necrosis, and mosaic pattern formation. These symptoms were found to be of great commercial importance even before viruses were discovered, for example, the appearance of flame-like streaks in tulip flowers called “tulip mania” which were sold at very high prices in Netherlands in

Table 3.1 The most frequently observed viruses, their hosts, and symptoms in compatible host–virus interaction

Virus	Host	Symptoms
Potato virus (PVY)	*Solanum tuberosum*	Leaf drop (LED)
	Nicotiana tabacum	Necrosis (N)
	Chenopodium quinoa	
Tobacco mosaic virus (TMV)	*N. tabacum*	Necrotic lesions (N)
	N. glutinosa	
	N. sylvestris	
Tomato mosaic virus (ToMV)	*Lycopersicon esculentum*	Leaf deformation (Ldef)
Cucumber mosaic virus	*Cucumis sativus*	Mosaic (M)
Alfalfa mosaic virus (AMV)	*Chenopodium album*	Yellow mosaic

the seventeenth century. It was later found to be a result of infection by *Tulip breaking virus* (Dekker et al. 1993).

Apart from external symptoms, internal symptoms or microsymptoms caused by virus infection are not uncommon. Some plants can react by forming special inclusion bodies in the cytoplasm of infected cell. For example, light microscopic studies have revealed the formation of pinwheel inclusion bodies in the cytoplasm of *Potyvirus*-infected cells. Symptoms of viral infection can occur on all parts of the plant like the root, stem, leaf, and flower, but most commonly they occur on the leaves of the susceptible hosts (Table 3.1).

Hypersensitive reaction (HR) is also a special type of host–virus relationship to avoid spreading of virus particles or nucleic acids. This mechanism is a survival strategy in which the infected cell dies before the virus can be translocated to other cells. Thus, virus infection might not lead to symptoms although the plants are susceptible to virus infection. Plants are thus able to confine virus particles at the site of infection. This trait of producing HR could be used in breeding procedures for resistance against viruses.

Viral symptoms can be greatly influenced through environmental factors. For example, TMV at lower temperature produces local chlorotic and necrotic lesions, but at higher temperature virus can be translocated in *Nicotiana tabacum* Xanthi plants. There may be absence of external symptoms although viral movement from cell to cell can be perceived in a special type of compatible host–virus relationship as illustrated in *Medicago sativa* infected by AMV (*Alfalfa mosaic virus*). The diagnosis of viruses in symptomless host can be detected by other specialized methods (Stange 2006).

3.3.2 Non-compatible Host–Virus Relationships

A non-comatible host-virus relationship develops when the host plant recognizes the virus. In this case, a series of defense reactions are induced in the host which obstructs the replication of virus and also movement within the host. When a host plant possesses extreme immunity, viruses cannot infect a host, external symptoms

do not appear, and virus cannot be detected. The resistance of host plants to viruses may be qualitative or quantitative. In the case of qualitative resistance, there is a special relationship between the host's resistance genes and viral genes which can be expressed as hypersensitivity or resistance to the spreading of viruses (Kiraly et al. 2008), whereas in quantitative resistance, specific relationship between their genes cannot be observed. This type of resistance is in the form of resistance to viral replication and spreading, field resistance, and tolerance to plant disease.

3.4 Methods of Diagnosis of Viruses

To establish a control program for any plant disease specifically viral disease, it must always be preceded with an approved and accurate diagnosis. Lack of correct information on the causal agents of viruses, their means of spreading to distances, and their survival strategies could lead to a total failure in an attempt to control plant disease, including viral diseases. For a correct and definitive diagnosis of a viral disease, several methods can now be used. But at the beginning of the study of plant virology, the causal viruses were identified and characterized through the external symptoms on plants (Purcifull et al. 2001; Lima et al. 2012). Because different viruses due to their synergistic effect may cause similar symptoms and same virus can cause different symptoms, it is now believed that the symptoms on plants may be an unreliable source of identifying a plant disease. The symptoms caused by viruses may vary according to the plant variety, environment, viral strain, and temperature. Some plant species show different symptoms of virus infections, but there are some which do not show any symptoms at all. For example, *Datura stramonium* hosts PVX (*Potato virus X*) but is resistant to PVY. It is therefore almost impossible to diagnose plant virus infection by just observing host symptoms. Still, for the denominations of plant virus, the original symptoms are of great importance, although several other methods are additionally used for a correct and definite diagnosis of a viral plant disease. For the identification of viruses, several laboratory methods have now been developed and adapted (Fig. 3.1). The important ones are discussed below.

3.4.1 Serological Assays: Traditional Molecular Method of Disease Detection

One of the easiest and specific methods involving a rapid and precise identification of plant viruses is serology (Purcifull et al. 2001). Because the viruses cannot be cultivated as other pathogens, bacteria, and fungi can be, therefore serological assays were developed for the identification and characterization of plant viruses. Pathogens can be detected by using polyclonal and monoclonal antisera and techniques such as enzyme-linked immunosorbent assay (ELISA), Western blots, immunostrip assays, dot–blot immune-binding assays, and serologically specific electron microscopy (SSEM). The first and most widely used immunodiagnostic

technique was ELISA in the 1970s. Because of its high-throughput potential, specificity, adaptability, economic advantage in the use of reagents, and sensitivity (Clark and Adams 1977), the virus particles at very low concentrations could be detected, and a large number of samples can be indexed in a relatively short period of time. These methods are relatively simple involving antigen-antibody reactions in vitro, and any sophisticated and expensive equipment is not required. The advent of ELISA has facilitated the use of serology for virus identification on a large scale, and, thus, ELISA can be used in a wide range of situations (Purcifull et al. 2001; Lima et al. 2012). Direct and indirect ELISA are the most frequently used methods for the diagnosis of plant virus diseases although different variations of this serologic technique have further been developed (Lima et al. 2012).

The following variations of the ELISA technique were successfully used for the detection: indirect ELISA or the plate-trapped antigen technique (PTA-ELISA), immune virus particle precipitation followed by ELISA (IP-ELISA), and a simple kit for plate-trapped antigen ELISA (Dorokhov and Komarova 2016).

Polyclonal antisera for many viruses and bacteria have been developed for commercial use or research labs and have been used in numerous protocols, but their frequent cross-reactivity inspired the development of more effective monoclonal antisera using hybridoma technology (with cell lines with specificity to single epitopes). Because of the difficulty in producing a good virus-specific antiserum, serology for plant virus identification and detection becomes a serious limitation. Purified plant viruses or different types of viral protein used for immunizing warm-blooded animals can be used for preparing most of the antisera used in plant virus identification and detection. For example, most plant viruses like PLYV can serve as good and effective antigens stimulating the production of specific antibodies that can be used in different serologic tests. ELISA procedures (using both monoclonal and polyclonal antibodies) and rapid detection kits are commercially available for numerous taxa.

3.4.2 Molecular Techniques or Nucleic Acid-Based Methods for Virus Detection

Usage of molecular techniques for plant virus identification and characterization is increasing throughout the world although serology has been used extensively for the same on a large scale. Several molecular methods have now been developed, out of which some are DNA based like fluorescence in situ hybridization (FISH), many PCR variants (PCR), real-time PCR (q-PCR), and DNA fingerprinting, while others are RNA based like reverse transcriptase PCR (RT-PCR) and nucleic acid sequence-based amplification (NASBA). Reverse transcriptase polymerase chain reaction has been shown to be a suitable method of research with RNA plant viruses (Lima et al. 2012). These enable a rapid and accurate detection and quantification of pathogens and therefore can overcome uncertain diagnosis or pathogen taxonomy. However,

reproducible and efficient protocols are needed for critical preparation of samples required during this method. To avoid the presence of inhibitory compounds that compromise detection, many published protocols for RNA and DNA isolation were developed. The primary compounds interfering with these methods include polysaccharides, phenolic compounds, or humic substances from plants or other substrates. Different genomes such as ssRNA, ssDNA, or dsDNA have been used for several different protocols developed for PCR-based methods. To extract nucleic acids from different types of plant material, many commercial kits have been specifically designed and widely used. However, these methods are not always effective with all types of plant material. Each combination of pathogen and plant needs to be evaluated before these can be adopted for regular detection. For example, loss of production may vary from 20% to 100% depending on which begomovirus is causing tomato yellow leaf curl disease (TYLCD), and molecular analysis seems to be the only way by which different begomoviruses can be distinguished (Davino et al. 2006). However, PCR and RT-PCR have become attractive and efficient methods for the diagnosis of plant virus diseases, because of their power to amplify a target nucleic acid present at an extremely low level in a complex mixture of heterologous sequences. Mumford et al. (2000) developed a novel real-time quantitative PCR assay for the detection and quantification of plant viruses.

3.4.3 Innovative Detection Methods

Because many pathogens like nematodes, fungi, bacteria, viruses, and viroids can simultaneously affect cultivated plants, therefore relatively novel approaches detecting different infections in the same plant and detecting pathogen at presymptomatic to early spread stages are highly desirable. Novel methods allow detection of pathogen infections when symptoms are still unclear and limited to a few plants only. Traditional methods, on the other hand, can detect pathogens only at a later symptomatic stage.

3.4.3.1 Lateral Flow Microarrays (LFM)

This method allows rapid, hybridization-based nucleic acid detection and utilizes strong and reliable host plus pathogen biomarkers discovered through transcriptomic approaches to easily visualize the colorimetric signal. These arrays are built on miniaturized lateral flow chromatography nitrocellulose membrane. This method minimizes the need for expensive laboratory instruments, has detection limits similar to microarrays, and needs less time for hybridization. Key plant metabolites of primary and secondary metabolism can be used as biomarkers for different environmental stresses or pathogen infections, and they have been widely identified through metabolomics. For example, highly interactive proteins (acting as possible indicators of plant health status) such as dehydrins or heat shock proteins upregulated by different environmental factors can be identified by an OMIC approach (Dandekar et al. 2010).

3.4.3.2 Methods Based on the Analysis of Volatile Compounds as Biomarkers

Many volatile organic compounds (VOCs) are emitted from leaf surfaces into their immediate surroundings that serve vital functions in growth, communication, defense, and survival (Baldwin et al. 2006). VOCs are low molecular weight biomolecules and terminal metabolites of the host plant. VOCs have a high vapor pressure and low boiling point which can indicate physiological health status of the plant. They generally exist in the gaseous phase under standard temperature and pressure. A new avenue of research is opened by VOC profiling which may detect mechanisms for "plant-to-plant" and "plant-to-insect" communication. It provides with an ability to frequently and noninvasively monitor the health status of high-value commodity crops. This method offers potential for immediate applications within the plant sciences gaining new insights into host responses to pathogens and abiotic stressors. Development of hardware and software tools leading to novel analytical methods and instrumentation is required to make and interpret these data sets, which is critical to bring these concepts into the field. Several studies have been done involving VOC profiling of plants using gas chromatography mass spectrometry (GC-MS) like investigations into *Cucumber mosaic virus* (CMV) through VOC profiling. CMV-infected cultivated squash (*Cucurbita pepo* cv. Dixie) plants showed an overall net increase in VOCs, but no major qualitative difference in VOC profiles could be identified in infected plants (Mauck et al. 2010). The altered VOC profile emitted by CMV-infected plants demonstrates that the plant is inducing a change in VOC profile in response to viral infection, a mechanism known as "supernormal stimulus."

A variety of techniques (Fig. 3.2) have been used to identify the changes occurring in host genetic expression due to virus infection. The most common ones are in situ hybridization for individual genes and global profiling of host transcripts using cDNA microarrays or oligonucleotides (Zhu et al. 2001). The microarray-based studies have been done on model host systems, e.g., *Nicotiana benthamiana* and *Arabidopsis thaliana*. *Nicotiana benthamiana* experiments have utilized a heterologous array from potato (*Solanum tuberosum*, also a member of Solanaceae). Heterologous arrays are microarrays containing cDNA sequences or oligonucleotides from plants providing the RNA for transcription profiles. A large number of ESTs from six solanaceous species potato, tobacco, pepper, tomato, petunia, and *N. benthamiana* were analyzed, and it was found that 51–81% of ESTs had significant sequence similarity. Only 16–19% transcripts did not match among Solanaceae, rice, or *Arabidopsis* (Rensink et al. 2005).

Engineered viruses different from wild type in terms of virulence can also be used to study plant responses to viral strains and help to analyze the specific viral protein functions. Another strategy to discover common or specific responses of host to diverse viruses is by comparative analysis of expression data from viral infections of plants with different pathogens or stress.

To determine the control of host responses and to study the effects on viral pathogenicity, the different plant mutants and genotypes are analyzed. Another approach is to reveal the expression of individual viral components under the control of promoters either inducible or constitutive for studying the gene expression in host cells or tissues.

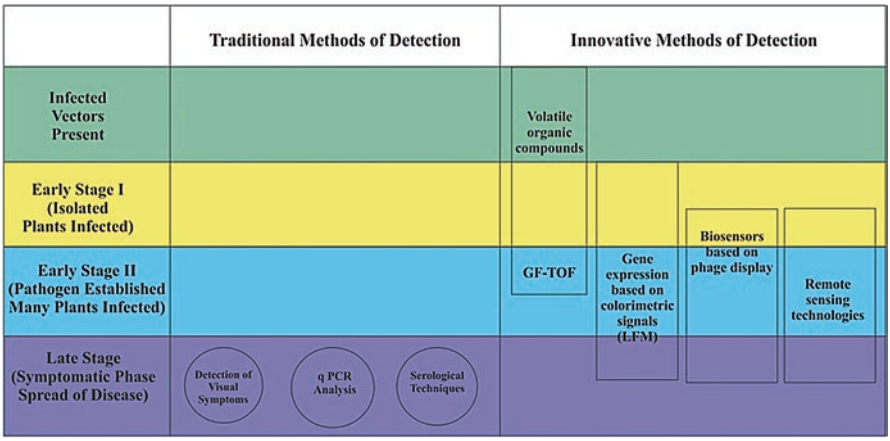

Fig. 3.2 Traditional and innovative methods of detection used for diagnosis of diseases in plants caused by viruses. Four disease stages are indicated along with their timing of use during plant disease progression

3.5 Transmission of Viruses

Agriculture is essential for continuous supply of food and other important products for human being. Therefore developing efficient strategies for protecting plants against pests and pathogens has been an important concern. Viruses are different from other plant pathogens because they require other organisms for their transmission. For instance, winged insects with sucking mouthparts help in transmission of most of the viruses from one place to another even at a distance (Vuorinen et al. 2011). Other insects like chewing insects are also responsible for the transmission of plant diseases especially those caused by plant viruses. The major insect vectors are aphids, whiteflies, and leafhoppers (Table 3.2). The viral transmission is completed in four steps: First is the acquisition of viruses from an infected plant. Second is the retention of virus in the vector by binding of virion to receptor-like elements in the digestive tract or circulation from the gut to the salivary gland. In the next step, virions are delivered from the retention sites to new site, and finally virions are deposited in a susceptible cell of the host plant. The transmission mode could be of two types depending on the length of the period during which the acquired virus can be transmitted to an uninfected plant by the vector. For persistent transmission few hours are required for acquisition of virus, and a retention time, varying from few hours to days, is required before the insect vector becomes viruliferous. Sometimes an intermediate mode called semi-persistent transmission occurs for viruses which require intermediate time period between acquisition and retention.

The transmission can also be classified as *non-circulative* or *circulative* depending on the route followed by the virus in the insect vector. In non-circulative transmission, the virus is retained in the anterior tract, i.e., the mouthparts or foregut of the digestive system. Viruses following this mode are generally nonpersistent or

Table 3.2 Main groups of insect vectors of plant viruses

Type of insect	Plant viruses
Aphids	Potyvirus
Whiteflies	Begomovirus
Leaf hoppers	Curtovirus
Plant hoppers	Oryzavirus
Thrips	Tospovirus
Beetles	Bromovirus

semi-persistent viruses. The efficiency of virus transmission increases or decreases as in semi-persistent viruses (where the acquisition time is longer) and nonpersistent viruses, respectively. The main difference is associated with the virus stability in the vector, so that virions get accumulated until the retention sites are saturated and the chance of later transmission increases. Aphids are unique type of insect vectors which transmit mainly nonpersistent viruses where the virions may rapidly be lost if the feeding period is extended beyond the limit. It is, therefore, difficult to control the spread of such viruses by insecticide treatments because both acquisition and inoculation occur during feeding only. In circulative transmission, the acquired virus must pass across the gut to reach the hemolymph after feeding on the infected plant. Finally it reaches the salivary glands and inoculated through saliva. This process might take few days due to latency period during which the virus crosses cellular barriers within the insect.

An important molecular interface which determines the acquisition from infected host plants and transmission to new hosts is the protein–protein interaction between plant viruses and insect vectors. These interactions are highly specific and open avenues for the control of insect vectors and viral transmission. Highly potential OMICS technology can be used to identify and validate the virus-interacting proteins in the vector and understand the innate immune responses to viral infection (Dietzgen et al. 2016).

3.6 Virus Accumulation and Movement in the Host

The virus accumulation in host plant requires replication and translation of viral gene sequences. The host further enhances their accumulation by providing factors for accumulation. The NAC (from the first word of these three genes: no apical meristem gene (NAM), Arabidopsis transcription activation factor gene (ATAF), cup-shaped cotyledon gene (CUC)) domain protein from tomato is found to interact with replication enhancer (REn) of geminivirus and hence participate in virus replication (Selth et al. 2005). NAC proteins also interact with Rep A (another viral protein) inhibiting the specialization of viruses (Xie et al. 1999). Interaction of NAC protein with viral coat protein is necessary in *Arabidopsis* during a resistance response (Ren et al. 2000).

Using yeast as an alternative host, screening for host factors affecting virus accumulation has been done which shows that the host gene involved in viral accumulation may vary from virus to virus (Panavas et al. 2005).

3.6.1 Viral Factors Involved in Plant Pathogenesis

The different virus factors, mainly proteins and nucleic acids, contributing to the pathogenesis of plant virus infections are discussed below:

1. **RNA replicase-related proteins/RNA-dependent RNA polymerase (RdRp):**

Some enzymes like viral RNA-dependent RNA polymerase or RNA replicase can affect viral genome replication and therefore viral accumulation and thus contribute to pathogenesis in an indirect way. For example, serial passages of singly mutated RNA replicase sequence of *plum pox virus* (PPV) in an experimental host, *Pisum sativum*, causes a drastic enhancement of disease symptoms which is associated with a notable increase in viral accumulation (Wallis et al. 2007). Like most viral proteins, RNA replicases can also lead to HR by functioning as elicitors of R-gene-driven effector-triggered immunity (ETI). For example, the helicase domain of the TMV RNA replicase (an avirulent replicase) induces HR (Erickson et al. 1999).

2. **Coat proteins/capsid proteins (CPs):**

Viral coat proteins or capsid proteins are the multifunctional proteins that, in addition to having a structural role (except some virus groups notably umbraviruses where the genomic RNA is not encapsidated by the coat protein), are involved in virus entry, disassembly, replication and translation of viral RNAs, movement or transmission of viruses, activation or control of antiviral resistance, symptom development, and host defense responses (Ni and Cheng Kao 2013). There has been a great advancement in the understanding of contribution of CPs to plant–virus interaction (Peng and Kao 2013).

Pathogen-derived resistance (PDR) can be engineered through mechanisms underlying the interaction of CP with host genes. RNA silencing of CP gene or inhibition of CP activity can mediate such resistance (Hafrén et al. 2010). It is not only the coat protein sequence but other sequences like those of MP and viral replicase that can also be used to engineer PDR. Although a tiny proportion of the transgenic crops carries the disease resistance traits, these engineered crops are of great interest in developing countries. Plant viral CPs like RdRps can also act as elicitors of R-gene-mediated HR. For example, *Tobacco mosaic tobamovirus* CP can function as an elicitor of hypersensitive response (HR) genes (Taraporewala and Culver 1997). Similarly the CPs of TMV and CMV act as avirulence factors that elicit resistance controlled by a dominant R-gene (Moffett 2009). Also a single amino acid substitution in the CP has been demonstrated to be responsible for conferring

the ability of TMV to elicit HR in *Nicotiana sylvestris* or to alter the symptom phenotype and host range in CMV-infected tobacco plants. Likewise antiviral defense generated upon viral infection can be enhanced through metabolic genes via CP production. For example, sulfur metabolism genes in the infected kenaf plants are upregulated by CP of *Hibiscus chlorotic ringspot carmovirus* (HCRSV).

Some kinds of CP interactions might target a redirection of host resources toward viral replication. And some CP can serve as an efficient suppressor of RNA silencing after binding to a host chaperone, thereby sequestering the silencing signal.

3. **Movement proteins (MPs):**

The viral pathogenesis is strongly influenced by the translocation of viruses from cell to cell throughout the plant body. An early event in the infective process is the movement of viruses from one cell to another. Viruses must cross the cell wall barrier to infect the adjacent cells and thus establish a systemic infection in plants. Derrick et al. (1992) described that TMV in *Nicotiana tabacum* and *Tobacco rattle virus* in *Nicotiana clevelandii* takes 4–5 h to move from one cell to another. Viruses first move to plasmodesmata (PD) from their replication sites on the periphery of cell and then pass through these channels entering the adjoining cell. This intercellular movement of virus occurs mainly through plasmodesmata in mesophyll and epidermal cells. Plasmodesmata are specialized intercellular organelles establishing cytoplasmic and endomembrane continuity between adjacent cells after crossing the cell walls. Although PD regulate the intercellular movement of macromolecules or macromolecular complexes, such as viral particles and ribonucleoprotein (RNP) complexes, they allow small molecules to diffuse between cells. This movement is mediated by viral encoding factors named movement proteins (MPs) (Waigmann et al. 2004). These facilitate the translocation of plant viruses among cells and through plant. During the first stage, these MPs bind to viral genome forming ribonucleoprotein complexes or to tubular structures which hold viruses allowing them to cross plasmodesmata. These then transport the virus through plasmodesmata from the epidermis to mesophyll and then to vascular bundles. There is still a poor understanding on how MP opens PD and how viral RNPs or virions pass through MP-gated or MP-formed tubule at PD. PD have a very small aperture with the cytoplasmic sleeve not more than 10 nm in diameter. Factors restricting the PD aperture include callose and pectic polysaccharide. Callose is biosynthesized at the cell wall by callose synthases, while it is degraded by β-1,3-glucanases. A positive correlation exists between β-1,3-glucanase expression and viral spread. And TMV has also been found to interact with an enzyme involved in de-esterification of pectic homogalacturonan, i.e., pectin methylesterase (PME) (Faulkner et al. 2008). MPs play a crucial role in host specificity for most of the plant viruses. Viral MPs have been shown to interact with products of R-genes to elicit HR response. During the hypersensitivity reaction (HR), callose (β 1–3 glycan) gets deposited in the plasmodesmata blocking the channels and restricting the viral movement from one cell to another. The *Potato virus X* (PVX) produces a protein interacting with

β-1,3-glucanase (callose-degrading enzyme) facilitating the movement of PVX through the plasmodesmata (Fridborg et al. 2003). Constitutive or tissue-specific expression of viral MPs may trigger typical viral infection symptoms such as abnormal sugar accumulation, diminished photosynthesis, chlorosis, and dwarfism. One of the most important strategies is blocking cell-to-cell or long-distance movement of the virus. It has been reported that a single-nucleotide substitution in MP gene which enhanced the viral movement efficiency of tymovirus led to greater viral accumulation and also increased severity of symptoms. Additionally, in TAV cucumovirus, the different levels of expression of MP can determine the difference in severity of symptoms between two virus strains (Moreno et al. 1997).

A coordinated action of host factors and virus-encoded movement proteins (MPs) is required for cell-to-cell movement of viruses through PD. More than ten host factors in addition to these cell wall enzymes have been found to be involved in intercellular movement of different viruses (Heinlein 2015).

Interaction of plant virus MPs has also been reported with the host protein promoting viral movement. These proteins can be localized in the nucleus, microtubules, and also plasma membrane which may be required for intra- or intercellular virus movement and affect the symptom development (Table 3.3).

Transgenic plants overexpressing these proteins have been used to understand the involvement of MPs in viral pathogenesis. These proteins interfere with the cytoplasmic communication channels as transgenically expressed MPs are located in the plasmodesmata, hence increasing the size exclusion limit of plasmodesmata further triggering alteration in metabolism and distribution of carbohydrates (Pallas et al. 2011).

Another such host apparatus is the cytoskeleton and its components, which further facilitate viral transport through plasmodesmata. It may act together with the endomembrane system of the host cells. To deliver viral MPs, vRNPs, and virions

Table 3.3 Plant viral movement proteins

Virus-interacting protein	Host protein	Role in viral movement	References
Pvx-tgb2	Tip I	Cell-to-cell movement	Fridborg et al. (2003)
TMV-MP	Calreticulin	Regulate cell-to-cell movement	Chen et al. (2005)
	ANK		Ueki et al. (2010)
	PME		Dorokhov et al. (1999)
PMTV-TGB-2	RME-8 family	Intracellular and intercellular movement	Haupt et al. (2005)
GRV-MP	Fibrillarin	Vascular transport intracellular movement – nucleus	Kim et al. (2007a, b)
Geminivirus – NSP	Protein kinase		
CaMV-MP	MPI-7	Susceptibility – cytoplasm	Huang et al. (2001)

to PD, viruses must hijack the endomembrane system, early secretory pathways, and/or the cytoskeleton network along with its motor proteins. The various viral MPs are transported via the ER to plasmodesmata, and actin/myosin filaments control the flow of proteins in ER membrane. In TMV, microfilaments assist in the cell-to-cell movement of virus, and microtubule-associated proteins degrade the viral movement proteins (Kragler et al. 2003). Calreticulin is a chaperone protein localized in the lumen of ER which helps in protein degradation via the proteasome. The overexpression of calreticulin in transgenic plants redirects *Tobacco mosaic virus* to microtubules from plasmodesmata, and cell-to-cell transport of virus is compromised (Chen et al. 2005).

Viral intercellular movement may be summarized into four major types on the basis of the characteristics of CP involvement and MP behavior:

1. The first type is illustrated through tobamovirus, bromovirus, and cucumovirus. The movement of viruses is in the form of the viral RNA-MP complex (vRNP). In the case of bromovirus and cucumovirus, the viral movement requires CP but is independent of the virion. Single dedicated MP of TMV however increases the size exclusion limit of PD for CP-independent viral RNP movement between cells.
2. Rod-shaped viruses such as hordeivirus-like viruses and potexviruses illustrate the second type. The three TGB (triple gene block) proteins encoded by these viruses plus CP coordinate the intercellular transport of vRNPs (for hordeivirus-like viruses) and virions or vRNPs (for potexviruses).
3. The third type including comovirus and nepovirus transverses and modifies the PD and is in the form of a virion guided by the MP-assembled tubules.
4. The last type illustrated by potyviruses assists the passage of virion by the formation of cone-shaped and not tubular structures at the PD.

3.6.2 Viral Long-Distance Movement

The virus moves from the mesophyll via bundle sheath cells, phloem parenchyma, and companion cells into phloem sieve elements (SEs) for long-distance or phloem-dependent movement. Following the source-to-sink flow of photoassimilates, the virus is then passively transported and unloaded from SEs to sink tissues at distant sites. It then results in further infection. PME seems to affect long-distance movement as well. In a PME-silenced tobacco line, there is a substantial delay in viral systemic transport. This happens because TMV movement out of the vascular system is blocked because of the significant degree of pectin methylesterase (PME) suppression in the vascular tissues (Chen MH and Citovsky 2003). However, the exact roles and underlying mechanisms of the host factors in viral long-distance transport are still far from understanding.

3.7 Responses of Plants to Viruses

Plant–virus interplay where the virus tries to take over plant resources for its multiplication and then counteracts the antiviral defenses raised by the plant often leads to manifestation of disease symptoms. The cross talk among different hormones governing the various pathways in antiviral defense is also disturbed through virus infection (Pallas and García 2011; Nagy and Pogany 2012). Thus the most common symptom of plant virus infections appears to the developmental abnormalities resulting from such disturbances. Different subcellular organelles, cells, and organs when adapting to the new requirements of virus replication and movement can interfere with normal plant homeostasis and therefore lead to disease symptoms. However, sometimes interactions among definite viral and host factors can lead to specific symptoms through viral infection as illustrated in the case of HR-related necrosis elicited by discrete viral elements or misregulation of auxin response factor 8 caused by several viral silencing suppressors leading to developmental defects. Still the interactions between numerous viral and host factors conditioned by multiple environmental conditions can lead to much more complex manifestations of many plant virus infections. Unique experimental designs based on systems biology and the development of powerful technological tools in the coming years are considered to open new chapters in understanding the molecular basis of these plant virus pathogenicity relationships. On viral attack, plant responses can be broadly classified into two categories: (a) cellular stress and (b) developmental defects. The changes in plant gene expression profiles due to viral infection are very much like the defense and stress responses. There may be induction of heat shock proteins (HSPs) during stress-like responses and pathogenesis-related (PR) genes under defense-like responses.

3.7.1 Cellular Stress

PR genes are the defense-related genes like PR-1, β-1 glucanase (PR-2), chitinase (PR-3) PR-4, thaumatin-like protein (PR-5), superoxide dismutase (SOD), and glutathione S-transferases (GT). Some other genes are also co-induced with the PR genes. For example, it has been observed that in *Arabidopsis thaliana* and *Nicotiana benthamiana*, few genes of WRKY family of transcription factors also get induced during viral infection.

Under stress conditions and also in normal growth and development, HSPs get induced. Their expressions also get induced by DNA and RNA viruses in the host plants. The first report of HSP70 induction in response to viral pathogen in pea embryos was demonstrated by in situ hybridization (Aranda et al. 1996). Further evidence was provided in *Arabidopsis* in response to different RNA viruses (Whitham et al. 2006) (Fig. 3.1).

3.7.2 Development Defects

Some of the genes of the host plants found to express themselves on viral infection may have a connection with the developmental defects and symptoms of disease. Viruses interfere in the signaling pathways and hence affect plant growth and development. The abnormal growth forms of virus-infected plants have led to the experiments revealing effects of viruses on hormone levels in host plants. Depending on host–virus combination, auxin, cytokinin, gibberellin, ethylene, and abscisic acid levels get altered on viral attack. For TMV and RDV (*Rice dwarf virus*), a link between auxin–gibberellin levels has been well established (Jameson 2000). The interaction of helicase domain of TMV 126 and 183 kDa replicase protein with IAA transcription factor (IAA26) was established (Padmanabhan et al. 2005). Silencing of IAA 26 resulted in TMV-infected plant phenotypes directing the loss of function of IAA 26 by TMV replicase. The P2 protein of RDV also affects gibberellic acid signaling (Zhu et al. 2005).

The effects of viral RNA silencing suppressors (RSS) on host gene expression cannot be neglected as they promote viral infections by interfering with host defense mechanisms and also regulatory miRNAs and trans-acting small interfering RNAs ultimately leading to developmental defects in host plants (Dunoyer and Voinnet 2005). To counter the defense system of plants, viruses have acquired a strategy by disruption of host antiviral silencing.

3.7.3 Abnormalities in Chloroplast

After studying viruses from around 12 families covering major genera and those responsible for devastating disease (having either sense ssRNA, antisense ssRNA, or ssDNA genomes), it has been confirmed that chloroplast abnormality is a common event across diverse plant–virus interactions. Chloroplast has been implicated as a common target of plant viruses for a long time. Typical photosynthesis-related symptoms, such as chlorosis and mosaic pattern, cause disruption in normal chloroplast function although the causes leading to development of viral symptoms are different (Rahoutei et al. 2000). For example, infection by CMV in *Nicotiana tabacum* cv. Xanthi associated with fewer grana and reduced chloroplasts led to severe chlorosis on systemic leaves (Roberts and Wood 1982). In accordance with the prevailing studies, the two main causes of virus-induced chloroplast symptomatology include ultrastructural alteration of chloroplast and the reduced abundance of proteins involved in photosynthesis. Typical chloroplast malformations include:

1. Overall decrease in the number and clustering of chloroplasts
2. Change in the appearance of chloroplast, such as swollen or globule or amoeboid-shaped chloroplast, chloroplast with membrane-bound extrusions, or chloroplast with a dynamic tubular extensions from chloroplast known as stromule
3. Broken envelope and irregular out-membrane structures such as peripheral vesicle, cytoplasmic invagination, and membrane proliferations

4. Any change in the content of the chloroplast such as large intermembranous sac, increase in the number and size of electron-dense granules/plastoglobules/bodies, enlarged or numerous starch grains, and small vesicles or vacuoles in stroma
5. Absence of stroma or grana stacks and distorted, loosened, or dilated thylakoid
6. Completely destroyed chloroplasts and disorganized grana scattered in the cytoplasm (Zhao et al. 2016)

Recently carried out studies reveal that chloroplast ultrastructure and symptom development are affected by viral factors, especially CPs, as shown in the work of Neeleman et al. (1991).

On comparison with healthy plants, virus-infected plant cells were seen to contain reduced amounts of chlorophyll–protein complex. No significant differences were observed in the photosystem I (PSI) reaction center upon TMV infection, but an inhibition of photosystem II (PS II) activity was observed by selectively decomposing the light-harvesting antenna complex of photosystem II. Likewise, among the stroma proteins, chlorotic tissues of *Cucumber mosaic virus* (CMV)-infected plants observed a loss in the activity of the small subunit of the enzyme ribulose-1,5-biphosphate-carboxylase-oxygenase (RuBisCO). A significant increase was measured in the enzyme activity of chlorophyllase and catalase vis-a-vis decrease in the chlorophyll content. This could be due to release of the enzyme chlorophyllase bound to the chloroplast inner membrane after the disorganization in the chloroplast. Rarely does virus replication occur on chloroplast, but still the products of viral infection like coat proteins are found to inhibit the photosynthetic activity. The interaction of CP with chloroplast might affect chloroplast function and stability, which gives rise to chlorosis. Similar to replicases and silencing suppressors, viral CPs have been recently shown to interfere with/modulate hormone signaling pathways.

Transcriptomic and proteomic analysis of expression of chloroplast proteins upon virus infection shall provide an insight into the molecular events during symptom expression. For example, in response to virus infection in susceptible plants, a majority of significantly changed proteins are identified to be located in chloroplasts or associated with chloroplast membranes. Most of them correlate with severity of chlorosis and are seen to be downregulated (Dardick 2007).

3.8 Viral Infection and Physiological Functioning of Host Plants

A successful infection by plant virus results from molecular network of interactions during viral infection process. Therefore understanding this complex molecular interplay between the host plant and the invading virus may assist in the development of novel antiviral strategies. Theoretically, viral infection in plants can be divided into several major steps including viral particle disassembly, viral genome translation (in case of + ssRNA viruses), cellular membrane modifications coupled with formation of VRC (viral replication complex), viral genome encapsidation,

cell-to-cell movement, and long-distance transport. Viruses are thus evolutionarily empowered with an ability to take over the host cellular pathways and manipulate the cellular components. Additionally, diverse host-encoded proteins or host factors are also present at each of these steps (Wang and Krishnaswamy 2012).

3.8.1 Virion Disassembly and Viral Genome Translation

3.8.1.1 Host Factors in Virion Decoating

Positive-sense RNA viruses do not encapsidate the virally encoded RNA-dependent RNA polymerase (RdRp; an absolute requirement for viral genome replication) in their virions. Physical barrier is a major bottleneck that plant viruses have to overcome to spread from cell to cell for the subsequent systemic invasion of their host. Thus, plant wounding is an obligatory condition for virus entry. Mechanical damage to the CW provides the virus with an opportunity to enter the cell. As soon as the virus gains entry into the plant cell, the first step toward a successful infection is the removal of viral shell or capsid (made of capsid protein subunits) so that the viral genome is exposed to cellular translation machinery to begin viral genome translation. Although viral particles are highly stable and can survive for extended periods under the harsh extracellular environment, there remains a poor understanding on how the stable virions disassemble in the environmentally friendly symplast of their host. Decoating is a passive process triggered by the change of pH and positively charged cation concentration in the cell as suggested in some of the early studies on TMV. This thus leads to removal of few CP subunits from 5′ end of the encapsidated RNA (Culver 2002) and thus leading to initiation of virion disassembly. Thus, an important, if not universal, strategy for plant positive-sense RNA viruses is to disassemble the cotranslational mechanism. In order to prevent the activation of host antiviral mechanisms (like RNA silencing triggered by ds RNA intermediates), to provide a scaffold for tethering the VRC, and to restrict the process of viral replication to a specific safe cytoplasmic site, viral replication is associated with the virus-induced intracellular membranous structures. The origins of such membranous structures are diverse and dependent on the type of virus (Laliberte and Sanfacon 2010). Example, tobamoviruses and *Tomato mosaic virus* (ToMV) recruit and modify the ER membrane for their genome replication in plants.

3.8.2 Viral Replication Complex Composition

All VRC components (in addition to remodeling of cellular membranes by viral proteins and co-opted host factors) must be present together at the site of replication to form a functional unit for catalysis of viral replication. The following constitutes the VRC present in the modified cellular membranes:

- Viral replicase proteins, such as RdRp and viral helicase
- A viral RNA template
- Diverse host proteins like heat shock proteins (HSPs) and RNA-binding proteins (RBPs) (Verchot 2012)

3.8.2.1 Host Factors in Viral Genome Translation

Viruses have evolved diverse strategies to recruit the host translation apparatus for quick and efficient translation of the viral genome because they lack functional ribosomes and their genomes do not encode other translation-required components. Translation is known to be a tightly regulated process including several phases, i.e., initiation, elongation, termination, and ribosome recycling. Wang and Krishnaswamy (2012) have discussed the possible mechanistic roles of eIF4E and its isoforms (best characterized host factors for plant potyviruses) in potyviral infection. Because the viral genome translation and replication of positive-sense RNA viruses are intimately linked by sharing the same site and the same template (often considered to be a coupled process), it is highly possible that these translation factors may contribute to viral genome multiplication (Kawamura-Nagaya et al. 2014).

3.8.2.2 Regulation of Viral Replication by Viral Protein Modifications

The functional diversity of the plants can be increased by posttranslational modifications (PTMs) of viral proteins in the infected cells. Various PTMs have been documented till date to affect viral replication in plants. Examples include phosphorylation (the most common and reversible protein phosphorylation which seems to negatively regulate viral replication), ubiquitination, SUMOylation, and other PTMS (like methylation which modifies eEF1A and thus affects tombusvirus replication) which have also been shown to affect viral replication (Li et al. 2014).

Viruses not only infect and damage the crops, but weed plants are also seriously infected. Keeping in view the considerable losses and destruction of crop plants, earlier investigations were conducted only on crop plants. But later it was observed that weeds also play an important role in infecting and spreading viral diseases and not just compete with crop plants for nutritional benefits. Thus, biological decline of weeds is considered to be an important strategy for promoting crop production, and the level of weeds is therefore maintained under economic threshold to sustain agriculture. Viruses can directly contribute to weed control by reducing their competitive ability. The investigations done earlier were focusing more on physiology and biochemistry of virus-infected crops although viruses can damage both crops & weeds and weed–virus interaction was almost not focused upon. The various physiological effects of viruses to crop plants and weeds reduce their growth and development. There are certain reports such as in *Datura stramonium*, where a reduction in photosynthetic pigment content (Chl-b) was detected in the leaves infected with CMV (*Cucumber mosaic virus*) and HeMV (*Henbane mosaic virus*). Depending upon the host–virus relationships, there was 30–80% reduction in shoot dry weight of test plants. Virus infections do have an important effect on germination characteristics of weeds. Viral infection influenced seed dormancy rather than viability of seeds in *Solanum nigrum* plants infected with Obpv (*Obuda pepper virus*) and PepMV (*Pepino mosaic virus*), whereas it was reverse in the case of chenopodium plants infected with SoMV. Considerable effect of viral infection on the nutrient uptake of

plants was observed in *S. nigrum* plants infected with TMV. Seed production is also reduced by TMV infection in *S. nigrum* plants (Takacs et al. 2014). Weeds can even function as sources of primary infection in the spreading of plant diseases and overwintering of plant viruses apart from its role as direct competitors for nutrients, light, and water. Weeds can also function as host plants of virus vectors. Therefore, in sustainable agricultural practice, the level of weeds is kept under economic threshold, and considerable efforts are taken to maintain biological diversity.

3.8.2.3 Interference of Viral and Plant Factors with Hormone Regulation

Hormones are those signal molecules which move around the plant to stimulate responses to different environmental stresses. Hormones like salicylic acid (SA), jasmonic acid (JA), and ethylene (Et) have long been known for their roles in tuning plant responses to biotic stresses. However, the other hormones known for their roles in plant growth and development like auxins (Auxs), brassinosteroids (BRs), cytokinins (CKs), and abscisic acid (ABA) are also known to be involved in plant–pathogen interactions (Denance et al. 2013). Certain hormones can prevail over others under specific circumstances by showing antagonistic or synergistic interrelations. For example, SA, JA, and Et, regulating defense pathways, exhibit antagonistic interactions.

On recognition of viral effectors by *R*-gene products, SA biosynthesis and signaling are activated which conditions incompatible interaction. It activates SAR in distal tissues. To limit viral propagation at the infection site, activation of the incompatible reaction occurs resulting in several responses like callose deposition, tissue disorganization, nuclear and nucleolar degradation, alterations in the shape and size of chloroplasts, accumulation of reactive oxygen species (ROS) and pathogenesis-related (PR) proteins, induction of the hypersensitive response (HR), and programmed cell death (PCD). Several examples of incompatible plant–virus interactions exist. For example, a parallel and significant increase in the SA and expression of PR genes is observed in the inoculated and systemic leaves of resistant tobacco plants after infection with *Tobacco mosaic virus* (TMV) (Baebler et al. 2014).

ABA appears to have multiple roles against the pathogens depending on the stage of infection in addition to its antagonistic roles in defense hormone pathways such as SA and JA/Et. In cases where pathogen overcomes the first line of defense, ABA helps in regulating plant defense at the early stages of infection by the mediation of stomatal closure against invaders or induction of callose deposition. But, if activated at later stages, ABA can suppress ROS induction and SA or JA signaling transduction, thereby negating defenses controlled by these two pathways (Ton et al. 2009). Although the involvement of ABA in biotic stress has been studied thoroughly, the roles of ABA in virus replication and movement are not well characterized. The involvement of ABA in virus interaction was first studied in the context of the effect of TMV on ABA accumulation in *N. tabacum* and tomato, which revealed that ABA increases callose deposition and limits virus movement (Fraser and Whenham 1989). The defensive role of ABA against viruses is mediated through inhibition of the basic β-1,3-glucanase which is responsible for the degradation of

β-1,3-glucan (callose). Subsequently *β*-1,3-glucan (callose) is deposited on plasmodesmata which strengthens them against virus movement (Mauch-Mani and Mauch 2005). ABA also affects plant defenses at the level of the RNA silencing machinery, which is considered to be a broader defense system against viruses. For example, ABA seems to partially control *ARGONAUTE1* (*AGO1*) levels and thus have direct and indirect links with this system.

3.8.2.4 Interference of Viral and Plant Factors with Cell Cycle and Gene Expression

Few viruses infect the host cells, which are dividing actively. As most of the plant cells do not divide actively, viruses develop mechanisms involved in alteration of host cell cycle. For example, Rep protein of geminiviruses interacts with a retinoblastoma-related protein family pRBR, involved in negative regulation of cell cycle. This interaction inhibits the activity of pRBR protein, cell enters S-phase, and host DNA replication machinery is produced which is required to reproduce the virus (Kong et al. 2000).

Plant viruses can also reprogram host gene expression just like the animal viruses. The evidence came from the reports of Havelda et al. (2008), where a connection between reprogramming of host gene expression and pathogenicity of viruses was provided. A correlation between the interruption of cellular gene expression (switch off mechanism) and viral symptom development was provided.

3.8.2.5 Effect of 5′ and 3′ NCRs (Noncoding Regions) on Viral Pathogen

The viral translation and replication process are influenced by NCR sequences (5′ NCR and 3′ NCR) of viral RNA. There was reduction in viral RNA replication along with effects on symptom development where 5′ and 3′ NCRs were altered. A correlation was observed between the viral movement and alteration in 5′ NCR conditioning the viral pathogenicity (Petty et al. 1990). When a single nucleotide from ORF near the 5′ end of RNA in hordeivirus barley stripe mosaic virus was changed, there was prevention of viral movement encoded by immediately adjacent gene via negative regulation of synthesis of viral replicase.

The presence of 4 repetitions of 14 nucleotide sequence in 3′ NCR of TVMV (polyvirus *Tobacco vein mottling virus*) reduces the gravity of symptoms of disease without affecting viral accumulation.

3.9 Defense Responses in Plants and Viruses

Although viruses are relatively simple genetic entities, resistance molecular mechanisms and viral diseases susceptibility are still not fully comprehended and understood. Several mechanisms exist in plants for disease resistance against virus infections, but it is very difficult to explain them for various pathosystems

separately (Brown 2015). An understanding of plant–virus interactions and molecular mechanisms of these interactions has been achieved through the unveiling of several model bacteria–plant systems. Gene theory proposed in the early 1970s has served as a model for many years, explaining how disease resistance is turned on against pathogens (Keen 1990). According to this theory, a single resistance gene (R-gene) encoded by the host recognizes the presence of avirulence (Avr) proteins and triggers a hypersensitive response of resistance (HR) leading toward rapid cell death. The most general R-gene types can be classified either as genes encoding protein nucleotide-binding leucine-rich repeat (NB-LRR) or genes encoding receptor-like kinase/receptor-like proteins (Rathjen and Moffett 2003). About a decade later, another model was proposed known as zigzag model (Jones and Dangl 2006). There are two distinct defense responses for the plant defense system in the zigzag model. The primary defense level is called PAMP/MAMP-triggered immunity (PTI), and the secondary defense level is called effector-triggered immunity (ETI). A basic defense mechanism presented by PTI is preventing invasion of the pathogen through cell wall thickening in response to specific structures or pathogen-associated proteins so-called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). Plants show susceptibility only when a pathogen successfully establishes both PTI response suppression and its pathogenic effector's facilitation. ETI, the second defense response level, is triggered when the products of R-gene directly or indirectly sense the presence of specific effectors called as Avr factors. Consequently, an effective ETI will keep the plants resistant; however, an insufficient ETI will lead to the establishment of disease, i.e., the susceptibility of the plant. Models of general resistance of most pathogens don't fit well with viral resistance because of intracellular parasitic nature of virus. For example, receptors of pattern recognition which serve as a component of major defense by triggering the first layer of resistance when a receptor of plasma membrane perceives a fungal or bacterial MAMP or PAMP (Tena et al. 2011) cannot play a role in plant viruses fighting because viruses do not express extracellular PAMPs. Pathogens, however, have evolved counter measures, such as delivering effector proteins into the plant cell to suppress host PTI. The defense and counter-defense between host and pathogen never end. Plants, in turn, have acquired resistance (R) proteins to recognize these pathogen effectors and trigger the ETI, a more robust and specific response. Plant viruses are, thus, both trigger and target of RNA silencing. This reinforces the concept that RNA silencing evolved as an antiviral defense mechanism and highlights the arms race between plants and plant viruses (Chen 2010). The major strategies developed by plants to counteract virus infections are:

Resistance (R) gene mediated
RNA silencing-based defense
Recessive gene-mediated defense

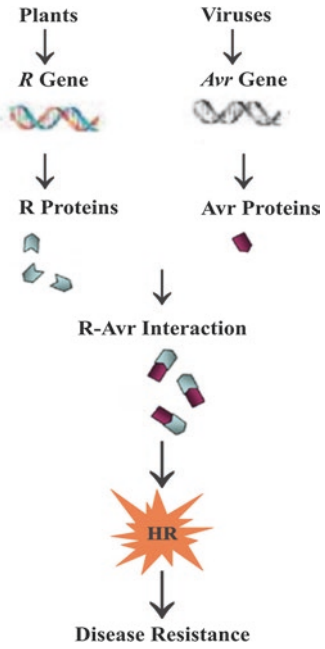

Fig. 3.3 A simplified representation of R-gene-mediated resistance in plants. R-genes (receptors) are expressed in plants which can bind to Avr gene products (elicitor protein) of the pathogen resulting in a cascade of transduction signals developed by receptor–ligand complex

3.9.1 Resistance (R) Gene-Mediated Response

More often it has been observed that viral disease symptoms are caused by toxic effect of some viral components. R-gene-mediated resistance which is the most intensively explored form of resistance toward the diverse bacteria, fungi, and viruses is frequently HR responsible and is an effective way to gain resistance against plant viruses. HR is the most common defense response to the viruses. R-genes (receptors) are expressed in the plant which can bind to the Avr gene products (elicitor protein) of the pathogen. HR response is an outcome of a cascade of transduction signals developed by receptor–ligand complex (Fig. 3.3). As the R-genes restrict the pathogen to the inoculated area, its spread to the entire plant is obstructed. The first viral R-gene to be cloned and characterized was from *Nicotiana* and *Tobacco mosaic virus* (TMV) is the first virus discovered and isolated from *Nicotiana glutinosa*, and its counterpart R-gene served as a model for studying HR-based resistance, systemic acquired resistance (SAR), and gene-for-gene theory (Holmes 1929). R-gene product RX_1 interacts with the capsid protein of *Potato virus X* to cause programmed cell death at the infection site. At the same time, R-gene can block virus replication before the generation of sufficient amount of capsid protein to cause necrosis. In some instances, it doesn't impede the viral spread throughout the plant, but systemic necrotic spots are generated. HR may be induced by any viral gene other than CP also which may or may not be capable of

impeding viral propagation. The cytoplasmic inclusion RNA helicase from *Soybean mosaic virus* (SMV) and RSV3 gene from *Glycine max* (Zhang et al. 2009) and the interaction between p50 helicase domain of TMV replicase and N gene from *Nicotiana glutinosa* (Padgett et al. 1997) are the examples of HR inducers being capable of hindering the virus propagation.

There are reports of the other type also where viral gene product inducing HR may not be capable of restricting viral propagation. Systemic necrosis of *Arabidopsis thaliana* is caused by TuMV (*Turnip mosaic virus*), which is the result of gene interaction between P_3 encoding region of virus and TuNI resistance gene.

3.9.2 Role of Volatile Organic Compounds in Plant Defense

The plant defense system includes the organization of communication between plants and between different parts of the same plant. VOCs emitted by a damaged plant act on the plant's own leaves and on the organs of neighboring plants, modifying intercellular communication. In the absence of physical contact, they use volatile organic compounds (VOCs) for communication. Theoretically, three responses are possible in a neighboring plant upon the release of VOCs as a signal of damage. First situation could be when there is no response by the neighboring plant. Secondly, plant could respond by creating conditions that promote plant–virus interaction (increasing sensitivity of plant, its sensitization). Finally, the plant can create conditions that prevent virus infection by increasing its antiviral resistance. Thus, we cannot define a negative or a neutral influence of VOCs in plant–virus relationships. Plants release terpenes, fatty acid derivatives, benzenoids, phenylpropanoids, and amino acid-derived metabolites. Many of these products are made more lipophilic before their release into the air by the removal or masking of hydrophilic functional groups through reduction, methylation, or acylation reactions (Pichersky et al. 2006). VOC-mediated signals are transmitted to neighboring plants promoting the survival of entire community. Such VOC-mediated signals protect plants not only against insects, bacteria, fungi, and nematodes but also viral pathogens.

We currently have a fairly complete understanding of the processes and metabolic pathways involved in the production of many VOCs, but we have an extremely limited understanding of how VOCs affect intercellular traffic and thus what impact VOCs have on the plant virus–host interaction. This modification of intercellular transport has a significant effect on pathogenic infections. The VOC-mediated process of preparing plants for a putative attack can be referred to as "priming" (i.e., the initiation of reactions before the impact of the pathogen). In general, the process of priming is related to plant immunity, whereby plants trigger their defenses in response to a signal or previous challenge so that they can react with increased severity (Holopainen and Blande 2012). However, when we consider the role of priming in the relationship of the virus and the plant host, the picture is ambiguous.

Because the life cycle of viruses include the intercellular transport, cell-to-cell movement of viral genome, and also long-distance spread throughout the plant,

therefore, unlike bacteria, fungi, oomycetes, and nematodes, viruses depend more on the state of intercellular transport, and their life cycle is completed in the symplast (Xu and Jackson 2010).

Volatile communication plays an important role in mediating the interactions between plants, aphids, and viruses in the environment. Thus, VOCs can also influence the virus–host–vector combination apart from regulating the life cycle of viruses. Because the plant cell is bounded by a rigid cell wall (preventing direct contact between adjacent cells and virus particles), there is no evidence of the participation of constitutively emitted VOCs in the innate immunity of plants to viruses.

Transgenic plants with increased VOC emission may be used as disinfectants to trigger and enhance a protective response against pathogens and plant-eating insects (Holopainen and Blande 2012).

3.9.3 RNA Silencing-Based Defense

In 1990, a defense mechanism was described where silencing of viral RNA was done as viruses are capable of overcoming complex defense barriers developed by host plants. This genetic surveillance system seems to be conserved in most eukaryotic organisms ranging from fission yeast to human beings. It is also known as RNA interference (RNAi) in animals and posttranscriptional gene silencing (PTGS) in plants (Carrington 2000). Intracellular presence of dsRNA triggers this system which ultimately leads to downregulation of the expression of genes that share substantial sequence homologies with this dsRNA trigger.

The silencing-induced dsRNAs are perceived by the RNA silencing machinery and then acted upon by a dsRNA-specific nuclease designated Dicer or Dicer-like (DCL) nuclease in plants (Fig. 3.4). It results into the formation of short RNA duplexes of 21–25 nucleotides known as small interfering RNAs or siRNAs (Hamilton and Baulcombe 1999). They function as key specific determinants of RNA silencing. Another nuclease, referred to as argonaute (AGO), recruits one strand of the siRNA duplex and guides it to ssRNAs containing complementary sequences to the siRNAs. siRNAs also help in amplifying the silencing process with the help of RNA-dependent RNA polymerases (RDRps). siRNA-complementary ssRNAs act as templates to synthesize more of dsRNAs, which are again processed by DCLs, and thus leading to an amplification in silencing process. Additionally a family of dsRNA-binding proteins (DRBs) having abundant differences in their structures and functions have been reported as partners of DCLs at various steps of RNA silencing cascade. As the function of RNA silencing is defense of host cells against virus invasion, few DRBs play an important role in antiviral defense not only in animals but also in plants. DRB_4 has been involved in defense against a large number of viruses in *Arabidopsis* (Qu et al. 2008). Thus four different families of proteins primarily constitute the plant RNA silencing machinery: DCLs, DRBs, AGOs, and RDRps.

The best understood RNA silencing pathway in plants is probably the *microRNA* (*miRNA*) pathway. In plants, an *MIR* gene is first transcribed to primary miRNAs

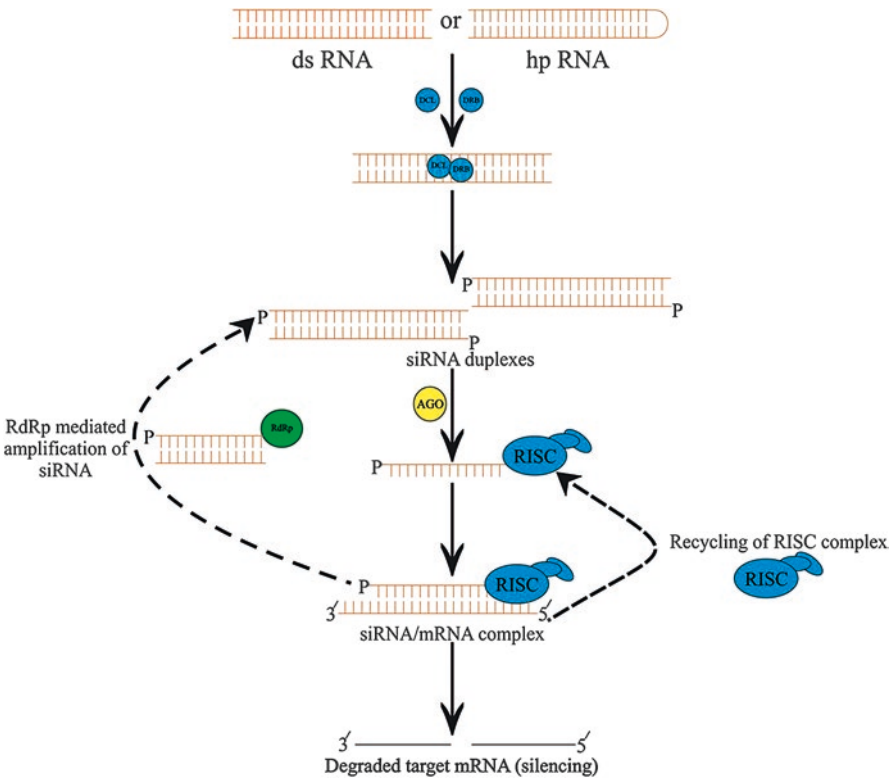

Fig. 3.4 An overview of RNA silencing cascade in plants. Both long dsRNA and hairpin RNA with a significant length of double-stranded region (hpRNA) can be processed by DCL/DRB complex into siRNA duplexes. This in turn mediates degradation of ssRNAs with the help of AGO and RISC complex. Additionally, siRNA could also serve as primers to prime the synthesis of new dsRNAs

(pri-miRNA) by DNA-dependent RNA polymerase II; pri-miRNAs form partially double-stranded hairpins through extensive intramolecular base pairs. These pri-miRNAs are processed by RNase III-like Dicer-like I endonuclease (DCL1) to generate miRNA precursors (pre-miRNAs). These are then sequentially processed by DCL1 to produce miRNA/miRNA duplex. The duplex moves to the cytoplasm, and mature miRNA (20–22 nucleotides) is selectively introduced into RISCs associated with AGO1. miRNA-programmed RISCs are directed to mRNAs with complementary sequences to mediate cleavage as well as translational repression and thus inhibit gene expression. RNA silencing serves as a major component in the antiviral defense mechanism; however, the strategy of R-gene-mediated resistance is effective against viruses as well as other phytopathogens (Nakahara and Masuta 2014).

3.9.4 Recessive Gene-Mediated Defense

Viruses being intracellular parasites are exclusively dependent on host mechanisms for their life cycle. After entering a plant cell, viral genome is released, and early viral proteins are translated. The virus also takes over some of the host functions. Because of limited number of genes coded by viral genome, a number of host factors are required to continue the viral cycle. Any alteration in the host factor or its absence can be an efficient strategy for plant defense and can be considered as a passive form of resistance. Such resistance mechanism has been frequently shown to be recessively inherited. More than half of plant virus resistances are recessively inherited, and many are still to be characterized. In the case of recessive inheritance resistance, the majority of genes have been identified in plant–virus pathosystems, and several recessive resistance genes have been characterized in bacterial and fungal pathogens research including *xa5* (a *Xanthomonas*-resistant gene in rice) and *mlo* (resistance gene for powdery mildew in barley). Much of the identified R-genes confer resistance to various potyviruses. Recessive r-genes conferring resistance of potyvirus have been identified and deployed for decades in various crops. For example, an essential host factor required for virus infection is translation factor of eukaryotes 4E (eIF4E) which plays a major role in host translation initiation by recruiting the ribosomal complex. Thus, natural variations in eIF4E provide an effective resistance against potyvirus infection in multiple crop species by preventing sequestration of virus. Thus, alteration in translation initiation factors (host factors) is a common strategy for developing viral resistance in plants (e.g., sbm1 in pea seed-borne mosaic virus of *Pisum sativum* and pot1 in PVY of *Solanum lycopersicum*). Another example of recessive resistance in tomato is the recently characterized ty5 which encodes messenger RNA surveillance factor pelo and confers resistance in *Tomato yellow leaf curl virus* (TYLCV). Pelo leads to impaired functionalities in protein synthesis and ribosome recycling phases which triggers viral infection suppression in resistant ty5 genotypes.

3.10 Virus Counter Defense Mechanism

Virus pathogenesis not only induces a resistance mechanism in the host but also suppresses it. Almost all plant viruses have developed a viral-encoded silencing suppressor-mediated counterdefense mechanism inhibiting RNA silencing at different steps. HC-Pro protein of potyviruses was the first identified silencing suppressor interfering with RNA silencing mechanism. The inactive forms of miRNAs (microRNAs) get accumulated on ectopic expression of HC-Pro protein which have negative regulatory function. HC-Pro also performs long-distance movement of viruses and also enhances pathogenicity of viruses (Saenz et al. 2002). To counteract the antiviral RNA silencing, most plant viruses have evolved silencing suppressor proteins that block one or more steps in the RNA silencing pathway.

3.11 Engineering Crops for Resistance Against Viruses

Geminiviruses are spread worldwide due to climate change, increasing insect vector population changes in crop cultivation, and changes in agricultural practices causing significant crop loss globally. Many of these crops are staple foods in tropical and subtropical regions; therefore developing resistance against geminiviruses is important socially and economically. The strategies used against geminiviruses include eradicating the vectors by using insecticides, but most of the times, the virus has already been transmitted to the plant even before the vector is killed. Also insecticides are extremely toxic and have adverse effects on the environment. Therefore, environment-friendly agricultural practices are to be raised. These aspects necessitate the development of natural resistance through genetically engineered crops providing more durable resistance to geminiviruses. The objective is to develop varieties by introducing selected nucleic acid sequences into plants either from the pathogen itself (pathogen-derived resistance, PDR) or not from the pathogen (non-pathogen-derived resistance) (Khan et al. 2014). Transgenic papaya cultivars were developed in 1998 against *Papaya ring spot virus* (PRSV) which is an RNA virus saving papaya industry from severe destruction in Hawaii. After herbicide- and insect-resistant crops, the third important transgenic crop having resistance against a virus is the RNA resistance papaya which is grown commercially. Currently, transgenic resistance is the most important research area to provide protection to plants against a large number of viruses.

3.12 Future Prospects

Viruses are obligate parasites infecting only living cells. The use of resistant cultivars is an effective strategy for reducing crop loss due to viral infection. Counter defense mechanism by virus encoding silencing suppressors gives rise to a battle between host and virus; ultimately variable symptoms are produced due to evolution of viral strains. The global transcriptomic and proteomic analyses have helped us to understand the plant–virus interaction at molecular level and also to acquire a broad vision of viral pathogens. Identification of host factors has long been one of the few major goals of virology research. The driving force comes from the obvious practical applications for the development of novel antiviral strategies and for beneficial biotechnological uses of viruses. Host factors may be targeted for the development of inheritable recessive genetic resistance in plants through advanced biotechnology, i.e., mutation, silencing, or downregulation of the host factor gene(s) (Wang and Krishnaswamy 2012).The challenge is to learn the influence of environmental factors, plant phenology, mode of viral entry into host, evolutionary adaptations of host and virus, and development of pathological process in the host for producing virus-resistant crops in the forthcoming years.

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Pathogen-Associated Molecular Patterns and Their Perception in Plants

4

Jitendra Singh Rathore and Chaitali Ghosh

Abstract

In plants, innate immunity, the first line of microbial recognition leading to active defense responses, relies on the perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Pattern recognition receptors (PRRs) enable plants to sense non-self molecules exhibited by microbes and raise proper defense responses or establish symbiosis. This recognition leads to PAMP-triggered immunity (PTI). Despite the numerous PAMPs recognized by plants, only a handful of PRRs are characterized. Most of them correspond to the transmembrane proteins with a ligand-binding ectodomain. PRRs interact with additional transmembrane proteins that act as signaling adapters or amplifiers to achieve full functionality. The crucial role of PRRs in antimicrobial immunity is demonstrated by the direct targeting of PRRs and their associated proteins by pathogenic virulence effectors. In recent years the importance of PRR subcellular trafficking to plant immunity has become apparent. PRRs traffic through the endoplasmic reticulum (ER) and the Golgi apparatus to the plasma membrane, where they recognize their cognate ligands. At the plasma membrane, PRRs can be recycled or internalized via endocytic pathways. By using genetic and biochemical tools in combination with bio-imaging, the trafficking pathways and their role in PRR perception of microbial molecules are now being revealed.

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Keywords
Pattern recognition receptor (PRR) · Pathogen associated molecular pattern (MAMPs/PAMPs) · Receptor like kinase (RLK) · Receptor like protein (RLP) · Effector triggered immunity (ETI) · PRR triggered immunity (PTI)

4.1 Introduction

Plants are sessile organisms and are in continuous exposure to various environmental signals. The perception of environmental signals and the ability to respond accordingly are essential for plants to survive. To defend themselves against a plethora of pathogenic microbes or pests, plants relies only on innate immunity as they lack specialized immune cells. The plant innate immunity has a two-tier perception system (Dodds and Rathjen 2010). The first layer of defense is mediated by surface-localized pattern recognition receptors (PRRs), leading to PRR-triggered immunity (PTI). PTI relies on the perception of the specific molecular patterns, which includes microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) or self molecules (damage-associated molecular pattern – DAMPs), that are released upon pathogen-induced cell damage (Boller and Felix 2009) (Table 4.1). MAMPs/PAMPs are conserved among pathogen and nonpathogenic bacteria; hence they are essential structures. MAMPs/PAMPs are recognized in plants by pattern recognition receptors (PRRs). These receptors are localized on the surface of plant cells, and their induction provides the first line of defense. PRRs are membrane localized and broadly classified as receptor-like kinase (RLK) or receptor-like protein (RLP). RLK and RLP have multidomain architecture, including extracellular ectodomain, transmembrane domain, and cytoplasmic domain. The major difference between RLK and RLP lies in the cytoplasmic domain which is essential for downstream signaling. As kinase is the key function of RLKs, therefore they have a long cytoplasmic domain, responsible for signaling. However, on the other hand, RLPs have a small cytoplasmic domain, and hence they depend upon other cytoplasmic proteins for downstream signaling. To cross the first line of defense, microbes like bacteria have developed a mechanism by which they directly inject effector proteins into the host cells. Type three secretion system (TTSS) in bacteria is the best studied example of this type. To overcome such conditions, plants have developed a second line of defense, which recognizes the effector proteins directly or indirectly through plant-resistant (R) gene product called effector-triggered immunity (ETI) (Jones and Dangl 2006). Most of the R proteins are intracellular immune receptor proteins and interact with effector proteins indirectly.

PTI's early cellular response includes the rapid generation of reactive oxygen species (ROS) and nitrogen oxide (NO), activation of mitogen-activated kinases, expression of immune-related genes, alteration in cell wall architecture and synthesis of pathogenesis-related (PR) protein and antimicrobial compounds. ROS and NO have antimicrobial effect, whereas NO is responsible for the cross-linking of the

Table 4.1 Pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and nematode-associated molecular patterns (NAMPs)

Name	Corresponding plant receptor (PRR)	References
PAMPs		
Beta-glycan (GE)	GEBP (putative receptor soybean)	Umemoto et al. (1997)
Flagellin	FLS2 (*Arabidopsis*)	Felix et al. (1999) and Gómez-gómez et al. (2001)
Lipopolysaccharide (LPS)	Not identified	Newman et al. (1995)
Chitin	CeBip and CERK1 (rice): AtCERK1 (*Arabidopsis*)	Kaku et al. (2006), Miya et al. (2007), and Shimizu et al. (2010)
Xylanase (ElX)	ElX (tomato)	Ron and Avni (2004) and Bailey et al. (1990)
Elongation factor TU (EF-Tu, elf18/elf26)	EFR (*Arabidopsis*)	Kunze et al. (2004)
Pep-13 (an oligopeptide of 13 amino acids from *P. megasperma*)	Not identified	Nurnberger et al. (1994)
Cellulose-binding elicitor lectin (CBEL) from *Phytophthora*	Not identified	Mateos et al. (1997), Sejalon-Delmas et al. (1997), and Gaulin et al. (2006)
Peptidoglycan (PGN)	Lym1 and Lym3 (*Arabidopsis*)	Gust et al. (2007), Erbs et al. (2008), and Willmann et al. (2011)
Bacterial cold shock proteins (RNP1 motif)	Not identified	Felix and Boller (2003)
Bacterial superoxide dismutase (Sod)	Not identified	Watt et al. (2006)
Activator of XA21 (Ax21)	XA21 and XA21D (*Oryza sativa*)	Song et al. (1995), Wang et al. (1998), and Gust et al. (2007)
Avirulence on Ve1 tomato (Ave 1)	Ve1 putative tomato receptor (*Solanum lycopersicum*)	de Jonge et al. (2012), Kawchuk et al. (2001), and Thomma et al. (2011)
DAMPs		
Systemin	Not identified	Narvaez-Vasquez and Ryan (2004)
Pep1 (23 aa part of a cytosolic protein from *Arabidopsis*)	PEPR1 (*Arabidopsis*)	Huffaker et al. (2006), Yamaguchi et al. (2006)
Oligogalacturonides (OGs)	WAK1 (*Arabidopsis*)	Brutus et al. (2010) and Nothnagel et al. (1983)
Cutin	Not identified	Schweizer et al. (1996) and Kauss et al. (1999)
Hydroxyproline-rich systemin	Not identified	Pearce and Ryan (2003) and Heiling et al. (2010)
Extracellular ATP (eATP)	Does not respond to nucleotide 1	Choi et al. (2014) and Jeter et al. (2004)

(continued)

Table 4.1 (continued)

Name	Corresponding plant receptor (PRR)	References
Plant elicitor peptides (Peps)	PEPR1/PEPR2	Pearce et al. (1991, 2001), Huffaker et al. (2006), Yamaguchi et al. (2010), Huffaker et al. (2011), Yamaguchi and Huffaker (2011), Huffaker and Ryan (2007), and Huffaker et al. (2013)
AtHMGB3	Not identified	Choi et al. (2016)
NAMPs		
Ascr#18	Not identified	Choi and Klessig (2016)

polymer of plant cell wall and hence provides strength against degradation caused by pathogen. To inhibit the pathogen multiplication, plants also produce PR proteins such as β-1-3 glucanase and chitinase. All these responses are sufficient to overcome most of the microbes or pathogens (Dou and Zhou 2012; Dodds and Rathjen 2010). Plants, defective in PRR or PTI signaling components, are often more susceptible to pathogens.

The general elicitors for PTI in plants were oligosaccharides or glycoproteins (Boller and Felix 2009). The increasing amount of genomic information leads to the identification of novel protein elicitors and their corresponding epitopes. Bacterial peptides like Flg22 and elf18 are derived from surface-associated flagellin protein (Flg) and translocation elongation factor Tu (Ef-Tu), respectively (Felix and Boller 2003; Zipfel et al. 2006). Apart from these two well-studied elicitors, bacterial glycoconjugates including peptidoglycan (PGN) which provides strength to the cell envelope of Gram-positive and Gram-negative bacteria also work as elicitor of plant innate immunity (Erbs et al. 2008; Willmann et al. 2011). Lipopolysaccharide (LPS) carrying lipid A moiety from the Gram-negative bacteria is a potent MAMP for mammals. It is responsible for activating pro-inflammatory responses via toll-like receptor 4 (TLR4) in mammals. Similarly, LPS from the outer membrane of Gram-negative and Gram-positive bacteria also evokes innate immune response in plants (Silipo et al. 2005; Erbs and Newman 2012). Recently, it has been discovered that *Arabidopsis thaliana* can sense LPS, specifically from *Pseudomonas* and *Xanthomonas*. Gene knockout studies showed that bulb-type lectin S-domain-1 receptor-like kinase LORE (SD1-29) is essential for the perception of LPS in plants. These results were further confirmed when LORE mutants were found hypersusceptible to *Pseudomonas syringae* infection. Further studies with chemically degraded LPS, isolated from *Pseudomonas* species, proved that LORE detected mainly the lipid A moiety of LPS. The heterologous expression of LORE in tobacco conferred sensitivity to LPS, which confirmed its key function in LPS sensing (Ranf et al. 2015). Oligosaccharide derived from fungus and oomycetes also act as MAMP/PAMP. Fungal chitin and its derivatives N-acetylglucosamine are responsible for induction of innate immunity in monocot and dicot plants.

Apart from bacteria and fungus, viruses and nematodes also target plants and cause pathogenicity. So far not a single conserved viral MAMP/PAMP has been

reported. However, plants generally defend themselves from viruses via RNA silencing mechanism (RNAi). To counter such defense mechanism, plant viruses developed RNAi silencing suppressors, many of which bind with double-stranded RNA (dsRNA) (which generally acts as PAMP) and attenuate RNAi mechanism (Csorba et al. 2009; Ruiz-Ferrer and Voinnet 2009). Nematodes are also reported to parasitize plants, but detailed perceived signal is not known. Recently, defense signaling molecules from different plant-parasitic nematodes have been identified. They belong to conserved nematode pheromone family called ascarosides. Ascr#18 is among one of them which induces innate immunity in different plants via activating defense genes, MAPKs, enhancing resistance against bacteria, viruses, fungi, and oomycetes.

Due to their sessile lifestyle, plants are subjected to biotic stresses as well as a multitude of abiotic stress factors (e.g., cold, excess water, increased salt concentrations). In animal systems, the C-type lectins represent a key player in the recognition of pathogens and the induction of the immune response, whereas C-type lectins in plants are rather rare. To recognize lectins in plants, the membrane-bound PRRs carry extracellular lectin domains, that are coupled to intracellular Ser/Thr kinase domains. These lectin receptor kinases (LecRKs) are further categorized into four types: G-, C-, L-, and LysM-type (Bouwmeester and Govers 2009; Cambi et al. 2005; Vaid et al. 2012, 2013; Singh and Zimmerli 2013). In plants the G-type LecRKs carry a lectin domain belonging to the *Galanthus nivalis* agglutinin (GNA) family. There are 32 G-type LecRKs identified from *Arabidopsis* and about 100 from rice. However it is not clear whether these lectin domains play a role in the interaction with pathogen or not (Vaid et al. 2012).

Currently 11 PRRs with known ligands have been characterized from different plant species. Flagellin sensing 2 (FLS2) and EF-TU receptor (EFR), resistant to *Xanthomonas oryzae* pv. *oryzae* (Xa21), detect the bacterial MAMP flagellin (flg22), elongation factor Tu (elf18), and the Ax21 sulfated protein (AxYS22), respectively. The LysM proteins LYM1, LYM2, and chitin elicitor receptor kinase 1 (CERK1) are responsible for the perception of bacterial peptidoglycans (PGN), whereas ethylene-inducing xylanase receptors (LeEIX1/2) sense fungal xylanase, and both chitin elicitor-binding protein (CEBiP) and CERK1 recognize fungal chitin (Borner et al. 2003; Kaku et al. 2006; Miya et al. 2007; Shimizu et al. 2010). The PRRs differ in their dependence on BRI1-associated kinase 1/somatic embryo receptor kinase 3 (BAK1/SERK3), a LRR-RLK identified as a co-receptor for the brassinosteroid insensitive 1 (BRI1), but now it is known to regulate multiple signaling pathways (Chinchilla et al. 2009). PRRs not only mediate MAMP-/PAMP-triggered immunity (PTI), the first layer of active defense in plants, but are also required for non-self-discrimination during symbiotic plant-microbe interactions (Boller and Felix 2009). This includes LysM receptor-like kinase 3 (LYK3) and NOD factor perception (NFP) perceiving bacterial nodulation factors (NF) and symbiosis receptor kinases (SYMRK) necessary for bacterial and fungal symbiosis (Gherbi et al. 2008; Limpens et al. 2003; Stracke et al. 2002; Madsen et al. 2011).

4.2 Bacterial PAMPs

4.2.1 Flagellin (Flg)

Flagellin is an essential structure of most of the bacteria as it provides motility and in some cases assists in binding to the host cells. It is an oligomeric proteinaceous structure made up of monomeric multidomain subunit of flagellin (Flg). In animals, flagellin is recognized by surface-localized toll-like receptors (TLR5) (Ramos et al. 2004). In plants such as *Arabidopsis* and tomato, it is conserved in the N-terminal of flagellin which elicits innate immune response. Studies in plants showed that only 22 amino acids of flagellin (Flg22) are responsible to activate immune response in plants (Felix et al. 1999). To decipher the receptor of Flg22, various genetic approaches have been applied in *Arabidopsis*. It has been observed that among various mutants of *Arabidopsis*, it is FLS2 mutant which is susceptible to pathogen. The gene responsible for coding FLS2 is located on chromosome number 5 in *Arabidopsis* and belongs to the RLK family. Further studies proved that it is LRR domain of FLS2 which is responsible for binding with Flg22 (Gomez-Gomez and Boller 2000; Bauer et al. 2001). However, it is not the same conserved domain of Flg22, which is recognized by all plants. In case of tomato (*Solanum lycopersicum*), it is Flg15, a shorter version of Flg22 from N-terminal, which elicits the immune response, whereas in rice it is the full-length flagellin which gives better immune response as compared to Flg22. Heterologous expression studies of FLS2 from *Arabidopsis* in tomato cells further proved its specificity for Flg22. Different plants perceived different epitopes of flagellin. For example, 15-amino-acid-long peptide from *E.coli* Flg has shown high response in tomato, whereas the same peptide is not able to elicit immune responses in tobacco (Newman et al. 2013). Differences in the perception of flagellin are not limited to different plants, but it is also observed in different species of the same family. In other studies, when FLS2 from tomato is expressed in *Nicotiana benthamiana* cells, they gained flagellin perceptions, specific for tomato (Robatzek et al. 2007).

4.2.2 Elongation Factor TU (EF-Tu)

Protein biosynthesis is essential for the survival of microorganisms where mRNA and ribosomes play a crucial role. During protein synthesis, elongation factors are associated with ribosomes. EF-Tu is among one of them and is present in abundance in the bacterial cells (Jeppesen et al. 2005). N-terminal of elongation factor is highly conserved and has elicitor property in plants. Either 28-amino-acid- or 16-amino-acid-long protein form N-terminus named elf28 or elf16, respectively, is responsible for elicitor property. Each elicitor is perceived by specific or common receptors in plants. EF-Tu is also perceived by EF-Tu receptor in plants (EFR) and is independent from the Flg, as FLS2 mutants are active against EF-Tu and elicit innate immune responses in plants (Kunze et al. 2004). Cross-linking assays in *Arabidopsis* cells confirmed that elf18 and flg22 recognized different receptors, whereas they

induced a small pool of genes. Further combined treatments of elf26/elf18 and flg22 did not show any additive effect of immune responses (Zipfel et al. 2006). Studies with EFR mutant further showed no response-like oxidative burst and increased ethylene synthesis, and resistance against *Pseudomonas syringe* pv. *tomato* (*Pst*) DC3000 was observed when challenged with EF-Tu derivatives. On the other hand *Arabidopsis* Col-0 and *fls2* mutant respond perfectly against EF-Tu elicitor. *N. benthamiana* does not have a perception system for Elf-Tu. Heterologous expression of EFR in *N. benthamiana* makes it active against EF-Tu elicitor and has been confirmed that it acts as the functional receptor (Zipfel et al. 2006). Further, the efficiency of *Agrobacterium tumefaciens (At)*-mediated transformation in EFR mutant has been increased as compared to the wild type. This indicates that EF-Tu recognition reduced the *Agrobacterium*-mediated transformation (Zipfel et al. 2006).

4.2.3 Peptidoglycan (PGN)

Gram-negative and Gram-positive bacteria cell envelopes have peptidoglycan which provides rigidity to their membranes. Since eukaryotes do not have PGN, therefore PGN becomes excellent target to host immune system (McDonald et al. 2005; Dziarski and Gupta 2006). PGNs are complex glycan structures which are hold together with oligopeptides. Chemically, they are composed of alternative N-acetylglucosamine (NAG) and N-acetylmuramic (NAM) with short peptides in between (Newman et al. 2013). Studies in tomato showed that pre-inoculation with *Staphylococcus aureus's* PGN reduced the bacterial infection in PGN treated tissues. *S. aureus* PGN was considered as an active elicitor as it induces extracellular alkalinization in cultured tobacco cells. No such response was observed in cultured tomato cells, indicating that PGN is perceived differently in *Solanaceae* (Felix and Boller 2003). Studies in *Arabidopsis* confirmed that PGN from Gram-negative and Gram-positive bacteria acts as an elicitor and evokes innate immune responses in plants (Gust et al. 2007; Erbs et al. 2008). Further, it has been shown that sugar backbone of Gram-positive PGN is responsible for the immune response in plants. Derivatives of PGN like muramyl dipeptide (MDP) or the muropeptide dimer are not responsible for triggering immune responses in plants, whereas these are easily perceived in insects and vertebrates (Traub et al. 2006). On other hand, PGN from two Gram-negative bacterial plant pathogens *Xcc* and *At* and their derivatives induces immune responses including ROS generation, PR1 gene expression, extracellular pH increase, and callous deposition (Erbs et al. 2008). Further it has been observed that derivatives like muropeptides are more effective as compared to the total PGN structures. Hence, we can say that PGN from Gram-negative and Gram-positive bacteria is perceived by different mechanisms. It could be due to different structure and recognition sites of muropeptides of human pathogen vs. plant pathogen. Studies with *Arabidopsis cerk1* mutant plant enhanced ROS response after treatment with PGN from Gram-negative *Pst* DC3000 and indicated its independent perception system for CERK1 (Newman et al. 2013).

4.2.4 Lipopolysaccharide (LPS)

Gram-negative bacteria have two-membrane architecture. It has an outer membrane followed by periplasmic space and an inner membrane. Each structure has its own function. Outer membrane contributes to the permeability as well as supports the growth of bacteria in unfavorable environments. LPS, a major component of the outer membrane, interacts with plants and plays a major role in inducing innate immune response (Newman et al. 1995; Bedini et al. 2005; Dow et al. 2000; Silipo et al. 2005). LPS consists of core oligosaccharides and a lipid O-polysaccharide part. Since like dissolves like, it is the lipid part which is embedded in the outer phospholipid bilayer of bacterial membrane and referred as lipid A. Lipid A is linked to the core oligosaccharide via 3-deoxy-D-manno-2-octulosonate (KDO). The core oligosaccharide is made up of short sugar and O-antigen. The composition of O-antigen is repeating oligosaccharide units (Raetz and Whitfield 2002). LPS of various phytopathogenic bacteria has O-antigen, composed of oligorhamnans (Bedini et al. 2002). To decipher the structure of LPS responsible to trigger immune response in plant, different O-antigen polysaccharides and different length of oligorhamnans were tested in *Arabidopsis*. It has been observed that PR genes including *PR1* and *PR2* were induced by tri-hexa- and nanosaccharides. Further, the increasing length of sugar and their coiled structure of O-antigen (synthetic) evoke plant innate immune responses ((Bedini et al. 2002). *Xcc* lipooligosaccaride (LOS) and its derivatives have shown the induction of *PR* genes in *Arabidopsis*. Therefore we can say that *Xcc* LOC and *Xcc* core oligosaccharides induce innate immunity in plants (Newman et al. 2013). However, in the case of tobacco, neither lipid A nor O-chain of LPS (*Xcc*) induces any innate immunity. Interestingly the inner core of the LPS was responsible for the induction of oxidative bursts (Braun et al. 2005). It has been observed that phosphorylation of lipid A affects its biological activity in mammalian system (Gutsmann et al. 2007). The same has been determined in *Arabidopsis* too. Studies with dephosphorylated *Xcc* LOS in *Arabidopsis* showed no localized induced responses (LIR) (Silipo et al. 2005). This result indicates that the phosphate group has its important function in the binding with specific receptor in plants. LPS also plays an important role in prime expression of defense response in plants, post bacterial infection. This includes synthesis of antimicrobial compound including feruloyl tyramine (FT) and p-coumaroyl tyramine (CT) (Newman et al. 2002, 2007; Conrath et al. 2006). Induced systemic response (ISR) was also observed in *Arabidopsis*, and it was the O-antigen of LPS responsible for such kind of response. This observation was further confirmed in an experiment where bacterial mutant lacking O-antigen was not able to induce ISR (van Loon et al. 1998). Apart from ISR, systemic acquired response is also observed in plants and is responsible for increase in salicyclic acid (SA) and related signaling (Schneider et al. 1996; Ryals et al. 1996). Recent study showed that it is not the necrotic lesion formation (which induces SAR in plants) but MAMPs, Flg, or LPS that is responsible for bacterial induction of SAR. Studies with *Pseudomonas aeruginosa* LPS, Flg, or nonhost bacteria in *Arabidopsis* showed induction of immune response such as accumulation of SA and *PR* gene expression as well as SAR marker,

flavin-dependent monooxygenase 1 gene in treated as well as in nontreated distal leaves (Mishina and Zeier 2006, 2007). The exact downstream signaling system acquired response is not known clearly.

LPS is known to induce immune response in dicots. Recent studies in monocots like rice with LPS from pathogenic or nonpathogenic bacterium induced defense-related gene as well as ROS. These results indicate that monocot and dicot may have a common mechanism (Desaki et al. 2006, 2012). However, LPS from some bacteria showed programmed cell death, which is not elicited in the case of dicot (Desaki et al. 2006). The exact mechanism for LPS perception in plant is not known. However, fluorescein-labeled *Xcc* LPS showed that they are internalized in *N. tabacum* cells, similar to the endocytosis process in mammals (Gross et al. 2005). Still there is no specific PRR identified for perception of LPS in plant.

Different groups are attempted to identify PRR for LPS in plants. Transcriptomic analysis of *Arabidopsis thaliana* cells post *B. cepacia* LPS treatment showed that there was no induction of callous synthesis genes (Livaja et al. 2008). Moreover, genes involved in ROS production were also not upregulated. However, LPS from *B. cepacia* induced *PR3* and *PR4* gene expression in *Arabidopsis.* Other sources of LPS like *E.coli* and *P. aeruginosa* induced *PR1* and *PR5* expression in *Arabidopsis* leaves (Mishina and Zeier 2007). Differences in these results could be due to different plant system as well as different sources of LPS (Newman et al. 2013).

Plants also have the ability to modify LPS. This property is observed when lipid A or structures within LPS are altered in symbiotic interaction with plants (Kannenberg and Carlson 2001). These changes are responsible for the increase in the resistance against bacteria or to inhibit activity of lipid A in inducing host defense (Newman et al. 2013). Acylation and phosphorylation of lipid A are responsible for the induction of immune response in plants (Silipo et al. 2008). Lipid A from *Halomonas magadiensis*, an extremophilic and alkalinophilic Gram-negative bacterium, has low degree of acylation and is known to inhibit *E. coli* lipid A immune response in mammalian cells (Ialenti et al. 2006). Studies showed that differences in lipid A structure like acylation made them act as agonist or antagonist (Munford and Varley 2006). *H. magadiensis's* lipid A acts as antagonist for the action of *E. coli* lipid A in *Arabidopsis*. LPS for the experimental studies in plants is prepared from bacterial culture and grown in artificial medium. Alteration in LPS structure could occur when they multiply in plants. These changes could be important for the recognition and downstream signaling point of view. Mass spectroscopy and transcriptomic analysis of bacteria isolated from plants may provide fruitful information in this direction. Lipid A-like molecules are absent in plants like *Arabidopsis*; however, the six orthologous genes for the synthesis of lipid A, out of nine in *E. coli*, are present in *Arabidopsis*. Knockout studies of these genes showed that mutations are viable; however they are able to produce lipid A-like precursors (Li et al. 2011). It may be possible that higher plants may acquire lipid A biosynthetic genes from Gram-negative bacterium via endosymbiosis, and hence lipid A may play an important role in the structure of mitochondria or chloroplast membrane (Newman et al. 2013). Lipid A-like molecules may be involved in signaling in *Arabidopsis*, but the mechanism of LPS perception in plant is still unknown.

4.2.5 XA21-Mediated Immunity (Ax21)

XA21 receptor is identified in rice; however its ligand Ax21 has been discovered recently. Ax21 is present in *Xanthomonas* spp., *Xylella fastidiosa*, and *Stenotrophomonas maltophilia* (a human pathogen), and hence it is conserved in nature and has an important biological role. Ax21 is composed of 194 amino acids, and only 17-amino acid (sulfated) peptide is responsible for its biological activity (Bogdanove et al. 2011). *Xa21* gene is located in chromosome number 11 and encodes receptor kinase-like protein, having LRR transmembrane (TM), juxtamembrane (JM), and cytosolic kinase domain. *XA21* gene is responsible for providing resistance to a large number of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) species (Song et al. 1995; Wang et al. 1996). *XA21* is among the seven members of gene family. Among the seven, *XA21D* is closest to *XA21* and provides resistance to plants. However, they differ in the degree of resistance as *XA21D* provides mild resistance. Structural LRR domain of *XA21* and *XA21D* is 99% identical. However, *XA21D* lacks transmembrane and kinase domain (Song et al. 1997; Wang et al. 1998). Different proteins are known to interact with *XA21*. For example, ATPase XB24 binds and promotes XA21 phosphorylation and keeps it in inactive state. Later binding of Ax21 to XA21 dissociates XB24 from XB21/XA21 complex and activates XA21 (Chen et al. 2010).

4.3 Fungal and Oomycete MAMPs

4.3.1 Chitin

Fungal chitin and β-glucan from the *P. megasperma* act as MAMPs. In fungus, branched β-glucan is cross-linked with chitin, whereas in oomycetes cross-linking is done with cellulose. Chitin and its fragments are responsible for inducing immune response in plants. Fungal chitin is recognized by CEBiP and CERK1 (Kaku et al. 2006; Shimizu et al. 2010). Studies in rice showed that RLP CEBiP binds with chitin fragments at cell surface and interacts with LysM-RLK OsCERK1 for downstream signaling (Miya et al. 2007). In *Arabidopsis*, three CEBiP-like proteins have been reported, and they are designated as LYS1, LYS2, and LYSM. Heterologous expression of each in tobacco BY cells, followed by test for binding ability with chitin oligosaccharide, showed that LysM2 (AtCEBiP) have the highest affinity (Shinya et al. 2012). Biotinylated $(GlcNAc)_8$ binding studies with CEBiP prove its cell surface localization. However, single or triplet knockout of LysM1, AtCEBiP, and LysM3 as well as overexpression of AtCEBiP suggests that they are not responsible for signaling in *Arabidopsis* (Shinya et al. 2012). Therefore, we may conclude that rice and *Arabidopsis* have different receptors for chitin perception. In *Arabidopsis*, five LysM RLKs1-5 have been reported (Lyk1, Lyk2, Lyk3, Lyk4, and Lyk5) (Wan et al. 2012). Lyk1 is also known as CERK1. Knockout studies have been performed to determine their role in chitin signaling. It has been observed that in Lyk4, mutant immune response has been reduced. Moreover, Lyk mutant plants were more susceptible to *Alternaria brassicicola* and bacterial pathogen *Pst*

DC3000 (Wan et al. 2012). Apart from this, lysine motif-containing proteins, LYP4 and LYP6, are responsible for binding with chitin as well as PGN and may function as dual PRR in rice. Therefore, in rice, overlapping perception system does exist for fungal and PGN. However, LYM1 and LYM3, which are orthologs of LYP4 and LYP6 in *Arabidopsis*, recognize only PGN and are not able to recognize chitin (Willmann et al. 2011).

4.3.2 Ethylene-Inducing Xylanase (EIX) and AVE1 Peptide

Tomato was resistant against *Verticillium dahliae* and *V. albo-atrum* (Kawchuk et al. 2001). Genes responsible for the resistance lie in Ve loci, *Ve1* and *Ve2*. Both of them encode the surface receptors which belonged to receptor-like protein (RLP) class. Studies showed that among Ve1 and Ve2, Ve1 was found responsible for developing resistance. Further knockout studies were done with BAK1, which confer more susceptibility to tomato against *Verticillium* infection (Fradin et al. 2009). A putative ligand for Ve1 was AVe1 (avirulence on Ve1 tomato) peptide. It was observed that Ave1 was conserved among many fungus and plant pathogenic bacteria like *Xanthomonas axonopodis* pv. *citri*. Ave1 was also found to have homology with plant natriuretic peptides (PNPs) which is responsible for maintaining homeostasis in stress conditions (Wang et al. 2011). Ave1 acts as an elicitor and induces immune response via Ve1-mediated downstream signaling. Hence, we can say that Ave1 peptide acts as MAMP, and Ve1 acts as PRR (Thomma et al. 2011). There are other PRRs in tomato including RLPs S1Eix1 and S1Eix2. They also have homology with tomato Ve1 and Cf PRRs (Ron and Avni 2004). They are known to recognize fungal ethylene-inducing xylanase. EIX1 (β-1-4-endoxylanase) is proteinaceous in nature having molecular weight of 22 kDa. It is isolated from *Trichoderma viride* and is responsible for inducing innate immunity in tomato and tobacco. The primary sequence of S1Eix1 and S1Eix2 is 81.4% identical and has the ability to bind with EIX. However, they have different functions, such as S1Ex2 when binds with Ex1 induces innate immune response, while binding of Ex1 with S1Ex1 inhibits plant defense (Bar et al. 2010; Ron and Avni 2004).

4.3.3 Damage-Associated Molecular Pattern (DAMP)

Plant-derived molecules in certain cases are responsible to induce immune response. This system is similar to mammals, where immune system can detect danger through DAMPS (Seong and Matzinger 2004; Boller and Felix 2009) and is responsible for inflammatory response. Systemin, which is an 18-amino-acid peptide, induces immune response in plants (tomato) (Pearce et al. 1991; McGurl et al. 1992). Systemin is derived from its cytoplasmic protein precursor, prosystemin (Narvaez-Vasquez and Ryan 2004). Upon cell damage, systemin is released, which then acts as DAMP. Earlier studies showed RLK SR160, which is an ortholog of BRI1 of tomato, acts as a receptor for systemin (Scheer and Ryan 1999, 2002).

Putative cytoplasmic peptide (Pep1) in *Arabidopsis* is responsible for activating defense genes and alkalization in cell culture. Pep1 is a 23-amino-acid-long peptide and has seven homologues, which are derived from PROPEP1-7 (Huffaker et al. 2006). Pep1 is perceived by PEPR1 receptor which belongs to LRR X1 subfamily (Yamaguchi et al. 2006). On the basis of primary sequence, a second receptor for Pep peptide was found and designated as PEPR2. *PEPR1* and *PEPR2* transcription occurred under different conditions including wounding Pep peptide and specific MAMP. Binding studies with peptide and receptor showed redundancy, as Pep1 and Pep2 both bind with PEPR1 and PEPR2, whereas PEPR1 binds with Pep3-6 (Yamaguchi et al. 2010).

Cutin and oligogalacturonides (OGs) released from plant cell walls also act as DAMPs (Denoux et al. 2008; Brutus et al. 2010; Schweizer et al. 1996). Wall-associated kinase1 (WAK1) acts as a receptor for OG (Brutus et al. 2010). However, receptor for cutin is still not known. OG is known to evoke immune responses in plants by activation of MAPK, ROS production, callous deposition, and calcium release in cytoplasm as well as activation of defense genes (Chandra et al. 1997; Denoux et al. 2008). Another elicitor which comes in DAMP category in plants is the extracellular ATP (eATP). Surface-associated receptor for eATP perception does not respond to nucleotide 1 (DORN1) (Choi et al. 2014). It has been observed that a mutant of DORN1 suppressed transcriptional response to wounding and upregulated genes post eATP application, which was wound-inducible (Tanaka et al. 2014). eATP induces innate immune response as it is able to activate MAPK, Ca^{2+} influx, synthesis of JA, and ethylene as well as induce defense-related genes (Jeter et al. 2004; Song et al. 2006; Tanaka et al. 2014).

In *Arabidopsis*, HMGB protein AtHMGB3 is reported to act as DAMP. In *Arabidopsis* there are 15 genes, responsible to encode HMG-box domain-containing protein. They are further divided in four groups: (1) HMGB-type protein, (2) 3X HMGB which contains three HMG boxes, (3) A-/T-rich interaction domain (ARID-HMG protein), and (4) SSRP1, structural-specific recognition protein 1 (Merkle and Grasser 2011). On the basis of domain structure and nuclear location, there are eight different types including HMGB1/2/3/4/5/6/12/14. Among these HMGB2/3/4 are cytoplasmic as well as nuclear localized (Launholt et al. 2006; Pedersen and Grasser 2010; Merkle and Grasser 2011). Recombinant AtHMGB3 when infiltrated in leaves induced MAPK activation and deposition of callus, induced defense gene, and developed resistance against *Botrytis cinerea* (Choi et al. 2016).

SA binds to AtHMGB3 through its conserved Arg and Lys residues and abolishes its DAMP activity (Choi et al. 2016). However, SA is a positive regulator of immune response in plants and provides resistance to biotrophic and hemi-biotrophic pathogen. JA is responsible for providing resistance against necrotrophic pathogen as well as insects (Vlot et al. 2009; Dempsey et al. 2011). Signaling pathway of JA and SA is antagonistic (Thaler et al. 2012). Necrotrophic pathogen caused infection and cellular damage, which leads to the release of AtHMGB3 into extracellular space, which activated JA-/ethylene-associated defense genes. However, biopathogenic pathogen infection increased SA levels which suppressed AtHMGB3

DAMP-mediated immune defense and activated assay-associated defense gene expression (Choi et al. 2016).

4.3.4 Nematode-Associated Molecular Pattern (NAMP)

Nematodes are known to parasitize both plants and animals (Lambert et al. 1999; Vercauteren et al. 2001; Kyndt et al. 2012). However, elicitor for plants is not known. Recently a group of defense signaling molecules from root-knot and cyst nematodes have been identified (Manosalva et al. 2015). They are conserved nematode pheromones called ascarosides. The most abundant ascaroside is Ascr#18. It is responsible to induce innate immune response in the plant including activation of MAPKs, defense genes, SA, and JA defense signaling pathways. It is responsible for enhanced resistance against viral, bacterial, fungal, and oomycete pathogen and root-knot nematodes in different monocot and dicot plants (Choi and Klessig 2016).

4.4 Binding of Bacterial PAMPs with Plant PRRs

Perception of PAMPs requires either homodimerization, heterodimerization, or heteromultimerization of PRRs. Recent advances in biochemical structure and genetic studies open the new window to know about the molecular mechanisms underlying PAMP binding to plant PRR.

4.5 Heterodimerization: Flagellin Perception in Arabidopsis

In *Arabidopsis*, bacterial flagellin is recognized by PRR, FLS2, which is LRR-RLK. Among full-length bacterial flagellin, the conserved 22 amino acids behave like epitope flg22 (Gomez-Gomez and Boller 2000). The FLS2 ectodomain has 28 LRRs and directly binds to flg22 (Chinchilla et al. 2006). FLS2 exists in dimer form even in the absence of elicitor, but its relevance is not clear yet (Sun et al. 2012, 2013a). Although FLS2 is conserved in most of the plant species, still there are lots of differences in specificity of recognition (Boller and Felix 2009). This has been proved in a study where a shorter peptide flg15 behaves as an agonist in tomato, whereas it acts adversely in *Arabidopsis* (Mueller et al. 2012; Bauer et al. 2001; Felix et al. 1999; Robatzek et al. 2007). Comparative and mutagenic studies of both FLS2 LRRs and flg peptides have identified possible LRRs involved in flg22 recognition (Dunning et al. 2007; Helft et al. 2011; Mueller et al. 2012). Further studies have shown that the N-terminal part of flg22 is responsible for receptor binding, whereas C-terminal part is a must for the activation of the immune response (Meindl et al. 2000; Sun et al. 2013a). Flg22 perception directs the FLS2 to form a complex with regulatory LRR/RLK and BAK1/SERK3 (Chinchilla et al. 2007; Schulze et al. 2010). This indicates the close proximity of FLS2 and BAK1 in the plasma membrane. It has been further proved by structural studies that the N-terminal of flg22

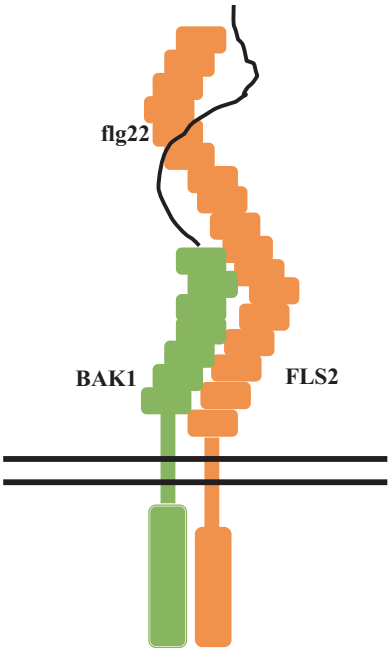

Fig. 4.1 The flg22 peptide perception in *Arabidopsis* directs the ectodomain of LRRs of FLS2 to form a stable heterodimer with the co-receptor BAK1

binds to the concave surface of FLS2 ectodomain and the C-terminal region of the FLS2-bound flg22 interacts with BAK1 ectodomain (Sun et al. 2013a). This interaction leads to the stabilization of FLS2-BAK1 dimerization, and thus FLS2-BAK1 heterodimerization is both ligand and receptor mediated as shown in Fig. 4.1. Hence, it is proven that flg22 binds to FLS2 first and later with BAK1; therefore BAK1 acts as a co-receptor for flg22 and is essential for signaling (Chinchilla et al. 2007; Sun et al. 2013a). Similar mechanism has been reported recently in *Arabidopsis* where LRR-RLK BRI1 (the receptor for the plant hormone brassinosteroid, which regulates growth and development) binds with BAK1 or the BAK1-related protein SERK1 (Santiago et al. 2013; Sun et al. 2013b). These results suggest that different LRR-containing RLKs (and RLPs) may be involved in similar heterodimeric complexes with BAK1 or related SERK proteins. The increasing number of newly identified LRR-RLKs and LRR-RLPs has shown their association with BAK1 and related SERK proteins. However some mechanistic differences exist between different plant species. For example, in rice (*Oryza sativa*) ortholog of BAK1, OsSERK2, forms a complex with the LRR-RLK XA21, and thus it confers resistance against *Xanthomonas oryzae* pv. *oryzae* (Chen et al. 2014). Due to the absence of confirmed ligand for XA21, it is not clear yet if the interaction between XA21-OsSERK2 is enhanced after PAMP perception or not (Bahar et al. 2014). FLS2-BAK1 dimerization is independent of kinase activity, whereas the association of OsSERK2 with the intracellular domains of ligand-binding receptors is kinase dependent (Schwessinger et al. 2011; Chen et al. 2014; Sun et al. 2013b).

4.6 Binding of Fungal PAMPs with Plant PRRs

Similar to bacterial PAMPs, fungal PAMPs also need homodimerization, and heteromultimerization.

4.6.1 Homodimerization: Chitin Perception in Arabidopsis

In *Arabidopsis thaliana* LysM-RLK CERK1/RLK1/LYK1 is required for chitin perception (Wan et al. 2008; Miya et al. 2007). Three extracellular LysM domains of CERK1 are responsible for the binding with oligomers of fungal chitin (Liu et al. 2012; Petutschnig et al. 2010; Miya et al. 2007). Seven to eight GlcNAc residues which constitute long chitin oligomers act as a bivalent ligand for CERK (Liu et al. 2012). This binding leads to the homodimerization of the CERK1 as shown in Fig. 4.2, which acts as an active receptor complex responsible for initiating chitin-induced immune signaling. However CERK1 is also able to bind with four to five GlcNac residues (shorter chitin oligomer), but their interaction does not induce CERK1 homodimerization, and hence does not trigger any immune responses (Liu et al. 2012). These studies highlighted that homodimerization of CERK1 is a must for signaling initiation, by bringing together CERK1 cytoplasmic domains, which contain an active kinase (Petutschnig et al. 2010), enabling intermolecular transphosphorylation. While CERK1 is the major chitin-binding protein in *Arabidopsis* and is strictly responsible for chitin-triggered immune responses, the other related LysM-RLK LYK4 is also known for chitin binding and is involved in chitin perception (Miya et al. 2007; Petutschnig et al. 2010; Wan et al. 2008, 2012).

4.6.2 Heteromultimerization: Chitin Perception in Rice

LysM-RLP CEBiP is the major chitin-binding protein in rice (Kouzai et al. 2014; Kaku et al. 2006). CEBiP is a GPI-anchored protein with three extracellular LysM domains along with a C-terminal tail (Hayafune et al. 2014; Kaku et al. 2006). Binding of long chitin oligomers with OsCEBiP leads to its homodimerization,

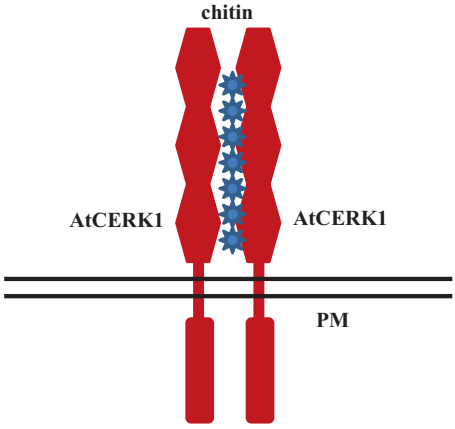

Fig. 4.2 Perception of seven to eight GlcNAc residues in *Arabidopsis* act as bivalent ligands and direct the formation of active receptor complex by homodimerization of AtCERK1

which resembles with that of chitin-binding mechanism shown for *Arabidopsis* chitin receptor AtCERK1 (Hayafune et al. 2014; Liu et al. 2012). As like other RLPs, CEBiP does not have cytoplasmic C-terminals responsible for signaling motifs, and therefore it requires assistance of additional proteins to initiate signaling (Kaku et al. 2006). In the presence of biologically active chitin, CEBiP is also known to form a hetero-oligomeric receptor complex with OsCERK1, which is the rice ortholog of AtCERK1 (Shimizu et al. 2010). OsCERK1 has only one single extracellular LysM domain and hence does not bind to chitin but is essential for chitin-mediated signaling (Shimizu et al. 2010). These results showed that the chitin perception system in rice requires hetero-oligomeric receptor complex formed by dimers of an elicitor-binding CEBiP (LysM-RLP) and a nonligand-binding signaling-active OsCERK1 (LysM-RLK) as shown in Fig. 4.3 (Kaku et al. 2006; Shinya et al. 2012). This confirmation of hetero-oligomer receptor form a sandwich type receptor for chitin oligomers (Hayafune et al. 2014). Apart from CEBiP (OsLYP4 and OsLYP6), the LysM-RLPs is also known for chitin binding and responsiveness (Liu et al. 2012). In *Arabidopsis*, CEBiP orthologs are not required for classical chitin immune responses, including ROS burst or immune gene expression (Shinya et al. 2012; Wan et al. 2012). However, the closest ortholog of CEBiP in *Arabidopsis*, AtLYM2, is known to bind chitin (Petutschnig et al. 2010; Shinya et al. 2012). The localization of AtLYM2 is in plasmodesmata and AtLYM2 is responsible for the closure of plasmodesmata in CERK1-independent manner but upon chitin perception chitin-induced. In addition AtLYMs also provides resistance to fungal pathogens (Faulkner et al. 2013; Narusaka et al. 2013). Therefore, in *Arabidopsis* some localized cellular responses which are initiated by chitin are also involved in hetero-oligomerization between a chitin-binding LysM-RLP (AtLYM2) and an unknown LysM-RLK

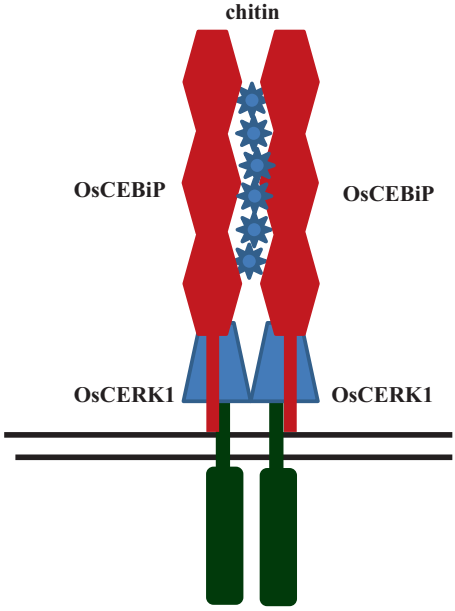

Fig. 4.3 Chitin perception in rice requires a multimeric receptor formed by dimers of OsCEBiP and OsCERK1

similar to CERK1. OsLYP4 and OsLYP6 (CEBiP paralog) are also known to bind with the bacterial cell wall component (peptidoglycan, PGN) (Liu et al. 2012). In *Arabidopsis*, AtLYM1 and AtLYM3 (orthologs of CEBiP) are known to bind with PGN and not with chitin (Willmann et al. 2011). Here CERK1 is also required for PGN-induced responses without binding with PGN itself (Willmann et al. 2011). These results show that CERK1 plays a multifaceted role, as it is able to function as a ligand-binding PRR for chitin and also as a positive regulator of PGN responses. In *Arabidopsis* the complex between AtLYM1, AtLYM3, and AtCERK1 has not been confirmed yet biochemically; however these results indicate that PGN perception system in *Arabidopsis* resembles with that of rice chitin receptor, which involves hetero-oligomeric complex of ligand-binding RLPs and RLKs.

4.6.3 Perception of Fungal Xylanase and Ave Peptide

In tomato the only RLP involved in PAMP perception receptor for the fungal ethylene-inducing xylanase (EIX) is LeEIX1/2, encoded by the genes *LeEIX1/2*, as shown in Fig. 4.4a (Ron and Avni 2004). Both LeEIX1 and LeEIX2 are reported to bind with EIX independently. However it is only LeEIX2 that can transduce the signal when expressed transiently in tobacco. In *Arabidopsis*, a few additional RLPs

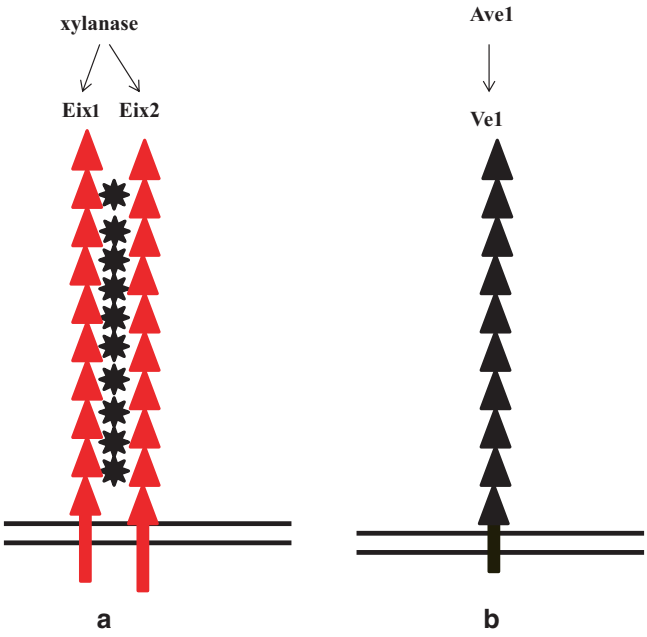

Fig. 4.4 (**a**) Perception of fungal xylanase by Eix1/2 PRR and (**b**) Ave peptide by Ve PRR in tomato

have been identified using reverse-genetic approaches, which linked to innate immunity. It has been reported that the chitin-inducible RLP52 gene mutant is more susceptible to adapted as well as non-adapted powdery mildews (Ramonell et al. 2005). Similarly, atrlp30 as well as atrlp18 mutants are found susceptible to the non-adapted bacterium *Pseudomonas syringae* pv. *phaseolicola 1448A* (Wang et al. 2008). In addition to this, tomato LRR-RLP Ve1 mediates resistance to fungus *Verticillium* by recognizing a peptide Ave1 which is conserved in several fungi and bacterium *Xanthomonas axonopodis* as shown in Fig. 4.4b. Ve1 is also reported to mediate resistance against *Fusarium oxysporum* (Monaghan and Zipfel 2012).

4.7 Plant Viruses and PRRs

In animal systems, viral patterns inducing PTI are well known, but nothing similar has been reported from plants so far. In plants, antiviral defense is mediated by well-studied posttranscriptional gene silencing of viral RNA as well as through effector-triggered immunity, which includes the recognition of virus-specific effectors by resistance proteins. In *Arabidopsis* the regulator BAK1 plays an important role for antiviral defense as *Arabidopsis* bak1 mutants show increased susceptibility to different RNA viruses during compatible interactions. It is also studied that crude viral extracts induce several PTI marker responses in a BAK1-dependent manner; however, purified virions don't. Hence, we may say that BAK1-dependent PTI contributes to antiviral resistance in plants; however specific PAMP for specific virus and its specific PRR are still not known, and further investigation is required in this direction (Korner et al. 2013).

4.8 Signaling

4.8.1 Activation of PRRs

The PRRs either contain a cytoplasmic kinase domain or are associated with RLKs. The binding of ligand to the extracellular domain of the receptor kinases leads to the activation of the intracellular kinase domain and which further phosphorylates the substrates, which contribute to intracellular signal transduction. Identified LRR-RLKs have an intracellular kinase domain with the potential for signaling, till they require dimerization with BAK1 (or a related SERK protein) post binding with ligand to transduce the signal as shown in Fig. 4.5.

BAK1 is a RD kinase, whereas most RLK PRRs (e.g., FLS2, EFR, XA21) are non-RD kinases, as they are having another amino acid in place of an otherwise conserved arginine residue in the catalytic loop of the kinase domain (Dardick et al. 2012). Non-RD kinases seem to be associated with immune functions across kingdoms, as the association between non-RD and RD kinases for the initiation of PTI is similar to *Drosophila* and humans (Dardick et al. 2012). It has been observed that

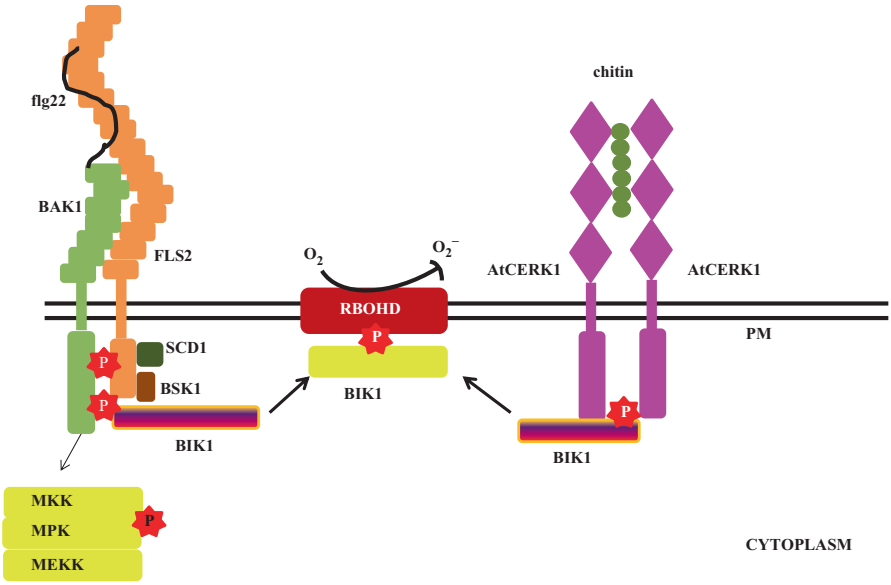

Fig. 4.5 The flg22 peptide perception in *Arabidopsis* phosphorylates the cytoplasmic domains of FLS2 and BAK1, as well as the receptor like cytoplasmic kinase BIK1. Phosphorylated BIK1 gets released from the receptor complex, and further phosphorylates and activates NADPH oxidase AtRBOHD. In addition to this, RLCK BSK1 and the endocytosis regulator SCD1 are also required for the Flg22-mediated ROS burst. Similarly, BIK1 also get phosphorylated followed by chitin perception and activation of AtCERK1 and is required for the ROS burst. The activation of MAPKs and other downstream substrates followed by flg22 perception is not clear yet

FLS2 or EFR kinase domains have weaker kinase activity as compared to other RD kinases (e.g., BAK1 or BRI1) (Schwessinger et al. 2011); therefore it may be possible that non-RD ligand-binding RLK PRRs require the association of a strong RD kinase (such as BAK1) to enhance the phosphorylation process and initiate signaling (Dardick et al. 2012). This has been further proved in a mutagenesis study where mutations that impair complex formation between FLS2 and BAK1 eliminate the phosphorylation of both proteins as well as initiation of downstream signaling (Sun et al. 2013b). Hence, it shows that kinase activities of FLS2 and EFR are essential for flg22- and elf18-mediated responses (Cao et al. 2013; Schwessinger et al. 2011). In *Arabidopsis*, CERK1 itself contains an RD kinase domain and therefore does not require BAK1 to initiate chitin-triggered signaling (Gimenez-Ibanez et al. 2009; Heese et al. 2007). However, signaling mediated by RD RLKs, including BRI1 or the PRRs PEPR1/PEPR2, still needs BAK1 (and other SERKs) (Gou et al. 2012; Krol et al. 2010; Li et al. 2002; Nam and Li 2002; Roux et al. 2011) indicating that SERK proteins are essential for the functioning of both RD and non-RD ligand-binding RLKs. SERKs act as kinase activity enhancers and are responsible for phosphorylation event in *cis* and *trans*, within PRR complexes. These phosphorylated PRR

complexes regulate the interaction with specific substrates and activation of specific signaling branches, leading to various downstream responses. This event is similar to the observed generation of docking sites for downstream substrates of animal receptor kinases driven by phosphorylation on specific residues (Lemmon and Schlessinger 2010). BAK1 forms a complex with both BRI1 (regulating growth) and FLS2 (regulating immunity) and contributes to signaling specificity in a phosphorylation-dependent manner (Schwessinger et al. 2011). Further studies are necessary to understand the sequence and nature of the phosphorylation events triggered after ligand perception and how they contribute to signaling initiation and specificity.

RLPs generally lack intracellular signaling domains and therefore depend on the association with other kinases for signaling. It has been observed that LRR-type and LysM-type RLPs act as PRRs in association with BAK1 (or other SERKs) and CERK1, respectively, to fulfill their role of signaling kinase domains activated, post ligand perception. In tomato in addition to CERK protein, LRR-RLK SOBIR1 was recently found in association with several LRR-RLP PRRs, such as Eix2, Ve1, and Cf4 (Liebrand et al. 2013). It has been reported that silencing of SOBIR1 expression compromises Cf4- and Vel-mediated responses which indicate that SOBIR1 is a must for the accumulation of Cf4 and Vel (Liebrand et al. 2013). Since SOBIR1 is localized in the cytoplasmic vesicles, they may be necessary for adequate trafficking and providing stability to LRR-RLP-containing complexes (Liebrand et al. 2013). In *Arabidopsis* also, SOBIR1 is a must for proper functioning of several RLPs involved in innate immunity, which indicates that SOBIR1 is a common regulator of LRR-RLP PRRs in different plant species (Jehle et al. 2013; Zhang et al. 2013). Till date it is not clear how SOBIR1 regulates LRR-RLP accumulation. It has been observed that in rice, PRR XA21 (an LRR-RLK) autophosphorylation at several Ser and Thr amino acids subjected to phosphorylation-dependent mechanism is controlling protein stability (Xu et al. 2006).

Apart from this, for the accumulation of XA21, several non-kinase proteins are also required. It has been reported that ubiquitin ligase XB3 and XB25 (plant-specific ankyrin repeat) proteins interact with XA21 in plants as shown in Fig. 4.6. These are further transphosphorylated by XA21 kinase domain (Jiang et al. 2013; Wang et al. 1996). XB3 or XB25 is crucial for immune response as the reduced expression of XB3 or XB25 results in reduced accumulation of XA21 which consequently compromises XA21-mediated immunity (Wang et al. 2006; Jiang et al. 2013).

Recently it has been observed that the additional RLKs can be a part of PRR complexes. The LRR-RLK BIR2 is found to interact with BAK1 even in the absence of PAMP perception (Halter et al. 2014). The nature of BIR2 is a pseudo-kinase, and it negatively regulates BAK1 interaction with FLS2. Later during Flg22 perception, BIR2 dissociates from BAK1 which ultimately allows FLS2-BAK1 dimerization and downstream signaling (Halter et al. 2014).

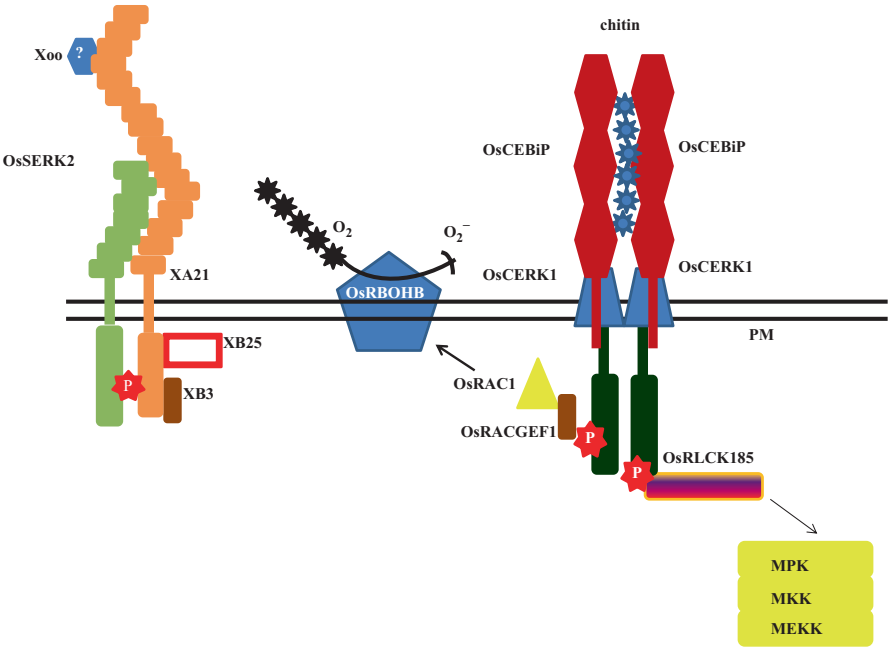

Fig. 4.6 For the stability of XA21 in rice, it requires the E3 ligase XB3 as well as the PANK protein XB25 and forms a constitutive complex with OsSERK2. However the exact mechanisms of signal transduction followed by perception of *Xanthomonas oryzae* pv. *oryzae* (Xoo) is not known. The heteromultimeric chitin receptor complex formed by dimeric OsCEBiP and OsCERK1 associates with various cytoplasmic proteins, which are required for signal transduction. Post chitin perception, OsCERK1 phosphorylates receptor like cytoplasmic kinase OsRLCK185, which gets dissociated from the receptor complex after phosphorylation and further responsible for the activation of MAPKs. In addition, OsCERK1 also activates the Os-RacGEF1/OsRac1 module, which is responsible for the activation of chitin-induced ROS burst

4.8.2 Downstream Events

For the downstream intracellular signaling, surface-associated PRRs and associated transmembrane proteins require cytoplasmic partners. Recently, receptor-like cytoplasmic kinases (RLCKs) have emerged as key substrates of PRR complexes and positive regulators of PTI signaling. In the absence of flg22 in *Arabidopsis*, RLCK BIK1 is associated with FLS2 and BAK1 (Lu et al. 2010; Zhang et al. 2010). Post flg22 perception, BIK1 gets phosphorylated by BAK1, which later phosphorylates both FLS2 and BAK1, and finally gets dissociated from the FLS2- BAK1 complex as shown in Fig. 4.5 (Lu et al. 2010; Zhang et al. 2010). BIK1 is also phosphorylated after elf18 perception and interacts with EFR and CERK1 and, therefore, plays a key role in downstream signaling (Lu et al. 2010; Zhang et al. 2010). This is further supported by mutagenesis studies, which showed that bik1 mutants are compromised in immune responses triggered by flg22, elf18, and chitin. Hence, they are more susceptible to the pathogenic fungus *Botrytis cinerea* and non-adapted bacterial pathogens

(Laluk et al. 2011; Veronese et al. 2006; Lu et al. 2010; Zhang et al. 2010). BIK1 is also reported to interact with PEPR1 and is involved in PTI amplification mechanism involving the gaseous hormone ethylene (Liu et al. 2013; Tintor et al. 2013; Zipfel 2013; Laluk et al. 2011). These results further highlighted the important role of BIK1 for the activation of PRR complexes in *Arabidopsis* innate immunity.

RLCK-VII subfamily contains more than 46 members, including BIK1, PBL1, PBL2, and PBL5. Among these, PBL1, PBL2, and PBL5 are known to regulate flg22-induced ROS burst (Liu et al. 2013; Zhang et al. 2010). It has been reported that BIK1 and PBL1 don't have any role in flg22-induced activation of MAPKs (Feng et al. 2012). However, MAPK activation via flg22 was suppressed upon expression of the bacterial *uridine 50-monophosphate transferase* AvrAC. The AvrAC inhibits phosphorylation in conserved residues located in the activation site of BIK1 and related kinases (Feng et al. 2012). These results suggest that additional BIK1-related proteins, including PBL2 and PBL5, may be required for this response.

OsRLCK185 is a substrate of OsCERK1 in rice and responsible for chitin- and PGN-induced immune responses (Yamaguchi et al. 2013). It has been observed that OsCERK1 phosphorylates OsRLCK185, which later partly dissociates from the OsCERK1 complex post chitin perception (Yamaguchi et al. 2013). Earlier it was identified that RLCK BSK1 acts as a substrate of brassinosteroid receptor BRI1 and also acts as a positive regulator of brassinosteroid responses (Tang et al. 2008). However, recently, it was found that BSK1was also associated with FLS2 and partially dissociated after flg22 perception. BSK1 was also required for a subset of flg22-triggered responses, but not for the activation of MAPK (Shi et al. 2013). BSK1 is a common positive regulator for both BRI1- and PRR-mediated signaling, but interaction of BIK1 with BRI1 acts as a negative regulator of brassinosteroid-triggered responses (Lin et al. 2013). After brassinosteroid perception, BRI1 phosphorylates BIK1 independently of BAK1, and this leads to the release of BIK1 from the BRI1 receptor complex (Lin et al. 2013). However antagonism exists between the BRI1 and FLS2 pathways, due to the indirect crosstalk by transcriptional regulator BZR1 and not caused by competition between common regulators at the plasma membrane (Malinovsky et al. 2014; Lozano-Duran et al. 2013; Albrecht et al. 2012; Belkhadir et al. 2012). Different PRRs recruit distinct RLCKs, such as BIK1, PBL1, PBL2, and PBL5, and are responsible for FLS2-mediated ROS burst, whereas only BKI1 and PBL1 are responsible for ROS burst mediated by PEPR1/2 activation (Liu et al. 2013; Zhang et al. 2010). Similarly, BSK1 is responsible for flg22-mediated ROS burst but not for elf18 (Shi et al. 2013). The involvement of different RLCKs for specific PRR responses indicates that the choice of specific RLCKs as PRR substrate constitutes another layer in the regulation of signaling, branching from PRR complexes. ROS burst and MAPK activation are the indicating responses to analyze signaling branching from PRR complexes, as both responses are traceable after PAMP treatment (<5 min) and constitute independent signaling (Ranf et al. 2011; Segonzac et al. 2011; Xu et al. 2014). The important role of RLCKs is in the initiation and specificity of PTI signaling and has initiated the search for different RLCK substrates. Recently NADPH oxidase, AtRBOHD, has been identified as direct target for BIK1 as shown in Fig. 4.5 (Kadota et al. 2014). It has been observed that AtRBOHD is the key enzyme responsible for the rapid production of

apoplastic ROS upon PAMP perception (Nuhse et al. 2007; Zhang et al. 2007). For example, flg22 treatment leads to the activation of BIK1, which directly phosphorylates and activates AtRBOHD, leading to the further ROS burst and subsequent immunity (Kadota et al. 2014).

Apart from RLCKs, other substrates of PRR complexes have been identified to play an important role in PTI signaling. In rice, chitin treatment leads to OsCERK1-mediated phosphorylation of the Rac GDP/GTP exchange factor 1 (OsRacGEF1), which further activates OsRac1 as shown in Fig. 4.6 (Akamatsu et al. 2013). Therefore the activation of the OsCEBiP/OsCERK1 complex and OsRacGEF1/OsRac1 module together is essential for chitin-triggered responses and resistance against fungal pathogens in rice (Ono et al. 2001; Suharsono et al. 2002; Akamatsu et al. 2013).

4.8.3 Attenuation

Constitutive or hyperactivation of immune responses is always detrimental for plant growth. Therefore PRR and their immediate downstream signaling must be tightly regulated before and post ligand perception. The exact mechanism for the attenuation of PRR activation is not known exactly. Regulation mechanisms ensure that PRR complexes return to their steady state and are ready to get activated in case of further pathogen attack. As discussed earlier, phosphorylation is the key for the activation of many PRR complexes. Therefore negative regulation of PRR complexes is possible by protein phosphatases. Several phosphatases have been shown to have their association with PRR and/or with associated kinases, to keep the complex in inactive form through dephosphorylation in the absence of ligand binding. In rice, XA21 is kept inactive before ligand binding through the association with ATPase XB24, which promotes XA21 autophosphorylation at specific Ser-Thr sites. Binding of Ax21 to XA21 leads to the disassociation of XB24, thus further allowing XA21 activation and immune responses. Post activation, a phosphatase XB15 known as POL-type protein phosphatase 2C (PP2C) acts on XA21 and dephosphorylates it, further leading to its inactivation as shown in Fig. 4.7. Recently, novel phosphatases have been identified that regulate pre-ligand and post-ligand binding in FLS2/EFR complexes. A key regulatory aspect of surface-associated RLKs is their degradation after post-ligand binding and their subsequent replenishment via de novo synthesis at the plasma membrane. It has been reported that post flg22 perception, FLS2 is subjected to endocytosis and degradation (Lu et al. 2011; Robatzek et al. 2006; Smith et al. 2014). FLS2 degradation is controlled by two E3-ubiquitin ligases, PUB12 and PUB13. They exist in a constitutive complex with BAK1 and are therefore recruited into the FLS2 complex after flg22 binding takes place as shown in Fig. 4.8. It has been observed that phosphorylated PUB12/PUB13 by BAK1 is responsible for polyubiquitination of FLS2, and this polyubiquitination further leads to FLS2 degradation as shown in Fig. 4.8. However the exact role of PB12 and PB13 in FLS2 endocytosis is currently unknown. The negative regulation mediated by PUB12/PUB13 is highlighted in a study which proved that loss of PUB12/PUB13 results in heightened flg22-induced responses and enhanced resistance to PtoDC3000 (Lu et al. 2011; Robatzek et al. 2006). Additional PUBs,

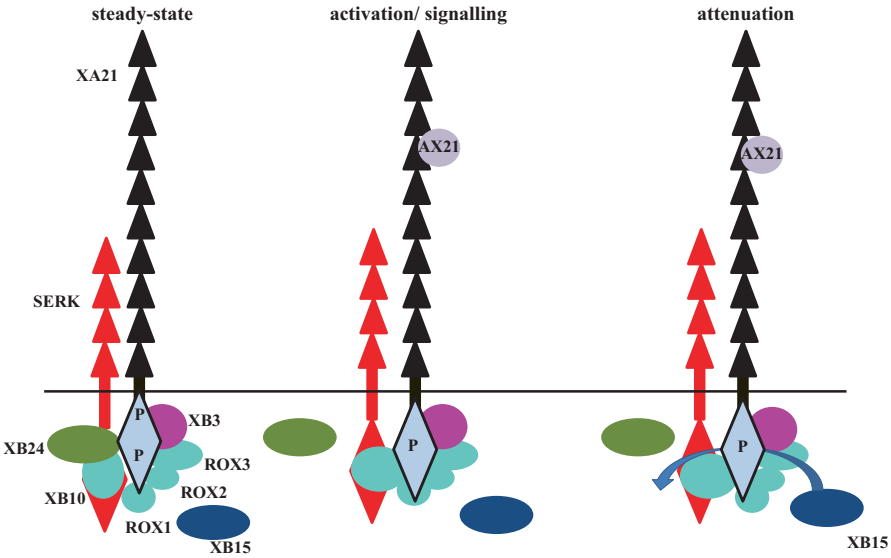

Fig. 4.7 The XA21 complex. In rice, constitutive interaction of XA21 has been observed with the E3-ligase XB3, the ATPase XB24, the PP2C XB15, the WRKY transcription factor XB10, the NOL1/NOL2/sun protein ROX2, the nudC-like nuclear migration factor ROX3 and the thiamine pyrophosphokinase ROX1. XB24 keeps XA21 in an inactive state by inducing auto-phosphorylation at specific Ser-Thr residues. Ligand binding is responsible for XB24 dissociation from XA21, followed by phosphorylation of XA21 at distinct residue(s), which further activates immune signaling. XB15, a phosphatase, further dephosphorylates XA21 and suppress the signaling. Phosphorylation is shown as labelled 'P', and arrows shows the direction of post-translational modifications

PUB22/PUB23/PUB24, were also reported as negative regulators of PTI responses, and their mechanism of regulation is still not known and therefore kept in separate clad forms, PUB12/PUB13 (Trujillo et al. 2008).

Degradation of FLS2, mediated by ligand-induced endocytosis, plays a key role to prevent continuous signaling from the activated surface-associated receptors (Smith et al. 2014). De novo synthesized FLS2 are incorporated into the plasma membrane to replenish the degraded FLS2, so that it restores the sensitivity of the cell for the upcoming pathogen attack (Smith et al. 2014). Hence appropriate PRR trafficking, after ligand perception, is essential for specific signal responses. During cytokinesis and cell expansion, DENN domain protein SCD1 is responsible for clathrin-mediated endocytosis (McMichael et al. 2013). It has been also reported that SCD1 also interacts with FLS2 and is essential for flg22-triggered immune responses (Korasick et al. 2010). Studies with chemical inhibitors of vesicular trafficking impaired flg22-triggered immune responses, suggesting that there is a potential link between endocytosis and flg22-mediated immune responses (Smith et al. 2014).

In *Arabidopsis* flagellin perception through FLAGELLIN-SENSITIVE 2 (FLS2) induces the activation of mitogen-activated protein kinases (MAPKs) and provides immunity. The precise molecular mechanism that connects activated FLS2 to

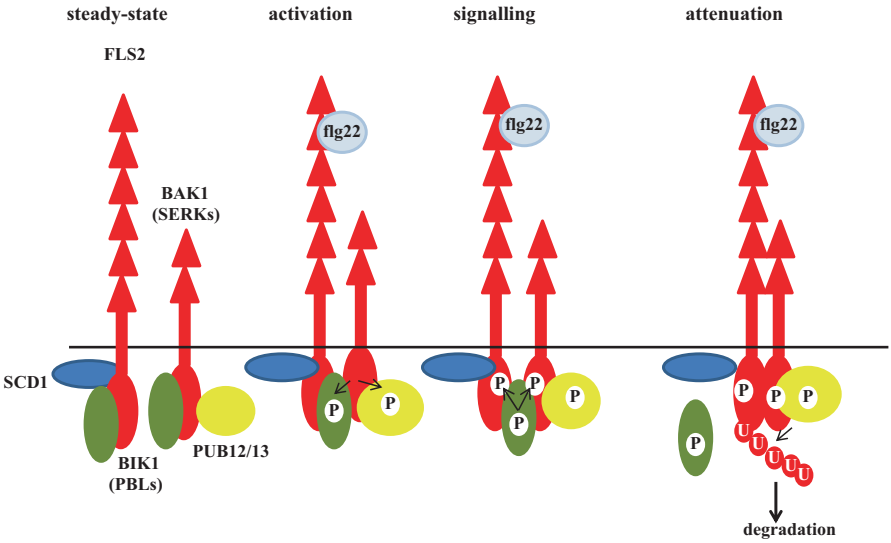

Fig. 4.8 The FLS2 heterodimeric complex. In *Arabidopsis*, constitutive interaction of FLS2 with BIK1 and related PBLs, as well as with SCD1 has been observed. BAK1 interacts with PUB12 and PUB13 constitutively and may associate with BIK1. Post flg22 binding, BAK1 interact with FLS2 and trans-phosphorylates BIK1 (and other PBLs) and PUB12/13. Phosphorylated BIK1 then trans-phosphorylates BAK1 and FLS2, resulting in a downstream signaling. Phosphorylated PUB12/13 then polyubiquitinate FLS2 resulting in its degradation and suppression of signaling. Phosphorylation is shown as labelled 'P', ubiquitination is as shown as labelled 'U', and arrows shows the direction of post-translational modifications

downstream MAPK cascades is still under investigation. Recently it has been identified that differentially phosphorylated MAP kinase also interacts with FLS2 (Mithoe et al. 2016). With the help of targeted proteomics and functional analysis, it has been observed that phosphorylated MKKK7 on specific serine residues negatively regulates flagellin-triggered signaling and basal immunity. MKKK7 is responsible for the attenuation of MPK6 activity and defense gene expression. Moreover, MKKK7 is also responsible for the suppression of the reactive oxygen species burst downstream of FLS2, indicating that MKKK7-mediated attenuation of FLS2 signaling occurs through direct modulation of the FLS2 complex (Mithoe et al. 2016).

In *Arabidopsis*, RLCKs have also been reported to play a key role in the regulation of plant innate immunity. The PBL13 kinase negatively regulates plant innate immunity to pathogenic bacteria *Pseudomonas syringae* and is also associated with RBOHD before pathogen perception (Lin et al. 2015). Hence these findings are consistent with the hypothesis that PBL13 acts to prevent inappropriate activation of defense responses in the absence of pathogen challenge. Similarly a recent study in rice related to functional characterization of four new rice RLCKs from subfamily VII – OsRLCK57, OsRLCK107, OsRLCK118, and OsRLCK176 – has been done. It has been observed that these OsRLCKs interact with the rice brassinosteroid receptor, OsBRI1, in yeast cell, but not the XA21 immune receptor. Silencing

of these genes decreased XA21 gene expression and compromised XA21-mediated immunity to (*Xoo*). Hence, this study demonstrated that these OsRLCKs negatively regulate BR signaling, while positively regulating immune responses by contributing to the expression of the immune receptor XA21 (Zhou et al. 2016).

4.9 Conclusions

Plant receptors for the bacterial PGN and the proteinaceous PAMP Flg and EF-Tu elongation factor and fungal PAMPs have been identified and well-studied; however those involved in LPS perception are not studied in details. One of the most complex signaling molecular machineries is the plant PRR immune complex. When encountered with danger, the simplest mechanism is an initiation of signal when a surface-localized transmembrane receptor is bound to its corresponding ligand. The complete mechanism involves co-receptors, regulatory proteins, and substrates that link PRR activation and negative regulation. Since phosphorylation is a key event to drive the initiation of signaling from PRR, it will be important to decipher whether phosphorylation at specific sites is the key for recruitment or dissociation of the specific signaling component to the receptor complex or to determine the fate of activated PRR. Therefore specific phosphorylated sites would regulate directly or indirectly the branching of signaling from PRR complex. The expression pattern of different proteins, comprising of PRR complexes at the cell, tissue, or organ level, is still unknown. We need to address more PAMP/PRR perception systems to understand how plants integrate the different signals during natural infection. This is particularly important, as infections are often multi-tropic in nature. While PAMP induces overlapping responses, certain PAMP combinations seem to act synergistically or even antagonistically. Our knowledge of molecular mechanisms involved during downstream PRR signaling is very limited. Genetic screening, coupled with interaction studies complemented by the addition of more effector targets, is needed to fulfill this gap. The understanding of the basis of PRR complex formation, organization, activation, and subsequent connection of downstream networking leads to actual immunity and will be a key challenge for the future. In conclusion we say that with the assistance of expanding bioinformatics and molecular biology tools, we will be able to identify novel PAMPs, understanding their perception and signaling in plants.

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Role of NBS-LRR Proteins in Plant Defense

5

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Abstract

The NBS-LRR proteins are encoded by one of the largest and most important gene family involved in disease resistance in plants. Many of these NBS-LRR proteins recognize effectors secreted by pathogens directly or indirectly that in turn activate downstream signaling pathways leading to activation of plant defense response against various classes of pathogens including bacterial, fungal, viral, nematode and insect. Defense response by NBS-LRR protein is a sophisticated strategy that induces effector-triggered immunity (ETI). The NBS-LRR proteins comprised of amino-terminal variable domain, a central nucleotide-binding site (NBS) and carboxy-terminal leucine-rich repeats (LRR) domain. The NBS domain binds and hydrolyzes ATP and primarily functions as a signal transduction switch following pathogen recognition. LRRs are highly adaptable structural domains that are involved in protein-protein interactions, and these LRRs can also evolve very different binding specificities. In the following chapter we have discussed in detail about the present knowledge pertaining to NBS-LRR class of proteins and their prospect in crop improvement against diseases.

Keywords

Plant Defense · NBS-LRR · R-Genes · Effectors · Phytopathogens

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5.1 Introduction

Plants are continuously attacked by a plethora of pathogens. To counter pathogen attack, plants have evolved an immune system, and the major part of this system is contributed by specificity determinants, which are the resistance (R) genes. R genes are set of genes that confer resistance to a wide variety of pathogens including bacteria, fungi, oomycetes, nematodes, and insects. Nucleotide-binding site-leucine-rich repeat (NBS-LRR) genes comprise the largest class in the category of all known R genes which encompasses more than 80% of characterized R genes (McHale et al. 2006; Meyers et al. 1999, 2003, 2005; Friedman and Baker 2007; Zhang et al. 2016; Song et al. 2017). The name NBS-LRR has been assigned due to a central nucleotide-binding domain, which is also known as the NB-ARC {nucleotide-binding adaptor shared by apoptotic protease activating factor 1 (APAF1), certain R genes and cell death protein 4 (CED4)} domain, and their C-terminal leucine-rich repeat (LRR) domain. NBS-LRR proteins form a subclass of the signal transduction ATPases with numerous domains (STAND) super family, a class of molecular switches that are involved in a variety of mechanisms, including immunity, apoptosis (e.g., APAF1 and CED4), and transcriptional regulation (Takken and Goverse 2012).

Plant NBS-LRR proteins show sequence resemblance with the members of the mammalian nucleotide-binding oligomerization domain (NOD)-LRR protein family{also called caspase recruitment domains (CARD), R (purine)-binding, transcription enhancer, pyrin, lots of leucine repeats (CATERPILLER) proteins}, which play crucial role in inflammatory and immune responses (Inohara et al. 2005; McHale et al. 2006). Evolutionary studies based on phylogeny to understand the origin and similarities between plant NBS-LRR and mammalian NOD proteins are inconclusive till date. Though a plain, convergent evolutionary origin was proposed initially (Ausubel 2005; McHale et al. 2006), the most recent study suggests that the plant NBS-LRR and mammalian NOD proteins may be a result of independent evolutionary events that occurred at least twice (Urbach and Ausubel 2017). Whether these events are due to horizontal gene transfer or due to some other mechanism, is yet to be determined.

NBS-LRR genes have been grouped into two classes, namely, TIR-NBS-LRR (TNL) and the non-TIR-NBS-LRR (nTNL). The last residue, D (aspartate) or W (tryptophan), of the conserved kinase-2 motif within the NBS domain helps in distinguishing TNL from nTNL by 95% accuracy (Meyers et al. 1999; Wan et al. 2012). Former class is different from the latter as the former class comprises a toll/interleukin-1 receptor-like (TIR) domain at the protein amino terminus (Bai et al. 2002; Meyers et al. 1999; Zhou et al. 2004; Zhang et al. 2016). In contrast, at the N-terminus, most nTNL genes encode a CC domain, and these nTNL genes are often called as CC-NBS-LRR (CNL) genes (Ameline et al. 2008; Meyers et al. 2003). Apart from CNL genes, a small group of nTNL genes that possesses a special N-terminal domain known as resistance to powdery mildew 8 (RPW8) domain has recently been discovered. RPW8 domain containing nTNL genes represents distinct class of NBS-LRR genes (RPW8-NBS-LRR, RNL) (Bonardi et al. 2011; Collier et al. 2011; Cannon et al. 2004; Shao et al. 2014; Xiao et al. 2001; Zhang et al. 2016).

Commonly, NBS-LRRs are intracellular multidomain proteins that recognize pathogen-derived effectors either directly or indirectly (Cesari et al. 2014; Dodds and

Rathjen 2010; Jones and Dangl 2006; Van der Hoorna and Kamoun 2008; Shao et al. 2016; Song et al. 2017). As per direct model, an NBS-LRR protein binds to the pathogen effector and serves as a substrate for the effector's enzymatic activity (Shao et al. 2016). According to indirect model, NBS-LRR recognizes the modifications of additional host protein(s). Further these modifications in the additional host proteins are targeted by the effector (Shao et al. 2016). Ultimately, the aim of NBS-LRR proteins is to sense and detect the respective pathogen effectors and virulence factors. This recognition leads to the activation of downstream signaling pathway, which results in various immune responses like hypersensitive response at the site of pathogen infection which provides immunity to the host plant (Bittle and Robatzek 2007).

5.2 Susceptibility and Resistance in Plants

Different pathogens such as viruses, bacteria, fungi, and nematodes are responsible to cause many destructive diseases in plants. As the diseases progress, visible symptoms can be seen, and in some cases severe infections can lead to the death of the plants. Often these symptoms include yellowing of leaves and stunted growth of plant, followed by necrosis at the infection site ultimately leading to cell death. To acquire resistance, plants need to suppress pathogen growth and replication at the site of infection.

Plant resistance in response to pathogen attack is associated with the rapid burst of reactive oxygen species (ROS), followed by a localized programmed cell death (PCD) called hypersensitive response (HR) at the infection sites, and increased expression of pathogenesis-related (PR) genes (Chisholm et al. 2006; Gururani et al. 2012; Hammond–Kosack and Jones 1996; Heath 2000; Morel and Dangl 1997; Shao et al. 2016; Takahashi et al. 2003). Other resistance responses include (but are not limited to) the activation of defense gene expression, induced biosynthesis, and accumulation of salicylic acid (SA) and jasmonic acid (JA) (Creelman and Mullet 1995). Hypersensitive response is an especially effective process in limiting pathogens (biotrophs) that require living host cells. Often HR is triggered when an appropriate R gene recognizes an effector or a pathogen elicitor gets recognized by suitable receptor (Minsavage et al. 1990; Nürnberger et al. 1994). The NBS-LRR class of protein play a very important role in both cases as major portion of R genes and immune receptors discovered till date belongs to NBS-LRR and LRR kinase (LRK) class of proteins in plant cell. A well-characterized example of HR mechanism through gene-for-gene interaction is provided by the tomato (*Solanum lycopersicon*) *Cf-9* gene, which confers resistance to races of the fungus *Cladosporium fulvum* expressing the *Avr9* gene (Van Kan and De Wit 1992).

5.3 NBS-LRR Gene-Mediated Resistance to Pathogens in Crop Plants

NBS-LRR gene-mediated resistance has several striking features for disease control. When the resistance is induced in a timely manner, then the signaling cascade can effectively halt pathogen growth with minimal collateral damage to the plant

(McDowell and Woffenden 2003). Most of the NBS-LRR genes exhibit high recognition specificity with elicitors. Specific amino acid changes in the NBS or ARC2 domain of various NBS-LRR R genes such as tomato *I-2*, potato *Rx*, and flax *L6* result in autoactivation and Avr-independent defense signaling (Tameling et al. 2006). Alternatively, a random in vitro mutagenesis of the domains in the R gene sequence can also result in pathogen recognition and subsequent recognition specificities of the selected gene variants. A subset of the mutant *Rx* gene sequences in potato was shown to confer resistance against the original viral strain as well as additional potato virus X strains and a second distantly related virus species (Farnham and Baulcombe 2006).

5.3.1 NBS-LRR Gene Family in Plant Genomes

Recent advancement in sequencing technologies holds the capacity to provide sequence information at a very high-throughput scale (Solexa, Roche; Illumina, single-molecule real-time sequencing). These technologies have opened new vistas to assess plant-microbe interactions. Their uses enable genome-wide analyses of NBS-LRR genes, and this analysis is based on the NB-ARC domain (Meyers et al. 2005). On the basis of the sequenced genomes of various plant species, hundreds of NBS-LRR genes have been identified (McHale et al. 2006). *Arabidopsis thaliana*, the first sequenced plant species, has 165 NBS-LRR genes including 52 CNLs, 106 TNLs, and 7 RNLs (Zhang et al. 2016). Plants that belong to Solanaceae family like *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) have more than twice the number of NBS-LRR genes than *Arabidopsis*, and plants of Solanaceae family possess more CNLs than TNLs (Consortium TG 2012; Guo et al. 2011; Kim et al. 2014; Jupe et al. 2012). It is considered that TNLs have evolved after the divergence of monocots and dicots (Goff et al. 2002; McHale et al. 2006; Meyers et al. 2003). The NBS-LRR class of R genes fit into rapidly evolving gene family, and the number of NBS-LRR genes differs among plant species (Clark et al. 2007; Rafiqi et al. 2009). For illustration, 198 NBS-LRR genes (138 CNLs, 55 TNLs, and 5 RNLs) were acknowledged in *Arabidopsis lyrata.* At the genus level, more differences in R gene composition have been observed. In recent times, identification of novel NBS-LRR genes has been possible with the help of *R* gene enrichment and sequencing (RenSeq) method (Andolfo et al. 2014; Jupe et al. 2013). Overexpression of these genes showed various phenotypes associated with basal defense response such as elevated SA level; therefore, the role of NBS-LRR proteins in basal defense has also been well characterized (Nandety et al. 2013). In this direction, genome-wide analysis of NBS-LRR genes may expand the scope to understand the role of these genes in plant disease resistance.

On the basis of the N-terminus, NBS-LRR proteins have been classified into two categories in plants which are TIR-NBS-LRR (TNL) and the non-TIR-NBS-LRR (nTNL). This classification is, however, not always precise. nTNL proteins comprise those NBS-LRR proteins which possess other domain at their N-terminus rather than TIR domain. NBS-LRR genes usually contain additional domains such

as CC or RPW8 at the N-terminus and an unpredictable number of LRR domains at the carboxy-terminus (Shao et al. 2014; Zhang et al. 2016).

The functionality of NBS-LRR proteins is dependent on below described domains:

5.3.1.1 TIR Domain

TIR is most common domain in the group of NBS-LRR proteins (Zhang et al. 2016). Many factors which interact with animal TLRs (toll-like receptors) also possess TIR domain, for example, MyD88. During these interactions, TIR domains interact physically (Riedl et al. 2005). Thus TIR domains facilitate heterodimerization in plants like that of some animal TLR receptors (Nandety et al. 2013; Williams et al. 2014). In plants, TIR domain participates in the detection of Avr proteins, e.g., interaction between the TIR domain of tobacco N protein and the tobacco mosaic virus (TMV) p50 protein. This interaction leads to hypersensitive response (HR). Noticeably, NRIP1 (the additional host protein) is requisite for this interaction (Nandety et al. 2013). Recent studies suggest the role of TIR domain in pathogen recognition and in signaling cascade (Williams et al. 2014). However, TIR domain is notably absent from most monocotyledons (Tarr and Alexander 2009).

5.3.1.2 CC Domain

The coiled-coil (CC) domain usually ascribed a role analogous to the TIR domain as it acts as a mediator in interactions with other essentials of the signaling cascade. It has been observed that the EDVID motif is conservative motif in the coiled-coil domain of all CC-NBS-LRR proteins (Collier and Moffett 2009) but exceptions are also present in case of RPS2, RPS5, and Dm3 proteins. Mutations in this motif cause turbulence in the intramolecular interaction in between NBS and LRR domains; as a result decreased resistance response against pathogen attack has been observed (Zhang et al. 2016).

The CC structure is made up of two or more α-helices with a super helical twist. It represents heptad repeat sequence $(abcdefg)_n$ where 'a' and'd' are hydrobhobic amino acid residues, while the 'e' and 'g' represent polar amino acid residues. The CC structure is the most common in NBS-LRR proteins present in both monocots and dicots. It has been studied that CC domain alone is able to induce cell death, for instances, the *Arabidopsis RPS5*, *RPS2*, *RPM1* and *activated disease resistance 1* (*ADR1*); *Nicotiana benthamiana N requirement* gene 1 (*NRG1*); and barley *mildew-resistance locus A 10* (*MLA10*) genes (Jacob et al. 2013). But in contrast, central NBS domain of potato Rx protein is sufficient to trigger cell death (Moffett et al. 2002).

R proteins have also been known to contain a CC domain which binds to target proteins of pathogen effectors, e.g., the CC domain of *A. thaliana* RPS5 protein interacts with PBS1, which is a target of *Pseudomonas syringae* AvrPhB effector (Ade et al. 2007).

Fig. 5.1 Subdomains of NBS domain, showing comparison in between plants and animals

5.3.1.3 RPW8 Domain

Apart from CNL genes, a small group of nTNL genes that possess a special N-terminal domain known as RPW8 (resistance to powdery mildew8) domain has recently been discovered. RPW8 domain containing nTNL genes which represent a distinct class of NBS-LRR genes, i.e., RPW8-NBS-LRR, RNL (Bonardi et al. 2011; Cannon et al. 2004; Collier et al. 2011; Shao et al. 2014; Xiao et al. 2001; Zhang et al. 2016). Species-specific duplication studies on five *Rosaceae* species, namely, *Malus domestica* (apple), *Fragaria vesca* (strawberry), *Pyrus bretschneideri* (pear), *Prunus mume* (mei), and *Prunus persica* (peach), clearly described RNL as a distinct class of NBS-LRR genes (Zhong et al. 2015).

5.3.1.4 NBS Domain

The NBS domain was identified in NBS-LRR proteins, and it shows similarity to the homologous sequences in the animal APAF-1 and CED-4 proteins (Van der Biezen and Jones 1998). NTPase activity is a characterizing feature of NBS domain. This domain is suggested to play a crucial role as a molecular switch in activating signal transduction. In the signaling cascade, reversible nucleotide binding leads to changes in the conformation of the NBS domain. The conformational changes in NBS domain lead to the activation/deactivation of the whole receptor (Tameling et al. 2006).

After human APAF-1 studies, it has become possible to speculate about the structure and function of the NBS domain in plants. Four subdomains, NB, ARC1, ARC2 and ARC-3, are found in NBS domain of the human APAF-1 protein (Fig. 5.1) (Riedl et al. 2005). ARC3 domain is absent in the plant R proteins; nevertheless a short linker connecting ARC2 with LRR is found in the plant R proteins. ARC1 is made up of a bunch of α-helices and ARC2 of α-helices rolled up in a winged helix fold. The spatial structure of the NBS domain differs, and it depends on whether it is combined with ATP or ADP (Tameling et al. 2002). Many conservative motifs such as the P-loop (Walker A or kinase 1), the RNBS-A, kinase 2 (Walker B), kinase-3a, RNBS-B, RNBS-C, GLPL, and RNBS-D have been identified in this domain (Wan et al. 2012). P-loop, kinase-3a, kinase 2, and GLPL conserved motifs are common in both TNLs and nTNLs subfamilies (Wan et al. 2012).

NBS domain has the capability to bind and hydrolyse ATP, and thus it confers switching of R protein from active to inactive state (Riedl et al. 2005). Mutations in the NBS domain are attributed to the autoactivation of resistance response in the absence of an elicitor, and this is characterized by an increased susceptibility to pathogen. Constitutive active resistance response is because of disturbance in ATP hydrolysis. Therefore, the binding with ATP rather than ADP seems necessary to

activate a receptor. It has been proven that the autoactivation mechanism of NBS-LRR protein is generally associated with the mutations in the NB and ARC2 subdomains (Takken et al. 2006). In spite of this, the ARC1 subdomain seems to be involved in binding to LRR domain; this has been proven after the evolution of loss of functional mutants. However, low stability of ARC1 domain is suggested for the cooperation of some additional factors which are necessary in the signaling cascade (Rairdan and Moffett 2006).

5.3.1.5 LRR Domain

The C-terminus of NBS-LRR protein is occupied by LRR domain. Tandem repeat of 20–30 amino acids containing a consensus sequence LxxLxLxxNxL is the essential structural element of LRR domain. In consensus sequence LxxLxLxxNxL, L is a leucine residue, N is asparagine/threonin/serine or cystein, and x is any amino acid (Bella et al. 2008; Kajava 1998; Matsushima et al. 2007; Stange et al. 2008). NBS-LRR protein with an LRR domain has to contain at least two LRR repeats. Horseshoe-shaped superhelix is the usual tertiary structure of LRR domain. Each repeat of amino acid forms other coils of the horseshoe-shaped super helix. It is proposed that the LRR domains constitute a platform for protein-protein interactions (Bella et al. 2008; Kobe and Kajava 2000). The tertiary structure of LRR usually has an inner surface composed of parallel β-strands. These parallel β-strands are composed of hydrophobic aliphatic residues of a consensus sequence and are assumed to be a site of interaction with other proteins, which in case of plant NBS-LRR proteins provides a crucial condition for the recognition of specific elicitors.

The outer part of the LRR domain is usually composed of α-helices which are connected with β-strands by β-turns (Bella et al. 2008). Consensus sequence LxxLxLxxNxL participates in interactions with microbe-associated molecular patterns (MAMPs) or microbe-induced molecular patterns (MIMPs), and this has been proven by the differences in the specificity of flax rust (*Melampsora lini*) effector recognition by the *P* and *P2* genes of flax (*Linum usitatissimum*).

After binding with a proper MIMP or MAMP, conformational changes within the LRR domain occur that lead to the dissociation of the LRR domain from the NBS domain (Wang et al. 2007). It is suggested that the dissociation of the LRR and NBS domains from NBS-LRR protein might not be required for the activation of the receptor (Rairdan and Moffett 2006; Van Ooijen et al. 2008). In contrast, rhythmic rounds of dissociation and reassociation could lead to the amplification of the signal originating from elicitor recognition (Rairdan and Moffett 2006).

5.3.1.6 Other Domains in NBS-LRR Protein

Despite the domains listed above, some additional domains are located at the N-terminus and more rarely at the C-terminus in many of the NBS-LRR proteins. One of the examples for such proteins is *Arabidopsis* RRS-1R that have WRKY domain at their N/C-terminal. In this protein plant WRKY transcription factors have been identified by the presence of WRKYGQK conserved motif situated at the N-terminus of the NBS-LRR protein. This WRKY transcription factor also has a

typical domain similar to Zn (Zinc) finger motif and plays an essential role in regulating the expression of genes that are involved in plant resistance (Eulgem and Somssich 2007; Ulker and Somssich 2004).

In many instances, WRKY transcription factors are directly affected by some NBS-LRR proteins. Alleles of barley MLA proteins detect the related Avr proteins of *Blumeria graminis* and then disengage HvWRKY1/2 transcription factor, which has been found to repress resistance genes (Liu and Coaker 2008). It has been observed that some NBS-LRR proteins also contain a domain having similar structure as that of a WRKY transcription factor; thus, it became feasible to affect gene expression directly. An instance of such protein is the RRS-1R receptor of *A. thaliana* which is one of the TIR-NBS-LRR proteins. RRS-1R receptor possesses a C-terminal WRKY domain and recognizes the PopP2 effector of *Ralstonia solanacearum* (Deslandes et al. 2002). WRKY domain may act as a suppressor of signaling cascade responsible for bringing resistance against a pathogen (Noutoshi et al. 2005); this has been proven by the analysis of *Arabidopsis thaliana* mutants, known as SLH1. These mutants possess alteration in single amino acid in the WRKY domain of the TIR-NBS-LRR-WRKY protein (Noutoshi et al. 2005).

5.4 Intramolecular Interactions

Structural analyses of plant NBS-LRR are dependent on domain-swapping experiments, mutant analysis, and 3D modeling of NBS-LRR protein. Overall, it seems that plant NBS-LRR proteins have complex interactions among their domains; however, the function of those interactions in pathogen detection and activation of signal transductions seems to vary with some degree among plant NBS-LRR proteins. The first evidence for intramolecular interaction of plant NBS-LRR protein domains came after the work on Rx and Bs2 R proteins from pepper (Leister et al. 2005; Moffett et al. 2002). It has been known that plant NBS-LRR proteins have complex interactions among their domains; however, the role of intramolecular interactions in pathogen detection and activation of signal transductions seems to vary with some degree among plant NBS-LRR proteins. Co-immunoprecipitation experiments in Rx demonstrate physical interactions between the CC and NBS-LRR domains and between the CC-NBS and LRR domains.

It is considered that interaction of Rx domains with domains from the closely related NBS-LRR proteins Bs2 and HRT (an *Arabidopsis* R protein that confers resistance to turnip crinkle virus) has also been detected, suggesting some degree of 'promiscuity'; however, such physical interactions are not sufficient to reconstitute functional signaling molecules (Rairdan and Moffett 2006). Further, the intramolecular interactions of RPS5 have been studied, and it was found that the intramolecular interactions of RPS5 are similar but not identical to those reported for Rx and Bs2. Co-immunoprecipitation experiments have revealed that the CC and LRR domains individually interact with the NBS domain, but not with each other (Moffett et al. 2002).

5.5 NBS-LRR Formation of Oligomers

Several plant NBS-LRR proteins are known to form oligomers; however, the function of the NBS domain in that oligomerization is yet to be resolved. For example, several domains of Rx are able to interact when expressed in trans, as are domains of Bs2, although it has been found that some of those interactions are 'preferentially' intramolecular (Leister et al. 2005; Moffett et al. 2002). Oligomer formation of full-length Rx in the absence of pathogen effector has not been detected, and thus the direct interaction of NBS domains has not yet been detected for these proteins (Moffett et al. 2002). It has also been observed that in the presence of pathogen effector, NBS-LRR proteins form oligomers but the TIR domain is the only domain directly associated with that oligomerization, e.g., in N protein of tobacco (Mestre and Baulcombe 2006). But later on, it has been observed that in case of RPS5, the amino-terminal CC domain of RPS5 also forms oligomers. It suggests that in case of plant NBS-LRR proteins, the amino-terminal domain may be involved in the formation of oligomers, which differs from APAF1 and CED-4. Finally, study on tobacco N protein has revealed that the formation of oligomers is necessary but not sufficient for disease resistance. In addition, formation of oligomers is an early event in pathogen detection because downstream signal transduction mutants do not affect the formation of oligomers (Mestre and Baulcombe 2006).

5.6 Major Classes of R Proteins Containing at Least One NBS and/or LRR Domain

Plant resistance genes can be classified into nine groups. This classification is based on the organization of their amino acid motif and their membrane spanning domains (Fig. 5.2 and Table 5.1). In the majority of R proteins, LRR domain is present, and it plays important role in recognition specificity (Gururani et al. 2012; Jones 2001).

Class-I: CC-NBS-LRR genes are the first major class of R-genes. Proteins encoded by NBS-LRR genes are found in cytoplasm. First NBS-LRR protein encoded by NBS-LRR gene was discovered as a cytoplasmic protein. It possesses a putative CC at the N-terminus, a NBS, and a C-terminal LRR. *RPM1* resistance gene of *Arabidopsis* is the example of the first major class of R-genes (*I-2*).

Class-II: The second class of resistance genes includes the cytoplasmic proteins which possess N-terminal TIR domain, a NBS motif, and C-terminal LRR domain. The tobacco *N* gene, *RPP5* gene, and flax *L6* gene are the examples of the second class of R-genes (Gururani et al. 2012; Lawrence et al. 1995).

Class-III: The third class of R-genes lacks NBS motif and consists of extra cytoplasmic leucine-rich repeats (eLRR). This eLRR is attached to a transmembrane domain (TrD). eLRRs are supposed to play main role for certain defense proteins like polygalacturonase-inhibiting proteins PGIPs (Gururani et al. 2012; Jones and Jones 1997), but still no direct role of eLRRs in pathogen recognition and in activation of defense genes have been observed (Gururani et al. 2012; Jones

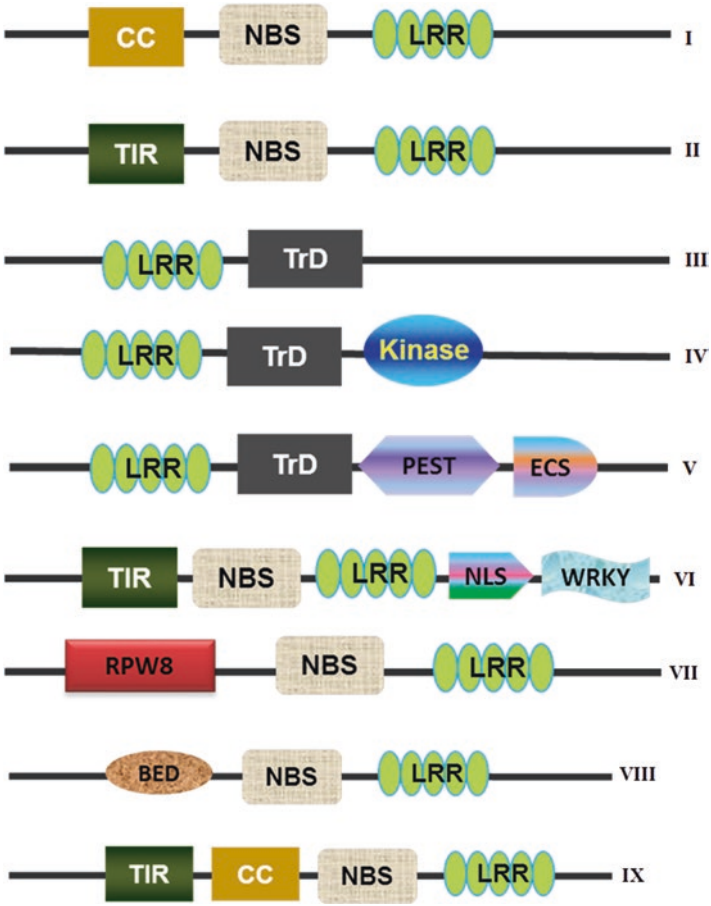

Fig. 5.2 Major classes of plant NBS-LRR genes with important domains: *LRR* leucine-rich repeats, *NBS* nucleotide-binding site, *ECS* endocytosis cell signaling domain, *TIR* toll/interleukin-1-receptors, *CC* coiled coil, *TrD* transmembrane domain, *PEST* amino acid domain, *NLS* nuclear localization signal, *WRKY* amino acid domain

2001). The *Cladosporium fulvum* resistance genes (*Cf-9*, *Cf-4*, and *Cf-2*) are the examples of this class.

Class-IV: The fourth major class of the resistance genes have an LRR extracellular domain, a transmembrane domain (TrD), and an intracellular KIN (serine-threonine kinase) domain (Gururani et al. 2012; Song et al. 1995). *Xa21* gene of rice which shows resistance against *Xanthomonas* is an example of this class.

Class-V: The fifth class encoding resistance genes contain the extracellular LRRs (putative), a Pro-Glu-Ser-Thr (PEST) domain, and an ECS (short proteins motifs). The PEST domain is involved in protein degradation, and it is found only in tomato *Ve2*, not in tomato *Ve1*. The ECS is supposed to target protein for receptor-mediated endocytosis.

Table 5.1 Major classes of plant NBS-LRR genes with important domains

S.No	Major R-gene classes	Domains											Examples
		LRR	NBS	RPW8	TIR	Kinase	CC	TrD	PEST	ECS	NLS	WRKY	
I	CC-NBS-LRR	+	+	–	–	–	+	–	–	–	–	–	*I2*, *RPS2*, *RPM1*
II	TIR-NBS-LRR	+	+	–	+	–	–	–	–	–	–	–	*N*, *L6*, *RRS1*, *RPP5*
III	LRR-TrD	+	–	–	–	–	–	+	–	–	–	–	*Cf-9*, *Cf-4*, *Cf-2*
IV	LRR-TrD-Kinase	+	–	–	–	+	–	+	–	–	–	–	*Xa21*
V	LRR-TrD-PEST-ECS	+	–	–	–	–	–	+	+	+	–	–	*Ve1*, *Ve2*
VI	TIR-NBS-LRR-NLS-WRKY	+	+	–	+	–	–	–	–	–	+	+	*RRS1*
VII	RPW8-NBS-LRR	+	+	+	–	–	–	–	–	–	–	–	*RPW8.1*, *RPW8.2*
VIII	BED-NBS-LRR	+	+	–	–	–	–	–	–	–	–	–	Poptr_1:787192
IX	TIR-CC-NBS-LRR	+	+	–	+	–	+	–	–	–	–	–	Reported in *Populus trichocarpa*

+ Present; – Absent

LRR leucine-rich repeats, *NBS* nucleotide-binding site, *ECS* endocytosis cell signaling domain, *TIR* toll/interleukin-1-receptors, *CC* coiled coil, *TrD* transmembrane domain, *PEST* amino acid domain, *NLS* nuclear localization signal, *WRKY* amino acid domain, *RPW8* resistance to powdery mildew8, "BED-type zinc finger domain"

Class-VI: *Arabidopsis RRS1-R* gene is an example of the seventh major class of R-genes. *RRS1-R* gene confers defense against bacterial phytopathogen *Ralstonia solanacearum*. Recently, *RRS1-R* gene has been included as a new member of the TIR-NBS-LRR class. TIR-NBS-LRR falls in R-protein class. RRS1-R has a C-terminal extension, along with a putative NLS (nuclear localization signal) and a WRKY domain (Deslandes et al. 2002, 2003). The WRKY domain comprises a 60 amino acid region.

Class-VII: The ninth class of R-genes contains RPW8 domain at the N-terminus, along with a NBS and LRR domain. *Arabidopsis RPW8.1* and *RPW8.2* genes are the examples of this class (Xiao et al. 2005).

Class VIII: This class of NBS-LRR genes contains a BED finger domain at their N-terminus (Kohler et al. 2007). This domain consists of about 50–60 amino acid residues which possess a shared pattern of cysteine and histidine amino acid residues which form a zinc-finger DNA-binding domain. This BED domain is present in rice *Xa1* gene and poplar's poptr_1:787192 (Bai et al. 2002; Kohler et al. 2008).

Class IX: Another class of NBS-LRR genes has been predicted through genome-wide study of NBS-LRR in *Populus trichocarpa* (Kohler et al. 2008). This class contains both TIR and CC domains at the N-terminus and was termed as TCNL (TIR-CC-NBS-LRR). Perhaps this may have emerged by gene recombination events as they contain both TIR and CC domains.

5.7 Instance of Plant NBS-LRR Genes Conferring Defense Against a Wide Range of Pathogens

Many plant R genes, which have been studied till now, confer resistance against a plethora of pathogens and for bulk of plant diseases; the genetics of susceptibility are less corporeal. Some of the examples of the plant NBS-LRR gene which confer resistance against pathogen attack have been discussed below.

5.7.1 Plant NBS-LRR Genes Conferring Resistance Against Bacterial Pathogens

It has been identified that both plant and animal bacterial pathogens transport virulence proteins into the host cytoplasm by means of the type-III secretion system (T3SS). T3SS is also known as injectisome (Gururani et al. 2012). This system enables Gram-negative bacteria to secrete and instill pathogenicity proteins into the cytosol of host cells (Galan and Collmer 1999). HR and pathogenicity (hrp) and HR and Conserved (hrc) genes encode T3SS. Mutations in hrp and hrc eliminate bacterial pathogenicity in susceptible host plants and the ability to bring out HR in non-host/cultivar-specific resistant plants. In rice, susceptible and resistant alleles of *Xa27* encode identical proteins. However, R allele expresses only when a rice plant

faces bacterial AvrXa27 and the product of AvrXa27 is a nuclear localized T3SS effector. *Xa27* express only in the environs of infected tissue; however *Os8N3* (dominant rice gene) is an exception. It is upregulated by a bacterial type-III effector protein; thus it bestows gene-for-gene-specified disease susceptibility (Kaloshian 2004). Some plant R genes may confer resistance against unrelated or distantly related bacterial pathogens (Zhao et al. 2005) and demonstrate the transport feasibility of non-host *R* gene in between maize and rice. It was proposed that maize *Rxo1* detects *Xanthomonas oryzae* pv. oryzicola, and thus *Xanthomonas oryzae* is able to cause bacterial streak disease. In contrast, *Rxo1* has also been found to confer resistance to the distinct pathogen *Burkholderia andropogonis* (causes bacterial stripe of sorghum and maize).This study indicates that the same gene may control resistance toward both pathogens and nonpathogens of maize. This gives an idea that an NBS-LRR gene could be transferred between distinctly related cereals.

5.7.2 Plant NBS-LRR Genes Conferring Resistance Against Fungal Pathogens

Diseases which are caused by fungus contribute most devastatingly toward crop yield loses in approximately all major crops (Wani 2010). It has been studied that the variation in sequence and copy number within the central LRR domain of the gene plays an important role in identifying recognition specificity (Brande et al. 2001). For instance, sequence alterations in tomato *Cf*-4 and *Cf-9* genes play a key role in determination of recognition specificity. These genes confer resistance in tomato plants against *Cladosporium* (biotrophic leaf mold pathogen). HR is triggered upon detection of the fungus-encoded Avr4 and Avr9 peptides (Brande et al. 2001). Recently, a virus-induced gene silencing method for the validation of *Ve*-mediated signaling exposed that downstream signaling cascade of *Ve1* needs *EDS1* (enhanced disease susceptibility 1) and *NDR1* (non-race-specific disease resistance 1) gene. Another example of fungal R genes is the *RPW8.2* (*Arabidopsis thaliana* gene) which is induced by powdery mildew (Wang et al. 2009). It might also have been involved in reducing and enhancing of oxidative damage to the host cell and callosic encasement of the haustorial complex (EHC) formation, respectively. Marking of RPW8.2 to the extra haustorial membrane (EHM) needs normal functioning of the actin cytoskeleton.

5.7.3 Plant NBS-LRR Genes Conferring Resistance Against Oomycetes Pathogens

Many diseases like sudden oak death and late blight of potato are caused by phytopathogenic oomycetes. Cloning and functional analyses of four *Rpi* genes, *Rpi-blb3*, *R2*, *Rpi-abpt*, and *R2*-like, revealed that these genes contain all signature sequence characteristics of LZNBS-LRR (leucine zipper nucleotide-binding site-leucine-rich repeat). Also, several functional *R* genes which confer resistance to late

blight have been cloned, and it has been observed that all of them belong to the NBS-LRR class of plant R genes (Bendahmane et al. 2000; Van der Vossen et al. 2003). Another instance of oomycetes R genes with NBS-LRR domain is Dm3 (downy mildew-resistance gene) (Shen et al. 2002). *Dm3* is present in *Bremia lactucae* and belongs to the large RGC2 (resistance gene candidate2) multigene family (McHale et al. 2006).

5.7.4 Plant NBS-LRR Genes Conferring Resistance Against Nematode Pathogens

Plant parasitic nematodes acquire nutrition from the cytoplasm of living cells. Basically, these are obligate parasites, and many ectoparasites and endoparasites come under the category of nematodes. Defense mechanism in plants against root-knot nematode was identified for the first time in *Lycopersicum peruvianum* Mill. (Watts 1947). *Lycopersicum peruvianum* Mill. is a wild variety of cultivated tomato (Watts 1947). Tomato *Mi* gene confers resistance to three root-knot nematodes that are *Meloidogyne arenaria*, *Meloidogyne incognita*, and *Meloidogyne javanica* (Gilbert and McGuire 1956). *Mi* gene-encoded protein having CC-NBS-LRR domains was transferred into cultivated variety of tomato. Positional cloning approach was used to isolate *Mi* gene. The defense mechanism triggered by *Mi* gene involves a HR response in the host (Dropkin 1969). Tomato *Mi* gene is the cloned root-knot nematode defense gene (Williamson and Kumar 2006). *Mi* gene participates in the resistance mechanism by following a gene-for-gene model. Similarly, the potato *Gpa2* gene shows resistance against *Globodera pallida* (potato cyst nematode). *Gpa2* gene is a member of the NBS-LRR gene family, and it also possesses a leucine zipper near its N-terminus. This *Gpa2* gene shows similarities to the *Rx1* gene in amino acid sequence. Studies indicate that *Rx1* gene confers defense in potato against potato virus X (Van der Voort et al. 1999).

5.7.5 Plant NBS-LRR Genes Conferring Resistance Against Viral Pathogens

Major characterized viral R genes from plants fall into the NBS-LRR class of R genes. For instance, resistance in tomato against TSWV (tomato spotted wilt virus) is associated with *Sw-5* gene. It has been studied that *Sw-5* gene is involved in broad and stable resistance (Rosello et al. 1998). *Sw-5* locus resistance allele encodes a CC-NBS-LRR resistance protein, and it has been studied that Sw-5 protein is similar to the Mi protein of tomato with the exception of 4 LZs at the amino terminus (Brommonschenkel et al. 2000). Another example is *RT4-4* gene which belongs to TIR-NBS-LRR class of NBS-LRR R gene and is generally involved in resistance response in common bean (*Phaseolus vulgari* cv. Othello) against viral attack (Seo et al. 2006). It functions across two plant families, which are Cucurbitaceae and Solanaceae.

5.7.6 Plant NBS-LRR Genes Conferring Resistance Against Insect

Resistance against insects has been studied since long in plants (Bent 1996; Dempsey et al. 1998; Panda and Khush 1995; Quisenberry and Clement 2002), and a number of single dominant resistance genes have been mapped (Venter and Botha 2000; Yencho et al. 2000). In staple crops such as wheat and rice, most of these mapped genes have been characterized.

To date, only some of the insect R genes belonging to NBS-LRR group of resistance genes were cloned. For instance, *Mi-1* confers defense to potato aphid (*Macrosiphum euphorbiae*) and whitefly (*Bemisia tabaci)*. Lettuce *Nr*-gene confers resistance against only one single species of aphid (*Nasonovia ribisnigri*) (Reinink and Dieleman 1989). Other examples include apple *Sd1* gene, which shows resistance against *Dysaphis devecta* (rosy leaf curling aphid) and melon *Vat* gene which confers resistance against the cotton/melon aphid *Aphis gossypii* (Kaloshian 2004).

5.8 Sensing of Effector Protein by NBS-LRR Protein

It is well known that all LRR domains perform the same kind of functions. They sustain an NBS-LRR protein in an auto-inhibited state when pathogen is absent in host cell and this domain also confers specificity in pathogen recognition. The amino-terminal half of the LRR is supposed to act in intramolecular signal transduction, and the carboxy-terminal half plays a role in pathogen detection. The structural support for the function of LRR domain came from elucidation of the tertiary structure of the LRR domain of wild emmer wheat (*Triticum dicoccoides*) *Lr10* gene (Sela et al. 2012). In number of NBS-LRR proteins, such as L, Mla, and Rx, LRR domains possess a large number of hyper variable amino acid residues and plays a major role in pathogen recognition (Dodds et al. 2006; Seeholzer et al. 2010). Studies on domain swapping and individual mutations indicate that pathogen recognition specificity resides partly in the C-terminal of the LRR domain (Bendahmane et al. 2000; Sela et al. 2012).

5.9 Activation

Pathogen presence is perceived by the LRR; thus intramolecular signal is expanded to the remainder of the protein. In the resting state, the LRR and TIR-CC domain of NBS-LRR protein interact with the NB-ARC domain. These domains together form a closed conformation. It has been proven through biochemical studies carried out in tomato I-2, flax M, Mi-1, and L6 and barley MLA27. In auto-inhibited state, ADP binds the NB-ARC domain, or ADP bounded form on NB-ARC domain is an auto-inhibited state (Tameling et al. 2002; Williams et al. 2014). The crystal structure studies of CED4 show that in open configuration the NB-ARC domain binds to ATP. In activated state, LRR is suggested to trigger a conformational change in the NB-ARC domain. This conformational change allows exchange of ADP for ATP

and helps in adopting more open conformation. Constitutive expression of NBS-LRR genes has been observed during disturbance in ATP hydrolysis activity. It has been proven by the studies on *I-2* mutants. Additional evidence comes from studies on flax rust NBS-LRR protein M. The wildtype M protein co-purified with ADP and an auto-active mutant (D555V) preferentially co-purified with ATP (Williams et al. 2014). It has earliar been studied that in the resting state of the NBS-LRR, part of the tobacco N protein was present in ATP-bound form, while interaction with the effector molecules triggered exchange of ATP for ADP (Ueda et al. 2006). Both events indicate that NBS-LRR protein activation takes place due to the changes in nucleotide-binding state.

Recently, an unexpected and additional enzymatic activity has been identified in the NB-ARC domain of NBS-LRR proteins: PSiP from corn, R1 from rice (NB), and RPM1 from *Arabidopsis*. It has been proven that ATP is hydrolyzed by this domain in anticipation of the nucleoside instead of diphosphate (Mayerhofer et al. 2016), thus indicating that phosphatase activity is associated with NBS domain. The phosphatase activity for NB is consistent and after activation of the NBS-LRR protein returns to an adenosine-bound state rather than an ADP bound conformation.

Once NBS-LRR protein gets activated, it acquires conformational changes in its structure. Conformational changes in NBS-LRR protein may expose different binding surfaces for the interactions with other proteins. Activation of an NB-LRR protein, and a subsequent change in conformation, may expose different potential binding surfaces allowing distinct interactions with other proteins. For instance, *I2* mutants having differences in their nucleotide-binding state (ADP, ATP, or vacant) illustrate diverse interaction patterns in a yeast two-hybrid assay (Lukasik-Shreepaathy et al. 2012).

5.10 NBS-LRR Signal Transduction

Gene-for-gene model is based on the physical interaction between R proteins and pathogen effectors. This physical interaction results in the plant defense responses and eventually leads to resistance (Keen 1990). The tomato non-NBS-LRR Pto protein kinase interacts directly with its cognate bacterial effector AvrPto within the serine-threonine kinase activation domain. Tomato Pto also interacts directly with second bacterial effector AvrPtoB. AvrPtoB has intrinsic E3 ubiquitin ligase activity. This direct interaction with effectors was demonstrated in NBS-LRR genes. This has led to the guard hypothesis, which predicts that an effector protein interacts with a host target, which is itself recognized by more than one NBS-LRR protein (Jones and Dangl 2006).

This is supported by *Arabidopsis* RIN4 protein. *Arabidopsis* RIN4 protein is an example of a host target for type-III bacterial effectors, which is recognized by at least two CNL R proteins (Mackey et al. 2002).Yeast two hybrid analyses of RPM1-RIN4 interactions proved that the amino-terminal domain seems to mediate the physical association between resistance proteins and pathogen effector targets (Young and Roger 2006). This is at least applicable for those resistance proteins

that use an indirect recognition mechanism (Young and Roger 2006). Therefore, it has been suggested that the amino-terminal domain of plant NBS-LRR proteins may be involved in both detection of the pathogen signal and activation of the downstream response.

RIN4 is also targeted by structurally unrelated bacterial effectors AvrRpm1 and AvrB. Phosphorylation of RIN4 caused by AvrRpm1 and AvrB activates the R protein RPM1. A third effector, AvrRpt2 is recognized by RIN4 inside the plant cell. This effector cleaves RIN4 at two sites, and this cleavage activates the NBS-LRR protein, RPS2. Consequently, activation of HR on pathogen shot triggers a resistance response called SAR. SAR results in the accumulation of salicylic acid (SA) throughout the plant and the consequent expression of a characteristic set of defense genes. The SAR makes plants more resistant to subsequent attack by a range of other pathogens (Glazebrook 2001). Some defense responses require jasmonic acid (JA) and ethylene (ET) as signal molecules. The discovery of new genes and mutants allows dissection of local and systemic signaling cascade networks. These further highlight the complex interplay between defense molecules such as nitric oxide, SA, reactive oxygen intermediates, JA, and ET (Thomma et al. 2001).

Feys and Parker (2000) detected two *Arabidopsis* mutant *ndr1* and *eds1*. The mutant *ndr1* and *eds1* suppress race-specific resistance to strains of the bacterium *Pseudomonas syringae*. Generally, EDS1 and NDR1 are independently required for the function of different NBS-LRR genes. TIR-NBS-LRR R genes are suppressed by EDS1, whereas a subset of non-TIR-NBS-LRR R proteins is suppressed by NDR1 (Joshi and Nayak 2011).

RAR1/SGT1/HSP90 {RAR1 (required for Mla-dependent resistance 1), SGT1 (suppressor of G2 allele of SKP1), and HSP90 (heat shock protein 90)} are the cytoplasmically localized signaling complexes, and these proteins regulate R gene-mediated resistance (Austin et al. 2002; Azevedo et al. 2002; Muskett et al. 2002). All components work together to stabilize various NBS-LRR R protein complexes. SGT1 is the essential component for the function of SCF (Skp1-cullin-F-box protein) E3 ubiquitin ligase complex. SCF E3 ubiquitin ligase complex targets proteins for degradation and this degradation is caused the 26S proteasome. Therefore, R gene-triggered defense is also mediated by the ubiquitin-proteasome pathway. The components of the mitogen-activated protein kinase (MAPK) cascades are also other key regulators in the defense mechanism (Asai et al. 2002). Importantly, SA-inducible defenses are negatively regulated by the MAPK, EDR1 (Frye et al. 2001), whereas MAPK4 seems to differentially regulate JA and SA signals (Petersen et al. 2000). These studies strongly suggest that MAPK modules participate in different plant disease resistance pathways. Ankyrin repeat protein, NPR1, is also an important feature of the systemic signaling. Initially, it was identified as an SA response regulator which participates in both ISR (induced systemic resistance) and SAR. On treating *Arabidopsis* seedlings with SA, movement of NPR1 to the nucleus was observed. In the nucleus, NPR1 binds to many TGA (TGACG DNA motif) class transcription factors and confers a feasible route to defense gene induction (Fan and Dong 2002).

5.11 Conclusions

In plant innate immunity, NBS-LRR protein-based defense of plants to various pathogens is a foremost area of interest. Evolutionary studies on interactions between plants and pathogens provide a key idea to modify important crops for pathogen defense. However, only few such interactions have been successfully decoded regardless of the abundant NBS-LRR genes present in plant genomes. Further research should be focused on detecting new NBS-LRR genes and their related pathogen effectors and molding these NBS-LRR proteins and their interactions. Such studies would make it feasible to modify the plant defense against a range of plant pathogens.

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Recent Advancement on Map Kinase Cascade in Biotic Stress

6

Monika Jaggi

Abstract

Mitogen-activated protein kinases (MAPKs) are cell-signaling enzymes that govern an extraordinarily discrete range of biological processes in eukaryotes. MAPK cascade has come up as one of the most well-studied signaling pathways in recent years. It plays a vital role in transmitting extracellular signals to the nucleus in response to various environmental stresses. A MAPK cascade composes of a three strata system where each stratum is phosphorylated by upper stratum. It is depicted as a MAP3K-MAP2K-MAPK module that serves as a link between upstream receptors and downstream targets. MAP2K being the middle point of this cascade converge all the signals from upstream MAP3Ks and target genome through downstream MAPKs. Occasionally, MAP4Ks also get employed in coupling upstream signaling components to the core MAPK cascade. MAPKs then direct various genes involved in stress responses as well as cellular and developmental processes. Therefore, in this chapter, an endeavor has been made to compile the role of MAPK cascade in biotic stress in plants.

Keywords

Elicitor-triggered immunity · PAMP-triggered immunity · Plant defense · Signal transduction

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6.1 Introduction

Plants are one of the most essential components of our ecosystem. They photosynthesize and convert light energy into chemical energy, thus a rich source of energy/food for most life-forms on earth including microbes capable of infecting plants. These microbes or phytopathogens when infectious result in biomass reduction, decreased fertility, or even death, thus a prime reason of human concern, as they pose an increasing threat to food security by decreasing the quality or quantity of crop production. In order to combat such infections, plants have developed different response systems at molecular, cellular, organ levels through evolution for effective protection. Plants are predominantly resistant to the majority of pathogens. Primarily, they are protected against pathogens by various physical barriers, e.g., cuticle and cell wall. Cuticle is a combination of cutin and waxes and adcrusted on the epidermis of most aerial plant organs (Yeats and Rose 2013). Besides preventing water loss and protecting against UV radiation, it also forms a barrier against pathogens. Cell wall provides both skeletal support and protection. Fungal pathogens can penetrate these barriers by mechanical rupture or enzymatic degradation but bacteria cannot. The latter instead make entry into plant cells via natural openings, e.g., stomata, hydathodes, wound sites, etc. (Melotto et al. 2008).

In addition to physical barriers, plants also possess a variety of antimicrobial compounds that are produced during pathogen attack. Only a few successful microbes that can rupture the preformed barriers then have to confront the plant immune system which is comprised of pathogen recognition and defense. The first layer of the plant immune system involves pathogen perception via the identification of conserved pathogen-associated molecular patterns (PAMPs) by plant pathogen or pattern recognition receptors (PRRs). PRRs are usually plasma membrane-bound receptor-like kinases (RLKs) or receptor-like proteins with extracellular domains allowing MAMP perception (Bohm et al. 2014). Sometimes PAMPs are of nonpathogenic origin too, hence alternatively known as microbe-associated molecular patterns (MAMPs). The latter are microbe-derived particles that are important for microbes, but that can be identified by plants. MAMPs can be proteins (e.g., bacterial flagellin and elongation factor Tu), carbohydrates (e.g., fungal chitin), lipopolysaccharides, etc. (Felix et al. 1999; Kunze et al. 2004; Albert 2013). There is another set of molecules which are plant degradation products arising from the activity of invading pathogens following pathogen attacks. These are known as damage-associated molecular patterns (DAMPs), for example, cutin monomers (Yeats and Rose 2013; Serrano et al. 2014), cell wall damage products (Hamann 2012), or endogenous peptides, such as AtPep1, which is derived from its precursor PROPEP1 (Huffaker et al. 2006). The recognition of all these molecules by the plant incites the pathogen-triggered immunity or pattern-triggered immunity (PTI), a sophisticated set of reactions meant for resisting against a pathogen attack (Boller and Felix 2009; Yamaguchi and Huffaker 2011). Pathogen perception can also ensue via the detection of pathogen effectors, which are molecules manufactured by the pathogens and carried in the extracellular matrix or into the plant cell to augment pathogen competence by, for example, counteracting the induction of

PTI. Plants that are incapable of recognizing these effectors are susceptible to a pathogen, while plants that can recognize the effectors via disease resistance proteins (R proteins) can induce an immune response called effector-triggered immunity (ETI). The coevolution of plants and pathogens and particularly their range of effectors and R proteins created the so-called zigzag model (Jones and Dangl 2006).

Plants are proficient in inducing a number of defense mechanisms upon pathogen infection, including blockage of nutrient transfer from the cytosol to the apoplast to inhibit bacterial multiplication (Chen et al. 2010; Wang et al. 2012), closing of stomata to curb entry of bacteria (Melotto et al. 2008; Sawinski et al. 2013), production and secretion of antimicrobial compounds including phytoalexins (Cowan 1999; van Loon et al. 2006; Ahuja et al. 2012; Bednarek 2012), generation of reactive oxygen species (ROS), which have toxic effects on pathogens (O'Brien et al. 2012), and a programmed cell death (PCD), referred to as the hypersensitive response (HR), at the site of infection to terminate pathogen expansion (Mur et al. 2008).

The inception of these defense mechanisms upon pathogen recognition depends on an intricate network of signaling pathways. Nearly all of the signal transduction mechanisms are inflected by protein phosphorylation and dephosphorylation that is regulated by protein kinases and protein phosphatases (Zolnierowicz and Bollen 2000). This swift and effective signaling system performs all obligations desired to interconnect the signaling with gene expression processes in coordination with the physiological status of the cell. Several protein kinases (PKs) involved in cellular signal transduction services have been identified in plants. PKs transform proteins by catalyzing the addition of monophosphate groups to the side chains of amino acid residues (usually serine, threonine, and/or tyrosine) in the protein backbone. This process is reversible with the help of an enzyme, phosphatase which is capable of removing phosphate group (Zolnierowicz and Bollen 2000). The plant PK superfamily embodies different classes of kinases grouped on the basis of their amino acid sequence similarity. Mitogen-activated protein kinases (MAPKs) are one of the most conserved and best- characterized protein kinase signaling pathways. MAPK was first unveiled by Sturgill and Ray (1986) as a microtubule-associated protein kinase. It comprises a family of serine/threonine protein kinases. The extracellular signals are read by the uppermost MAPKKKs and are transferred to substrates through MAPKs (Hardie 1999). In plants, the first MAPK was observed in alfalfa, and pea succeeded by cloning of MAPKs from *Arabidopsis thaliana* (Mizoguchi et al. 1993) and *Nicotiana* (Wilson et al. 1996). Both environmental stresses and development signals are capable of triggering MAPK signaling cascade (Sinha et al. 2011; Xu and Zhang 2015), and the latter also have a significant role in plant disease resistance signaling (reviewed by Meng and Zhang (2013)). However, the molecular mechanisms of how signal perception is transduced to MAPK activation remain elusive. Recently, Cheng et al. (2015) reported a novel plant immune pathway where proteases secreted by pathogens stimulate a previously unknown signaling pathway in *A. thaliana* involving the Gα, Gβ, and Gγ subunits of heterotrimeric G-protein complexes, which function upstream of a MAPK cascade. This chapter mainly emphasizes the involvement of mitogen-activated protein kinases (MAPKs) in biotic stress.

6.2 General Framework of MAPK Cascade

MAPK cascade is one of the most widely studied signaling pathways, which eukaryotic cells use to adapt in response to extracellular stimuli. These are immensely conserved signaling modules. Typically, a MAPK cascade consists of three kinases, a MAPKKK (MAP3K or MEKK), a MAPKK (MAP2K or MEK or MKK), and a MAPK (MPK), which phosphorylate and therefore activate each other in a specific way. It is a three-tier system where each tier is phosphorylated by upper tier. The uppermost tier is composed of MAPKKKs that phosphorylate two amino acids in the S/T-X3−5-S/T motif of the MAPKK activation loop. The middle tier consists of MAPKKs that activate a MAPK through double phosphorylation of the T-X-Y motif in the activation loop. These converge all the signals from upstream MAP3Ks and target genome through downstream MAPKs. MAPKs are serine/threonine kinases capable of phosphorylating a wide range of substrates, including other kinases and/or transcription factors. The fourth level of kinases, named MAPKKKKs (or MEKKKs), may act as adaptors linking upstream signaling steps to the core MAPK cascades. Interactions between kinases within a MAPK cascade occur through docking sites present in the kinases and/or with the help of external scaffolding proteins (Hamel et al. 2006; Ichimura et al. 2006).

MAPK proteins possess a highly conserved kinase domain enclosing 11 subdomains. Depending on the amino acid motif present at the phosphorylation site between the sub-domains VII and VIII, they are grouped as TEY and TDY activation motifs. The TEY subtype can be further formulated into three groups, A, B, and C, whereas the TDY subtype forms a more distant group D (MAPK Group 2002). Group A MAPKs are mostly associated with developmental processes and are activated in response to biotic and abiotic stresses, whereas group B members are implicated in pathogen defense and abiotic stress responses (Rodriguez et al. 2010). Group D members possess a C-terminal common docking (CD) domain that may act as a docking site for MAPKKs. The former TEY subtype contains a phosphorylation motif in the activation loop, while TDY subtype has motif within its active site buried at its domain surface. The N-terminal domain of MAPK protein has 135 residues which are aligned largely in the form of β-sheets and a glycine-rich loop named as phosphate anchor ribbon, while the C-terminal domain is of 225 amino acids bearing a catalytic base, Mg^{2+} binding sites, and the phosphorylation lip (activation loop). The sequence of phosphorylation and activation loop affects the substrate specificity. The TXY(XD/E/P) motif is a dual phosphorylation site, and phosphorylation of both residues is a prerequisite for the activation of this cascade.

MAPKKs could be split into four groups (A, B, C, and D) based on sequence similarities. Members of groups A and B carry eight to nine exons, while groups C and D possess only one exon. Kinases in group B are depicted by a nuclear transfer factor (NTF) domain (MAPK group 2002) which increases the nuclear import of cargo proteins and hence actively involved in cytoplasmic-nuclear transport. Different members of the same group are activated in the presence of different stimuli (Xu et al. 2008a; Zhou et al. 2009).

MAPKKKs are highly divergent and constitute the largest and most complex group of MAPK cascade. Rao et al. (2010) categorized MAPKKKs from rice and *Arabidopsis* into three subgroups, i.e., Raf, ZIK, and MEKK. Raf family members are characterized by the presence of a long N-terminal regulatory domain and a C-terminal kinase domain. In contrast, ZIK family members largely have N-terminal kinase domain. MEKK family members possess relatively a less conserved protein structure with kinase domain located either at N- or C-terminal or central part of the protein. Ubiquitin-interaction motif and ACT domain, which has a regulatory role in a wide range of metabolic enzymes, are seen only in the members of Raf family from rice and *Arabidopsis* (Rao et al. 2010).

6.3 MAPK Substrates

Presently, it is a great challenge to link a given Protein Kinase (PK) to its substrate with high specificity. In higher eukaryotes, nearly one-third of all proteins are governed by phosphorylation by PKs. Although plants are known to contain more than 1000 PKs, the substrates of only a few of them have been correctly identified. MAPK substrates have been the most extensively studied. An estimated 50% of the MAPK substrates are transcription factors. It was reported that MAPK phosphorylation regulates transcription factors by altering their activity, localization, and/or stability. A regular MAPK has an active site and a common docking site. Both sites are placed nearby and are involved in recognition and binding of target proteins. MAPK phosphatases and scaffolding proteins play a regulatory role in MAPK signaling specificity, location, and duration.

To identify potential MAPK substrates, direct protein-protein interaction and activity screens were used in various studies (Bethke et al. 2009; Zhang et al. 2016a). Feilner et al. (2005) used protein microarray and identified 48 in vitro substrates for MPK3 and 39 substrates for MPK6. Twenty six substrates were found common to both MAPKs. Similarly, 570 in vitro substrates for 10 different MAPKs were identified by employing *Arabidopsis* protein microarrays (Popescu et al. 2009). There was an overlap of 30–40% between the MPK3 and MPK6 substrates suggesting some functional redundancies of the two MAPKs. In *Arabidopsis*, ACS6 (1-aminocyclopropane-1-carboxylic acid synthase), PHOS32, bHLH speechless, VIP1, EIN3, WRKY53, WRKY33, MKS1, and ERF104 have also been identified as MAPK substrates (Andreasson and Ellis 2010). Sheikh et al. (2016) characterized WRKY46 as a substrate of AtMPK3. The type 3 effector NopL of *Sinorhizobium* sp. strain NGR234 is a newly identified mitogen-activated protein kinase substrate (Ge et al. 2016). MKS1 was reported to interact with MPK4 in a yeast 2-hybrid analysis and has a putative role in plant defense (Andreasson et al. 2005). The MPK4-MKS1 interaction was validated in planta by co-immunoprecipitation, and it was found that MKS1 further interacted with two transcription factors, WRKY29 and WRKY33. Later on, it was reported that MPK4 is found in complexes in vivo with PAT1, a constituent of the mRNA decapping machinery (Roux et al. 2015). PAT1 is also phosphorylated by MPK4, and, upon flagellin PAMP treatment, PAT1

accumulates and localizes to cytoplasmic processing (P) bodies which are sites for mRNA decay. Pat1 mutants exhibit dwarfism and derepressed immunity dependent on the immune receptor SUMM2. Since mRNA decapping is a critical step in mRNA turnover, linking MPK4 to mRNA decay via PAT1 provides another mechanism by which MPK4 may rapidly instigate immune responses (Roux et al. 2015). There is another study by Li et al. (2015) which demonstrates the negative regulation of plant defense by MPK4 via ASR3 (*Arabidopsis* SH4-related3), a trihelix transcriptional repressor. ERF104, an ethylene response factor, is specifically phosphorylated by MPK6 in vitro and is involved in defense responses (Bethke et al. 2009).

6.4 MAPK Modules in Biotic Stress

As discussed previously, plants have multilayered defense mechanism to protect themselves from pathogens (Jones and Dangl 2006). The first mechanism involves pattern recognition receptors (PRRs), which recognize conserved PAMPs. Under stress, these receptors (PRRs) get activated and stimulate convergent intracellular signaling pathways, which initiates the establishment of PAMP-triggered immunity (PTI). Defense responses in PTI are, in general, transient and are not associated with HR cell death. The other mechanism is known as effector-triggered immunity (ETI) which is essentially quantitatively stronger and longer-lasting than PTI. It often results in HR death.

6.4.1 MAPKs in PAMP-Triggered Immunity (PTI)

MAPKs play key roles in PTI signal mechanism by transducing signals from PRRs to downstream targets. The bacterial flagellin receptor FLS2, which identifies a conserved 22-amino-acid peptide (flg22) from flagellin (Gomez-Gomez and Boller 2000); the bacterial elongation factor EF-Tu receptor EFR, which acknowledges a conserved 18-amino-acid epitope (elf18) of EF-Tu (Zipfel et al. 2006); and the chitin receptor CERK1 from *Arabidopsis* (Miya et al. 2007; Wan et al. 2008) are among few well-characterized plant PRRs. Both flg22 and elf18 can provoke a strong but transient stimulation of MAPKs in *Arabidopsis*, including MPK3, MPK6, MPK4, and MPK11 (Asai et al. 2002; Zipfel et al. 2006; Suarez-Rodriguez et al. 2007; Ranf et al. 2011; Roux et al. 2011; Bethke et al. 2012). Upon activation by PAMPs, both FLS2 and EFR form heterodimers with BRI1-associated kinase (BAK1), a positive regulator of PTI (Chinchilla et al. 2007). Mutation in BAK1 leads to significant decline of flg22- and elf18-triggered activation of MPK3, MPK6, and MPK4 (Heese et al. 2007; Roux et al. 2011). BKK1 was reported to be essential for flg22- and elf18-induced MAPK activation, and it is thought to act collaboratively with BAK1 in the PAMP signaling (Roux et al. 2011). At present, molecules connecting the receptor complexes and MAPK cascades are still enigmatic. One possible regulator downstream of the FLS2-BAK1 complex is the

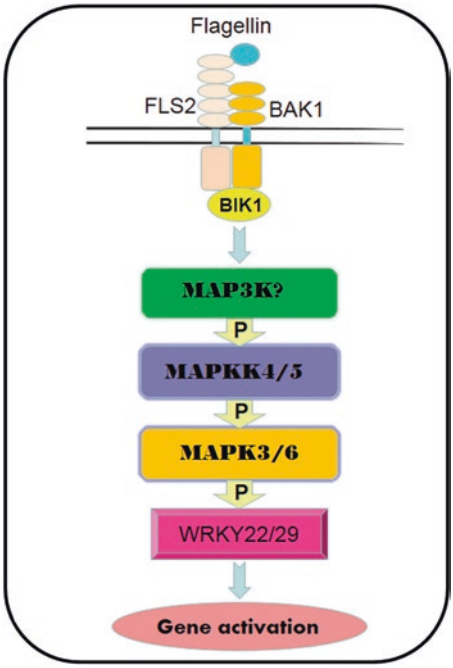

Fig. 6.1 Plant mitogen activated protein kinase (MAPK) cascade in response to flagellin in *Arabidopsis*

cytoplasmic protein kinase BIK1 (*Botrytis*-induced kinase 1), which interacts with and is phosphorylated by the FLS2-BAK1 complex and is needed for flg22-triggered PTI responses (Lu et al. 2010).

The first complete MAPK cascade in regulating plant defense against the bacterial pathogen, MAP3K1-MAPKK4/MAPKK5-MAPK3/MAPK6-WRKY22/WRKY29, was reported to be downstream of flagellin receptor kinase (FLS2 LRR) (Asai et al. 2002). Later studies revealed normal MPK3/6 activation in MAP3K1 mutant plants after flg22 treatment indicating that MAP3K1 is not upstream of MAPKK4/MAPKK5 (Ichimura et al. 2006; Nakagami et al. 2006; Suarez-Rodriguez et al. 2007). Instead, there are redundant orthologs of MAP3K1 in the flg22-elicited activation of MAPK3 and MAPK6. As mentioned above, an elicited FLS2 complex with BAK1 induces MAPK signaling cascade (Fig. 6.1). Another flg22-activated MAPK cascade, MAP3K1-MAPKK1/MAPKK2-MAPK4-MKS1, mediates jasmonate- and salicylate-dependent defense responses (Qiu et al. 2008). This cascade negatively regulates plant immunity as the mutants of either MAP3K1 or MAPK4 resulted in more resistance to pathogens. It was proposed that MAPK4 along with its substrate MAP kinase substrate 1 (MKS1) and WRKY33 controlled the expression of camalexin biosynthetic enzyme, phytoalexin deficient 3 (PAD3). Upon microbial attack, WRKY33 is released from this complex and attaches to a PAD3 promoter and activates its expression (Qiu et al. 2008). The production of camalexin was compromised in *mpk3* and *mpk6* mutants depicting a role of MPK3/6 in defense responses. MAPKK3 participates in jasmonate-mediated developmental signaling and pathogen defense responses through activation of MAPK6 (Takahashi et al.

2007). The interplay between the positive upregulation of defense responses via MAPK3/6 and the negative regulation via MAPK4 likely enables the tight control of defense responses to promote or restrict growth and make appropriate death versus survival decisions.

6.4.2 MAPKs in Effector-Triggered Immunity

ETI and *R* gene signaling also involves MAPK cascades as evidenced by various studies in tobacco and tomato. Wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK) were found to get activated by tobacco mosaic virus (TMV) infection in plants that express the N resistance gene (Zhang and Klessig 1998). Both of these MAPKs also got induced in transgenic tobacco cells expressing the Cf-9 resistance gene. Loss-of-function experiments demonstrated that SIPK, WIPK, and their upstream MAPKK2 are required for N-gene-mediated TMV resistance. Another well-studied example is the tomato Pto-mediated ETI where overexpression of tomato MAP3K, MAPKKKα, increased the development of HR lesions, whereas its silencing led to the inhibition of HR (del Pozo et al. 2004). Later, MAPKK1-NTF6 and MAPKK2-SIPK were proposed to act downstream of Pto resistance gene (Ekengren et al. 2003). AvrPto and AvrPtoB effectors showed interaction with FLS2 and BAK 1. AvrPto can cease the binding of BAK1 to FLS2, while AvrPtoB mediates polyubiquitination and proteasome-dependent degradation of FLS2. In this way, both effectors negatively regulate MAPK signaling.

Many bacterial pathogens employ the HopAI1 effector, which has a unique phosphothreonine lyase activity that dephosphorylates MAPKs (Li et al. 2007). It was deciphered that inducible expression of HopAI1 inactivated the MAPK3 and MAPK6 resulting in the complete suppression of PAMP-induced genes and ROS burst. Another effector molecule $HopPtoD_2$ from *P. syringae* pv. tomato is known to possess phosphatase activity. Its expression in tobacco cells led to a decline in cell death induced by expression of the constitutively active MAPKK variant $NtMEK2^{DD}$. However, $HopPtoD_2$ does not inhibit flg22-mediated MAPK activation in *Arabidopsis*.

An interesting example of an opportunistic interaction between plants and bacteria is where pathogens manipulate host MAPK signaling and hijack AtMPK3 by *Agrobacterium tumefaciens*. The activation of MPK3 in response to flg22 or *Agrobacterium* results in the phosphorylation, subsequently followed by nuclear translocation of the host protein VIP1 (virE2-interacting protein 1). Agrobacteria hijack this nucleocytoplasmic shuttle system (VIP1) to transfer their T-DNA into the host nucleus, where it integrates into the host genome (Djamei et al. 2007). Because VIP1 does not only serve as a nuclear shuttle for the pathogenic T-DNA complex but can also induce the expression of defense genes, nuclear VIP1 would be counteracting *Agrobacterium* invasion. *Agrobacterium* targets nuclear VIP1 for proteasome degradation by secreting VirF effector, which helps in ceasing VIP1-induced plant defense genes activation.

Stomata play an essential role in plants as they are involved in various physiological functions like photosynthesis, respiration, and transpiration and also responsible

for gas exchange. But sometimes, various pathogens use them as an entrance gate to enter into their hosts. Pathogen-induced stomatal closure restricts the invasion of various pathogens. Therefore, stomata are important in providing protection against both biotic and abiotic stresses. MAPKs have been implicated in both stomatal development and function. In drought stress, stomatal closure mediated by ABA involves MKK1, MPK3, and MPK6 (Hamel et al. 2006; Gudesblat et al. 2007). Previous studies have also indicated crosstalk between ABA and defense signaling. The MAPK cascade, comprising YODA-MAPKK4/MAPKK5-MAPK3/MAPK6, plays an important role in regulating stomatal development (Bergmann et al. 2004; Wang et al. 2007; Bayer et al. 2009; Popescu et al. 2009). MAPK9 and MAPK12 were reported to be downstream of H_2O_2 in regulating ABA-induced stomatal closure (Jammes et al. 2009). It was reported that mapk9-1/12-1 double mutants were highly susceptible to *Pseudomonas syringae* DC3000 compared to wild-type plants. These results suggested that the regulation of stomatal apertures by MAPK9 and MAPK12 contributed to the first line of defense against pathogens (Jammes et al. 2011). Whether pathogen-induced and ABA-induced stomatal closures are signaled via a common MAPK cascade remains to be elucidated.

Fungal pathogens are also known to induce MAPK cascades (Izumitsu et al. 2009). *Phytophthora infestans* attack led to the swift transcriptional induction of MAP3K19, MAPKK9, and MAPKK4, whereas *Botrytis cinerea* infection led to enhanced transcriptional activation of MAP3K18, MAP3K19, MAP3K20, Raf43, ZIK2, and ZIK8, suggesting the distinct signaling pattern in response to bacterial and fungal pathogen attack. Cardinale et al. (2002) described the activation of SIMK and SAMK by various fungal elicitors. Two alfalfa MAPKs, MMK2 and MMK3 (Medicago MAPK2 and MAPK3, respectively), were shown to be activated by fungal elicitors. Nevertheless, a fungal biocontrol agent *Trichoderma asperellum* was shown to induce systemic resistance in plants through a mechanism that employs jasmonic acid (JA) and ethylene (ET) signal transduction pathways resulting in activation of a Trichoderma-induced MAPK (TIPK) gene in cucumber (Shoresh et al. 2006). MAPKK1 is involved in defense responses including flg22-induced activation of MAPK4 (Asai et al. 2002; Meszaros et al. 2006). MAPKK7 is a positive regulator of systemic acquired response (Zhang et al. 2007). MAPKK7/9 is hypothesized to regulate cell death during pathogen defense. Several other groups reported proteins, e.g., NLPs (necrosis and ethylene-inducing peptide1-like proteins) triggering MAPK activation and inducing defense responses (Qutob et al. 2006).

6.5 Defense Studies from Different Plant Sources

Recently, a lot of information has been gathered where microbial attack led to the activation of MAPKs in different plant genera as described below. Ectopic expression of Cotton GhMPK11 decreased disease resistance through the gibberellin signaling pathway in transgenic *Nicotiana benthamiana* (Wang et al. 2016). In watermelon, transient expression of CIMPK1, CIMPK4-2, and CIMPK7 in *N. benthamiana* resulted in enhanced resistance to *B. cinerea* and upregulated expression

of defense genes, while transient expression of CIMPK6 and CIMKK2-2 led to increased susceptibility to *B. cinerea* (Song et al. 2015). Approximately 74 MAP3Ks, 9 MAPKKs, and 19 MAPKs were identified in maize followed by functional studies (Kong et al. 2013; Liu et al. 2013). The role of ZmMKK1 in pathogen defense has been deciphered by Cai et al. (2013) where it played a differential function in necrotrophic versus biotrophic pathogen defense responses. Overexpression of ZmMPK5 in tobacco- induced increased resistance to viral pathogens and activated the expression of PR genes, e.g., PR1a, PR4, PR5, and EREBP (ethylene-responsive element binding protein) (Zhang et al. 2013). Pan et al. (2012) reported the involvement of ZmMPK17, a novel maize group D MAP kinase gene, in multiple stress responses. In *Gossypium*, GhMAPK6a played a negative role during pathogen infection as its overexpression increased sensitivity to the bacterial pathogen *Ralstonia solanacearum*. Another MAP2K from *G. hirsutum*, GhMKK1, showed increased susceptibility to the same pathogen by lowering the expression of PR genes, but no interaction between MAPK6a and MKK1 was observed (Lu et al. 2013).

Polyamines generated in plants play important roles in several developmental and physiological processes. AtMPK3 and AtMPK6 are known to play a positive role in the regulation of putrescine biosynthesis leading to bacterial pathogen defense in *Arabidopsis* (Kim et al. 2013). The *Hordeum vulgare* signaling protein MAP kinase 4 (HvMPK4) had a similar role to AtMPK4 as both showed negative regulation of SA in pathogen defense. HvMPK4 mutant lines detected elevated SA levels and were more resistant to hemibiotrophic fungal pathogen *Magnaporthe grisea* (Abass and Morris 2013). NtMKP1 (tobacco MAP kinase phosphatase) antisense lines exhibited increased resistance against a necrotrophic pathogen, *B. cinerea*, and lepidopteran herbivores, *Mamestra brassicae* and *Spodoptera litura*. It was suggested that NtMKP1 negatively regulates wound response and resistance against both necrotrophic pathogens and herbivorous insects through suppression of JA or ET pathways via inactivation of MAPK (Oka et al. 2013). Lately, NtMPK2 has been reported to positively regulate tobacco defense responses to *P. syringae* pv. tomato DC3000 (Zhang et al. 2016b).

6.6 Function of MAPK Activation in Plant Defense Responses

Plants respond differentially to invading pathogens and activate a variety of defense mechanisms that often includes stomatal closure, ROS generation, defense gene activation, phytoalexins accumulation, cell wall modification, and HR cell death. These defense responses are combined by a complex signaling network that involves MAPK cascades. In this section, the role of MAPKs in controlling the synthesis and/or signaling of defense hormones, reprogramming gene expression, and driving metabolic flow to antimicrobial metabolite synthesis, among other defense responses, will be discussed.

6.6.1 Defense Hormone Synthesis and/or Signaling

Phytohormones ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) are an important class of signaling molecules involved in plant defense signaling (Wang et al. 2002; Broekaert et al. 2006; Browse 2009; Vlot et al. 2009; Spoel and Dong 2012). SA is usually involved against biotrophs or hemibiotrophs, while JA and ET are mostly important against necrotrophs. Upon microbial infection, plants activate MAPK cascade, which in turn modulates levels of these phytohormones. Plant MAPK cascades have been involved in both the regulation of defense hormone biosynthesis and the signaling events downstream of hormone sensing. Recent studies revealed that a subset of MAPKs in plants, represented by tobacco SIPK/Ntf4/WIPK and *Arabidopsis* MPK3/MPK6, plays key roles in regulating pathogen-induced ethylene biosynthesis (Liu and Zhang 2004; Han et al. 2010; Li et al. 2012). The ethylene signaling pathway is well characterized, and several works demonstrated that phosphorylation of ACS2 and ACS6 by MPK3 and MPK6 is a key step for ET production (Guo and Ecker 2004; Stepanova and Alonso 2009). Binding of ethylene to its receptors, ETR1, ETR2, ERS1, ERS2, and EIN4, results in the inactivation of the negative regulator CTR1, which leads to the derepression of the positive regulator EIN2, the stabilization of the EIN3/EIL transcription factors, ethylene-responsive gene expression, and eventually ethylene responses (Stepanova and Alonso 2009).

SA plays a pivotal role in plant defense responses, mainly through its downstream components, NPR1, and three redundant transcription factors, TGA2, TGA5, and TGA6 (Vlot et al. 2009; Spoel and Dong 2012). A current model advocates that NPR3 and NPR4 are SA receptors that govern NPR1 levels, ensuing cell death or survival depending on SA concentration (Yan and Dong 2014). AtMPK3 and, to a lesser extent, AtMPK6 were seen to play a principal role in SA-mediated stimulation of plants for disease resistance (Beckers et al. 2009). When plants were given benzothiadiazole (a functional analog of SA) treatment, it resulted in the accumulation of mRNA and inactive proteins of MPK3 and MPK6. Unlike MPK3/MPK6, the MPK4 is considered a negative regulator of SA signaling because *mpk4* mutant or mutation of its upstream MKK1/MKK2 and MEKK1showed elevated levels of SA, constitutive PR-1 expression, and SAR (Petersen et al. 2000; Kong et al. 2012). Lately, SUMM2, an R protein, was reported to be essential for activation of SA responses in the *mpk4* mutant, indicating that the constitutive SA responses in the *mpk4* mutant are induced by the SUMM2-mediated signaling pathway (Zhang et al. 2012). Additionally, MPK4 substrate 1 (MKS1), a substrate of MPK4, is also required for full activation of SA responses in the mpk4 mutant (Andreasson et al. 2005), but no interaction of MKS1 with SUMM2 has been detected (Zhang et al. 2012), proposing that SUMM2-mediated activation of SA responses may be independent of MKS1.

JA is another important defense hormone activated during pathogen infection, herbivore attack, and mechanical wounding (Browse 2009; Gfeller et al. 2010). In tobacco, WIPK and SIPK were shown to be vital for wounding- and herbivore-induced JA production, whereas overexpression of WIPK was seen to be sufficient

to induce JA accumulation (Seo et al. 1999, 2007; Wu et al. 2007). However, in the conditional gain-of-function NtMEK2^{DD} plants, activation of WIPK and SIPK was insufficient to induce JA accumulation (Kim et al. 2003). It is possible that these two MAPKs are needed but not sufficient to induce JA synthesis. Later, AtMPK6 was reported to be an important regulator of the JA signaling pathway (Takahashi et al. 2007). AtMPK6 together with its upstream AtMKK3 was shown to be involved in JA-dependent negative regulation of JIN1/MYC2 expression and root growth. MPK4 was also implicated in the JA signaling pathway. The MPK4 deficient lines exhibited constitutive activation of SA-dependent defenses but compromised the expression of JA-responsive genes (Petersen et al. 2000). Interestingly, removing SA in the NahG/mpk4 double mutant failed to reverse the suppression of JA-inducible gene expression, suggesting that MPK4 positively regulates JA-inducible responses independent of its negative regulation of SA signaling (Petersen et al. 2000).

6.6.2 Activation of Defense Genes

MAPKs can activate defense genes via direct phosphorylation of downstream transcription factors. In *Arabidopsis*, MPK6 interacts with and phosphorylates ERF104 that activates defensin genes PDF1.2a and PDF1.2b (Bethke et al. 2009). Intriguingly, the interaction of MPK6 and ERF104 is rapidly lost in response to flg22, and this complex disruption requires MPK6 activity, suggesting that phosphorylation of ERF104 by MPK6 in response to flg22 leads to the release of ERF104 from MPK6 and defensin gene activation (Bethke et al. 2009). Meng et al. (2013) identified ERF6, another *Arabidopsis* ERF family member, as a new substrate of MPK3/MPK6. Phosphorylation of ERF6 by MPK3/MPK6 leads to stabilization of the ERF6 protein, which further activates the expression of multiple defense-related genes, including PDF1.1, PDF1.2a, PDF1.2b, ChiB, and HEL. The positive and negative roles for soybean MPK6 in regulating defense responses have also been reported (Liu et al. 2014).

6.6.3 Phytoalexin Biosynthesis

Phytoalexins are also known to play essential roles in plant defense (Hammerschmidt 1999; Dixon 2001; Ahuja et al. 2012). Camalexin (3-thiazol-2′-yl-indole) is the major phytoalexins produced in *Arabidopsis* and related Brassicaceae species (Tsuji et al. 1992; Ahuja et al. 2012). Conditional expression of constitutively active variants of MKK4 (MKK4^{DD}), MKK5 (MKK5^{DD}), or the functionally interchangeable NtMEK2 (NtMEK2^{DD}) from tobacco, all of which specifically activate MPK3/MPK6 in *Arabidopsis*, leads to camalexin induction and coordinated upregulation of multiple genes, including PAD2, CYP71A13, and PAD3, that encode enzymes in the camalexin biosynthetic pathway (Ren et al. 2008; Mao et al. 2011). Expression of constitutively active MKK9, which also activates MPK3/MPK6, induces camalexin accumulation (Xu et al. 2008b; Su et al. 2011). Consistent with the essential

roles of MPK3/MPK6 in regulating camalexin biosynthesis, pathogen-induced camalexin accumulation is almost completely abolished in a rescued mpk3/mpk6 double mutant (Ren et al. 2008), whereas mutations in two MAPK phosphatases (MKP1 and PTP1), which target MPK3/MPK6, lead to constitutive camalexin accumulation (Bartels et al. 2009).

Biochemical data revealed that WRKY33 is a substrate of MPK3/MPK6, and the MPK3/MPK6 phosphorylation sites in WRKY33 are required for its function in vivo (Mao et al. 2011). Interestingly, MPK3/MPK6 also controls the pathogen-inducible expression of the WRKY33 gene, and the latter can bind to its own promoter, suggesting a potential MPK3/MPK6-mediated positive feedback regulatory loop that controls WRKY33 expression (Mao et al. 2011). WRKY33 was also reported to control camalexin production through its interaction with another MAPK, MPK4 (Qiu et al. 2008). It is likely that pathogen-responsive MAPK cascades in different families of plants are responsible for regulating the biosynthesis of different types of phytoalexins. The OsMKK4-OsMPK6 module in rice was shown to control the chitin elicitor-induced production of diterpenoid phytoalexins by regulating the expression of their biosynthetic genes (Kishi-Kaboshi et al. 2010). Tobacco SIPK/Ntf4 and WIPK also phosphorylate and thus activate the WRKY33-related NbWRKY8, which further induces the expression of 3-hydroxy-3-methylglutaryl CoA reductase2 (HMGR2), a key gene for the production of isoprenoid phytoalexins (Ishihama et al. 2011). Tobacco HMGR is one of the first few defense genes known to be highly induced by SIPK/WIPK activation (Yang et al. 2001; Kim and Zhang 2004).

6.6.4 Hypersensitive Response Cell Death

ETI frequently results in HR cell death, a process associated with MAPK activation, ROS generation, metabolic reprogramming, and SA accumulation (Greenberg and Yao 2004; Coll et al. 2011). Pharmacological studies using kinase inhibitors suggest that SIPK and WIPK activation in tobacco is involved in HR-like cell death (Zhang et al. 2000). Potential MAPKKKs upstream of the tobacco NtMEK2-SIPK/Ntf4/WIPK module include MAPKKKα and MAPKKKε (del Pozo et al. 2004; Melech-Bonfil and Sessa 2010). In the search for signaling events downstream of MAPKs in mediating HR cell death, ROS generation was found to be associated with MAPK-induced cell death (Ren et al. 2002).

6.6.5 PAMP/Pathogen-Induced Reactive Oxygen Species Burst

ROS is considered as an important and common messenger generated in various stresses and known to trigger the expression of many MAPKs. PTI is also associated with rapid ROS production, known as a ROS burst (Apel and Hirt 2004; Torres 2010). PAMP/pathogen-responsive MAPKs were believed to function downstream of early ROS burst in signaling plant immunity, because defense-related MAPKs,

including *Arabidopsis* MPK3, MPK6, and MPK4 and tobacco SIPK and WIPK, can be activated by exogenously added H_2O_2 (Pitzschke et al. 2009). Recently, MAP3K7 was characterized to suppress the ROS burst downstream of FLS2. It was demonstrated that MAP3K7 negatively regulates flagellin-triggered signaling and basal immunity by attenuating MPK6 activity (Mithoe et al. 2016). The role of ROS-mediated MAPK signaling in plants has been reviewed by Siddhi et al. (2015) where they suggested that ROS can activate a similar MAPK cascade in different stresses and can exert differential responses accordingly.

6.6.6 Stomatal Immunity

Plants have an ability of closing their stomata as an immune response to pathogens. MAPKs have been cited in both ABA- and PAMP/pathogen-induced stomatal closure (Pitzschke and Hirt 2009). Gudesblat et al. (2007, 2009) suggested a role of MPK3 in stomatal immune response. Guard cell-specific silencing of MPK3 compromised PAMP/bacteria-induced stomatal closure but does not affect the stomatal closure induced by ABA. MPK6 was reported to modulate NO production by phosphorylation and activation of nitrate reductase NIA2 (Wang et al. 2010). Above results indicate that MPK3 regulates stomatal immunity by promoting NO synthesis, which has been implicated to be required for PAMP/pathogen-induced stomatal closure (Melotto et al. 2006).

6.7 Conclusion

In recent years, MAPK cascade has shown its universality among eukaryotes and has demonstrated its significance in multitude of stress elements. Multifunctionality and signaling specificity of MAPKs can be conferred by their ability to phosphorylate different substrates. For example, in *Arabidopsis*, MPK3/MPK6 can regulate camalexin production, ethylene biosynthesis, NO generation, and defensin gene expression by phosphorylating WRKY33, ACS2/ACS6, NIA2, and ERF104 proteins, respectively (Bethke et al. 2009; Han et al. 2010; Wang et al. 2010; Mao et al. 2011). The identification of additional MAPK substrates will extend our understanding of MAPK functions in plant disease resistance. One major void in our understanding of plant defense signaling is the connection(s) between the receptors/sensors and MAPK cascades. So, it's very crucial to identify these missing links in elucidating the functions and underlying molecular mechanisms of MAPKs in plant innate immunity. In light of these realities, new experimental strategies are needed to generate conditional mutant systems for functional analyses which might reveal the fine-tuning mechanisms in plant innate immunity system. The complete MAPK cascades have been identified in a few biotic and abiotic stresses, but many are still missing. So there is a great requisite to decode the complete signaling cascade with specific underlying mechanisms using novel approaches and strategies. This will help in engineering the MAPK cascades and their utilization in crop improvement.

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Role of Phytohormones in Plant Defense: Signaling and Cross Talk

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Vibha Gulyani Checker, Hemant Ritturaj Kushwaha, Pragati Kumari, and Saurabh Yadav

Abstract

Plants, being sessile throughout their life cycle, are vulnerable to various kinds of abiotic and biotic stress conditions. They have evolved sophisticated mechanisms to detect precise environmental change and respond with an optimal response, thereby minimizing damage and conserving resources for growth and development. The response of plants towards these stresses are dynamic and complex. A defense response is initiated via modulation of molecular events, which involves interplay of signaling molecules including phytohormones. Phytohormones are small endogenous, low-molecular-weight molecules, which trigger an effective defense response against both biotic and abiotic stresses. Apart from defense signaling, these phytohormones are also regulators of growth, development, and physiological processes. The phytohormones such as auxins, cytokinins (CKs), gibberellins (GAs), salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and brassinosteroids (BRs) respond to stress via synergistic and antagonistic actions often referred to as signaling cross talk. These phytohormones coordinate with each other in a harmonious manner and respond to developmental and environmental cues. All defense response in plants are the result of interplay of many genes and gene families nicely orchestrated in

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a network. Various phytohormones are known to play important role in almost all the process through the modulation of genes. Further, through the optimal mix of phytohormones, plants maintain homeostasis and adapt to the environmental changes. This is only possible by an efficient and systemic cross talk between various phytohormones which help plants to maintain a critical balance between growth and environmental response. This chapter would assist plant biologist in further understanding the ability of the plants to perceive, synthesize, and respond to phytohormones in response to environmental stresses.

Keywords
Signaling · Phytohormones · Jasmonates · Biotic stress · Plant defense · Cross talk

7.1 Introduction

Plants, throughout their life cycle, are entrenched to one place, which makes them vulnerable to various kinds of stress conditions. These stresses can be classified as abiotic and biotic stress. A high percentage of the crop production is said to be affected due to the persisting stresses (Boyer 1982). All stresses are often interrelated and cause physiological, biochemical, and molecular changes which are finally reflected in terms of its declined growth and productivity. Over the years, major progress has been made using tools and techniques of molecular biology, to understand the comprehensive view of abiotic and biotic stress perception, response, and tolerance in plants. Apart from various abiotic stresses, plants also have to face various biotic challenges such as pathogenic microbes and herbivorous insects. In order to defend against these challenges, plants rely on their immune system and preformed defense systems. Preformed defense system, which is the first hurdle for an invading organism, includes various chemical compounds (Osbourn 1996), cuticle layers, thick cell walls, needles, thorns, or trichomes. The principal components of cell walls are cross-linked high-molecular-weight polysaccharides which resist the physical penetration (Carpita and McCann 2000). Also, these cell walls are the dynamic reservoirs of antimicrobial proteins and secondary metabolites which prohibit the growth of many pathogens.

Plants also possess multiple layers of innate immune system which detects and limits pathogen expansion. One layer of this system uses pattern recognition receptors (PRRs) present on the surface of the plant cell to analyze for molecules containing signature patterns conserved in microbes known as pathogen-/microbe-associated molecular patterns (PAMPs/MAMPs). Detection of these PAMPs/MAMPs triggers pattern-triggered immunity (PTI). PTI prevents further pathogen establishment. However, some pathogens have developed effector proteins that overpower PTI. These pathogens are suppressed via other layers of innate immune system – effector-triggered immunity (ETI). Plants have evolved intracellular resistance (R) proteins which induce ETI to regain resistance. These R proteins recognize their

pathogen-encoded effectors directly or indirectly. The responses initiated by ETI are more rapid and intense than PTI. ETI also turns local infection into global defense by forming necrotic lesions which restricts movement of pathogen from the infection site (Fu and Dong 2013). Both types of signaling mechanisms (PTI and ETI) activate a league of defense responses in the affected tissue, like generation of reactive oxygen species (ROS), increase in intracellular Ca^{2+} concentrations, activation of mitogen-activated protein kinases (MAPKs), increased expression of various defense-associated genes, and production of antimicrobial compounds (Chisholm et al. 2006). The rise in intracellular calcium is sensed by calcium sensors which in turn bind to cis-elements of promoters of genes conferring tolerance. These calcium sensors may also bind DNA-binding proteins controlling these stress-responsive genes. The increase in intracellular calcium further activates various kinases and phosphatases which can phosphorylate or dephosphorylate transcription factors controlling stress-responsive genes. Many defense-related proteins have been identified in various plant species like PR-1, β1,3-glucanase (PR-2), chitinases (PR-3, PR-4, PR-8, PR-11), thaumatin (PR-5), proteinase inhibitors (PR-9), ribonuclease like defensin (PR-10), lipid transfer protein (PR-14), NBS-LRR proteins, glycoproteins, WRKY proteins, and catalases.

Plants, in order to survive through biotic stresses, have evolved more sophisticated mechanisms in order to perceive external signals which allow them to prepare for an optimal response (Fig. 7.1). Small endogenous, low-molecular-weight molecules commonly known as phytohormones regulate the defensive response against biotic stresses. The phytohormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), brassinosteroids (BRs), auxins, cytokinins (CKs), and gibberellins (GAs), respond to stress via synergistic and antagonistic actions often referred to as signaling cross talk (Mauch-Mani and Mauch 2005). Over the years, analysis done using large-scale transcriptome analyses has supported the existence of cross talk between various signaling networks (Davletova et al. 2005). Apart from defense signaling, these phytohormones are also regulators of growth, development, and physiological processes. These phytohormones act while maintaining a balance in highly complex network in response to development and environmental cues. Like animal hormones, these phytohormones are transported from one location to the other for mediation in various processes (Davies 2010). Under stress conditions, the production, distribution, or signal transduction of these hormones is affected leading to morphological, molecular, and physiological changes which prepare plants to withstand the stress conditions (Eyidogan et al. 2012). The phytohormones, being lowest level transducers, lead to the stress signal activation of signaling cascade which further initiates the response mechanism (Harrison 2012).

We have summarized the roles of phytohormones in various signaling mechanisms, in response to various stress responses. Further, it also focuses on the crucial, delicate, and complex cross talk of these phytohormones in response to various biotic stresses, in various plant species. This chapter would assist plant biologist in further exploring the crucial role of phytohormones in response to biotic stresses.

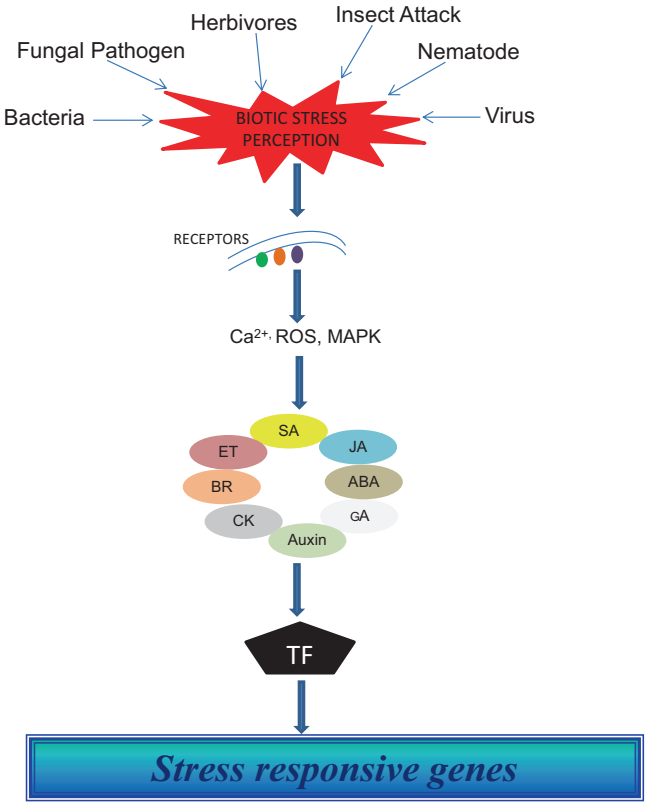

Fig. 7.1 Phytohormones are watchdogs of stress and cross talk to regulate the defense response against biotic stresses

7.2 Role of Phytohormones in Biotic Stress via Different Signaling Pathways

7.2.1 Brassinosteroids (BRs)

One of the most ubiquitously present steroids, brassinosteroid (BR), is an endogenous plant growth-promoting hormone. Initially reported as organic extract (Mitchell et al. 1970), brassinosteroids (BRs) are localized in almost all parts of the plants, namely, seeds, fruits, young vegetative tissues, and pollens, and are known to affect cellular proliferation and expansion (Clouse and Sasse 1998; Sakurai et al. 1999). The BRs on the basis of the number of carbons in their structure are classified as C27, C28, and C29 (Vardhini 2013a, b). Till date, 60 BR-related compounds have been identified (Haubrick and Assmann 2006), but brassinolide (BL), 28-homobrassinolide (28-HomoBL), and 24-epibrassinolide (24-EpiBL) are most widely known BRs, which are used in various physiological and experimental

studies (Vardhini et al. 2006). These steroids are either present in free form or in conjugation with various sugars or fatty acids (Bajguz and Hayat 2009). BRs are involved in the vascular differentiation (Ashraf et al. 2010) and xylem formation in epicotyls (Zurek et al. 1994). Analyses suggest the role of BRs in providing the resistance against various biotic and abiotic stresses (Bajguz and Hayat 2009; Gomes 2011). In rice and tobacco, BR treatment has been observed to enhance the disease resistance (Nakashita et al. 2003). Thus, it has been hypothesized that BRs by triggering the accumulation of apoplastic H_2O_2 further upregulated the antioxidant system, thereby inducing the stress tolerance in plants (Jiang et al. 2012). A cell surface receptor kinase, brassinosteroid-insensitive 1 (BRI1), is known to perceive the BRs in plants. Various bioassays have revealed that BRs bind to the extracellular domain of the BRI1 receptor, which is plasma membrane-localized leucine-rich repeat (LRR) receptor of a serine/threonine (S/T) kinase (Friedrichsen et al. 2000). This binding initiates the dissociation of BRI1 from the negative regulator BIK1, activation of the co-receptor BRI1-associated receptor kinase 1 (BAK1) and its heterodimerization with *BRI1*, phosphorylation of the BRI1-interacting signaling kinase *(BSK1)*, and activation of the BSU1, a protein phosphatase (Lin et al. 2013). The signal is then transmitted to the cytoplasm where a protein kinase, brassinosteroid-insensitive 2 (BIN2), is inhibited, which is the negative regulator of BR biosynthetic pathway (Fariduddin et al. 2014), and transcription factors like BZR1 and BES1/BZR2 are activated. These transcription factors move to the nucleus and activate BR-responsive genes by binding to their promoter. Further BAK1 is involved in the regulation of microbe-induced cell death (Kemmerling et al. 2007), and even interacts with various pattern recognition receptors (PRRs), and is a part of PAMP-triggered immunity (PTI) (Fradin et al. 2009; Chaparro-Garcia et al. 2011). It is also suggested that BAK1 can function in control of cell death and innate immunity independent of BR (Chinchilla et al. 2009). The complexity of BR response pathway was studied in *Arabidopsis* and the authors demonstrate that this key player can act antagonistically and synergistically. Researchers fine-tuned the BR pathway and concluded that BAK1 acts as a mediator for synergistic activities of BR on PTI but also supports a scenario that BR can control plant defense independent of BAK1 (Belkhadir et al. 2012). Lozano-Duràn et al. (2013) and Shi et al. (2013) further provided insights into BR suppression of PTI responses independent of BAK1. Another feather to BR cap of signaling molecules was added as the study conducted by Lin et al. (2013) identified the receptor-like cytoplasmic kinase Botrytis-induced kinase 1 (BIK1) which shares the BR and PTI pathways.

BRs were regulated in plants leading to the various responses depending upon various environmental conditions. Studies in recent past have highlighted that exogenously given BRs exerted a positive effect on the resistance of various crops to a broad range of pathogens (Khripach 2000). This was further supported in small-scale disease trials by Nakashita et al. (2003). The group reported a varying (local, as well as systemic) positive effect of BL on disease tolerance to distinct leaf pathogens in tobacco and rice plants. Exogenous BR application was also shown to confer resistance to barley from several *Fusarium* diseases (Ali et al. 2013). Apart from these positive effects, BR can have negative impact on disease resistance. It was

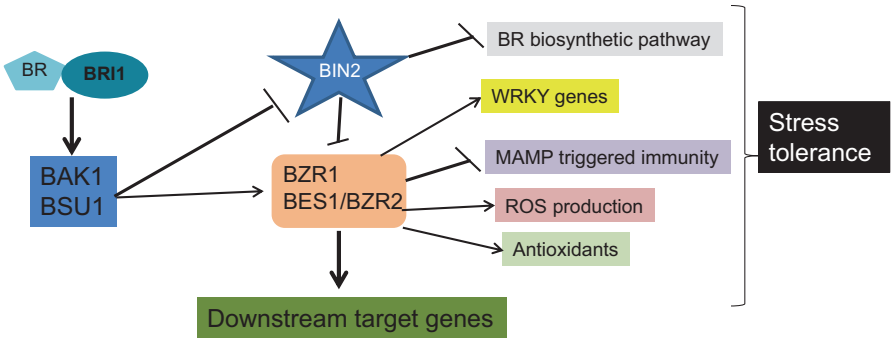

Fig. 7.2 Multifaceted roles of BR in plant pathogen interaction

seen that exogenous BL application either could not alter resistance of *Arabidopsis* to *Pseudomonas syringae* pv. tomato (Pto) and *Alternaria brassicicola* (Albrecht et al. 2012) or makes rice hypersusceptible to the *Pythium graminicola* and *Meloidogyne graminicola*, the root pathogens (De Vleesschauwer et al. 2012; Nahar et al. 2013). In the case of *P. graminicola*, it was even recommended that the pathogen seizes the host BR machinery, as virulence factors to promote infection (De Vleesschauwer et al. 2012). Thus, BRs are an important class of regulators that merge immune system function with normal growth and developmental programs.

The narrow range of BR concentrations can also influence the production of ROS (Baxter et al. 2014). BR-induced ROS production plays an important role in stress tolerance. BRs can also stimulate antioxidant production and scavenge ROS (Bajguz and Hayat 2009; Fariduddin et al. 2013). They can also influence disease resistance by fine-tuning secondary metabolite production. The repressive effect of BZR1 and BES1 on glucosinolate production in *Arabidopsis* was observed (Guo et al. 2013).

BR therefore plays multifaceted roles in plant-pathogen interactions (Fig. 7.2), ranging from modulation of PAMP perception, activation of stress-responsive gene, fine-tuning oxidative metabolism, and production of secondary metabolites. However its role in making the plant resistance or susceptible depends on plant-attacker combination. It further emphasizes the fact that plant hormone interaction in pathogen defense cannot be generalized (Bruyne et al. 2014). Recently, BRs are found to be the master regulators of gibberellic acids (GAs), which are related to the growth and development of vascular plants (Unterholzner et al. 2015). In rice, GA metabolism is found to be modulated by the BRs (Tong et al. 2014). Following stress perception, BRs can cross talk among defense signaling pathways with a range of hormones, such as SA, JA, ABA, auxins, and GA.

7.2.2 Abscisic Acid (ABA)

The phytohormone, abscisic acid (ABA), plays prominent role in various aspects of plant growth which include germination, dormancy, and seed development. Several

analyses have shown its role on developing stress response in plants towards abiotic and biotic stresses. ABA accumulates after infection and can have positive and negative effect on defense responses depending on the environmental conditions. It mostly acts antagonistically with SA/JA/ethylene making the plant susceptible against disease and herbivore attack. ABA promotes stomatal closure as a physiological response to various environmental stresses. ABA thus induces preinvasive defense by inhibiting the entry of pathogens through these passive ports (Ton et al. 2009). This is proven by the analysis of susceptibility toward *Magnaporthe grisea* in rice plant due to ABA application (Koga et al. 2004). It has been found that viral infection in plants leads to the increased ABA concentration in tobacco (Whenham et al. 1986). Plants react to the infection once it is established. SA, JA, and ethylene might not be activated during initial exposure to stress, but at this time ABA acts as a key endogenous factor in playing important role by inducing stomatal closure. It can even antagonize their induction, thereby modifying future responses against potential pathogens. Postinfection, ABA signaling cascade initiates events to strengthen the resistant phenotype through callose accumulation to prevent pathogen invasion in attacked plants. The rise in ABA concentration has been shown to have negative effect on the ET production (LeNoble et al. 2004); they thus interact in antagonistic manner in plant-pathogen interaction. Similar response has been observed in case of jasmonic acid (JA) (Staswick et al. 1992). Overall, by interfering with the biotic stress signaling, ABA affects the resistance of plants toward various diseases in negative manner (Mauch-Mani et al. 2005). Exogenously applied ABA increased the virulence of *P. syringae* pv. tomato on *Arabidopsis* plants (de Torres et al. 2007). Furthermore, application of ABA suppressed the transcript accumulation of defense genes like *PDF1.2* (*plant defensin 1.2*), *CHI* (*basic chitinase*), *HEL* (*hevein-like protein*), and *LEC* (*lectin-like protein*), thereby increasing susceptibility of *Arabidopsis* plants to the fungus, *Fusarium oxysporum* (causal agent of wilt), and to the bacteria, *Erwinia chrysanthemi* (causal agent of bacterial wilt), infection (Asselbergh et al. 2008). Similarly the mutants defective in ABA biosynthesis and perception demonstrate the negative effect of ABA in disease resistance. For example, tomato mutants showed reduced pathogen growth after *B. cinerea* infection (Audenaert et al. 2002). Similarly, *ABA-deficient Arabidopsis* mutant, *aba1-1*, induced HR-like defense response at the site of *Peronospora parasitica* inoculation. Such response was not observed in wild-type plants (Mohr and Cahill 2007). ABA-deficient mutant, *aba3-1*, failed to close stomata upon exogenous application of pathogen-derived elicitors suggesting the involvement of ABA-mediated signaling in closure of stomata. Evidences suggest that exogenous application of ABA bestowed resistance against fungal pathogens in *Arabidopsis* (Adie et al. 2007) and barley plants (Wiese et al. 2004). The increased resistance observed in these plants is because of reduced pathogen colonization as a result of ABA-mediated callose biosynthesis or inhibition of its degradation (Jacobs et al. 2003; Rezzonico et al. 1998). Also, there are evidences that show correlation between the nitrogen status and ABA levels in plants (Wilkinson and Davies 2002). Therefore,

ABA in plants, not only associated with growth and development but also with various levels of stress signaling.

7.2.3 Ethylene (ET)

In plants, the growth, development, and tolerance to many biotic stresses are dependent upon various hormones including ethylene. It regulates the flower fertilization, senescence, fruit ripening, and organ abscission in various plant species. The perception and signal transduction of ethylene are conserved among the plants, which show its relevance in the plant development and survival mechanisms. The ethylene induced during the stress acts as trigger due to its autocatalytic mechanism of ethylene synthesis in a stressed plant. The ethylene production is controlled by mitogen-activated protein kinase (MAPK) phosphorylation cascades (Takahashi et al. 2007). The ethylene pathway is a well-studied pathway in plants. It requires S-adenosylmethionine (SAM) which is also a precursor in other pathways and is widely found in plant tissues. SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5′-methylthioadenosine (MTA) in the reaction catalyzed by ACC synthase in a rate-limiting step of the pathway. During the high rate of ethylene production, levels of l-methionine remain largely unchanged due to the presence of 5′-methylthioadenosine (MTA) (Abeles et al. 1992). The ACC produced is oxidized to ethylene and various other products in combination with oxygen, catalyzed by ACC oxidase. The presence of ethylene leads to the induced expression of gene encoding ethylene response sensors 1 and 2 (ERS1, ERS2) and ethylene resistant 2 (ETR2) proteins. Ethylene is sensed by five ER-localized receptors which are divided into two subfamilies. The subfamily I includes ethylene response 1 [ETR1] and ethylene response sensor 1 [ERS1], while subfamily II includes ETR2, ERS2, and ethylene insensitive 4 [EIN4]. Ethylene signaling also includes some downstream elements like constitutive triple response (CRT1), ethylene insensitive 2 (EIN2), ethylene insensitive 3 (EIN3)/ethylene insensitive-like protein 1(EIL1), and ethylene response factors (ERFs). The major factors governing ethylene signaling are ethylene response factors (ERFs). However, it is suggested that ethylene insensitive 3 (EIN3) induces ERF 1 gene expression to activate defense response. Thus ERF1 acts downstream of EIN3 (Solano et al. 1998). Several ERF subfamily members are associated with biotic stress tolerance, for example, it has been shown in rice and *Arabidopsis* that ERF1 binds to GCC box (AGCCGCC) in promoters of biotic stress-inducible genes (Xu et al. 2007; Cheng et al. 2013). In rice ERF 922 has been shown to be a negative regulator of defense against rice blast fungus *Magnaporthe oryzae* (Liu et al. 2012). It was observed that overexpression of ERF 922 enhanced ABA levels in rice which could be the cause of enhanced susceptibility to fungus (Koga et al. 2004). In several cases, ERFs are associated with long-distance leaf-to-leaf and leaf-to-root signaling to equip the plant against future pathogen attack (Dey et al. 2014). In a study conducted on rice roots, treatment with *Pseudomonas* isolate EA 105 induces the expression of ERF1 in distal

uninfected leaves and enhances resistance to *M. oryzae* compared to noninfected plants (Spence et al. 2014). Further in the absence of ethylene, EIN2 is repressed by constitutive triple response (CTR1). The repression of EIN2 is relieved by ETR1 upon perception of ethylene. CTR1 interacts and phosphorylates the cytosolic C-terminal domain of EIN2 and prevents EIN2 from signaling in absence of ethylene (Ju et al. 2012). The rate of ethylene biosynthesis has been found to be enhanced under the biotic stress (Van Loon 1984; Abeles et al. 1992). Interestingly, ethylene signaling mechanism in plants shows novel negative feedback mechanism. This is because ethylene is essential for plant response to the stress and prepares the plants for tolerance mechanism which is essential for their survival. But excessive ethylene production due to persisting stress conditions leads to the inhibition of growth and development in plant, which if continued for long duration leads to the plant death. Hence, it is considered that a tight regulation of ethylene homeostasis is maintained for the plant survival during stress conditions and its recovery for growth later. Ethylene interacts with other phytohormones like jasmonic acid and salicylic acid and affects the signaling response. It may affect SA-mediated defense responses, positively and negatively depending on the different lifestyles of the pathogens (Derksen et al. 2013). The gain of function and loss of function of ethylene-related genes affect the susceptibility toward pathogens in plants. In rice, overexpression of ACS2 (1-aminocyclopropane-1-carboxylic acid synthase) resulted in high level of ethylene and increased resistance to *M. oryzae* and *R. solani* (Helliwell et al. 2013). RNAi silencing of OsEIN2b in rice plants increased susceptibility to *M. oryzae* infection (Seo et al. 2011).

7.2.4 Jasmonates

Jasmonates are one of the most widespread class of phytohormone in the plant kingdom. Jasmonic acid was first of all isolated as methyl jasmonate from the essential oil of *Jasminum grandifloram*. Vick and Zimmermann (1984) provided insights into biosynthesis of JA. The family, jasmonates, includes various other compounds, namely, 12-oxophytodienoic acid (12-OPDA), methyl jasmonate (MeJA), and amino acids conjugated to the jasmonic acid such as JA-leucine and JA-isoleucine. Jasmonic acid is the most studied and characterized member of the jasmonate family in plants (Avanci et al. 2010). The members of the jasmonate family have been analyzed for their role in development of fruits, senescence, reproductive process, various secondary metabolisms, and direct and indirect defense responses against pathogen (Wasternack 2007). Methyl jasmonate (MeJA) has been earlier shown to be involved as signaling molecule which mediates the intra- and interplant communications, thus altering the plant defense responses (Seo et al. 2011; Wasternack 2007). This is due to the ability of MeJA to diffuse through the membranes and its volatile nature. MeJA has also been observed to play major role in regulating the reproductive process in plants (von Malek et al. 2002). The biosynthesis and signaling pathways of the jasmonate have been studied in detail in *Arabidopsis* and tomato

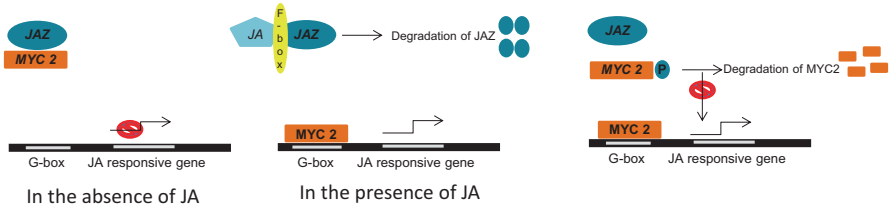

Fig. 7.3 (**a**, **b** and **c**) Role of JAZ and MYC2 in JA-responsive gene expression. In the absence of active JA, JAZ proteins interact with transcription factor JIN1/MYC2 and inhibit expression of JA-responsive genes. In the absence of active JA, JA binds to its receptor which is a F-box protein CORONATINE INSENSIVE 1 (CoI1) and mediated the 26 S-mediated degradation of JAZ, thereby allowing MYC2 to upregulate the expression of JA-responsive genes. (**c**) Fine-tuning of JA responses by MYC2

(Kazan and Manners 2008). Jasmonates play a role in the growth control, shoot and root tissue elongation, and floral bud formation (Maciejewska and Kopcewicz 2003). Apart from that, jasmonates also contribute to the regulation of the biosynthesis of many secondary metabolites such as phenylpropanoids, alkaloids, terpenoids, and antioxidants. During pathogen attack, especially necrotrophic fungal and bacterial infection, jasmonates lead to the expression of defense-related genes (Mei et al. 2006). JA biosynthesis has been found in the roots of the plants (Pedranzani et al. 2003), but evidence also suggests its presence in the leaves (Mueller et al. 1993). In sorbitol-treated tomato plant, endogenous levels of OPDA, JA, and MeJA were observed in leaves (Abdala et al. 2003). The activity of jasmonates is also associated with the slowing of activity of the photosynthetic apparatus. The hormone also plays an important role against attack by herbivores like caterpillars, mites, thrips, beetles, etc. The transcription factor JASMONATE INSENSITIVE/MYC2 (JIN1/MYC2) plays an important role in JA-responsive gene expression. JA-regulated stress responses are also mediated by ethylene-responsive factors. The JA-responsive marker gene PLANT DEFENSIN 1.2 (PDF 1.2) is regulated by ethylene-responsive factors ERF1, ERF2, and ERF5. A repressor protein JASMONATE-JIM DOMAIN (JAZ) plays a prominent role in JA-mediated stress response. In the absence of active JA, JAZ proteins interact with transcription factor JIN1/MYC2 and inhibit expression of JA-responsive genes. Once the JA pathway is activated, i.e., JA-Ile binds to its receptor which is a F-box protein CORONATINE INSENSIVE 1 (CoI1) and mediates the 26 S-mediated degradation of JAZ, thereby allowing MYC2 to upregulate the expression of JA-responsive genes. Recent studies have also highlighted the fine-tuning of JA responses by MYC2. A posttranslational modification of MYC2 at Thr 328 stimulates its transcription activity. The modified MYC2 is unstable and degraded by plant U-box protein (PUB 10), thereby facilitating turnover of MYC2 (Antico et al. 2012). The model depicting above interactions is shown in Fig. 7.3.

7.2.5 Salicylic Acid (SA)

SA is a plant phenolic compound synthesized by plants to regulate defense mechanism against biotrophic and hemibiotrophic pathogens and is derived from the shikimate-phenylpropanoid pathway (Sticher et al. 1997). Detection of phytopathogens stimulates the synthesis of SA. The pivotal role of salicylic acid in conferring disease resistance was first demonstrated in *Arabidopsis* and cucumber plants when levels of the hormone were detected prior to development of local and systemic resistance (Malamy et al. 1990). Since then its role in biotic stress has been established in all plant species. Activation of SA pathway at the site of infection triggers a defense response in distal parts of the plant.

The constitutive SA accumulation often leads to reduction of plant fitness (Ishihara et al. 2008), by mobilizing resources and energy away from growth and reproductive process. Hence, its biosynthesis and signaling are under tight control of the cell. SA is synthesized in the chloroplast and then transported to the cytosol where it signals immune responses. The export of the hormone is mediated by a chloroplast membrane-localized member of the multidrug and toxin (MATE) transporter family EDS5 (or SID1). Inside the cytoplasm, SA can be subjected to various modifications that usually render it inactive (Dempsey et al. 2011). These modifications are an important check to regulate the level of biologically active SA in the cytoplasm such as glucosylation of SA at its hydroxyl group generates a glucoside (SAG), whereas glucosylation at its carboxyl group produces an ester (SGE). SAG moves to the vacuole and behaves as a nontoxic storage from which it can be hydrolyzed following pathogen attack to release free active SA. Methylation of SA produces methyl SA (MeSA) which is a mobile phloem signal that travels from the infected leaf to the systemic tissues, where it activates defense mechanism after being converted back to SA (Fig. 7.4) (Park et al. 2007). Formation of SA-amino acid conjugates is another adjustment strategy (Dempsey et al. 2011) as salicyloyl-aspartate (SA-Asp) has been identified in plants. There are a number of physiological processes that are influenced by SA. In plants, SA is required for the initiation of various stress symptoms and hypersensitive-response (HR)-like cell death. The PR genes are a diverse class of genes encoding antimicrobial proteins and increasing resistance against broad spectrum of pathogens. The expression of various pathogenesis-related (PR) proteins encoding genes has also been observed with the exogenous application of the SA (Malamy et al. 1990). SA is also required for establishing the systemic acquired resistance (SAR) with its accumulation in the distant tissues in the condition of stress in plants (Vernooij et al. 1994). Earlier, significantly high number of leaves and increased biomass have been observed in wheat seedlings, when treated with SA (Hayat et al. 2005). Various analyses provided an indication that SA is related to several processes, and therefore it is assumed that it has a role in biotic stress conditions. Yalpani et al. (1994) have suggested that exposure to ozone and the ultraviolet light leads to the accumulation of SA. The ozone induces oxidative stress levels; thus, SA induces antioxidant defenses in plants showing it may have some role in other abiotic stress conditions. Also, analysis suggests that there is an overlap of ozone and pathogen-induced resistance

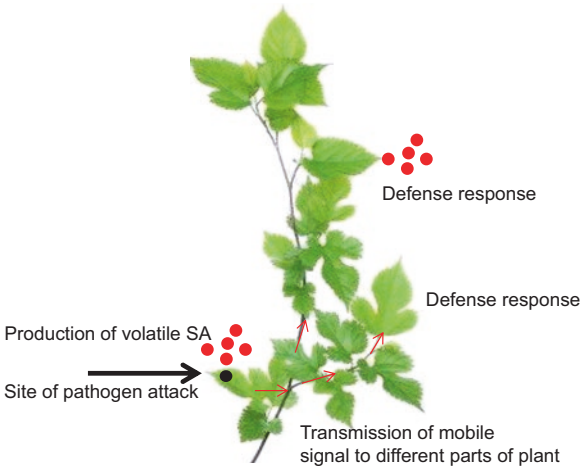

Fig. 7.4 Activation of SA pathway at the site of infection triggers a defense response in distal parts of the plant. Methylation of SA produces methyl SA (MeSA) which is a mobile phloem signal that travels from the infected leaf to the systemic tissues, where it activates defense mechanism after being converted back to SA

pathways which confirms the role of SA in plants (Sharma et al. 1996). SA leads to the induced expression of receptor protein kinase (RPK) which is known to initiate the response to the signal (physical or chemical) in plants (Bassett et al. 2005). Further, SA has been hypothesized to be the direct or indirect regulator of calcium-mediated signaling pathways. This is evident from the analysis in which SA was observed to induce expression of certain calcium-dependent protein kinases (CDPKs) in plants (Leclercq et al. 2005). Owing to the role of SA in inducing many crucial genes in signaling pathways, it is assumed that SA is a crucial part of the complex signaling transduction networks. The members of NON-EXPRESSOR OF PR GENE (NPR family) are proposed SA receptors. The implementation of SA action is monitored through action of SA marker gene PR-1 whose activation requires the positioning of the SA-dependent transcriptional enhanceosome to its promoter (Rochon et al. 2006). The enhanceosome contains the members of TGA2 clade of bZIP transcription factors (Zhang et al. 2003) and the transcriptional coactivator (NPR1) (Rochon et al. 2006). Recent studies provide insights into mode of action of this SA receptor-NPR1, which is central to activation of SA defense genes. TGA2 is a transcriptional repressor and its inactivation is brought about by NPR1. The N-terminal region of NPR1 contains a BTB/POZ domain that interacts with TGA2 repression domain and negates its function (Boyle et al. 2009). NPR1 harbors in its C-terminal region a transactivation domain which contains two cysteines (Cys^{521} and Cys^{529}) required for the activating function of the enhanceosome (Rochon et al. 2006). NPR1 binds specifically to SA, which in turn regulates conformation of NPR1 by depolymerizing it to a dimer. This causes release of the C-terminal transactivation domain from the N-terminal autoinhibitory BTB/POZ domain. SA binding to NPR1 relieves sequestration of NPR1 transactivation domain (Fig. 7.5). Further, nearly 30 SA-binding proteins (SABs) with variable affinities for SA have been identified. SA signals its effects by interacting with these SABs. SA research is even benefitting agriculture as pretreating plants with high concentration

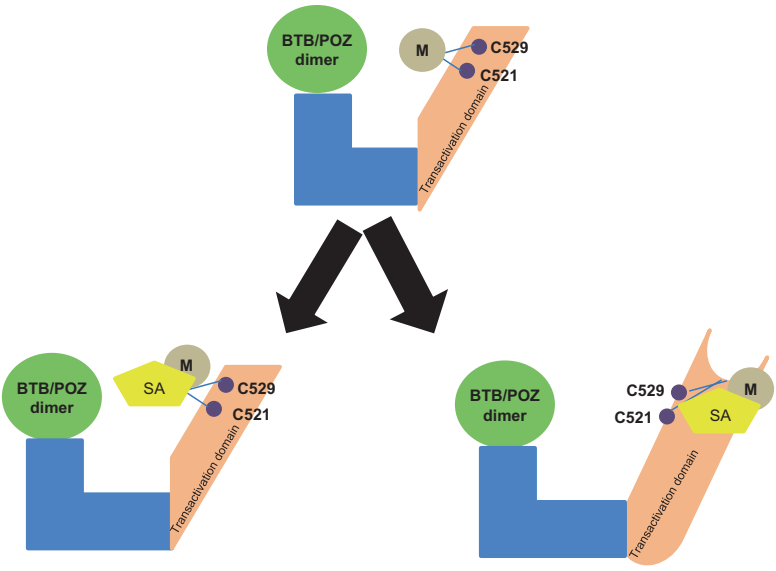

Fig. 7.5 SA binding to NPR1 relieves sequestration of NPR1 transactivation domain. The N-terminal region of NPR1 contains a BTB/POZ domain that interacts with TGA2 repression domain and negates its function. NPR1 harbors in its C-terminal region a transactivation domain which contains two cysteines (Cys^{521} and Cys^{529}). Binding of SA to the cysteines in the transactivation domain disrupts its interaction either due to steric hindrance or SA-induced conformational change

of SA can initiate defense response which can later be activated rapidly by subsequent infection (Dempsey and Klessig 2017).

7.2.6 Gibberellin (GA)

The plant phytohormone, gibberellins, is one of the large families of diterpenoid compounds. It is not only produced by higher plants but also by fungi and bacteria (MacMillan 2001). It is widely known to promote some of the essential processes related to floral development, flowering time, elongation of stem, trichome development, and seed germination (Davies 2010). Various environmental stimuli lead to the change in the GA concentrations in plants which further affects various processes (Davies 1995). The GAs are synthesized in the plastids by methylerythritol phosphate pathway from trans-geranylgeranyl diphosphate. Several analyses suggest that GA as a signaling component plays an important role in the disease susceptibility and resistance in plants. The first report for the role of GA came from the work of Zhu et al. (2005). They demonstrated that one of the capsid proteins of the rice dwarf virus interacts with plant ent-kaurene oxidases resulting in decreased GA levels in the infected plants. The DELLA proteins are a class of nuclear growth-repressing proteins. They are the negative regulators of GAs and have been found

regulating the immune response modulated by JA and SA in plants. DELLAs proteins regulate the balance of SA/JA signaling during plant immunity, supporting JA perception and/or signaling, and restricting SA biosynthesis and signaling (Navarro et al. 2008). These proteins have also been shown to regulate the expression of various genes which are known for producing ROS detoxification enzymes (Achard et al. 2008). Thus, indirectly GAs can be assumed to play a role in the oxidative stress related to salinity and osmosis. GA signaling plays an important role in cell wall development and represses cell wall relaxation by altering the expression of xyloglucan endotransglucosylase/endohydrolases (XTHs) and expansins. Loosening of cell wall is undoubtedly important for cell growth, but it also allows pathogen entry and nutrient leakage. GA is also responsible for modification of carbon and energy metabolism and decrease production of antimicrobial compounds or increase nutrient efflux favoring microbes. DELLAs have been found to play an important role in plant immunity by regulating cell cycle-dependent expression of immunity-conferring genes. The rice mutants having defective GA receptors were found to accumulate high levels of GA and showed better resistance to fungus *Magnaporthe grisea* known for causing blast, than the wild types (Tanaka et al. 2006). Under flooding conditions, rapid internode elongation takes place in plants. The elongation is the result of upregulation of ethylene response factor (ERF) domain proteins SNORKEL1 and SNORKEL2 which leads to the direct or indirect increase in the levels of GAs in plants (Hattori et al. 2009). Interestingly, the GA signaling pathways have been found to be modulated by other phytohormones (Fu and Harberd 2003). Through many biological studies utilizing mutant lines and gain of function and loss of function and plants impaired in gibberellic acid signaling, it is observed that GAs are important regulators of plant growth including biotic stress (Colebrook et al. 2014). Exogenous application of GA in rice decreases resistance to the hemibiotrophic rice pathogens *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (Qin et al. 2013). Decreased level of GA by overexpressing a GA-deactivating enzyme in rice resulted in low levels of both salicylic acid and gibberellic acid, but the resistance to *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* was enhanced and reverse result was obtained by loss of function and plants became more susceptible (Yang et al. 2008). Similar to the classic defense hormones jasmonic acid, salicylic acid, and ethylene, gibberellic acid acts as multifaceted regulators of plant immunity. This depends upon the plant species and pathogen type and also on plant pathogen interaction (Bruyne et al. 2014).

DELLA proteins interact with transcription factors and regulate plant growth and development. Many groups have shown that DELLA proteins compete with JAZ, a transcriptional repressor of JA to bind to MYC2. Binding of DELLA proteins relieves MYC2 from JAZ, which is now free to enhance JA-responsive gene action. This increases resistance against necrotrophic pathogens (Navarro et al. 2008). However, GA exerts its control as in the presence of GA, DELLA proteins are degraded, and JAZ proteins bind to MYC2, thereby inhibiting JA-responsive gene action. Figure 7.6 explains this interaction. Therefore GA may disable JA-mediated stress response against pathogen invasion. DELLAs seem to positively mediate JA-responsive gene expression by blocking JAZ proteins (Hou et al. 2010) and also

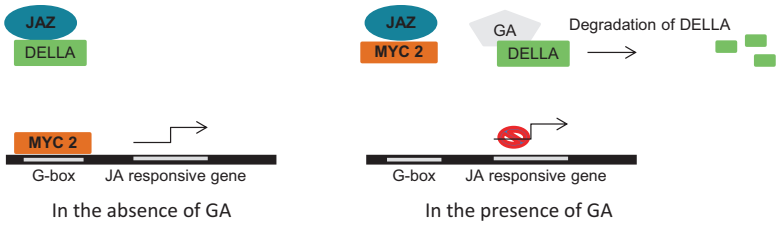

Fig. 7.6 Control of GA over JA-responsive gene action. In absence of GA, the DELLA proteins are stabilized and bind to JAZ. This frees MYC2 which can activate JA-responsive gene action. In presence of GA, DELLA proteins are degraded, JAZ proteins binds to MYC2, and JA-responsive gene action is blocked

by acting as JA-responsive regulators. To defend against pathogens, plants may co-opt for a JA signaling cascade to regulate DELLA levels in planta as a DELLA protein RGL3 is also known to be induced in a MYC2-dependent fashion by JA. DELLA proteins therefore form an important node of multiple hormone signaling pathways as they are main centers controlling multiple environmental signals and developmental cues. DELLA proteins also interact with BR-regulatory TF *brassinazole resistant 1* (BZR1). They inhibit BR signaling cascade by sequestering BZR1 into inactive protein complexes. This in turn regulates numerous defense-related genes controlled by BZR1 (Wang et al. 2014).

7.2.7 Cytokinins

The role of phytohormone, cytokinins, has been poorly understood in plants although there are certain reports which show its role in defense response against some pathogens. Apart from that, its role has been shown in the growth of root, shoot and inflorescence, seed development, senescence in leaves, and stress response (Muller and Sheen 2007). The cytokinins when applied with auxins have been found to trigger callus differentiation and induce cell proliferation in shoots. It also contributes to the sink strength, nutrient translocation, and grain yield of various plants. In developing tissues like cambium, shoot apex, and root tips, cytokinins have been found in abundance. Whole genome expression analysis *of Arabidopsis infected with Plasmodiophora brassicae* (known to cause clubroot disease in plants) showed downregulation of gene playing role in cytokinin homeostasis, namely, cytokinin synthases and cytokinin oxidases/dehydrogenases, which shows its role in the clubroot disease in *Arabidopsis* (Siemens et al. 2006). The phytohormone cytokinins have also been found to regulate the nitrogen metabolism in plants by enhancing the activity of nitrate reductase (Sykorová et al. 2008). The activities of the enzymes involved in the photosynthesis have been observed to be enhanced by the root-derived cytokinin signals (Sakakibara et al. 2006). In plants, cytokinins are perceived through a well-conserved multistep histidine-to-aspartate phosphorelay system which is known as two-component signaling systems (Schaller et al. 2011).

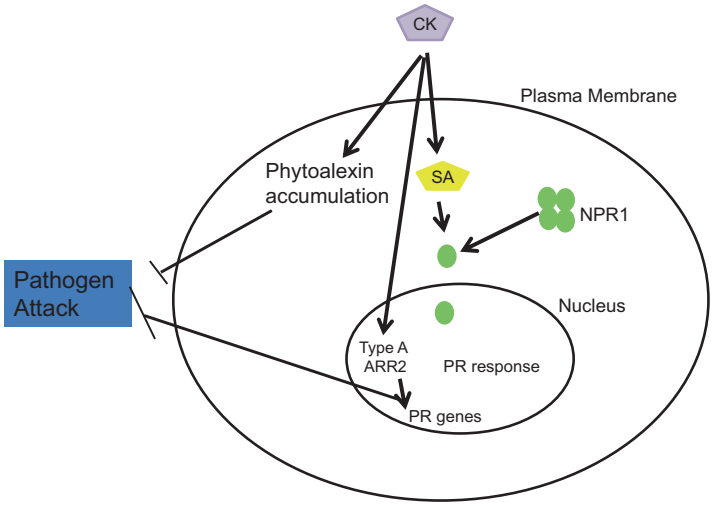

Fig. 7.7 Role of cytokinin in plant defense response. Arrows indicate positive regulation, and blocked arrows indicate negative regulation

The two-component system in plants is a well-studied and characterized signaling system specifically in *Arabidopsis* and rice. It is comprised of histidine-containing phosphotransfer proteins, receptors, and response regulators (Argueso et al. 2010). Specifically, type B response regulator, which functions as a transcription factor, regulates the expression of genes regulated by cytokinins. Analyses also suggest that cytokinins are the endogenous negative regulator of senescence (Singh et al. 1992). In *Glycine max*, the decrease in the cytokinin content has been observed with the onset of senescence (Noodén et al. 1990). Similar results have also been observed in *Arabidopsis* in high-throughput analysis (Breeze et al. 2011). These results suggest that cytokinins delay senescence in plants. Cytokinins have been proposed to induce synthesis and accumulation of phytoalexins in SA-independent manner. The hormone accumulated post pathogen attack and also helps the plant to cope against infection by affecting the priming response through SA-mediated signaling and inducing PR gene expression (PR-1, PR-3, PR-4, PR-5). Further a component of CK signaling pathway type B, ARR2 is required to regulate the activity of SA receptor NPR1 (O'Brien and Benkova 2013). The role of cytokinin in plant defense response is depicted in Fig. 7.7.

7.2.8 Auxins

Auxins are low-molecular-weight organic compounds, and it constitutes one of the most important and diverse groups of phytohormone generally found in all the plants. They are involved in large number of developmental processes such as shoot architecture control, vascular development and lateral root formation by controlling

the cell division (Woodward and Bartel 2005). Auxins have also been shown to control the senescence, respond to various pathogens, and develop abiotic stress response in plants (Fu and Wang 2011; Wang et al. 2010). Apart from that, it also regulates the formation of fruits (De Jong et al. 2009). Indole acetic acid (IAA) is one of the most abundant endogenous auxins in plants and is synthesized via tryptophan (Trp)-independent and Trp-dependent pathways (Zhao 2010). In *Arabidopsis*, rice, and soybean, auxin stimulates the expression of auxin response genes (Javid et al. 2011). With the mediations and cross talk with other phytohormones, auxins play an important role in the biotic and abiotic stress response (Fahad et al. 2015). Apart from its positive role, auxins have been established to weakening of the defense response in plants. Analysis shows that its exogenous application promotes the diseases caused by Pst DC3000, *Pseudomonas savastanoi*, and *Agrobacterium tumefaciens* (Navarro et al. 2006; Chen et al. 2007). The signaling initiated by auxins regulated the response of cell to varied levels of auxins that are formed by auxin metabolism and transport. The TIR1/AFB family receptors are known for the auxin signaling (Dharmasiri et al. 2005; Kepinski and Leyser 2005). The phytohormone auxins can move long distances from highly active young tissues to the roots through the phloem (Marchant et al. 2002). Apart from that, it also moves to the short distances from cell to cell and is regulated by specific influx and efflux carrier proteins (Muday and Rahman 2008). Under various environmental stress conditions, inverse interactions between ROS and auxins have been observed, but the auxin-responsive gene is still unidentified. The impact of oxidative stress has been observed in auxin stability due to oxidization of IAA via peroxidase activity (Kawano 2003). Auxin acts in a mutually antagonistic fashion with SA and shares many commonalities with JA during plant defense. Recent evidences suggest that upon infection some pathogens either produce auxin themselves or increase auxin biosynthesis as a part of the plant's defense and developmental machinery (Valls et al. 2006; Kazan and Manners 2009; Bielach et al. 2017).

7.3 Cross Talk in Various Phytohormones

All the processes and responses in plants are the result of interplay of many genes and gene families suitably orchestrated in a network. In plants, growth, development, and response to various environmental cues go hand in hand. Various phytohormones are known to play important role in almost all the process through the modulation of genes. Further, through the balancing of phytohormones, plants maintain homeostasis and adapt to the environmental changes. This is only possible by an efficient and systemic cross talk between various phytohormones. SA, JA, and ET work together in a harmonious manner with BR, auxin, cytokinin, and GA in mediating plant response to biotic challenges. Plant defense response doesn't depend solely on any one hormone rather all the phytohormones work with each other regulating defense response positively or negatively (Fig. 7.8). There are evidences which suggest JA and SA signaling intersect in a negative manner against necrotrophic pathogens and herbivores (Glazebrook 2005). NPR1 is a key

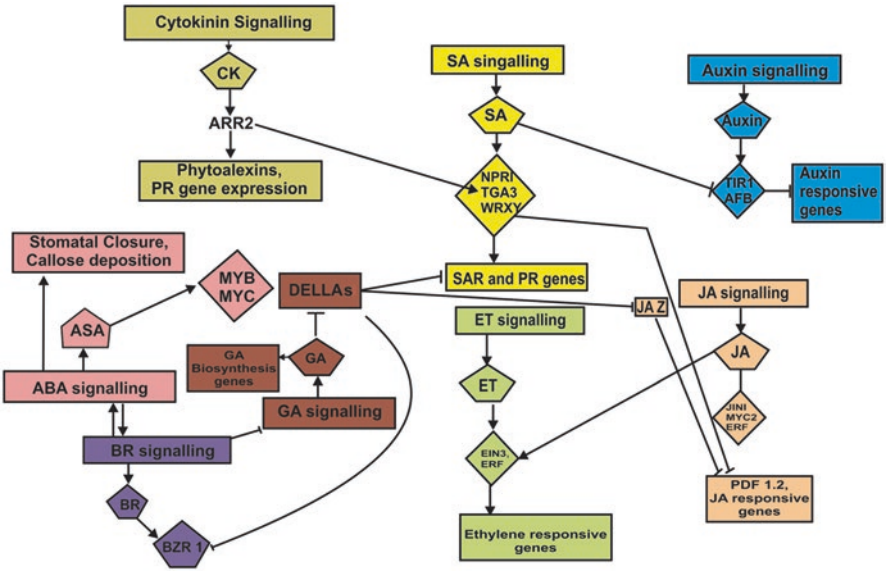

Fig. 7.8 Cross talk of phytohormones in biotic stress tolerance. ABA, SA, JA, and ET are key players in stress response. GA pathway produce DELLAs as an intersecting point. Auxins and CK pathway participate in biotic stress response in a SA-dependent manner. Arrows indicate positive regulation, and blocked arrows indicate negative regulation

intersecting player in the antagonistic talks of SA and JA. SA assisted in downregulation of JA-responsive genes like lipoxygenase 2 (LOX2), plant defensin 1.2, and vegetative storage protein (VSP). This suppression was abolished in *npr-1 mutants*. The WRKY transcription factor 70 family also plays prominent role in antagonistic interactions of SA and JA. The recombinant WRKY-70 overexpressing line displayed constitutive responsive expression of PR genes and repression of JA-responsive PDF1.2 (Li et al. 2004). Similar studies in *mpk4* (Map Kinase 4) mutants of *Arabidopsis* proved antagonistic interactions of SA and JA (Petersen et al. 2000). A few reports also prove their synergistic interactions.

JA and ET work together in a synergistic manner and regulate synthesis of defense genes postinfection. Both the phytohormones work cooperatively to induce or stabilize EIN3 and help the plant against necrotrophs by developing root hairs (Zhu et al. 2011). The synergy of JA interaction was observed in tomato plants where genes encoding proteinase inhibitors were induced in response to wounding (O'Donnell et al. 1996). Their cooperative action is even required for induction of ERF1-induced expression of PR genes (Lorenzo et al. 2003). Their antagonistic actions are also proved against insects and herbivores. The JA-activated *MYC2* represses the downstream functions of EIN3, and in a similar fashion, *MYC2* inhibits JA-regulated genes.

Auxins are known as key players in regulating plant growth and development, but several evidences suggest that auxins advance disease susceptibility and

resistance against biotic challenges requires repression of auxin signaling. SAR reduces auxin-responsive genes. The treatment of *Arabidopsis* plants with a SA analog benzothiadiazole S-methyl ester (BTH) caused repression of auxin-responsive genes (Wang et al. 2007). Furthermore, SA signaling suppresses expression of transport inhibitor resistant 1 (TIR1) and auxin signaling F-box genes (AFB) and stabilizes auxin repressor protein (AUX/IAA) which represses further auxin action.

The pivotal role of cytokinins in biotic stress response has been demonstrated by several groups (Reusche et al. 2013; Wang et al. 2007). Recombinant *Arabidopsis* plants with stabilized CK levels displayed improved resistance against hemibiotrophic pathogen *Verticillium longisporum* (Reusche et al. 2013). CK and SA signaling cascades intersect to regulate plant defense as it has been seen that SA defense responses are promoted by cytokinin-activated transcription factor (Wang et al. 2007). The cooperativity between SA and CK was also observed in rice plants as increased resistance was observed against rice blast fungus in an *Os NPR1*- and *WRKY 45*-dependent manner (Jiang et al. 2013). GA and JA also interact as DELLA proteins are known to interact with key repressors of JA signaling JAZ1, thereby preventing JA-mediated suppression of transcription. JA is also known to regulate the expression of repressor of GA1–3 (RGL3), which positively regulates JA-mediated response against necrotrophs by competing with MYC2. BRs can cross talk among defense signaling pathways with a range of hormones such as SA, JA, ABA, auxins, and GA. BR is known to increase resistance against pathogens in a SA dependent as well as independent manner. It also negates JA-induced resistance in rice plants. BRs can also cross talk with auxins, and this BR-auxin bidirectional interplay can have effects on BR signaling cascade in disease and resistance. BR is also known to interact with GA. It suppresses GA biosynthesis genes and activates GA repressor genes. Known interactions also exist between BZR1 transcription factor and DELLA proteins further mediating cross talk between BR and GA.

There are several evidences which validate the cross-talk hypothesis of the plant phytohormones which seems to be true in the sense that plants maintain a critical balance between growth and environmental response in which all the phytohormones play important role. In conclusion, this is just the beginning of unraveling the complex phytohormones signaling cascades. The dissection of plant immunity web comprising of different hormones will help to understand the roles played by each one of them both as an antagonistic and synergistic way. So, elucidating the mechanisms of plant hormone interaction and solving the plant immune network will help fundamental understanding of how plants comprise of orchestrated immune system function. The phytohormone intricate web may be a target for crop improvement. The yield may be increased by the disease resistance plants by transgenic approaches. The methods employed will comprise of either strengthening resistance of signaling pathway by modulating the antagonistic pathway or synergistic pathways.

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8 Insights into the Role of WRKY Superfamily of Protein Transcription Factor in Defense Response

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Abstract

Plants are constantly challenged by a variety of biotic and abiotic stresses. To combat these challenges, plants have developed intricate mechanisms to perceive external signals and respond with the proper physiological and morphological changes. Generally, plants regulate the expression of many stress-related genes by activating or repressing their transcription upon signal perception and transduction of the external stimuli. The WRKY transcription factors comprise a large family of plant-specific zinc-finger-type regulatory proteins and regulate many plant defense responses to diverse biotic and abiotic stresses. WRKY proteins possess either one or two WRKY domains, a 60-amino-acid region that contains the amino acid sequence WRKYGQK, and a zinc-finger-like motif. In spite of the strong conservation of their DNA-binding domain, the overall structures of WRKY proteins are highly divergent and can be categorized into distinct groups, which might reflect their different functions. Based on the number of conserved WRKY domains and the features of the zinc-finger motif, the WRKY superfamily can be divided into three distinct groups: I, II, and III. Previous studies have demonstrated that WRKY transcription factors participate in regulating defense gene expression at various levels, partly by directly modulating immediate downstream target genes, by activating or repressing other TF genes, and by

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regulating WRKY genes. WRKY proteins also seem to be involved in other plant-specific processes, such as trichome development and the biosynthesis of secondary metabolites. In this chapter, we will focus our attention to the role of WRKY TFs in plant defense response.

Keywords
Biotic stress · Plant defense · Transcription factor · WRKY · Zinc-finger protein

8.1 Introduction

The global climate change leads to the dynamic interaction between climatic and biological factors. It is not only confined up to modification of physiology and resistance of plants, but rather it also modifies the rates and stages of pathogen development, which will ultimately lead to the shifting of host-pathogen physiological interactions. Pathogen would be following the hosts and may infect vegetation of natural plant communities which were previously not exposed to the more aggressive strains. Therefore, new combinations of species are evolving. So the emphasis is shifted to develop new strategies to cope up with these biotic constraints in present scenario. However, the nature orchestrated by plants with an inherent defense system can be generally divided into two levels: the first is PAMP-triggered immunity (PTI), which confers resistance to most pathogens, and the second begins in the cytoplasm and mainly relies on the recognition of microbial effectors which is called effector-triggered immunity (ETI). Bostock (2005) reported that both PTI and ETI activate local as well as systemic defense responses, modulated by jasmonic acid (JA) and salicylic acid (SA). Activation of these two pathways extensively shares downstream signaling pathway, which in turn induces expression of defense gene and their corresponding defense responses (Tsuda et al. 2013). The response leads to adaptive plasticity of plants, which is mainly achieved by enforcement of a network of various transcription factors (TFs). TFs are multimers of polypeptide mediating different cellular responses through recognizing the specific cis-regulatory DNA sequences at the promoters of their targets genes (Franco-Zorrilla et al. 2014) and the rearrangement of the multimeric subunits leading to different functions through their differential expression patterns (Berk and Schmidt 1990). The binding of TFs with *cis*-elements of stress-related genes results in either overexpression or suppression of these genes, which may improve the plant's tolerance potential against different biotic stresses. Approximately 6% of the plant genome encodes for TFs, and among all, WRKY TFs are one of the largest families of transcriptional regulators in plants (Eulgem and Somssich 2007; Bakshi and Oelmüller 2014), involved in regulation of various physiological processes. WRKY TFs are emerging players in plant signaling, which regulate diverse cellular programs by relaying extracellular signals to intracellular responses and involved in multiple defense responses, development, metabolism, etc. The reprogramming of

WRKY network under biotic stress efficiently deteriorates the pathogens, and at the same time, it restricts defense responses, which can be detrimental for plant growth, development, and reproductive fitness. However, in the contemporary time of scientific advancement, enormous role of WRKY TFs in abiotic stresses has also been revealed.

In the present chapter, we emphasize on WRKY TFs and their action on downstream regulation of different molecular switches under biotic stress. This will provide important insights in understanding of regulatory networks and its associated functions to develop strategies for crop improvement and value addition in plants, which could be useful to the humankind.

8.2 WRKY Domains and Classification

WRKY proteins constitute a novel family of plant-specific TFs and are characterized by the presence of WRKY domain which consists of ~60 amino acid residues at the N-terminus and a zinc-finger-like motif C-C-H-H/C at the C-terminus (Rushton et al. 1996). The WRKY domains contain the conserved heptapeptide "WRKYGQK" also referred to as the "signature sequence" at the N-terminus of DNA-binding domain. WRKY proteins bind to W-box elements containing the consensus motif TGACC/T, which occur either as single hexamers, TTGACC/T; as palindromic sequence, TGACC/T-A/GTCA; or as tandem repeats, TGACC/C--TGACC/T in the promoter of target genes (Eulgem et al. 1999; Yang et al. 1999). WRKY proteins have been categorized into three groups based on the number of WRKY domains and the type of their zinc-finger-like motif (Kumar et al. 2016). Generally, group I member contains two WRKY domains both at N- and C-terminal and C2H2-type zinc-finger motif (C–X4–5–C–X22–23–H–X1–H), and group II has one WRKY domain with C2H2-type zinc-finger motif. Group II members have been further divided into subgroups a–e based upon additional amino acid motifs present outside the WRKY domain. Group III also has one WRKY domain but with C2HC-type zinc-finger motif (C–X7–C–X23–H–X–C) at C-terminal (Eulgem et al. 2000). It has been reported for group I WRKY proteins from *Arabidopsis thaliana*, parsley (*Petroselinum crispum*), and sweet potato (*Ipomoea batatas*) that sequence-specific DNA binding occurs at the C-terminal of WRKY domain, but not the N-terminal domain (Ishiguro and Nakamura 1994; Agarwal et al. 2011) (Table 8.1).

The first WRKY TF has been identified as DNA-binding protein (SPF1) from *Ipomoea batatas* and shown to regulate gene expression in sucrose inducibility (Ishiguro and Nakamura 1994). WRKY proteins have been identified in a wide range of plants due to successive duplication events, resulting in large gene families including up to 74 members in *Arabidopsis* (Ülker and Somssich 2004), >109 in rice (Shimono et al. 2012), 197 in *Glycine max* (Schmutz et al. 2010), 66 in papaya, 104 in *Populus*, 68 in sorghum (Pandey and Somssich 2009), and 45 in barley (Mangelsen et al. 2008). WRKY TFs play a broad-spectrum regulatory role as a positive and negative regulator to control gene expression (Eulgem and Somssich 2007).

Table 8.1 WRKY TFs involved in plant defense and its associated regulatory pathways

S. No.	Gene/pathway	Action/role	Plant species	Tolerance/resistance	References
1.	*AtWRKY18*	Modulation of defense-related genes	*Arabidopsis thaliana*	*Pseudomonas syringae*	Chen and Chen. (2002)
2.	*AtWRKY3* and *AtWRKY4*	Regulation of SA and JA-/ET-mediated signaling pathway	*Arabidopsis thaliana*	*Pseudomonas syringae* and *Botrytis cinerea* A	Lai et al. (2008)
3.	*AtWRKY33*	Reduced expression of the salicylate and jasmonate defense genes	*Arabidopsis thaliana*	*Botrytis cinerea*, *Pseudomonas syringae*	Zheng et al. (2006)
4.	*AtWRKY6*	Regulation of genes associated with leaf senescence	*Arabidopsis thaliana*	*Pseudomonas syringae*	Robatzek and Somssich. (2001)
5.	*AtWRKY7*	Reduced expression of defense-related genes	*Arabidopsis thaliana*	*Pseudomonas syringae*	Kim et al. (2006)
6.	*AtWRKY70*	Involvement of R-gene	*Arabidopsis thaliana*	*Hyaloperonospora parasitica*	Knoth et al. (2007)
7.	*BnWRKY33*	Induction of salicylic acid, jasmonic acid, ethylene, and glucosinolate synthesis pathway	Oilseed rape	*Sclerotinia sclerotiorum*	Wang et al. (2014)
8.	*BrWRKY12*	By upregulation of defense-related proteins and jasmonic acid signaling pathway	*Brassica rapa*	*Pectobacterium carotovorum*	Kim et al. (2013)
9.	*FaWRKY1* and *WRKY75*	Uncoupled to PATHOGENESIS-RELATED (PR) gene expression but strongly associated with oxidative burst and glutathione S-transferase (GST) induction	Strawberry	*Colletotrichum acutatum*	Encinas-Villarejo et al. (2009)
10.	*GhWRKY15*	Through increased RNA expression of pathogen-related genes, NONEXPRESSOR OF PR1, ET biosynthesis, and antioxidant enzymes POD and APX	*Gossypium hirsutum*	*C. gossypii*, *Fusarium oxysporum*, and *Rhizoctonia solani*	Yu et al. (2012)
11.	*GhWRKY25*	Through co-expression of large number of resistance- and defense-related genes	*Gossypium hirsutum*	*Magnaporthe grisea*, *Blumeria graminis*, *and M. oryzae*	Liu et al. (2015)

12.	*GhWRKY27a*	Through increased RNA expression of pathogen-related genes, NONEXPRESSOR OF PR1, ET biosynthesis, and antioxidant enzymes POD and APX	*Gossypium hirsutum*	*Rhizoctonia solani*	Yan et al. (2015)
13.	*MaWRKY1* and *MaWRKY2*	Upregulation of defense-associated genes	*Poplar tomentosa* Carr	*Dothiorella gregaria* Sacc	Ye et al. (2014)
14.	*MrWRKY30*	Uncoupled to PATHOGENESIS-RELATED (PR) gene expression but strongly associated with oxidative burst and glutathione S-transferase (GST) induction	*Muscadinia rotundifolia*	*Peronospora parasitica*	Jiang et al. (2015)
15.	*OsWRKY22*	Through co-expression of large number of resistance- and defense-related genes	Rice	*Magnaporthe grisea*, *Blumeria graminis*, *and M. oryzae*	Abbruscato et al. (2012)
16.	*OsWRKY45*	By upregulation of defense-related proteins and jasmonic acid signaling pathway	*Oryza sativa*	Brown planthopper (BPH, *Nilaparvata lugens*)	Huangfu et al. (2016)
17.	*OsWRKY53*	Upregulation of defense-related genes	*Oryza sativa*	*Magnaporthe grisea*	Chujo et al. (2007)
18.	*OsWRKY77*	Through enhanced expression of defense-related PR1, PR2, and PR5 genes	*Oryza sativa* L.	*Pseudomonas syringae*	Lan et al. (2013)
19.	*PtoWRKY60*	Upregulation of defense-associated genes	*Poplar tomentosa* Carr	*Dothiorella gregaria* Sacc	Ye et al. (2014)
20.	*PtrWRKY89*	Through enhanced expression of defense-related PR1, PR2, and PR5 genes and downregulating the marker gene of SA and JA pathways	*Populus trichocarpa*	*Pseudomonas syringae*	Jiang et al. (2016)
21.	*SpWRKY1*	Enhanced expression of SA- and JA-associated genes (*NtPR1*, *NtPR2*, *NtPR4*, *NtPR5*, and *NtPDF1.2*), as well as various defense-related genes (*NtPOD*, *NtSOD*, and *NtPAL*)	*Solanum pimpinellifolium* L3708	*Phytophthora nicotianae*	Li et al. (2015)
22.	*VvWRKY1*	Involvement of SA- and JA-/ET-mediated signaling pathway	*Vitis vinifera*	*Downy mildew*, *Plasmopara viticola*	Marchive et al. (2013)

8.3 Structure of DNA-Binding Domain of WRKY Proteins

The first structure of the C-terminal WRKY domains of AtWRKY1 and AtWRKY4 protein of *Arabidopsis thaliana* revealed that the WRKY domain consists of a four-stranded antiparallel β-sheet and zinc-binding pocket formed by the coordination of zinc atom with the conserved two cysteine and histidine residues (Yamasaki et al. 2005). The crystal structure of the C-terminal of WRKY domain of AtWRKY1 consists of five β-strands, with DNA-binding residues located at *β2* and *β3* strands (Duan et al. 2007). The N-terminal region of the β-strand consisting of WRKY signature sequence partly protrudes from one surface of the protein, thereby enabling access to the major DNA groove, and binds to its cognate W-box. Recently, solution structure of WRKY domain with W-box-binding site has been determined, and it revealed that four-stranded β-sheet enters the major groove of the DNA in an atypical mode where the plane of the sheet is nearly perpendicular to the helical axis of DNA (Yamasaki et al. 2012). In the WRKYGQK signature sequence, the tryptophan residue forms the core of the structure, while all the other amino acids (RKYGQK) are directly involved in DNA binding. The glycine residue helps in bending of the strand and thus enables deep penetration into the DNA groove. Recognition of the W-box sequence occurs mainly through the hydrophobic interaction with the methyl groups of thymine (T) bases of the DNA strand. Mutations in the residues involved either in DNA binding or in Zn binding significantly impaired the DNA-binding activity due to the disruption of the tertiary structure, which is important in DNA binding (Duan et al. 2007; Yamasaki et al. 2013).

8.4 Regulation of WRKY TFs

8.4.1 Autoregulation and Cross-Regulation

WRKY proteins are involved in diverse pathways and regulate the expression of downstream target genes either as a positive or negative regulator. The regulation of WRKY-dependent signaling pathways is very extensive and complex. In response to the internal or external stimuli, WRKY TFs trigger the expression of the target genes by binding to their W-box elements in the promoter regions. W-box elements are also present in the promoters of the majority of the WRKY genes, and this suggests that they are regulated via specific feedback mechanisms (autoregulation by themselves or cross-regulation by other WRKY TFs) (Eulgem and Somssich 2007; Rushton et al. 2010). For example, chromatin immunoprecipitation (ChIP) analysis of PcWRKY1 of parsley (*Petroselinum crispum*) revealed that it binds not only to the W-box of its own promoter but also has affinity toward binding the promoters of PcWRKY3 and marker gene PcPR1 (Eulgem et al. 1999; Turck et al. 2004). Likewise, WRKY33 expression is activated by the MAPK3/6, and it autoregulates its expression via a positive feedback loop by binding to its own promoter (Mao et al. 2011). WRKY18, WRKY40, and WRKY60 act as a negative regulator of ABA signaling and could directly bind to the W-box in the promoter region of their

respective genes and thus repress the expression of all three WRKY genes (Chen et al. 2010; Yan et al. 2013). The above finding suggests the importance of autoregulation and cross-regulation of WRKY TFs in maintaining the homeostasis of WRKY protein expression in the cell during abiotic and biotic stress conditions.

8.4.2 Regulation of WRKY TFs by MAP Kinases

Some WRKY TFs are also regulated via MAPK (mitogen-activated protein kinase) pathway (Adachi et al. 2015). WRKY TFs act downstream of various MAPKs to regulate defense-related plant genes (Phukan et al. 2016). The AtMPK3, AtMPK6, and AtMPK4 get activated during both biotic and abiotic stresses (Banerjee and Roychoudhury 2015). Group I WRKY TFs are the first protein, which gets phosphorylated by MAP kinases in response to PAMP-triggered MAPK signaling. Two WRKY proteins AtWRKY22 and AtWRKY29 act downstream of the bacterium flagellin receptor FLS2, are upregulated by a PAMP-induced MAPK cascade, and contain multiple W-boxes within their respective promoters. AtWRKY33, involved in the production of phytoalexin during pathogen attack, forms a complex with MPK4-MKS1 (MPK4 substrate) in the nucleus. Upon infection MPK, MKK (MAP kinase kinase), and MEKK (MAP kinase kinase kinase) are activated. The activated MPK4 phosphorylate MKS1, which lead to the dissociation of the MPK4-MKS1-WRKY33 complex, and AtWRKY33 was released. Then AtWRKY33 binds to the promoter of the target gene PAD3 (phytoalexin deficient 3) that is required for the synthesis of antimicrobial compound camalexin (Qiu et al. 2008). WRKY33 could be phosphorylated by two other MPKs, MPK3 and MPK6, which led to binding to its own and the PAD3 promoters in response to *B. cinerea*. Mao et al. (2011) had shown that in *wrky33* mutant, camalexin production was abolished and mutation in the phosphorylation sites of WRKY33 also had the same effect. Taken together, these results suggested that AtWRKY33 works downstream of the MPK3/MPK6 and phosphorylation of WRKY is important for the production of camalexin upon bacterial infection. In rice, OsWRKY33 is phosphorylated by BWMK1 (blast- and wounding-activated MAP kinase 1) and binds to the promoter of PR genes during salicylic acid-dependent defense responses (Koo et al. 2009). OsWRKY53 suppresses herbivore-induced defense in rice by negative feedback modulation of MPK3/MPK6 activity (Hu et al. 2015). Therefore, phosphorylation and activation of WRKY proteins by MPKs is an important regulatory mechanism which increases the capacity of WRKYs to bind to the promoters of target gene which are involved in the plant defense responses.

8.4.3 Regulation of WRKY TFs via Histone Modification

A few WRKY TFs have been shown to be regulated by histone-modifying complex. *AtWRKY70* gets activated by the *Arabidopsis* homolog of trithorax (ATX1) leading to nucleosomal histone H3K4 trimethylation which results in the activation of

SA-responsive gene PR1 and JA-responsive gene *THI2.1* (*THIONIN2.1*). This finding suggests that *PR1* and *THI2.1* genes are the downstream targets of WRKY70 and regulated epigenetically (Alvarez-Venegas et al. 2007). In response to senescence, H3K4 dimethylation and H3K4 trimethylation by histone methyltransferase occur at 5′ end and coding regions of *AtWRKY53*gene (Ay et al. 2009). Another example of histone modification has been shown in two type III WRKY TFS, *AtWRKY38* and *AtWRKY62*. During bacterial infection, HDA19 (histone deacetylase 19) removes acetyl group from histone tails and represses transcription of *AtWRKY38* and *AtWRKY62* and thus negatively regulates basal defense (Kim et al. 2008). Similarly, methylation at the promoter of *AtWRKY40* inhibits expression of *ABI5* and negatively regulates ABA signaling in seed germination and post-germination growth (Shang et al. 2010). Wang et al. (2012) showed that the protein encoded by chromatin remodeling linker histone H1gene (*MaHIS1*) and MaWRKY1 could interact and regulate physiological processes like fruit ripening and stress responses in banana. MaHIS1 has also been shown to be induced by other factors like JA, ABA, and hydrogen peroxide and under cold stress.

8.4.4 Interaction of WRKY TFs with Other Factors

8.4.4.1 VQ Proteins

It has been reported in the literature that many interacting partners like coactivators regulate the expression of many WRKY TFs. One of the interacting partners is VQ protein, which is a group of cofactors containing a short conserved VQ-related motif (FxxxVQxLTG). The conserved valine and glutamine residues in the conserved motif are important and required for the interaction with the C-terminal domain of WRKY TFs. In *Arabidopsis* and rice, 34 and 40 VQ members were identified, respectively, and shown to be involved in disease resistance and in the plant response to environmental stresses (Cheng et al. 2012; Kim et al. 2013). The first VQ proteins were identified as a MPK4 substrate (MKS) in *Arabidopsi*s by using a yeast two-hybrid assay. The VQ protein MKS has been shown to form complex with AtWRKY25 and AtWRKY33, which are involved in the regulation of plant defense responses (Andreasson et al. 2005). Binding of VQ proteins with WRKY TFs changes the binding affinity of the latter for the nucleotides flanking the conserved W-box. It has been shown that C-terminal domain of AtWRKY33 interacts with two VQ proteins, SIGMA FACTORBINDINGPROTEIN 1 (SIB1) and SIB2, to regulate plant defense response against necrotrophic pathogens Lai et al. (2011). These results demonstrate that VQ proteins are crucial cofactors in regulating WRKY-mediated gene expression (Cheng et al. 2012; Chi et al. 2013).

8.4.4.2 Calmodulin (CaM) Proteins

CaM binds to the conserved Ca^{+2}-dependent calmodulin-binding domain (CaBD) (DxxVxKFKxVISLLxxxR) present in WRKY group II members like *AtWRKY7* (Park et al. 2005). Increasing concentration of calcium triggers the interaction of CaM and WRKY members over WRKY-WRKY interaction (Chi et al. 2013).

8.4.4.3 14-3-3 Proteins

14-3-3 proteins specifically bind to phosphoserine and phosphothreonine and regulate many processes like plant development, plant defense, and stress responses (Roberts 2003; Denison et al. 2011). They function as homo- or heterodimers and each dimer binds two substrates. In *Arabidopsis*, seven WRKY members including WRKY6, WRKY16, WRKY18, WRKY19, WRKY27, WRKY32, and WRKY40 have been identified as putative interacting partners for 14-3-3 proteins by tandem affinity purification tag assay (Chang et al. 2009). 14-3-3 proteins interact and phosphorylate AtWRKY18 and AtWRKY40 to regulate ABA signaling (Shang et al. 2010; Shen et al. 2003). These results suggest that 14-3-3 proteins might have potential roles in regulating biotic and abiotic stress responses via WRKY TFs (Chang et al. 2009; Rushton et al. 2010; Chi et al. 2013).

8.5 WRKY TFs in Defense Response

The plant innate immunity is mainly responsive to two interconnected pathways termed PTI or ETI (Jones and Dangl 2006). PTI is initiated by the recognition of molecular patterns of pathogens and activates MAP kinase cascade pathway and defense-related genes, while ETI is associated with plant disease resistance (R) proteins that activate defense reactions upon specific recognition of pathogen effectors (Chisholm et al. 2006). PTI and ETI activate local as well as long-distance defense reactions like systemic acquired resistance (SAR) (Durrant and Dong 2004; Bostock 2005).

8.5.1 Interaction of WRKY TFs with SA and JA Signaling Pathway

SA and JA are two important signaling molecules in defense response. JA-dependent plant defense pathways are activated by necrotrophic pathogens, whereas SA-dependent defenses are triggered by biotrophic pathogens. JA and SA signaling pathway act antagonistically in regulating defense response (Koornneef and Pieterse 2008). During the past few years, much attention has been focused on TFs involved in the regulation of gene expression upon pathogen challenge. Expression profiling studies have revealed that a large set of the *WRKY TF* gene family members are responsive to pathogen challenge and regulate plant defense responses either as a positive or negative regulator (Eulgem and Somssich 2007). Expression of WRKY genes has been shown to get induced by pathogen infection and pathogen elicitors or by SA treatment in a number of plants (Agarwal et al. 2011). In *Arabidopsis* and rice, more than 75 and 109 WRKY genes have been reported (Shimono et al. 2012). Upon infection, pathogens induce SAR leading to accumulation of SA. Many WRKYs are positively regulated by SA through the receptors NPR1 and its paralogues NPR3 and NPR4 (Wang et al. 2006; Fu et al. 2012; Wu et al. 2012). A few WRKY genes including WRKY18, WRKY38, WRKY53, WRKY54, WRKY58, WRKY59, WRKY66, and WRKY70 bind to the W-box sequences in the promoter

region of NPR1 genes in *Arabidopsis*; this suggests that WRKY genes act upstream of NPR1 genes and involved in the positive regulation of WRKY TFs during pathogen-induced signaling (Wang et al. 2006; Ishihama and Yoshioka 2012). Many WRKY TFs are common component in the SA-/JA-mediated plant defense pathway (Koornneef and Pieterse 2008; Thaler et al. 2012). For example, WRKY70 works at a convergence point for maintaining balance between SA- and JA-mediated signaling pathways as well as also plays a crucial role for R-gene-mediated resistance. Overexpression of AtWRKY70 induces the expression of SA-induced PR genes and acts as a positive transcriptional regulator of SA signaling while for JA-responsive pathways acts as a negative regulator. Overexpression of AtWRKY70 improved resistance to biotrophic pathogen *Erysiphe cichoracearum* and necrotrophic bacteria *Erwinia carotovora* (Ecc) but reduced resistance to fungal necrotroph *Alternaria brassicicola*. Similar dual roles have also been observed for WRKY53. It positively regulates plant defense response during *P. syringae* infection, while its mutant displayed delayed symptom development toward *Ralstonia solanacearum* (Murray et al. 2007; Hu et al. 2008). Moreover, during *P. syringae* infection, WRKY11 and WRKY17 have shown to positively regulate the JA biosynthesis pathway genes, *LOX2* and *AOS*, while negatively regulate the expression of WRKY70 (Li et al. 2004, 2006; Journot-Catalino et al. 2006). AtWRKY53 was reported to positively regulate the basal defense response during *P. syringae* infection while negatively regulate during JA and ethylene signaling pathway (Murray et al. 2007).

Three WRKY TFs of subgroup IIa, WRKY18, WRKY40, and WRKY60, function in a partly redundant way in regulating plant disease resistance. Xu et al. (2006) showed that double mutants *wrky18wrky40* and *wrky18wrky60* and the triple mutant *wrky18wrky40wrky60* were found to be more resistant to *P. syringae* infection but susceptible to *B. cinerea.* In other studies, *Atwrky18/Atwrky40* double mutants showed resistance toward avirulent powdery mildew fungus *Golovinomyces orontii*, and complementation of WRKY40 in this mutant partially restored susceptibility (Pandey et al. 2010). The HvWRKY1 and HvWRKY2 homologs of AtWRKY18 and AtWRKY40 in barley act as a suppressor of PAMP-induced basal defense, leading to resistance against virulent pathogen *B. graminis.* During infection, fungal effector AVR10 is recognized by the resistance protein MLA (mildew resistance locus A) in the cytoplasm followed by interaction of HvWRKY 1 and 2 with activated MLA10 in the nucleus (Shen et al. 2007). In addition, AtWRKY33 is another example and was known to act as a positive regulator of resistance to the necrotrophic pathogens *Botrytis cinerea* and *Alternaria brassicicola*, while overexpression leads to susceptibility to *Pseudomonas syringae* DC3000. However, loss of function mutant of AtWRKY33 showed increased resistance toward *R. solanacearum* (Zheng et al. 2006; Birkenbihl et al. 2012). Similarly, WRKY3 and WRKY4, which are structurally similar proteins, confer resistance to necrotrophic pathogens (Lai et al. 2008).

Few of the WRKY TF members act as negative regulator of defense signaling including AtWRKY7, AtWRKY38, AtWRKY62, and AtWRKY52. AtWRKY11, AtWRKY17, AtWRKY38, and AtWRKY62 negatively regulate basal defense response toward bacterial pathogen *P. syringae.* Interaction of AtWRKY38 and AtWRKY62 with HDA19, a positive regulator of plant basal disease resistance,

leads to inactivation of defense repressing WRKY38 and WRKY62 TFs (Journot-Catalino et al. 2006; Kim et al. 2008). Expression of AtWRKY62 is induced by SA and JA in a NPR1-dependent manner. Loss of function mutant of AtWRKY62 resulted in enhanced expression of JA-response genes, while overexpression of AtWRKY62 inhibited JA-response gene expression (Mao et al. 2007). In other study, overexpression of WRKY62 leads to elevated transcript levels of PR1 gene, whereas in Atwrky62 mutant, PR1 gene is downregulated (Kim et al. 2008). In addition, AtWRKY48 and AtWRKY8 also negatively regulate basal resistance to *P. syringae* (Xing et al. 2008; Chen et al. 2010). Additionally, WRKY8 was also involved in negative regulation of crucifer-infecting tobacco mosaic virus (TMV-cg) (Chen et al. 2013). AtWRKY48 mutants showed increased expression of PR1 genes found to be associated with reduced bacterial growth, whereas *AtWRKY48* overexpressor lines showed the opposite phenotypes. *AtWRKY58* acts downstream ofNPR1, negatively regulating SAR (Wang et al. 2006). Some WRKY proteins exist as chimeric proteins like AtWRKY52 which possesses TIR-NBS-LRR (Toll/interleukin-1 receptor-nucleotide-binding site-leucine-rich repeat) domain in combination with group III-type WRKY domain and mediates R-gene-based resistance toward bacterial wilt *Ralstonia solanacearum*. The physical interaction of *AtWRKY52*/RRS1 with its cognate bacterial effector PopP2 within the plant cell nucleus has been suggested to inactivate the WRKY domain of RRS1 to activate defense mechanisms by derepression (Deslandes et al. 2003). *AtWRKY16* and *AtWRKY19* also contain NBS-LRR domain reported in *Arabidopsis*.

8.5.2 Overexpression/Downregulation of WRKY TFs for Biotic Stress Tolerance

Till date WRKY TFs have been reported from many plant species suggesting its importance in regulating plant defense response. Overexpression of many WRKY TFs from rice like *OsWRKY13*, *OsWRKY31*, *OsWRKY45*, *OsWRKY53*, and *OsWRKY47* showed enhanced resistance to fungal pathogen *Magnaporthe grisea*, the causal agent of the devastating rice blast disease (Ryu et al. 2006; Wei et al. 2013). In rice, OsWRKY13, an ortholog of AtWRKY70, is reported to have similar functions. Overexpression of *OsWRKY13* activates the genes related to SA pathways but reduces the expression of genes in JA pathway (Qiu et al. 2007). Overexpression of *OsWRKY3* led to elevated expression of NPR1, PR1b, phenylalanine ammonia-lyase (ZB8), and peroxidase (POX22.3), suggesting that it works as a transcriptional regulator in SA- or JA-dependent defense signaling pathway (Liu et al. 2005). Overexpressor transgenic lines of OsWRKY53 showed resistance against blast disease and induced the expression of PR proteins and peroxidase enzymes (Chujo et al. 2007). Overexpression of *OsWRKY89* showed more tolerance to the rice blast fungus white-backed planthopper (*Sogatella furcifera*), a rice herbivore (Wang et al. 2007). Lan et al. (2013) showed that overexpression of *OsWRKY77* in *Arabidopsis* led to enhanced resistance toward *P. syringae* suggesting its function as a positive regulator of plant defense. The OsWRKY45 showed improved

resistance to rice blast fungus and might work independent of NPR1-mediated SA signaling (Shimono et al. 2007). In addition OsWRKY45 is found to negatively modulate the resistance of rice to the brown planthopper *Nilaparvata lugens* (Huangfu et al. 2016). Silencing of *NaWRKY3* and *NaWRKY6* in *Nicotiana attenuata* made plants highly susceptible to lepidopteran herbivore (Skibbe et al. 2008). *CaWRKY1* from pepper (*Capsicum annuum*) negatively regulates plant defense, as silencing of this gene led to decreased growth *of Xanthomonas* (Oh et al. 2008), whereas constitutive overexpression of *CaWRKY40* resulted in enhanced resistance toward *Ralstonia solanacearum* (Dang et al. 2013). Shi et al. (2014) suggested that the overexpression of *GhWRKY39* may positively regulate the plant response against bacterial *R. solanacearum* and bacterial pathogen *R. solani.* Transgenic tobacco plants overexpressing *GhWRKY15* displayed more resistance toward viral and fungal infections and showed induced expression of NPR1 gene (Yu et al. 2012). *GhWRKY25* overexpression resulted in enhanced sensitivity to the fungal pathogen *Botrytis cinerea* by reducing the expression of SA or ET signaling-related genes and inducing the expression of genes involved in the JA signaling pathway (Liu et al. 2015). GhWRKY27a-overexpressing plants conferred reduced resistance to *R. solani* infection as demonstrated by severe disease symptoms in transgenic lines (Yan et al. 2015). Additionally, a number of WRKY TFs which are important players of plant immunity have been found in different plant species, for example, VvWRKY1 and VvWRKY2 from grapevine (*Vitis vinifera*), *PtrWRKY89* from *Populus trichocarpa*, and MaWRKY1 and MaWRKY2 from *Musa* spp. (Marchive et al. 2013; Jiang et al. 2014; Shan et al. 2016).

8.6 Conclusion and Future Perspectives

In this chapter, we have focused on the most recent advances on WRKY TFs. Over the last two decades, significant progress has been made in order to understand the role of WRKY TFs. Current information suggests that the WRKY superfamily of TFs is composed of different types of proteins that have been implicated in plant developmental processes and pathogen-induced defense response. New finding illustrates that they participate in regulating a plethora of genes at various levels, by working as positive or negative regulator, by direct activation of downstream target genes, and by activating or repressing other TF genes. WRKY TFs are itself regulated by a highly intricate mechanism in plants, and they are required to maintain normal cellular homeostasis under normal condition. One WRKY protein is found to regulate several plant processes at a time, and the mechanisms of regulation are not yet clear. Extensive study of these TF families is needed for better understanding of the signaling pathways involved in WRKY-mediated regulation of defense and developmental processes. In the future it would be exciting to explore "how WRKY TF networks exert their functions on DNA/chromatin level" which will certainly allow us to open new vistas of diverse metabolic pathways, their cross-linking, and overall cellular physiology of plants under biotic stress conditions.

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Small Noncoding RNA-Based Regulation of Plant Immunity

9

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Abstract

Plant pathogens trigger massive changes in plant gene expression in the host as a result of transcriptional reprogramming. This activates several defense-related pathways such as hormonal imbalances, signal transduction, induction of defense-related proteins, ROS generation, small RNA expression, etc.; small RNA regulates myriad biological processes in several eukaryotes constituting a vital group of gene expression regulators. Among all, plants utilize small noncoding RNA machinery as a crucial means to respond and defend against pathogens by regulating immune-responsive genes. In turn, phytopathogens have evolved various effector molecules such as proteins and recently discovered sRNAs of fungal origin delivered into host cells to suppress plant immunity, to counter-defend the effect of host small RNA machinery. The significance of the small RNA-mediated plant defense response during plant-pathogen interaction

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have been well-established. Here, we discuss findings on noncoding small RNAs (sRNAs) from plants and pathogens, which regulate host immunity and pathogen virulence.

Keywords
Small RNA · Plant immunity · Effector molecules · Phytopathogen · miRNAs

9.1 Introduction

Multicellular organisms (plants and animals) are evolved with various immune systems against invading pathogens (Ausubel 2005). PAMP-triggered immunity (PTI) is the first line of defense mediated by recognition of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) via transmembrane pattern recognition receptors (PRRs). Effector-triggered susceptibility (ETS) is achieved by microbes which provide effector molecules into the host cells to successfully suppress PTI. Consecutively, several plant species have developed a category of immunity activated by resistance (R) proteins that acts in response to pathogen effector proteins and attenuates PTI seizure. The disease resistance developed as a consequence of hypersensitive response (HR) at the site of infection is a type of immunity called effector-triggered immunity (ETI). In this coevolutionary perspective, natural selection compels pathogens and plants to diversify their effector and resistance genes, respectively (Ronald and Beutler 2010). Recently, host-encoded small RNAs are shown to be involved in PTI and ETI (Jin 2008). These tiny regulatory small RNAs act by causing either transcriptional gene silencing (TGS) or posttranscriptional gene silencing (PTGS) to a set of pathogen or host genes (Baulcombe 2004). The sRNA-based TGS is triggered by DNA methylation and histone modifications, while PTGS is mediated by sRNA involved in mRNA cleavage or translational repression (Schramke and Allshire 2004). MicroRNAs (miRNAs), natural antisense transcript-derived small interfering RNAs (nat-siRNAs), trans-acting small interfering RNAs (ta-siRNAs), heterochromatic small interfering RNAs (hc-siRNAs) or repeat-associated small interfering RNAs (ra-siRNAs), and long small interfering RNAs (lsiRNAs) (Vazquez et al. 2010) are the major classes of sRNA reported so far in eukaryotes. The current chapter focuses on the role of sRNAs during plant immunity upon exposure to various phytopathogens.

9.2 sRNA Biogenesis Pathway

sRNAs are 20–40 nucleotide long, noncoding RNAs existing in most of the eukaryotic organisms and control expression of the genes either at transcription or posttranscription level. The biosynthetic mechanism of sRNA generation has been broadly investigated in the model plant *Arabidopsis*. Rising reports have recommended the significance of both forward and reverse genetics for outlining the cellular proteins

that are implicated in the biogenesis and function of small RNAs (miRNAs and siRNAs). The noncoding genomic regions generally harbor the plant miRNAs. This is contradictory to animal miRNAs, which are occasionally processed from introns of protein-coding genes (Baskerville and Bartel 2005). RNA polymerase II transcribes miRNA genes to generate imperfect fold-back structure called primary miRNA (pri-miRNA). The pri-miRNA is processed into a stem-loop precursor miRNA (pre-miRNA) and then diced as a duplex encompassing the mature miRNA and a passenger strand called miRNA. DDL (DAWDLE) protein together with HYL1 (HYPONASTIC LEAVES 1) and zinc finger protein SE (SERRATE) acts upon the pri-miRNA to form pre-miRNA. The DCL1/DCL4 protein further mediates the processing of pre-miRNAs to form miRNA duplex (Fahlgren et al. 2007). miRNA duplex is then 2′O methylated at 3′ end by HEN1 (HUA ENHANCER 1) and is exported to the cytoplasm by an exportin homolog, HST (HASTY) (Fig. 9.1). Processed mature miRNA strand is selectively integrated into AGO-containing RISC complex to cause either cleavage or translational repression of target mRNA (Brodersen et al. 2008).

The major information of the process of posttranscriptional gene silencing (PTGS) was furnished by Boulcombe and Hamilton who identified the degraded RNA products as small RNA species (siRNA) of ~25 nucleotides. siRNAs form and accumulate as double-stranded RNA molecules and were first detected in plants undergoing either co-suppression or virus-induced gene silencing and were also undetectable in control plants. In contrast to miRNAs, siRNAs are derived from perfectly paired double-stranded RNA (dsRNA) precursors. These dsRNA precursors are produced either from antisense transcription or by the action of a cellular RNA-dependent RNA polymerase (RDR). Four different types of siRNAs have been reported in plants till now, viz., natural antisense transcript (NAT)-derived siRNAs (nat-siRNAs), heterochromatic siRNAs (hc-siRNAs) or repeat-associated siRNAs (ra-siRNAs), trans-acting siRNAs (ta-siRNAs), and long siRNAs (lsiRNAs).

9.3 Role of Noncoding sRNA in Bacterial, Viral, and Fungal Pathogenicity

Absence of DCL proteins makes bacterial sRNA different from other eukaryotic sRNAs. Bacterial sRNAs are heterogeneous in length, varying from 50 to 300 nucleotides, and regulate the stability and translation efficiency of target mRNAs through short and imperfect base pairing (10–25). Although in the recent past, several high-throughput RNA-seq studies have identified potential sRNAs in phytopathogenic bacteria such as *Agrobacterium tumefaciens* (Wilms et al. 2012), *Pst* (Filiatrault et al. 2010), *Xanthomonas campestris* (Schmidtke et al. 2012), and *Xanthomonas oryzae* pv. *oryzae* (Liang et al. 2011), however their defined function in pathogenesis is still unclear. Recently, genome-wide transcriptome analysis identified sRNA in *X. campestris* pv. *vesicatoria*, as the causal agent of bacterial spot disease in tomato and pepper. Gene deletion analysis demonstrated that the noncoding sRNAs

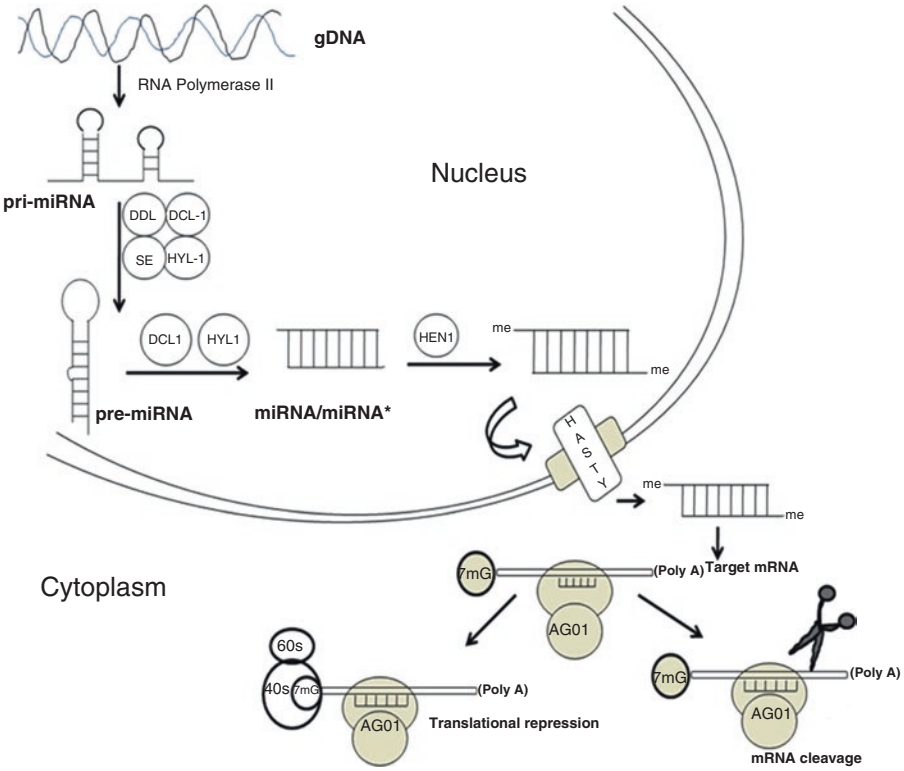

Fig. 9.1 miRNA biogenesis pathway in plants

sX12 and sX13 contribute to virulence (Schmidtke et al. 2013). sX13 promotes synthesis of HrpX and regulates the expression of other proteins putatively involved in motility, signal transduction, posttranscriptional and transcriptional regulation, and virulence. Bacterial noncoding sRNAs operate with RNA-binding protein complexes such as clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) system, regulatory protein Hfq, and CsrA/RsmA RNA-binding protein (Wiedenheft et al. 2012; Karkute et al. 2017). CRISPR-Cas is an exclusive prokaryotic adaptive immune system, similar to eukaryotic RNAi defense (Horvath and Barrangou 2010). CRISPR-Cas system produces small crRNAs to identify target DNA/RNA by short base pairing in the presence of PAM sequence. Hfq is a RNA-binding protein that acts as a global posttranscriptional regulator by binding to bacterial sRNAs to inhibit translation or promote degradation of target mRNAs (Vogel and Luisi 2011). Hfq protein is reported in many bacteria such as *Pectobacterium carotovorum*, *A. tumefaciens*, *P. syringae*, *Ralstonia solanacearum*, and *Xanthomonas spp*. In *A. tumefaciens*, Hfq binds to sRNA AbcR1, which regulates expression of atu2422, an mRNA component of ABC transporter. The *hfq* mutant exhibits overproduction of several other ABC transporter components and shows ectopic phenotypes of delayed growth, reduced

motility, altered cell morphology, and, most significantly, attenuated virulence (Wilms et al. 2012).

In addition, numerous reports have revealed that plant sRNAs are directly involved in bacterial disease responses. Among all, miRNAs were the first to be recognized to contribute in plant immunity during bacterial infections. Plants tested with pathogenic bacteria showed differential changes in miRNA accumulation (Jagadeeswaran et al. 2009). Virus-responsive sRNAs have also been investigated during several plant-virus interactions (Pumplin and Voinnet 2013). Nonetheless, in contradiction to bacterial infections, direct proof for the specific role of endogenous sRNAs in plant antiviral immunity has been incomplete due to interruption of the function and the biogenesis of plant sRNAs by viruses. Similar to a bacterial suppressor that persuaded transcriptional subjugation of miR393, viral infections may also change miRNAs at the transcription level (Table 9.1) (Bazzini et al. 2009). Additionally, other viral proteins may interrupt miRNA (miR156, miR167, miR171, and miR390) accumulation (Feng et al. 2012; Jay et al. 2011). Nevertheless, variations in accumulation of miRNA-derived tasiRNAs have also been linked with phenotypic variations during plant viral infections (Yifhar et al. 2012). Viral infections can alter hc-siRNAs production and modify the RNA-directed DNA methylation (RdDM) pathway which could reactivate transposons and transcription of silenced genes leading to negative plant defense responses (Dowen et al. 2012). In spite of the fact that viruses cause extensive effects on host sRNAs, some specific miRNA families seem to be directly implicated in antiviral immunity. Markedly, numerous host sRNAs are reported to be involved in regulation of host resistance genes.

Flagellin is perceived by *Arabidopsis* which limits *Pseudomonas* invasion, although detailed mechanism is unclear. Gene expression profiling of *flg22*-exposed *Arabidopsis* seedlings leads to build up of three auxin receptors such as TIR1, AFB2, and AFB3, which are targets of miR393 (Navarro et al. 2006). This suggests that miR393 play a role in regulating defense responses during *Pseudomonas invasion*. miR393a-overexpressing lines showed enhanced *P. syringae* pv. *tomato* (Pst) DC3000 resistance (Table 9.1) (Navarro et al. 2006). During infiltration with *Agrobacterium tumefaciens*, increase of miR393 was also observed. Together, these results suggest that miR393a is clearly involved in ETI via auxin signaling. In addition to miR393, miR160 and miR167 were also induced upon nonpathogenic *Pst* DC3000 hrcC inoculation and *flg22* treatment (Li et al. 2010). miR398 which targets a cytochrome c oxidase subunit V (COX5) and two superoxide dismutases (CSD1,CSD2) was shown to be reduced in the plants exposed to avirulent strains such as PstDC3000 avrRpm1 and PstDC3000 avrRpt2 (Jagadeeswaran et al. 2009). Expression of miR398 is downregulated in oxidative stress, promoting accumulation of CSD1 and CSD2 (Sunkar et al. 2006). miR773 is also reported to be involved in PTI which targets the mRNA coding for DNA methyltransferase 2 (DMT2) (Li et al. 2010). During *Agrobacterium* infection, reduced tumor formation results from RNAi-mediated gene silencing of DMT2 and a different DNA methyltransferase (DMT1) (Crane and Gelvin 2007). In tomato and legumes, a group of miRNA families have been reported to be directly involved in ETI by controlling many R genes of the NBS-LRR class (Zhai et al. 2011). In an attempt to discover other sRNAs

Table 9.1 Plant small RNAs involved in immunity

Small RNA	Target(s)	Host(s)	Pathogen(s)	Reference(s)
miR156	Ta3711, Ta7012, TaGAMYB1, and TaGAMYB2	*Triticum aestivum*	Fungus	Xin et al. (2010)
miR158	PPR gene	*Brassica napus* and *B. rapa*	Viruses and fungus	He et al. (2008)
miR159	MYB33, MYB65, and MYC101	*Arabidopsis* and *T. aestivum*	Bacteria and fungus	Zhang et al. (2011a) and Xin et al. (2010)
miR160	ARF10, ARF16, ARF17, ARF16, and a B3 DNA binding, Domain-containing protein	*Arabidopsis*, *M. esculenta*, and *O. sativa*	Bacteria and fungus	Li et al. (2010, 2014) and Pinweha et al. (2015)
miR162	DCL1	*Arabidopsis*	Viruses	Azevedo et al. (2010)
miR164	NAC1	*O. sativa* and *T. aestivum*	Fungus	Li et al. (2013) and Xin et al. (2010)
miR166	Ta3711, Ta7012	*T. aestivum*	Fungus	Xin et al. (2010)
miR167	ARF8, ARF6	*Arabidopsis* and *T. aestivum*	Bacteria and fungus	Fahlgren et al. (2007) and Gupta et al. (2012)
miR168	AGO1	*Arabidopsis*, *N. benthamiana*, and *O. sativa*	Bacteria and viruses	Varallyay et al. (2010) and Wu et al. (2015)
miR169	Nuclear transcription factor Y subunit A-3 (putative)	*O. sativa*	Fungus	Li et al. (2013)
miR171	SCL	*T. aestivum*	Fungus	Gupta et al. (2012)
miR172	MADS box (putative)	*O. sativa*	Fungus	Li et al. (2013)
miR390	TAS3	*Arabidopsis*	Bacteria	Zhang et al. (2011a)
miR393	TIR1, AFB2, AFB3, AFB1	*Arabidopsis* and *T. aestivum*	Bacteria	Navarro et al. (2006)
miR393b	MEMB12	*Arabidopsis* and *N. benthamiana*	Bacteria	Zhang et al. (2011b)
miR396a-5p	GRF	Solanaceae	Fungus	Chen et al. (2015)
miR398	CSD1, CSD2, COX5, SOD1, and SOD2	*Arabidopsis*, *Hordeum vulgare*, and *O. sativa*	Bacteria and fungus	Jagadeeswaran et al. (2009), Li et al. (2010, 2014)

(continued)

Table 9.1 (continued)

Small RNA	Target(s)	Host(s)	Pathogen(s)	Reference(s)
miR399	PHO2	*Citrus*, *Solanum melongena*	Bacteria and fungus	Yang et al. (2013)
miR403	AGO protein genes	*Glycine max*	Fungus	Guo et al. (2011)
miR408	Copper protein	*Arabidopsis* and *T. aestivum*	Bacteria and fungus	Zhang et al. (2011), Feng et al. 2012) and Gupta et al. (2012)
	Plantacyanin			
	Laccase copper			
	Protein and copper			
	Ion-binding protein			
	Genes			
miR444	MADS box	*T. aestivum*	Fungus	Gupta et al. (2012)
miR472	CC-NBS-LRR	*Arabidopsis*	Bacteria	Boccara et al. (2014)
miR482	NBS-LRR	*S. lycopersicum* and *Gossypium hirsutum*	Viruses and fungus	Shivaprasad et al. (2012) and Zhu et al. (2013)
miR773	DMT2, MET2	*Arabidopsis*	Bacteria	Li et al. (2010)
miR825	Remorin, zinc finger, Homeobox family, Frataxin-related	*Arabidopsis*	Bacteria	Fahlgren et al. (2007)
miR827/ miR1138	eIF-4b	*T. aestivum*	Fungus	Gupta et al. (2012)
miR1507	NBS-LRR	*M. truncatula*	–	Zhai et al. (2011)
miR1510	AGO protein genes	*Glycine max*	Fungus	Guo et al. (2011)
miR1535	AGO protein genes	*Glycine max*	Fungus	Guo et al. (2011)
miR1885	TIR-NBS-LRR	*Brassica napus*	Virus	Wroblewski et al. (2007)
miR2001/ miR2005/ miR2006/ miR2008/ miR2011/ miR2012	Unknown	*T. aestivum*	Fungus	Xin et al. (2010)
miR2013	Receptor N	*N. tabacum* and *T. aestivum*	Viruses	Li et al. (2012)
miR2109	NBS-LRR	*Medicago*		Zhai et al. (2011)
miR2118	NBS-LRR	*Medicago*, *S. lycopersicum*, *G. hirsutum*	Viruses	Shivaprasad et al. (2012)
miR5300	Solyc05g008650, tm-2	*S. lycopersicum*	Fungus	Ouyang et al. (2014)
miR6019/ miR6020	TIR-NBS-LRR	*N. tabacum*	Viruses	Li et al. (2012)
miR7695	OsNramp6	*O. sativa*	Fungus	Campo et al. (2013)

(continued)

Table 9.1 (continued)

Small RNA	Target(s)	Host(s)	Pathogen(s)	Reference(s)
miR9863	Mla1	*H.vulgare*	Fungus	Liu et al. (2014)
nat-siRNAATGB2	PPRL	*Arabidopsis*	Bacteria	Katiyar-Agarwal et al. (2006)
AtlsiRNA-1	AtRAP	*Arabidopsis*	Bacteria	Katiyar-Agarwal et al. (2007)
Bc-siR3.1	PRXIIF	*Arabidopsis* and *S. lycopersicum*	Fungus	Weiberg et al. (2013)
Bc-siR3.2	MPK2 and MPK1	*Arabidopsis* and *S. lycopersicum*	Fungus	Weiberg et al. (2013)
Bc-siR5	WAK	*Arabidopsis* and *S. lycopersicum*	Fungus	Weiberg et al. (2013)
TMV vsiRNA	CPSF30, TRAPa	*Arabidopsis*	Viruses	Qi et al. (2009)
Y-Sat siRNA	CHLI	*N. tabacum*	Viruses	Shimura et al. (2011) and Smith et al. (2011)
PC-sRNA8a/ PC-sRNA8b	HSP90	*P. persica*	Viruses	Navarro et al. (2012)
vd39/vd40	CalS11-like and CalS12-like	*S. lycopersicum*	Viruses	Adkar-Purushothama et al. (2015)
vdsiRNA	SolWD40	*S. lycopersicum*	Viruses	Avina-Padilla et al. (2015)

specifically induced in a pathogen response (*P. syringae*), Katiyar-Agarwal and coworkers (2007) identified a class of small RNAs (lsiRNAs). Of the six lsiRNAs identified, five were induced in response to *Pst* (avrRpt2) infection where AtlsiRNA-1 is the most functionally characterized lsiRNA.

In the recent past, more focus has been given to identify and characterize sRNAs in eukaryotic plant pathogens such as fungus involved in pathogenic development and virulence. An inclusive sRNA expression study in *M. oryzae* has identified two class of sRNAs linked with pathogenesis (Nunes et al. 2011). The sRNAs of one class were mapped to transfer RNA (tRNA) loci which are enriched in the appressoria region, while the other classes of sRNAs were mapped to several kinds of genomic. A second study reported that 24 nts is the primary size of sRNAs spotted from *M. oryzae* under physiological stress conditions and *in planta* during infection of rice (Raman et al. 2013). *M. oryzae* sRNAs regulate a subset of mRNAs post-transcriptionally, including an effector gene, ACE1. A hybrid between a polyketide synthase and a nonribosomal peptide synthetase is encoded putatively by ACE1, which probably functions in secondary metabolite production, and it is tightly controlled during the start of appressorial penetration (Fudal et al. 2007). Similarly, sRNAs from the entomopathogenic fungus *Metarhizium anisopliae* (Zhou et al. 2012b) and the white mold fungus *Sclerotinia sclerotiorum* (Zhou et al. 2012a)

showed differential regulation during sclerotia conidiogenesis and assembly, respectively. These results lend support to the belief that fungal endogenous sRNAs play a vital role in regulating virulence and developmental processes of fungal pathogens.

Additionally, plant miRNAs regulate phytohormones homeostasis by regulating the expression of their target transcripts (Sunkar and Zhu 2004). miR408 has been identified as negative controller of plantacyanins and laccase (Abdel-Ghany and Pilon 2008). Although the accurate function of plantacyanins in plants is unidentified, they have been, however, proposed to function in lignin formation, cell-to-cell signaling, and stress responses (Kim et al. 2003). Therefore, differential regulation of miR408 in susceptible and resistant wheat cultivars infected with *Puccinia graminis* f. sp. *tritici* pathotype 62G29-1 might lead to plantacyanin-mediated perturberance of lignin biosynthesis as a result of HR (Table 9.1) (Gupta et al. 2012). Similarly, miR2118 targets TIR-NBS-LRR in cotton infected with *Verticillium dahliae* (Yin et al. 2012), while pbe-SR23 and pbe-SR3 have been predicted to target TIR-LRR in *Populus* upon infection with *Dothiorella gregaria* (Chen et al. 2012). Very recently, Campo and co-workers (2013) have shown Osa-miR7695-mediated negative regulation of natural resistance-associated macrophage protein 6 (OsNramp6) in disease resistance, while illustrating the presence of a novel regulatory network that integrates miRNA function and mRNA processing in plant immunity. Overexpression of Osa-miR7695 in rice has resulted in resistance to blast fungus.

Yin and co-workers (2012) carried out studies on inclusive identification of miRNAs in two cotton cultivars, viz., Yi-11 (*Gossypium hirsutum*, *Verticillium*-sensitive cultivar) and Hai-7124 (*Gossypium barbadense*, *Verticillium*-tolerant cultivar), after infection with *Verticillium* fungus. Among identified miRNAs, three miRNAs, viz., Ptc-miR1444, Ptc-miR1448, and Ptc-miR482, target the cleavage of PPO gene (polyphenol oxidase) and disease resistance protein genes which regulate biotic and abiotic stress resistance in plants (Lu et al. 2008). Chen and co-workers (2012) suggested that there were as many as 74 conserved miRNA belonging to 37 miRNA families and 27 novel miRNAs in *Populus* infected with *D. gregaria*. In contrast to the results obtained in galled loblolly pine stems infected with the fungus *Cronartium* where miR156 were significantly repressed (Lu et al. 2007), pbe-miR156aee was found to be induced upon infection with *D. gregaria* in *Populus*. The distinctive expression pattern of miRNAs in the same family can be linked to different function in different species under different sets of pathogens. Consequently, to develop an improved understanding of the regulatory role of miRNAs on their target genes during pathogen stress, further experimental confirmation of miRNAs is essential. Taking together the previous studies, all the fungi found responsive to miRNAs target several genes simultaneously, and each target gene is involved in controlling numerous physiological and biochemical processes. Therefore, regulation and cross talk of gene expression during pathological development is an actively growing area to develop better understanding of disease pathogenesis. Nevertheless, taking into account the cases uncovered here of siRNAs related to plant immunity during bacterial and viral infections, together with the emergent information generated around sRNAs, more siRNAs could also be involved in regulating viral and bacterial stress responses.

9.4 Pathogen sRNAs Act as Effectors to Suppress Host Immunity

Pathogen effectors are molecules of pathogen origin, which are conveyed into host cells to overpower host immunity. Till now, almost all the studied effectors are protein in nature. Interestingly, a recent study in *Botrytis cinerea* (Bc) pathogen revealed that sRNA could also act as effector. This pathogen is a devastating pathogen having wide host range and can infect 200 or more different plant species. It was observed in *Arabidopsis thaliana* and tomato that during infection the Bc-sRNAs are delivered into host cells where they silence plant immunity genes. In both *Arabidopsis* and tomato hosts, more than 70 Bc-sRNAs have been identified to be probable effectors based on *in planta* expression and target gene predictions, for which three sRNA effectors have been demonstrated experimentally to silence host plant immunity genes by overpowering host RNAi machinery. The successful infection of *B. cinerea* in host plants can be ensured by silencing of host immune genes (Weiberg et al. 2013). The Bc-sRNA effectors share similar features with host sRNAs and are favorably sorted into *Arabidopsis* AGO1 (AtAGO1) protein and thus exploit the host RNAi machinery by loading into host AGO1 to silence host immunity genes. In support of this, the *Arabidopsis* mutant ago1-27 was less susceptible to *B. cinerea*, because the Bc-sRNA effectors were no longer functional in guiding the host gene silencing without the suitable AGO protein (Weiberg et al. 2013). It was the first report of inhibition of host immunity where pathogenic sRNAs act as effectors. Further research will reveal whether this novel sRNA-based virulence pathway exists in other plant eukaryotic pathogens or not. Undeniably, an aggressive fungal pathogen, *Verticillium dahliae*, might have developed a comparable tactic of hijacking the host plant RNAi machinery to repress host immunity. Similar to that observed during *B. cinerea* infection, the *Arabidopsis* ago1-27 mutant was more resistant against *Verticillium* spp., whereas several other *Arabidopsis* RNAi mutants exhibited improved vulnerability (Ellendorff et al. 2009).

9.5 sRNA Biogenesis Proteins in Eukaryotic Plant Pathogens

The key components of the RNAi pathway are the proteins DCL, AGO, and RDR which are encoded by most of the eukaryotic pathogens. Several RNAi-dependent phenomena in *N. crassa* and other fungal species have been studied. Quelling, meiotic silencing of unpaired DNA (MSUD), the silencing of repeat-induced point (RIP) mutation, sex-induced silencing (SIS), DNA repair and homologous recombination, and DNA methylation are examples of RNAi-dependent phenomena. However, it is unclear whether RNAi pathway machinery are directly essential for pathogenicity in eukaryotic plant pathogens. The *M. oryzae* genome encodes three putative AGOs (MoAGO1–MoAGO3), two DCLs (MoDCL1 and MoDCL2), and three RDRs (MoRdRP1–MoRdRP3). MoDCL2 is required for transgene-induced gene silencing and sRNA-directed transposon silencing (Kadotani et al. 2004).

Although there are no reports of any RNAi mutant strains, including *Mordrp*, *Modcl1*, *Modcl2*, and *Modcl1dcl2*, *Modcl2* shows somewhat reduced growth—that illustrates obvious phenotypes associated with pathogenicity (Kadotani et al. 2004). In contrast, obvious defects in sporulation and growth have been observed in the zygomycete fruit rot pathogen *Mucor circinelloides dcl2* and *dcl1* mutants, respectively (Nicolas et al. 2007). An *ago1* mutant of *M. circinelloides* depicts defects in asexual spore production.

Very divergent RNAi pathways are active in fungus; for example, in plants and animals, the miRNA pathways are generally conserved but not in fungi. Instead, at least four different milRNA biogenesis pathways, including both DCL-dependent and DCL-independent pathways, are proposed in *N. crassa*, and miRNA-like RNAs (milRNA) have been identified in fungi (Zhou et al. 2012). It is imperative to deduce the existence of similar sRNA biogenesis pathways in oomycete and fungal pathogens. Moreover, DCLs appear to be functionally superfluous in sRNA processing in the majority of fungus (Weiberg et al. 2013). The probable existence of varied sRNA biogenesis pathways in these organisms represents a challenge for researchers in the dissection of RNAi pathways and characterization of regulatory sRNAs in pathogenicity.

9.6 Conclusion and Future Perspectives

RNAi pathways and sRNAs play a vital role in regulating plant immunity. sRNAs from eukaryotic and bacterial pathogens are also significant regulators of pathogenicity. Since the RNA molecules are known to act as effectors in suppressing host immunity, so it would not be surprising if in plants some of them also serve as PAMPs to trigger PTI. It would be fascinating to examine whether and how extensive noncoding RNAs are in plant pathogens that can activate host innate immune responses or control pathogen virulence in plants. Several reports support the belief that sRNAs can translocate in between organisms, including between the pathogens and host cells, and can even stimulate cross-kingdom RNAi. Hence, this will continue to be a dynamic and pertinent research topic in near future.

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Transcriptomic Studies Revealing Enigma of Plant-Pathogen Interaction

10

Zahoor Ahmed Wani and Nasheeman Ashraf

Abstract

Plants being sessile organisms encounter numerous attacks by pathogens and pests with different lifestyles and modes of attack. In response, plants undergo cellular reprogramming in order to perceive these attacks and activate specific defense pathways. Plants possess extensive regulatory mechanisms which come into play during defense responses so as to coordinate the perception and activation of pathways specific to the type of pathogen in question. Further, many small molecule hormones play pivotal role in defense pathways and cross communicate with each other, thereby helping plant to finely regulate its response. This suggests that plant defense is controlled by intricate transcriptional regulatory network, therefore urging the need to develop genome- and transcriptome-based strategies to unravel these mechanisms. Transcriptomics has fuelled a better understanding of many biological processes and can therefore be used for understanding the host-pathogen interactions as well. Transcriptome analysis can provide more comprehensive picture of the pathways that come into play in response to different pathogens and also decipher the cascade of transcriptional events involved. This may also help in identifying the regulatory nodes in the transcriptional networks and understanding the hierarchical relationship between them. These resources in turn will help in understanding of the complex architecture of plant/host defense system which will have a long-term impact and value for crop improvement.

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10.1 Introduction

Plant-pathogen interactions are battles of attack and counterattack which are fought with highly sophisticated means for the survival of an individual. During this discourse plants respond by dynamic structural rearrangements within and around the attacked cells followed by reprogramming of cellular metabolism. In animals there are two forms of immune responses called as innate and adaptive immunity. Innate immunity forms a first line of defense against invading pathogens and is also a key element for the deployment of adaptive immunity. Plants lack adaptive immune system and depend entirely on innate immunity for defending themselves against pathogen attack.

Plants have developed a two-tier innate immune system to combat the invading pathogenic microbes. The first-tier immune system is designated as PTI (PAMP/pattern-triggered immunity), which is activated upon perception of conserved molecular structures called microbe-/pathogen-associated molecular patterns (MAMPs/PAMPs) by plasma membrane-bound receptors known as plasma membrane-localized pattern recognition receptors (PRRs). For example, PRRs in *Arabidopsis thaliana* are AtFLS2 (FLAGELLIN SENSING 2) and AtEFR (EF-TU receptor) that recognize bacterial flagellin and elongation factor-Tu (EF-Tu), respectively (Zipfel et al. 2006). The pathogen/microbe in turn acquires a number of mechanisms imparting them virulence to suppress the host immune system by activating various effector proteins (Dou and Zhou 2012). To counter this acquired virulence mechanism of pathogen/microbe, the plants have evolved the second tier of the innate immune system known as effector-triggered immunity (ETI). In this system the host/plant recognizes – directly or indirectly – such effector proteins, resulting in initiation of effector-triggered immunity (ETI). During PTI and ETI, plants come up with an array of immune responses such as reactive oxygen species (ROS) generation, cellular Ca^{2+} release, MAP kinase (MAPK) activation, phytohormone production, and reprogramming of transcriptional mechanism, which collectively contribute to immunity. The signaling components are similar in both the immune systems, PTI and ETI, but with distinct activation dynamics and amplitudes (Tsuda and Katagiri 2010; Tsuda and Somssich 2015).

Earlier, knowledge about various cellular processes including pathogen stress was gained by working on individual genes in context of a particular process. But recent developments have shown that cellular processes are controlled by highly connected gene networks. Therefore, the function of an individual gene should be understood in the context of its complex interplay with other gene products (Dittrich et al. 2008). Also existence of cross talk between various processes and pathways has been revealed. For example, many biotic and abiotic stress pathways have been shown to have some overlap. Further, many development-related genes are shown

to play a role in other cellular processes like stress responses (Chung et al. 2008). Therefore, in order to gain a broader understanding of biological processes, we cannot study genes in isolation but in the context of other genes. We have to move beyond the single gene approach and pave way to the study of genome or at least transcriptome of organisms as a whole. This will allow developing a wide picture of gene characteristics. Further, more needs to be achieved in less period of time. Food- and health-related problems are appealing the scientific community to do better and come up with more comprehensive understanding of biology and provide solutions to the problems. Here genomics and transcriptomics come to our rescue as these have the potential to address many such problems.

In recent years transcriptomic studies have changed the whole scenario of our understanding of the molecular approaches/mechanisms of cells and tissues in health and diseases. This provides essential tools to fully understand the molecular basis of various agronomic traits and to manipulate them for human benefits (Harlizius et al. 2004). The field of transcriptomics allows the simultaneous analysis of thousands of genes and their interactive networks to understand the architecture of genomes. Recent technological advances in the field of transcriptomics have seen a paradigm shift enabling the analysis of organisms in terms of genome organization, expression networks, and interaction (Hocquette 2005). This has led to and will further extend substantial and rapid advances in our understanding of the molecular basis of various processes including stresses (Mathers 2004). Such approaches may assist in illuminating the mechanism as it enables the simultaneous discovery and study of many biological processes and genes involved in such processes. Another importance is that it helps to capture the structure-function relationships of genes. Also, the genomic, proteomic, and metabolomic studies in combination will provide the link between the relatively static genome and the highly dynamic physiological processes. In this chapter we will focus on how transcriptomics leads to resource generation and subsequently development of understanding about how plants perceive and respond to pathogen stress. It will also throw light on how transcriptomic studies lead to unraveling the plant signaling and defense pathways.

10.2 Tools and Techniques Used in Transcriptomic Studies

There are various techniques used for the transcriptome-based studies, viz., real-time quantitative PCR (qPCR), northern blot, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), microarrays, bead arrays, and RNA sequencing. The qPCR and northern blot techniques are generally best for analyzing relatively small number of transcripts in a large set of samples; however, the RNA sequencing and microarrays offer genome-wide surveys of the transcriptome. The RNA sequencing technique generates expressed sequence tags (ESTs) from a given RNA sample without prior knowledge of the genes. In RNA sequencing, ESTs are created by sequencing the extreme ends of randomly isolated transcript cDNA, while as in Serial analysis of gene expression (SAGE) and Massive parallel signature sequencing (MPSS), 15-nucleotide tags and 17- to 20-nucleotide

tags are used, respectively. In case of microarrays and bead arrays, mRNAs of interest are hybridized with a large number of probes spotted on a suitable substrate. These can be in the form of rectangular chips for microarrays or beads in fiber-optic bundles in the case of bead arrays (Schena et al. 1995; Kuhn et al. 2004). These strategies are complementary to each other.

The development of EST libraries associated with "differential gene expression" (DGE) technologies is increasing and provides an idea about many biological processes (Marshall 2004). However, to generate ESTs related to a particular biological function like defense-related, development of suppression subtractive hybridization (SSH) technique (Diatchenko et al. 1996) might be helpful. In addition to these, there are various bioinformatics tools for *in silico* analysis of the transcriptomic database available to elucidate the identity and function of genes, promoter analysis of genes, interactome analysis to study the co-expressed networks of genes and transcription factors, etc. to analyze plant development as well as stress response.

10.3 Resource Generation

10.3.1 Gene Inventory and Function

Genome sequencing is a key step which may lead to our understanding of genetic organization and functions of genes. However, the genomes of most plants are quite large making genome sequencing very expensive. In contrast the sequencing of purified mRNA (mRNA-seq) can be used for the identification of genes and for providing information such as gene expression pattern and indications of epigenetic regulation. Thus mRNA-seq datasets can provide information about gene sequences and gene expression as well (Bancroft 2013). Transcriptome, as we define it, represents the complete set of RNAs encoded by a genome of a specific cell or an organism at a specific time or under specific conditions. Thus generation of sequence tags by transcriptomic techniques including next-generation sequencing (NGS) by 454 and Illumina platforms can be used as an effective method of gene discovery. cDNA libraries constructed from various tissues and the sequencing followed by annotation of the genes obtained thereof can lead to the discovery of genes associated with the specific tissues (Wellmer et al. 2006). Also construction of subtracted libraries from plants subjected to various stresses has led to the identification of many stress-related genes. Many studies have been reported where genes associated with perception of pathogen signals and other signaling pathway components were identified using transcriptomic approach.

Over the last few years, the plant EST database generation is observing a surge at an exponential rate, and these EST databases have become a major source of plant sequence data. At present there are more than two million plant-derived ESTs from various species available at public databases. These data have provided a rich resource for gene discovery and annotation (Rudd 2003). Additional information can be obtained from these collections by comparing ESTs from multiple species (Fulton et al. 2002; Vincentz et al. 2004). A large number of databases from

Table 10.1 Major resources/database for plant EST data

Name of database/ organization	URL	References
NCBI dbEST	http://www.ncbi.nlm.nih.gov/dbEST/	Boguski et al. (1993)
NCBI UniGene	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene	Pontius et al. (2003)
PlantGDB	http://www.plantgdb.org	Dong et al. (2004)
Sputnik	http://sputnik.btk.f	Rudd et al. (2003)
TIGR	http://www.tigr.org/tdb/tgi/plant.shtm	Ouyang and Buell (2004)
Genoplante	http://genoplante-info.infobiogen.fr/	Samson et al. (2003)
Gene Expression Omnibus	http://www.ncbi.nlm.nih.gov/geo/	Edgar et al. (2002)
ArrayExpress	http://www.ebi.ac.uk/microarray-as/ae/	Brazma et al. (2003)
Genevestigator	http://www.genevestigator.com/	Zimmermann et al. (2004) and Hruz et al. (2008)
The Botany Array Resource (BAR)	http://bbc.botany.utoronto.ca/	Toufighi et al. (2005)
ATTED-II	http://atted.jp/	Obayashi et al. (2007)
AtGenExpress/PRIME	http://prime.psc.riken.jp/	Akiyama et al. (2008)
PlantTFDB	http://planttfdb.cbi.pku.edu.cn/	Jin et al. (2014)

different sources with different experimental conditions per dataset have been developed in the last couple of years (see Table 10.1).

Recently van Verk et al. (2011) developed gene co-expression networks by using publicly available *Arabidopsis* microarray datasets. They reported that many genes previously reported in literature to be relevant for stress responses fit current models of stress gene regulation. Despite this potential, the resources available for comparative EST analyses in plants remain limited and dispersed. Further studies on different plant-pathogen model systems based on bioinformatics approach to increase the resource database will benefit further characterization of the genes, regulatory factors, and signal transduction pathways involved in plant defense.

10.3.2 Generation of Functional Molecular Markers

The RNA-seq or transcriptome data has helped in the development of molecular markers from the transcribed regions of the genome. Among the important molecular markers that can be developed from transcriptome are single-nucleotide polymorphisms (SNPs) (Rafalski 2002) and simple sequence repeats (SSRs) also called as microsatellites (Varshney et al. 2005). Putative functions of molecular markers can be deduced using homology searches (BLASTX) with protein databases and are therefore known as "functional markers" (FMs) (Gupta and Rustgi 2004). FMs have some advantages over random markers (RMs) because FMs are completely linked

to the desired trait allele. Functional markers can be derived from the gene responsible for the trait of interest and target the functional polymorphism in the gene, thus allowing selection in different genetic backgrounds. These markers will be helpful to identify loci controlling important traits like disease resistance and can be used for the development of improved or disease-resistant cultivars.

Simple sequence repeat (SSR) markers are widely used in plants and have various uses including development of linkage map, quantitative trait loci (QTL) mapping, and marker-assisted selection. They are also used in evolutionary studies (Cavagnaro et al. 2010; Zhu et al. 2012). SSRs are tandem arrays of one to six nucleotides and occur in genomes of all prokaryotes and eukaryotes (Buschiazzo and Gemmell 2006; Kelkar et al. 2008). These tandem arrays have a high rate of mutation, and therefore, the number of repeat units varies, thereby resulting in highly polymorphic SSRs. Isolation of SSRs by traditional methods is a costly and labor-intensive process. With the development of NGS technologies using 454 or Illumina platforms, the sequencing of large portions of plant genomes can be done easily at low costs, thus allowing the rapid development of molecular markers, including microsatellites or SSRs (Ekblom and Galindo 2011). Recent studies have shown the efficient use of NGS technology for discovery of SSR loci in plants. Further, it has an advantage of developing SSR markers associated with functional genes and therefore represent specific phenotypes (Li et al. 2002).

SSRs are widely used in crop breeding programs, as large numbers of molecular markers linked to disease resistance traits are available in most crop species (Miah et al. 2013). For example, SSRs and SNPs also form an important class of functional molecular markers. NGS technology has potential to identify biologically significant SNPs. These techniques identify SNPs from plant populations (different varieties/genotypes) associated with a particular trait like disease resistance. Hence these can be used as toolbox aiding in selection and management of important traits like disease resistance in plant populations.

10.4 Unraveling the Defense Signaling Pathways Using Transcriptomics

The advent of whole transcriptome sequencing techniques presented a new dimension to biological studies and brought a paradigm shift from single gene analysis to whole transcriptome analysis across a wide spectrum of biological systems. Instead of focusing on single gene studies, transcriptomics allows understanding of whole transcriptome changes across a spectrum of biological conditions, resulting in a massive accumulation of gene database. Transcriptomics helps in gaining information about the default and responsive expression states of the individual cells and tissues so as to understand how these states collectively form a functioning organism. Transcriptional profiling has become a component of a biologist's toolbox (Brady et al. 2006). Plants establish multiple interactions with different microorganisms in natural environments; therefore multi-species transcriptomics may lead to the discovery of key plant and microbial genes regulating these multi-species cross

talks. Such transcriptomic studies will be pivotal in plant defense signaling research in elucidating the key mechanisms by which plants respond to microbial infections and the counterresponse of microbes to plant defense signals. Defense responses are regulated by various signaling molecules including phytohormones [salicylic acid (SA), ethylene, jasmonic acid (JA)], nitric oxide (NO), reactive oxygen species (ROS), phytoalexins, etc. which vary as per the type of organism that interacts with the plant. The activation of a specific set of genes resulting in defense response is very much determined by the type of attacker encountered (De Vos et al. 2005; Mur et al. 2006; Ranjan et al. 2014). ROS and NO are important signaling molecules during the hypersensitive response (HR) and work together to bring about localized plant cell death as a defense response. SA has been observed to be involved in defense response against many plant pathogens including viruses, bacteria, biotrophic fungi, and phloem-feeding insects (De Vos et al. 2005; Glazebrook 2005). Ethylene and JA signaling pathways work synergistically against insects, necrotrophic fungi, and bacteria as a defense response (Reymond et al. 2004; De Vos et al. 2005; Bodenhausen and Reymond 2007; Ranjan et al. 2014). Reymond et al. (2004) estimated that 67–84% of *Arabidopsis thaliana*'s transcriptional responses to *Pieris rapae* were JA-mediated. Several of these genes code for proteins involved in anti-insect defenses. However, when *Arabidopsis* was given combined stress of drought and herbivory, the plant defense genes against biotrophic pathogens (e.g., *PR2*, *PR5*, *RLP39*, *RLP41*, *WAK3*) were downregulated (Olivas et al. 2016). This was in conformity with various reports that abiotic stresses have a negative impact on plant defense against pathogens (Suzuki et al. 2014; Ramegowda and Senthil-Kumar 2015). For plants exposed to combined stress imposed by *B. cinerea* and *P. rapae*, there was initial upregulation of *ERF104* and *BAP1* gene. ERF104 encodes a transcription factor that is involved in ET-mediated responses through interaction with MPK6 (Bethke et al. 2009), and BAP1 encodes a negative regulator of plant defenses and is required for growth homeostasis under normal conditions (Yang et al. 2007). Several key regulatory proteins functioning as a molecular switch between SA and JA cross talk have been identified in *Arabidopsis* (Li et al. 2004). Furthermore, PYL4 and PYL5 have been identified as components of the cross talk between the JA and ABA signaling pathways (Lackman et al. 2011). Thus in-depth understanding of the type of response and the genes expressed has been achieved with the help of transcriptomic approaches. Various defense genes involved in different signaling pathways as elucidated by various transcriptomic-based studies are given in Table 10.2.

Further, transcriptomic studies have shown that plants have evolved a powerful regulatory mechanism by cross talk among hormonal signaling pathways and pathways involving metabolism, development, and reactive oxygen species synthesis, to effectively adapt to the complex stress situation (Narusaka et al. 2003; Brady et al. 2006; van Verk et al. 2011; Schenk et al. 2012; Maleck et al. 2000). However, unraveling the complexity of the mechanisms underlying these molecular cross talks and their role in the plant's response to the hostile environment is a challenge in the field of molecular plant-microbe interactions, and transcriptomics hold an important portfolio in this aspect.

Table 10.2 Defense signaling genes involved in various signaling pathways

Signaling pathways	Genes involved	References
MAPK pathway	MAPKKK, MEKK1, MKK4/MKK5, MPK3/MPK6, WRKY22, WRKY29, etc.	Asai et al. (2002)
JA pathway	MYC2, JAZ2, JAZ3, JMT, AOS, WRKY18/53, WRKY54/70, OPR3, etc.	McGrath et al. (2005) and Thines et al. (2007)
ET pathway	ACS2, ACS4, ACS5, ACS6, ACO, ETR1, EIN2, EIN3, EOL1, ETO1, etc.	Chang (2003), Leon-Reyes et al. (2009), and An et al. (2010)
SA pathway	EDS1, PADA, ICS1, PBS3, WRKY28, WRKY46, etc.	Broderson et al. (2006) and van Verk et al. (2011)

10.5 Identification of Promoter Elements and Their Targets

Promoters are the regions on the DNA that regulate the gene expression at the transcriptional level and identification of the promoter elements, and their targets are crucial for improving the understanding of gene regulation. Promoters of protein-encoding genes often contain a "core promoter," which is a region located ~40 bp upstream of the transcriptional initiation site and comprises the TATA box, which is the binding site for the transcription initiation factor TFIID TBP (TATA-box-binding protein) subunit (Molina and Grotewold 2005). Upstream of the core promoter lie the proximal and distal regions of the promoter containing different regulatory sequences such as enhancers, silencers, insulators, and *cis*-acting elements that act as binding sites for the basic transcriptional machinery involved in the initiation and regulation of transcription (Lee and Young 2000; Hernandez-Garcia and Finer 2014). The modulation of gene expression during transcription by proximal promoter elements is straightforward due to their close proximity to the core promoter. The distal promoter elements involve DNA folding mediated by conformational changes in the three-dimensional structure of DNA and chromatin (Hernandez-Garcia and Finer 2014). Numerous genes induced in response to pathogen attack have been identified, and their promoters have also been characterized. These promoters contain specific cis-regulatory elements {W boxes, Box S (GCC-like elements), and D box} which are involved in inducing an anti-pathogen molecular cascade (Rushton et al. 2002). In recent times, many regulatory interactions between transcription factors (TFs) and the promoters of their target genes have been determined by various methods, for example, in vitro (by electrophoretic mobility shift assay and yeast one-hybrid) (Cai et al. 2008) or in vivo (by chromatin immune precipitation) (Aerts et al. 2008; Calo and Wysocka 2013). Recently two pathogen-responsive *cis*-elements, PRE2 and PRE4, were identified from the promoter region of OsWRKY13 in rice. The two *cis*-elements negatively regulate gene expression without pathogen challenge and positively regulate gene expression after pathogen-induced protein binding (Cai et al. 2008). However, it has been observed that a large number of genes in eukaryotes are not regulated by single promoters but multiple alternative promoters. For instance the MAP kinase gene *OsBWMK1* and the *LAGGING GROWTH DEVELOPMENT 1* (LGD1) genes in rice are differentially

Table 10.3 Pathogen-inducible promoters isolated from plants

Promoters	Plant species	References
Defensin promoters	All plants	Kovalchuk et al. (2010)
CaMV 35S	Cauliflower mosaic virus	Odell et al. (1985)
OsPR10a	Rice (*Oryza sativa*)	Hwang et al. (2008)
Germin-like GER4	Barley (*Hordeum vulgare* L.)	Himmelbach et al. (2010)
PPP1	Tobacco (*Nicotiana tabacum*)	Peng et al. (2004)
hsr203J	Tobacco (*Nicotiana tabacum*)	Pontier et al. (1994)
str246C	Tobacco (*Nicotiana tabacum*)	Gough et al. (1995)
gst1	Potato (*Solanum tuberosum*)	Martini et al. (1993)
sgd24	Tobacco (*Nicotiana tabacum*)	Malnoy et al. (2003)
Ypr10	*Malus domestica*	Pühringer et al. (2000)
VpSTS promoter	Chinese wild (*Vitis pseudoreticulata*)	Xu et al. (2010)
PR2	Parsley (*Petroselinum crispum*)	van de Löcht et al. (1990)
PRE2 and PRE4	Rice (*Oryza sativa*)	Cai et al. (2008)
EL117	Parsley (*Petroselinum crispum*)	Kirsch et al. (2001)

expressed due to usage of alternative promoters (Koo et al. 2009; Thangasamy et al. 2012). Synthetic promoters developed by combinatorial engineering of *cis*-elements of the native promoters have been pivotal in signaling and transcriptional activation. Synthetic promoters responsive to pathogen attack demonstrate that defense signaling is largely conserved across species at the promoter level (Rushton et al. 2002). An extensive review on the importance of synthetic promoters reported *in planta* has been recently published by Dey et al. (2015). Similarly minimal promoters containing pathogenesis-related elements (PR1), salicylic acid-responsive elements (SARE), jasmonic acid-responsive elements (JARE), and ethylene-responsive elements (ERE) involved in pathogen stress response were characterized using *Agrobacterium*-mediated transient expression assay (Hernandez-Garcia and Finer 2014).

Defense response promoters have great biotechnological applications in developing transgenic crop plants which show disease resistance. Though constitutive promoters, like CaMV 35S promoter from the cauliflower mosaic virus (Odell et al. 1985), have been commonly used in developing transgenic plants, they can have metabolic cost associated which may eventually impact the traits of interest like yield and biomass. The ideal pathogen-inducible promoters would be rapidly activated by a wide array of pathogens and deactivated under disease-free conditions. So far various pathogen-inducible promoters have been isolated from numerous pathogen-responsive genes in plants as given in Table 10.3.

A large database of plant promoters is available from different sources like PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002), PlantProm (http://mendel.cs.rhul.ac.uk/) (Shahmuradov et al. 2003), PLACE (http://www.dna.affrc.go.jp/PLACE/) (Higo et al. 1998), PlantPAN (http://PlantPAN. mbc. nctu.edu.tw.) (Chang et al. 2008), TRANSFAC (http://transfac.gbf-braunschweig.de) (Wingender et al. 1996), and Eukaryotic Promoter Database (EPD) (http://www.epd.isb-sib.ch) (Perier et al. 1998). However, there is a need to

increase the current toolbox of promoters and cis-elements to gain an understanding of the gene regulation and action in basic and applied studies. Genome-wide transcriptome analyses using high-throughput sequencing technologies will not only lead to better understanding of regulation of gene expression but also to the identification of novel promoters and *cis*-elements in plants.

10.6 Transcription Factors as Drivers of Transcriptional Regulatory Networks

Transcription factors (TFs) and transcriptional regulatory networks play key roles in both development and stress responses by acting as on/off switches for gene transcription. Multiple signaling pathways regulate stress response, and there is significant overlap between the gene expression patterns induced in response to different stresses (van Verk et al. 2011; Jin et al. 2015). TFs act as molecular switches in these multiple signaling pathways and by integrating and rewiring these pathways (Jin et al. 2015). There are almost 1500 genes encoding transcription factors (Czechowski et al. 2004) in *Arabidopsis*, and among these about 350 genes are involved in regulation of these defense-signaling pathways as per the *Arabidopsis* transcriptional regulatory map (ATRM) (Jin et al. 2015). The four prominent families of TFs involved in stress response are ethylene-responsive-element-binding factors (ERF), basic leucine zipper (bZIP) domain, WRKY proteins, and MYB proteins.

ERF proteins belong to subfamily of the APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP). ERF proteins share a conserved 58–59-amino acid domain (the ERF domain) that binds to two similar cis-elements: the GCC box. This box is found in several PR (pathogenesis-related) gene promoters and confers ethylene responsiveness. The ERFs are also involved in dehydration and cold responsive gene expression moderated by the C-repeat (CRT)/dehydration-responsive element (DRE) motif. There are about 124 ERF proteins in *Arabidopsis* (Riechmann et al. 2000). It is reported that octadecanoid-responsive-Catharanthus-APETALA2-domain proteins (ORCAs) form a link between JA and the production of secondary metabolites for defense (van der Fits and Memelink 2000).

bZIPs form a large family of transcription factors in plants, and there are 75 members in *Arabidopsis* (Jakoby et al. 2002). TGA/*octopine synthase (ocs)*-element-binding factor (OBF) proteins (a member of bZIP) bind to the *activation sequence-1 (as-1)/ocs* element and regulate the expression of some stress-responsive genes such as the *PR-1* and *GLUTATHIONE S-TRANSFERASE 6* (*GST6*) genes (Lebel et al. 1998; Chen and Singh 1999). *Arabidopsis* has seven members of the TGA/OBF family, which play roles in different stress responses.

WRKY proteins form a large family with 74 WRKY proteins identified in *Arabidopsis* and 109 in rice, 66 in papaya, 104 in poplar, 68 in sorghum, and 38 in the moss *Physcomitrella patens* (Eulgem et al. 2000; Robatzek and Somssich 2002; Qu and Zhu 2006; Pandey and Somssich 2009). WRKY proteins share a conserved 60-amino acid domain (WRKY domains) containing the amino acid sequence WRKYGQK and a zinc fingerlike motif. WRKY family members show enhanced

expression in response to a range of pathogens, defense signals, and wounding (reviewed in Eulgem et al. 2000). WRKY transcription factors bind to W box [(T)TGAC(C/T)] (Rushton et al. 1996) which forms a major class of cis-acting elements responsible for the pathogen inducibility of many plant genes (Rushton et al. 1996; Wang et al. 1998). It was reported that the expression profiling of *Arabidopsis* WRKY genes revealed that majority of them were differentially regulated in response to SA treatment or infection by a bacterial pathogen (Dong et al. 2003). Recently co-expression analysis showed that the WRKY transcription factors involved in SA-mediated defense signaling and the co-expression network comprises W22/29, W11/25, W18/53, W30/33/40, W18/53, W48, W28/46, and W22, while in JA-mediated defense signaling, the co-expression network comprises W25/33, W54, W46, W11/48, W28, W18/40, and W53/70 (van Verk et al. 2011). The role of WRKY transcription factors in plant defense has been extensively reviewed by Pandey and Somssich (2009). Some WRKY genes involved in biotic stress responses in plants are given in Table 10.2.

MYB TF family is involved in regulating many processes like responses to biotic and abiotic stresses, development, differentiation, metabolism, defense, etc. MYB proteins have a highly conserved MYB DNA-binding domain at N-terminus and 50–53-amino acid repeats encoding three a-helix structures (Lipsick 1996). However, C-terminus is the activation domain and varies significantly between MYB proteins. This variation results in a wide range of regulatory roles of MYB gene family (Jin and Martin 1999; Dubos et al. 2010; Muthamilarasan et al. 2014). Co-expression analysis of *Arabidopsis* transcription factors showed involvement of MYB15 and MYB32 in SA signaling pathway; MYB2, MYB29, and MYB15/95 in JA signaling pathway; and MYB36/38, MYB39/65, MYB43/55, and MYB61 in ET signaling pathway (van Verk et al. 2011). Some MYB genes involved in biotic stress responses in plants reported so far are given in Table 10.4.

10.7 Role of Small RNAs in Plant Immunity

Small RNAs are 20–40-nucleotide-long noncoding RNA molecules, generated by endoribonucleases DICER or DICER-like (DCL), and are present in most eukaryotic organisms. They regulate gene expression at either transcriptional or posttranscriptional level (Katiyar-Agarwal and Jin 2010) in many biological processes including development, metabolism, and biotic and abiotic stress responses (Katiyar-Agarwal and Jin 2010; Westermann et al. 2016). Recently, mobile small RNAs (sRNAs) have been indicated to have a critical role in the regulation of transportation of small regulatory molecules across the cellular boundaries, between the host and its interacting microbial partner in plant-microbe interactions (Westermann et al. 2016). Some of the sRNA and their targets reported so far, derived from the plant and microbial partner (pathogen) under different pathosystems, are given in Table 9.1 (Chap. 9).

Study of regulatory mechanisms involving small RNAs in plant defense is an emerging field, and with the advent of new technologies like high-throughput

Table 10.4 Some WRKY and MYB proteins and their plant defense response in plants

Name of the transcription factors	Plant species	Functions	References
AtWRKY16	*Arabidopsis thaliana*	Increase the expression of PR genes	Chen and Chen (2002)
AtWRKY18	*Arabidopsis thaliana*	Increase the expression of PR genes	Robatzek and Somssich (2002)
AtWRKY70	*Arabidopsis thaliana*	SA- and JA-induced resistance pathways	Li et al. (2004)
AtWRKY38 and AtWRKY62	*Arabidopsis thaliana*	Contribute negatively to basal resistance toward this bacterial pathogen	Kim et al. (2008)
AtWRKY3 and AtWRKY4	*Arabidopsis thaliana*	Induce plant resistance toward necrotrophic pathogens	Lai et al. (2008)
OsWRKY31	*Oryza sativa*	Enhanced resistance to fungal blast	Zhang et al. (2008)
OsWRKY13	*Oryza sativa*	Activates SA-biosynthesis and SA-response genes while suppressing JA signaling	Qiu et al. (2008)
OsWRKY03	*Oryza sativa*	Defense regulation	Liu et al. (2005)
PcWRKY1	*Petroselinum crispum*	Induces the expression of PR10 class gene, PcPR1-1, and represses PcWRKY3	Turck et al. (2004)
OsWRKY13	*Oryza sativa*	Regulates rice resistance to bacterial blight and fungal blast	Wen et al. (2003)
MYB1	*Nicotiana tabaccum*	Plant defense response against TMV	Liu et al. (2004)
TiMYB2R-1	*Thinopyrum intermedium*	Plant defense response against *Gaeumannomyces graminis*	Liu et al. (2013)
AtMYB44	*Arabidopsis thaliana*	Plant defense response against aphid	Liu et al. (2010)
AtMYB060/and AtMYB094	*Arabidopsis thaliana*	Biotic stress response	Cominelli et al. (2005)
MTF1	*Arabidopsis thaliana*	Regulates susceptibility against *Agrobacterium*	Sardesai et al. (2014)
AtMYB30, AtMYB44, AtMYB108/BOSI1	*Arabidopsis thaliana*	Plant defense response	Buscaill and Rivas (2014)
Yellow seed1 (y1)	*Zea mays*	Plant defense response against *Colletotrichum sublineolum*	Ibraheem et al. (2015)
AtMYB108	*Arabidopsis thaliana*	Biotic stress response	Mengiste et al. (2003)
TaPIMP1	*Triticum aestivum*	Plant defense response against *Bipolaris sorokiniana*	Zhang et al. (2012)
AtMYB15, AtMYB34, AtMYB51, and AtMYB75	*Arabidopsis thaliana*	Plant defense response against insect herbivore	Cheong et al. (2002) and Johnson and Dowd (2004)

(continued)

Table 10.4 (continued)

Name of the transcription factors	Plant species	Functions	References
AtMYB102	*Arabidopsis thaliana*	Plant defense response against insect herbivore *Pieris rapae*	De Vos et al. (2006)
OsJAMyb	*Oryza sativa*	Plant defense response against *Magnaporthe oryza*	Cao et al. (2015)
AtMYB96	*Arabidopsis thaliana*	Biotic stress response	Seo and Park (2010)
AtMYB72	*Arabidopsis thaliana*	Induced systemic resistance mediated by beneficial fungi and bacteria	Segarra et al. (2009)

next-generation sequencing, many more endogenous plant sRNA and pathogen-derived sRNAs will be identified in the future. Characterization of these small RNAs and their target genes will help in revealing new dimensions in plant defense signaling pathways and will ultimately lead to the development of effective tools for controlling diseases in plants.

10.8 Conclusion

Transcriptional reprogramming is important in the context of plant defense system, major challenge being to discriminate between gene expression associated with MTI and that orchestrated by effectors (Lewis et al. 2015). It is quite important to understand the responses of plant hosts to microbial infections so as to develop strategies for disease control. Since plant responses are complex, system-level transcriptomic studies will help in understanding these responses (Van Verk et al. 2013; Jin et al. 2015; Lewis et al. 2015). This will lead to understanding of whole transcriptome changes across a spectrum of biological conditions, resulting in development of comprehensive gene database. The latest transcriptomic tools able to determine gene expression pattern would result in development of knowledge-base about the up- or downregulation of genes in response to various stresses (Brady et al. 2006). Co-expression gene networks and transcriptional regulatory networks could be built using bioinformatics approach, therefore adding to this knowledge-base (Van Verk et al. 2011; Jin et al. 2015). Fifty years ago no one could have guessed that we would have the tools to answer these questions. We need to follow global approach well suited for the analysis of plant-pathogen interactions, and in this context integration of genomics, proteomics, and metabolomics with statistics will refine our understanding of how the transcriptome gives rise to biological form and function.

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Proteomic Studies Revealing Enigma of Plant–Pathogen Interaction

11

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Abstract

Pathogen attack is an intricate stimulus that induces stepwise defence response, namely, pathogen recognition, signal transduction and accomplishment of resistance/defense. These steps employ an array of proteins, interacting among themselves to sense the pathogen and produce antimicrobials antagonistic to pathogen growth. In order to gain insights in molecular mechanism of plant–pathogen interaction at the biochemical and cellular level, deciphering the proteins that are involved in this cellular medley is a prerequisite. Proteomics, one of the important subjects of "OMICS" generation, has played a principal role in the identification of these proteins. Proteomics aims at identification and quantification of the proteins mediating a specific cellular process. While the current proteomic studies give valid information about these processes, they also emphasize upon the significance of post-translational modifications. The information on sequence and post-translational modifications of proteins is then used to further decipher the biological processes using bioinformatics, genomics, cell biology, biochemistry and other areas of life sciences. We present a brief overview of the proteomic studies related to host–virus, host–bacteria and host–fungus interaction. We also provide the current stage of information on the techniques applied in proteomics and also the future challenges in this area of biological science.

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11.1 Introduction

All plant species, whether wild or cultivated, are persistently challenged by a variety of phytopathogens. Understanding the plant immune system has been a challenging task for plant biologists. Plants have multiple pathways that function in defence system against pathogens. The recognition of the pathogen and early signalling events are extremely rapid, consistent and specific. Subsequently, signalling cascades comprising of a large number of proteins and signalling pathways are activated which lead to production of antimicrobial compounds (Lodha et al. 2013). Recent results reveal that cellular responses to pathogen attack are dependent on phytohormone signals that are highly regulated and complex. Both positive and negative crosstalk occurs among themselves, in order to regulate appropriate defence pathway. Plants deploy two modes of defence: constitutive and inducible. Inducible defence covers a wide range of molecules, including various chemicals like secondary metabolites, inhibitors of necessary cellular pathways and digestive enzyme inhibitors like protease inhibitors. Although the basic phases in plants during pathogen attack remains to be the same, the response can alter greatly when host and pathogen combinations are different. The exact mechanism and strategies adopted by various plant species to ward off pathogens is not very well understood. However, the complicated mechanisms of plant–pathogen interactions and plant defence are being revealed anew due to considerable progress made in recent years on identifying differentially regulated genes and proteins in pathogen attack. Proteomic studies of plant–pathogen interaction to measure differential expression of proteins and their function in defence have opened up Pandora's box of novel information for plant pathologists.

In the past two decades, proteomics has emerged as a powerful tool for investigation of biological systems. Descriptive proteomics aims at identification of proteins present in an organism under a given condition. Thus structural and functional changes in an organism under different sets of conditions could be explained in terms of differential abundance of proteins under those conditions. Proteomics also studies the post-translational modifications of proteins which fine-tune the functions and structures of many proteins. With advancements in the techniques as well as expansion of our knowledge about protein functions and protein–protein interactions, other subfields of proteomic such as interactomics and secretomics have emerged out to be very crucial and are providing interesting information regarding

protein structure and function (Gonzalez-Fernandez and Jorrin 2010). While interactomics aims to determine protein–protein interactions, secretomics focuses upon only those proteins which are secreted from an organism. This chapter discusses the modern techniques of proteomics and its contributions unravelling the complex mechanism of different plant–pathogen interactions.

11.2 Tools of Proteomics

Proteins are structurally and functionally diverse biomolecules and are of immense importance in biocatalysis. The term proteomics in simplest terms is the study of the "proteome" (Wilkins et al. 1996). The proteome is the total protein of a defined biological space at a given time under specific conditions. Thus, proteomic studies explain the quantity, time, location and purpose of the proteins that are synthesized in an organism. Proteomics is performed by separating the proteins present in a biological specimen on two-dimensional polyacrylamide gels and then identification of differentially expressed spots using Sanger sequencing or mass spectrometry-based sequencing. In recent times it has also been possible to directly separate proteins on high-pressure liquid chromatography. Then protein identification and sequencing may be done by mass spectrometry (Fig. 11.1).

11.2.1 Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

2D-PAGE is a high-impact and popular method for separation of individual protein from a complex mixture. It is based on the two independent properties of proteins, isoelectric point (pI) and molecular size. Initially, proteins are separated on gels on the basis of their isoelectric points. Thus the technique is called as isoelectric focusing (IEF). All proteins have positively and negatively charged amino acids, and at one particular pH value, the net charge on the protein becomes zero. This is called isoelectric point of that protein. In isoelectric focussing, a pH gradient is made across the gel using electric current, and each protein comes to lie at a particular position in the gel where pH is equal to the isoelectric point at which the net charge on protein molecule is zero and it cannot move in the electric field.

The proteins are solubilized generally in highly concentrated urea solution, reducing agents and chaotrophs. Because of variable ion contents among different types of samples, the optimization of IEF buffer and the electrical profile is required for each type of sample. The pH gradient in gel is created using either carrier ampholytes, or alternatively immobilized pH gradient (IPG) gels can be used. Most modern researches use commercial IPGs for highly reproducible results. In the second dimension, protein molecules are separated on the basis of molecular weights (MW). This technique is called SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In order to visualize the separated protein spots, the gel is stained with different stains viz. Coomassie Brilliant Blue (CBB), silver stain or SYPRO

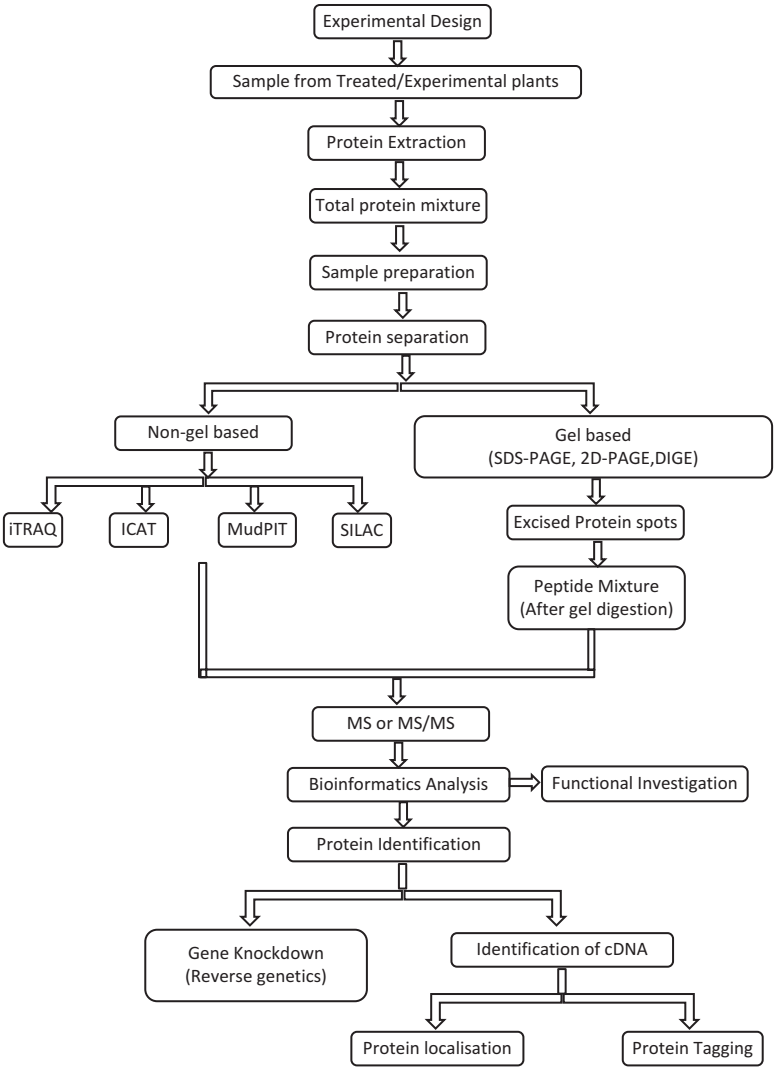

Fig. 11.1 Schematic flow chart to show steps involved in proteomic studies of plant–phytopathogen interaction

stain (Duley and Grover 2001; Nat et al. 2007). Although silver stain is highly sensitive and can detect proteins in the nanogram range, it has few drawbacks also. Silver staining is incompatible with the protein's microchemical preparation and identification by mass spectrometric techniques, staining of proteins is nonstoichiometric, and the assessment of silver staining is highly subjective. Another stain, SYPRO Ruby stain, which is ruthenium complex based is used for luminescent detection of protein molecules. It is reproducible, linear and highly compatible with the protein identification by mass spectrometry (White et al. 2004).

Digital images obtained from 2D-PAGE could be analysed using softwares such as Melanie, PDQuest, Phoretix, Progenesis, Z3 or Z4000 (Righetti et al. 2004). The spots of interest are excised and digested with sequence-specific proteases before subjecting to identification by mass spectrometry (Zhu et al. 2003; Rose et al. 2004). Thus with the help of 2D-PAGE, proteins could be quantified, and their molecular weight, isoelectric focusing point and post-translational modifications (PTMs) could be characterized (Gorg et al. 2004; Wittmann-Liebold et al. 2006). The 2D-PAGE holds the potential in thrust areas of research such as de novo sequencing and protein identification from unsequenced organisms, identification of modified proteins and protein isoforms (Rogowska-Wrzesinska et al. 2013). 2D-PAGE is especially useful for studying protein modifications and to find prognostic or diagnostic biomarkers in various disease states. The protein modifications can be identified on 2D gels by looking for protein spots close to other protein spots with isoelectric point (pI) and molecular weight shift characteristic of a post-translational modification.

11.2.2 Fluorescent Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)

2D DIGE is a versatile and advantageous technique of protein separation. It is an advanced form of 2D PAGE in which two or three protein samples can be compared simultaneously on the same gel. It can be used to study protein regulation between control and experimental samples. In this technique, the proteins in each sample are first covalently tagged using spectrally distinct, fluorescent cyanine dyes (e.g. Cy2, Cy3 and Cy5, etc.). These dyes are designed by matching charge and size to nullify its effect on the relative migration of proteins during electrophoresis. These dyes are pH and photostable and are highly sensitive for detection (Westermeier 2006). Each dye reacts with the N-terminal amino group present in proteins or with the amino group of lysine. After labelling with two different dyes, the two protein mixtures used for comparison are mixed together and run on a single two-dimensional (2D) gel. The dye-labelled samples are then analysed individually by gel scanning at different wavelengths. Thus every protein in one sample superimposes with its differentially labelled but identical counterpart in other sample. Proteins that are common in both samples appear as "spots" with a fixed ratio of fluorescent signals, whereas proteins that differ between the samples differ in fluorescence intensity. Scanning of the gel at two different wavelengths indicates that whether any individual spot is associated with molecule of only one dye rather than two (Unlu et al. 1997). Then various softwares, specifically designed for 2D-DIGE analysis, are used to analyse the resulting images (Marouga et al. 2005). Using these image analysis programmes, volume ratios are generated for each spot. Volume ratios explain the intensity of a particular spot in each test sample, and thus changes in the protein abundance level can be identified and quantified.

The 2D-DIGE technique possesses all advantages of 2D and additionally eliminates gel-to-gel variation, provides high resolution and is more sensitive and

reproducible (Gao 2014). Also the samples can be multiplexed on the same gel thereby reducing the number of gels required and limiting the experimental variations. The 2D-DIGE uses an internal standard; therefore, differences as low as 10% in protein expression can be quantified reliably (McGregor and Dunn 2006). The analysis of digital images of 2D-DIGE significantly improves the statistical assessment of proteome variation.

11.2.3 ICAT (Isotope-Coded Affinity Tags)

Isotope-coded affinity tag (ICAT) is a gel-free technology used for quantitative proteomics. In this technique the proteins present in samples are collected from two different experimental conditions (e.g. pathogen-challenged plant tissue extracts and control) and can be identified and quantified on the basis of chemical labelling agents (Gygi et al. 1999; Nat et al. 2007). The two different samples are first tagged with two different ICAT reagents. ICAT reagents consist of three functional elements: (i) a thiol-reactive group used for the selective labelling of reduced Cys residues, (ii) a biotin affinity tag that allows selective isolation of labelled peptides and (iii) a linker synthesized in either an isotopically normal ("light") or "heavy" form (utilizing ^{2}H or ^{13}C). Linker incorporates the stable isotope tags. Under denaturing conditions, protein disulphide bridges are reduced, and the free sulphhydryl groups of the proteins from the samples are labelled/tagged, respectively, with the isotopically "light" or "heavy" forms of the reagent. The samples are then mixed and cleaved enzymatically to generate peptide fragments. Then isolation of tagged peptides is done using avidin-affinity chromatography, and their analysis is carried out by microcapillary liquid chromatography-electrospray tandem mass spectrometry (Gygi et al. 1999; Nat et al. 2007). Peptides/proteins are identified using MS/MS analyses of all the individual fractions, and then observed MS/MS spectra are searched in protein sequence database. Thereafter the relative abundances of the peptide is obtained by observing the ratio between the signal intensities for the unfragmented isotopically "light" and "heavy" forms of the same peptide. Hence the protein can be identified from which it was derived, in the original samples. In this technique, relative quantities of the protein components present in mixtures can be determined in a single automated operation. This technique gives a measure of changes in protein levels induced by different stress conditions both quantitatively as well as qualitatively. ICAT is widely used to identify proteins associated with centrosomes that accumulate in abnormal ways in cancer cells and tumours, ICAT. This technique eliminates the need for 2-DE; however, major limitations of this technique are (i) selective detection of proteins which have relatively high cysteine content and (ii) difficulties in the detection of acidic proteins (Gygi et al. 2000; Zhou et al. 2002).

11.2.4 ITRAQ (Isobaric Tagged for Relative and Absolute Quantitation)

ITRAQ technology is a variation of ICAT. In ITRAQ proteins can be quantified from different sources in a single experiment (Ross et al. 2004; Agarwal et al. 2006; Zieske 2006; Lund et al. 2007). In this technique, the proteins are differentially labelled with different isotope tags that can be up to four, at the peptide level so that every peptide generated from the digestion of a complex sample is labelled. This method is based on covalent labelling of N-terminus and side chain amine of peptides from protein digestion with isotopic tag of varying mass. The labelled peptides are then separated by nanochromatography and analysed by tandem mass spectrometry (MS/MS). The data obtained after fragmentation is used in database search to identify the labelled peptides and hence the corresponding proteins. The low molecular mass reporter ions also generate from the fragmentations of attached tag. This data is used in quantification of peptides and the proteins from which they originate by using softwares such as i-tracker and jTraqX.

The iTRAQ technology is more advantageous as it includes the ability to multiplex several samples, quantification and simplified analysis with more analytical precision and accuracy (Agarwal et al. 2006; Lund et al. 2007; Zieske 2006). Two to four different samples can be used for comparisons in one MS-based experiment. The iTRAQ labelling strategy is not dependent on cysteine, so it eliminates the limitation of ICAT technology. The iTRAQ potentially covers a large range of the proteome by tagging tryptic peptides, and generally all of them possess primary amine groups (Agarwal et al. 2006; Ross et al. 2004; Zieske 2006).

11.2.5 MudPIT (Multidimensional Protein Identification Technology)

Another alternative to gel electrophoresis is MudPIT, an online 2D ion-exchange/reversed-phase HPLC method. In this technology peptides are separated systematically, depending on charge present on the molecule in the first dimension and on hydrophobicity of the molecule in the second (Veenstra and Smith 2003). The protein samples are first subjected to digestion using sequence-specific enzymes such as trypsin and endoproteinase lysC. Secondly, the mixtures of peptides obtained are separated by two orthogonal separation systems – (i) strong cation exchange (SCX) and (ii) reversed-phase high-performance liquid chromatography (RP-HPLC) (Issaq et al. 2005; Washburn et al. 2001). Peptides from the RP column are then subjected to MS analysis and searched for similarities in the protein databases (Washburn et al. 2001). Using this method, high-complex peptide mixtures can be analysed in a single experiment. Thus MudPIT technique provides a complete list of proteins present in a specific protein sample. It is fast, sensitive and highly reproducible. This method has added advantage of analysis of proteins of all functional and physical classes. Therefore, it is used for identification of protein complexes, cataloguing of proteins in cells and organisms at a large scale, profiling of proteins in membranes and

organelles, determination of post-translational modifications (PTMs), protein ubiquitination in diverse plant species and quantitative analysis of protein expression (Yates et al. 2005, Cantin et al. 2006, Speers and Wu 2007; Maor et al. 2007). MudPIT can catalogue proteins in pathogens such as protozoa, bacteria and viruses if their sequences appear in the databases. Using the MudPIT approach, more than 2000 proteins can be identified in any particular sample (Hernandez et al. 2012). Although MudPIT experiments have several advantages over gel-based methods, it is simultaneously a relatively lengthy process as the number of fractions produced take too much time to analyse by MS using the reverse-phase gradient (Anguraj-Vadivel 2015).

11.2.6 Mass Spectrometry (MS)

MS is a well-accepted analytical tool used to distinguish molecules on the basis of their mass-to-charge ratios (m/z) and thus plays a central role in the field of proteomics (Zhu et al. 2009, 2010). Mass spectrometers consist of an ion source, a mass analyser and a detector. Ion source converts analyte molecules into gas-phase ions, and mass analyser separates ionized analytes on the basis of m/z ratio. Then the number of ions at each m/z value becomes recorded in the detector. In advanced MS analysis, two soft ionization methods, i.e. electrospray ionization (ESI) (Fenn et al. 1989) and matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp 1988; Tanaka et al. 1988) are used. In both ESI and MALDI, ionization of large and nonvolatile analytes, such as proteins and peptides, is done with minimal fragmentation. Thus, biomacromolecules may be analysed with high throughput and much better sensitivity (Feng et al. 2015).

In protein samples, proteins or peptides are first fragmented using enzyme trypsin, and then liquid chromatography is used for separation of fragments obtained. After separation, the samples undergo ionization by ESI or MALDI method on a high-resolution mass spectrometer where peptide masses can be measured to three or four decimal places (exact mass) with high degree of accuracy. Gaseous ions are separated using mass analyser, and mass peaks are registered by the detector. The pattern of mass peaks is then searched against the database using search algorithms to identify similar patterns already reported in other proteins, and depending on the degree of similarity of peptide mass peak pattern, protein sequence and function can be predicted. This technique is called “peptide mass fingerprinting”.

If required, the peptides can also be sequenced de novo, i.e. in any peptide, the sequence of amino acids can be determined without referring to any database. For this, first, a peptide having a specific mass is fragmented by using collision-induced dissociation, and then it is sent through another mass analyser which generates a set of fragment peaks. The amino acid sequences of the peptides are inferred using this exact mass of these peaks, using de novo sequence search algorithms or software. There are two categories of algorithms: database search algorithms and de novo search algorithms. The database algorithms include ProLUCID, SEQUEST, Mascot, PEAKS Protein ID, Phenyx OMSSA, MyriMatch, ByOnic, SIMS, MassWiz, etc.

(Lodha et al. 2013; Xu et al. 2015), while DeNoS, PEAKS, CycloBranch and Novor are de novo search programmes (Lodha et al. 2013; Ma 2015; Novak et al. 2015). Some other softwares used in protein identification are ESI prot 1.0, Medicwave Bioinformatics Suite, ProteoIQ, VIPER and Decon2LS, PatternLab for proteomics, MSight and Spectromania (Lodha et al. 2013).

Mass spectrometry has been much improved by the invention of the time-of-flight mass spectrometry (TOF-MS), which is most commonly used with MALDI and relatively non-destructive method for the conversion of proteins into volatile ions. MALDI is an ionization technique which is used for analyses of biomolecules and large organic molecules based on embedding samples in a matrix from which they are desorbed by laser light. Nowadays this technique has been an indispensable tool to analyse biological samples and has various applications in the field of bio-analysis, diagnostics, drug discovery, environmental analysis etc. In proteomics, the main applications of MS are (i) identification of a protein from its peptide fragments, (ii) determination of protein folding and interactions, (iii) cataloguing protein expression, (iv) identification of sites of protein modification, (v) detection of PTMs in complex biological proteins and (vi) quantification of a given sample protein (Han et al. 2008; Lodha et al. 2013).

11.2.7 Protein Microarray

Protein microarray, also known as a protein chip, is a powerful technology for the study of hundreds or thousands of proteins simultaneously as it possesses the potential for giving fundamental information on proteins, analytes, ligands, receptors, various interactions based on antibody affinity and partners involved in binding. Protein microarray thus permits high-throughput analysis (Romanov et al. 2014). In protein microarrays, antibodies or other affinity reagents, e.g. short peptides, polysaccharides, aptamers, allergens or synthetic small molecules, are first arrayed on a chip surface that may be of glass or plastic or silicon. Then the cell lysate is passed over this surface resulting in the binding of antigens to their cognate antibodies. The bound antigens are screened either using fluorescently tagged or radioactively labelled proteins. Secondary antibodies against each antigen of interest can also be used for screening. There are three types of protein microarrays, (i) analytical, (ii) functional and (iii) reverse-phase microarrays, which are used in biochemical activity studies of proteins. Analytical microarrays are used in protein profiling of a complex mixture. In analytical microarray, binding affinities, specificities and the expression levels of the various proteins present in the mixture can be measured. The most common analytical microarrays are antibody microarrays (Bertone and Snyder 2005).Various interactions such as interaction of protein molecules with protein or DNA or RNA or phospholipid or with some other small molecules can be studied using functional microarray analysis (Hall et al. 2004; Zhu et al. 2001). Reverse-phase protein microarray (RPA) is used to determine the presence of altered proteins that may occur in diseased tissue. RPAs can also be used, specifically, for characterization of post-translational modifications occurred in the tissues as a

result of disease (Speer et al. 2005). The major applications of microarrays are in protein–protein interaction analysis, host–microbe interaction analysis, biomarker identification, biochemical pathway mapping, detection of infectious diseases, drug screening, development of vaccines, enzyme–substrate profiling and immunoprofiling (Zhu et al. 2012; Romanov et al. 2014; Moore et al. 2016). Thus it has become the significant tool for both classical as well as functional proteome analysis (Lodha et al. 2013).

11.2.8 Gel-Based Versus Non-gel-Based Proteomic Tools: Advantages and Limitations

Since two decades of the proteomics era, gel-based proteomics has gained immense importance for various studies pertaining to (i) the proteomic changes during plant growth and development and (ii) analysis of responses to various biotic and abiotic stimuli. The most widely used gel-based technique is 2D gel electrophoresis, in which ~2000 protein spots can be distinguished and processed before the identification by mass spectrometry (Vadivel 2015). Using combination of narrow range pH gels, more than 5000 distinct protein spots can be resolved in 2-D gels (Hoving et al. 2000; Fey and Larsen 2001). These gels provide an exhaustive information about the protein sample analysed by providing quantitative maps of intact proteins. This technique can be used for visualization of small sized sample of proteins (even less than 0.1 ng of protein per spot) (Smith 2009), detection of differential protein expression between two or more biologically relevant conditions and separation of protein isoforms (Rogowska-Wrzesinska et al. 2013). Although gel-based techniques are widely used, still there are some limitations, e.g. insensitivity to low-abundant proteins, inability to characterize the entire proteome in one gel and poor reproducibility. Because any given proteome may be much complex and 2D PAGE techniques have separation limitations, only fraction of the proteome can be analysed (Zhu et al. 2003). Instead, 2D-PAGE provides a map of intact proteins, and changes in protein expression level, isoforms or post-translational modifications can be detected easily. Due to post-translational modifications, isoelectric point (e.g. in phosphorylations) or relative mass (e.g. glycosylation or truncation) may change, and thus mobility of protein molecules may vary on a 2DE gel. Therefore, different isoforms of the same protein may be visualized as different spots on the 2DE gel. Gel-based methods are time-consuming and expensive too. Most of these limitations are addressed by gel-free proteomics techniques.

In proteomics, gel-free technology is more suitable for the analysis of proteins found in less abundance in complex samples. With liquid chromatography (LC) system, proteins and peptides can be separated in complex samples, very efficiently. Multidimensional chromatographic separation has further advantages in separation and identification of peptides. The advanced MS systems considerably improve the protein identification qualitatively as they are more sensitive and give more accurate protein quantitation results. However, gel-free MS-based proteomics requires considerable investment in expensive MS instruments and associated infrastructure and

requires expert personnel to run the facility. Thus both gel and non-gel based of these approaches are of great importance, but no single approach can provide the whole information (qualitative as well as quantitative) of all protein components in any given complex mixture (Abdallah et al. 2012). So both approaches have their own advantages and disadvantages and are complementary to each other. They may be used in parallel so that one can get a more exhaustive information of protein expression and interactions in a specific physiological condition.

11.3 Understanding Plant–Pathogen Interaction in Light of Proteomics

In order to understand the disease resistance mechanism, the specific proteins expressed during plant–pathogen interaction must be identified. Use of tools of genomics, proteomics and high-throughput sensitive instruments has remarkably increased the knowledge of such interactions. Proteomics for studying plant–pathogen interactions started with the pioneering work of Ekramoddoullah and Hunt in 1993. They studied protein profiles of two varieties of *Pinus lambertiana* (sugar-pine) (susceptible or resistant variety to pine blister rust fungus *Cronartium ribicola*). Since then various plant–pathogen interactions have been studied using the proteomic tools. Here we summarize the important plant–virus, plant–bacteria and plant–fungus interaction studies carried out using proteomics.

In a successful plant defence response, plants induce different complex pathways against pathogen to inhibit the growth of pathogen. In general, two types of defence responses are triggered in plants during pathogenic attack, local and systemic (Lodha and Basak 2012; Hammond-Kosack and Jones 2000; Schenk et al. 2009). Local response recognizes the pathogens on the cell surface (Zipfel 2008; Hammond-Kosack and Jones 2000; Schenk et al. 2009; Mur et al. 2008). The second type of recognition pattern is a systematic or long-distance response. It induces defence signals that are not just located locally but spread to distant systemic tissues and is thus called as systemic acquired resistance (SAR). Activation of SAR takes place by salicylic acid (SA)-mediated pathway which subsequently elevates the level of other stress-responding molecules such as ethylene, jasmonic acid (JA), nitric oxide (NO) and pathogenesis-related (PR) proteins (Lodha et al. 2013). The signalling pathways which are activated by different stress factors have been widely documented (Pieterse and Van-Loon 2004). Efforts are on to elucidate this complex defence system by comparative analysis of the proteins present in specific tissues, cells or cellular compartments during control conditions or under phytopathogenic stress.

11.3.1 Plant–Virus Interactions

Plant–virus interaction is one of the most studied biological relationships and is still an open area of research due to lacunae in our understanding of such interactions. Viruses are true parasites as they lack the cellular machinery necessary for

independent survival and replication outside a living cell. Proteomics has emerged as a promising tool to identify different class of proteins involved in plant–virus interactions. Cooper et al. (2003) and Brizard et al. (2006) isolated several virus–host protein complexes using size exclusion chromatography from the rice yellow mottle virus (RYMV)-infected rice plants (*Oryza sativa)*. Further identification of various types of proteins participating in metabolism functioning (e.g. glycolysis, malate and Krebs cycles), plant defence strategy (e.g. peroxidase, superoxide-dismutase) and in protein synthesis (e.g. molecular chaperones, elongation factors) were studied with the help of mass spectrometry.

The plant–virus interactions start with transmission of the viruses into the plant cells through mechanical means which brings a healthy plant in close proximity to a diseased (infected) plant or transmission through an effective vector (highly mobile elements) playing a major role in virus infection, followed by replication. They subsequently travel through plasmodesmata to nearby cells by local movement (cell to cell) and ultimately they circulate, systemically after reaching vascular tissues. This initiates circulation through vascular movement starting from phloem tissues to the sink tissues of the host leading viruses to establish systemic infection with the help of several cycles of replication. Viruses are dependent on plant proteins to carry out mode of infection and also are influenced by the counteraction against the infection emerging from plant host proteins. By means of several transcriptional tools, genes encoding these plant host proteins have been charted out from several plant–virus interactions (Table 11.1) and are extensively studied by researchers in this area. Diaz-Vivancos et al. (2006) reported about the variation in antioxidative system mapping levels of enzymatic activity and protein expression within leaf apoplast of *Prunus persica* cv. GS305 (peach) infected with on plum pox potyvirus (PPV). De-Blasio et al. (2015) studied interaction of host plant potato and its pathogen potato leafroll virus (PLRV) which produces a read-through protein (RTP). They carried out translational read-through of the amber stop codon to better understand the interaction via affinity purification along with quantitative MS (mass spectrometry) to form protein networks for a PLRV mutant (incapable of producing read-through domain (RTD)) in order to compare it with known wild-type PLRV protein interaction network.

As a result, PLRV–plant interactions were classified into four different categories: category I includes plant proteins along with nonstructural viral proteins interacting with assembled coat proteins; category II elaborates about plant proteins in association with both RTD and coat protein; category III includes plant proteins in close association with RTD; and category IV explains those plant protein which show close affinity for virion but lacking RTD.

Several other studies were carried out on plant–virus interaction which demonstrate that photosynthetic electron transport in photosystem II (PSII) was reduced which ultimately affects oxygen-evolving complex (Rahoutei et al. 1999, 2000; Perez-Bueno et al. 2004). This reduction in level of functioning of oxygen-evolving complex leads to large multiplication and accumulation of virus in the plant host. Another proteomic study on interaction of rice yellow mottle virus with its natural host showed expression of proteins involved in maintaining oxidoreduction

Table 11.1 Proteomic studies on plant–virus interaction

Plant species	Virus	Proteomic approach	References
Capsicum annuum	Tobacco mosaic virus	2D-PAGE, MALDI-TOF MS	Lee et al. (2006)
N. tabacum	Pepper mild mottle virus	2D-PAGE, N-terminal sequencing	Perez-Bueno et al. (2004)
Oryza sativa	Rice yellow mottle virus (RYMV)	SDS-PAGE, Nano-LC-MS/MS, 2D-PAGE, MALDI, LC-MS/MS	Ventelon-Debout et al. (2004)
Prunus persica, and *P. serotina*	Plum pox virus	SDS-PAGE, IEF, MALDI-TOF	Diaz et al. (2006)
Vigna mungo	Mungbean yellow mosaic virus	MALDI-TOF TOF-MS	Kundu et al. (2011)
Lycopersicon esculentum	Tomato yellow leaf curl virus	LC-MS/MS, 2D-PAGE, MALDI, LC-MS/MS	Pakkianathan and Murad (2014)
Beta vulgaris	*Beet necrotic yellow vein virus* (BNYVV)	MS/MS spectra	Kimberley et al. (2014)
Arabidopsis thaliana	*Tobacco etch virus (TEV, genus Potyvirus)*	Twin-strep-tag and identification by affinity purification followed by mass spectrometry analysis (AP-MS)	Martinez et al. (2016)
Lycopersicon esculentum	PMMoV-S	2D-PAGE, MALDI	Casado-Vela et al. (2006)
Nicotiana benthamiana	PMMoV-S	2D-PAGE, MALDI	Perez-Bueno et al. (2004)

environment; the generation and detoxification of reactive oxygen species were identified and were presumed to maintain an oxidoreduction environment which favours viral replication (Brizard et al. 2006). Several other categories of differentially expressed proteins which are actively involved in translation, elongation factors, chaperones and chaperonins and proteins involved in proteomic turnover were reported in other plant–virus interaction study (Brizard et al. 2006). Proteomic analysis performed in order to identify proteins involved to study the interaction between *Oryza sativa* (rice) and rice yellow mottle sobemovirus (RYMV) (Delalande et al. 2005) showed expression of a phenylalanine ammonia-lyase, chaperonin-60 (mitochondrial) along with aldolase C; however, their exact role and mechanism of these proteins in defense is still not clear. Recently a proteomic study has identified approximately 16 proteins from the interaction of host tomato fruits (*Lycopersicon esculentum*) with TMV. Most of the proteins involved in interaction were pathogenesis related (PR), and several others were antioxidative enzymes, and their expression probably helped in resistance of plant against viral infection (Casado-Vela et al. 2006).

Although proteomic approaches have identified several different categories of proteins, their probable role and mechanism in plant virus interaction is still

unachieved. Proteomic assay of various cell type involved in viral movement either it be leaf parenchyma or phloem tissue will only resolve this issue.

11.3.2 Plant–Bacterium Interactions

Plants are attacked by several groups of bacteria. Phylogenetic diversity of bacteria, on the basis of their interaction with plants, can be categorized into commensals (acquire nutrients in a manner the host remains unaffected), mutualists (helpful in plant growth) and pathogenic (acquire nutrients and plant health is negatively affected) (Newton et al. 2010). All three classes of bacteria have developed exceptionally modified physiology to interact with plants that reflects their requirement (Martin et al. 2003; Boller and Felix 2009). Environmental stresses result in changes in patterns of structural as well as transporter proteins, toxins and enzymes of bacteria, thus enabling them to adapt to the changed environment (Boller and Felix 2009). The cell wall degradation in plants aggravates plant defense strategy as signals received through cell surface proteins, polysaccharides, lipopolysaccharides and degradative enzymes after infection (Newton et al. 2010). Proteins of plant-associated bacteria (PAB) are studied in plants, in the form of bacterial responses to active biomolecules or natural plant extracts, or secretome analysis in order to study the virulence (vir) factor of the pathogenic bacteria (Guerreiro et al. 1997; Corbett et al. 2005; Gourion et al. 2006; Chung et al. 2007). As per Mehta and Rosato (2001), differentially expressed proteins, including a sulphate-binding protein, were studied by NH_2 terminal sequencing when *Xanthomonas axonopodis* pv. *citri* was cultivated in the host *Citrus sinensis.* Similar studies helped in identification of differentially expressed proteins, including a sulphate-binding protein, by NH_2 terminal sequencing (Table 11.2). Jones et al. 2004, analysed the proteomic and transcriptomic profiles of*Arabidopsis thaliana* during early response to *Pseudomonas syringae pv. Tomato* strain DC3000. Their experiments suggested that bacterial challenge generally induce the enzymes peroxiredoxins (PrxA, B and IIE) and the antioxidants glutathione S-transferases (GSTs F2, F6, F7 and F8) which potentially lead to specific post-translational modifications. They also concluded that individual members of these families may be specifically modified depending upon the degree of virulence of the DC3000 strain and outcome of the interaction. Similar studies were carried out in *Medicago truncatula* (model legume) in order to detect its response to pathogenic bacterium *Pseudomonas aeruginosa*. This proteomic analysis revealed significant changes in expression of 154 proteins, out of which 21 are related to defense and stress responses. Genome-based identification of types of proteins and effective toxins which are directly related with plants are referred as effectors and are quite significant to researchers in this area (Hogenhout et al. 2009).

In innate immune response, membrane proteins known as pattern recognition receptors (PRRs) are capable to identify pathogen-associated molecular patterns (PAMPs) of the invading pathogens (Gomez-Gomez and Boller 2000). As a result of PAMP recognition, there is activation of systemic acquired resistance (SAR), and this leads to production of resistance (R) proteins which results in effector-triggered

Table 11.2 Proteomic studies on plant–bacteria interaction

Plant species	Bacterial pathogen	Proteomic approach	References
Arabidopsis thaliana	*Pseudomonas syringae*	2D-PAGE, MALDI-TOF MS/MS	Jones et al. (2004), (2006)
Oryza sativa	*Xanthomonas oryzae* pv. *Oryzae*	2D-DIGE, MALDI-TOF MS	Mahmood et al. (2006) and Chen et al. (2007)
Oryzae sativa	*Xanthomonas oryzae*	2D-PAGE/MudPIT and MALDI-TOF/MS or nESI-LC-MS/MS	Wang et al. (2013)
Lycopersicon hirsutum	*Clavibacter michiganensis* ssp. *michiganensis*	2D-PAGE, MALDI-TOF MS/MS	Coaker et al. (2004)
Medicago truncatula	*Streptomyces meliloti*	2D-PAGE, MALDI-TOF MS/MS	Mathesius et al. (2003)
Brassica oleracea	*Xanthomonas campestris* pv. *Campestris*/parasite)	2D-PAGE, MALDI-TOF MS, MS/MS	Andrade et al. (2008)
Glycine max	*Bradyrhizobium japonicum*/symbiont	2D-PAGE,LCMS/MS, LTQ-Orbitrap MS	Delmotte et al. (2010)
A. thaliana	*Methylobacterium extorquens*/epiphyte	2D-PAGE, LC-MS/MS, MALDI-QqTOF MS/MS	Gourion et al. (2006)
Pisum sativum and *Vicia cracca*	*Rhizobium leguminosarum Biovar viciae* (symbiont)	2D-PAGE, microarray	Karunakaran et al. (2009)
Chrysanthemum	*Erwinia chrysanthemi* (soft rot)	2D-PAGE, MALDI-TOF-MS, LCQ ion trap MS	Kazemi-Pour et al. (2004)
Potato	*Pectobacterium atrosepticum*	2D-PAGE, MALDI-TOF-MS	Mattinen et al. (2007)
Medicago truncatula	*Streptomyces meliloti* strain	LC-MS/MS	Larrainzar et al. (2007)
Rice (var. Co43)	*P. fluorescens* KH- 1	2-DE, MS, LC-MS/MS	Kandasamy et al. (2009)

immunity (ETI) followed by hypersensitive response (HR) and leading to programmed cell death (Jones and Dangl 2006). Many reference data sets of proteomics have been established which identifies proteins for various PAB, via tools as two-dimensional gel electrophoresis (2-DGE) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques which identify proteins for various PAB (both pathogenic and mutualist) (Rosen et al. 2004; Chung et al. 2007; Anderson et al. 2006; Bosch et al. 2008). Basal defense system in the form of PAMPs and plant responses are induced, and such responses are in the form of flagellin protein, cold shock proteins and elongation factor (EFTu), chaperones and chaperonins (Newton et al. 2010; Rosen et al. 2004; Jacobs et al. 2012). A broad study of transcriptomics elaborates about pathogenic bacteria involved in pathogenicity and hypersensitivity and shows different secretion systems for colonization leading to

host cell death (Buttner and Bonas 2010). Proteomics is important to understand the reaction mechanism behind pathogenesis as well as symbiosis (mutualism). This will further open up new areas of research in protein-based plant–microbe crosstalk.

11.3.3 Plant–Fungus Interactions

Proteomics has been extensively used to understand and characterize the key elements involved in plant–fungus interactions. In recent past, quite a few studies have been performed, which describe proteomic studies of fungi-infected plants. The main focus of such studies had been to analyse, identify and quantify the proteins present in the host plant during successful or unsuccessful resistance to fungal pathogen. In the recent years, a wide variety of fungal proteins involved in successful pathogenesis have also been explored (Gonzalez-Fernandez and Jorrin-Novo 2010, 2012).

The pathogenic fungal/host plant protein after separation in gels can be identified using bioinformatics tools. Since availability of high-quality genome data is limited to only those organisms which have been sequenced so far, the proteomic analysis and identification of proteins are also limited to these plants. A list of important plant–fungus studies using proteomics is enlisted in Table 11.3.

The pathogenic fungi adopt different strategies for infecting the host plant. The necrotrophic fungi kill the host tissue and thereafter obtain nutrients from necrotic host cells. The biotrophic fungi colonize the living host cells either intracellularly or intercellularly and obtain nutrients from living host tissues. Hemibiotrophic fungi exhibit both biotrophy and necrotrophy in a two-phasic manner (Lo Presti et al. 2015). Due to difference in mode of infection, different types of proteins are involved in different plant–fungus interactions. Therefore, it is imperative to study individual interactions separately. The host plant in response to the invading pathogenic fungi elicits a host defense response. Both local as well as systemic immunity could be triggered by the host plant (Schwessinger and Ronald 2012). The plants show a two-tier innate immune response involving the localized plasma membrane proteins as well as the intracellular receptors (Lo Presti 2006; Dodds and Rathjen 2010; Asai and Shirasu 2015). During pathogenesis, intracellular proteins and the secreted proteins of fungi are either upregulated or downregulated, thereby enabling the fungi for pathogenesis. Considering this, numerous proteomic efforts have been done to identify and characterize these proteins. Bohmer et al. (2007) carried out pioneering research to create a proteome map from mycelia of phytopathogenic fungus *Ustilago maydis*, during its morphological transition from bud stage to filamentous stage. Greenville-Briggs et al. (2005) performed a parallel study of transcriptome and proteome of *Phytophthora infestans* so as to identify proteins/genes upregulated during appressorium formation in the germinating cysts of the fungus. Five different protein-expressing genes were identified which are methionine synthase (*Pi-met1*), a ketol-acid reductoisomerase (*Pi-kari1*), a tryptophan synthase (*Pi-trp1*), an acetolactate synthase (*Pi-als1*) and a threonine synthase (*Pi-ts1*). The

Table 11.3 Proteomic studies on plant–fungal pathogen interaction

Plant species	Fungal pathogen	Proteomic approach	References
Zea mays	*F. verticillioides*	2D-PAGE, MALDI-TOF-MS, LCQ ion trap MS	Campo et al. (2004)
O. sativa	*Rhizoctonia solani*	2D-PAGE, ESI Q-TOF MS, MS/MS	Lee et al. (2006)
Triticum aestivum	*Puccinia triticina*	2D-PAGE, MALDI-QqTOF MS/MS	Rampitsch et al. (2006)
A. thaliana	*Plasmodiophora brassicae*	2D-PAGE, MALDI-TOF-MS	Devos et al. (2006)
A. thaliana	*Fusarium elicitor*	2D-DIGE, MALDI-TOF MS	Chivasa et al. 2006
Triticum aestivum	*Fusarium graminearum*	2D-PAGE, LC-MS/MS	Zhou et al. (2006)
Brassica napus	*Sclerotinia sclerotiorum*	2D-PAGE, ESI-q-TOF MS/MS	Liang et al. (2008)
Fagus sylvatica	*Phytophthora citricola*	SDS-PAGE, 2D-PAGE, ESI, LC-ESI	Valcu et al. (2009)
Arabidopsis thaliana	*Alternaria brassicicola*	2D-PAGE, LC-MS/MS	Mukherjee et al. (2010)
Humulus lupulus	*Verticillium albo-atrum*	2D-PAGE, de novo sequencing	Mandelc et al. (2013)
Triticum aestivum	*Septoria tritici*	HPLC, MS/MS	Yang et al. (2013)
Triticum aestivum	*Rhizoctonia solani*	Nano-LC-MS/HPLC	Anderson et al. (2016)
Humulus lupulus	*Verticillium* nonalfalfae	LC-MS/MS	Flajsman et al. (2016)
Triticum aestivum	*Puccinia striiformis* f. sp. *tritici*	Nano-LC-ESI-MS/MS	Demirci et al. (2016)
Solanum tuberosum	*Phytophthora infestans*	LC-MS/MS	Larsen et al. (2016)
A. thaliana	*PGPR Paenibacillus Polymyxa*	2D-PAGE, LC-MS/MS	Kwon et al. (2016)

proteomes of both the pathogen and its host plant potato were evaluated during disease development.

Rampitsch et al. (2006) compared the proteome of a susceptible line of wheat which was infected with leaf rust to mock-inoculated wheat using 2DE followed by MS analysis. They observed 22 differently expressed proteins which included proteins with known and hypothetical functions.

Another approach is the study of fungal proteins for the analysis of exoproteome, also called secretome. In this context, Phalip et al. (2005) identified 84 fungal secreted proteins. They grew *Fusarium graminearum*, on hop (*Humulus lupulus*), and analysed the secretome using 2D-PAGE/MS. Some of the fungal proteins involved in plant–pathogen interaction are methionine synthase and threonine synthase (*Phytophthora infestans*/*Solanum tuberosum*); chitinase, serine proteinase, leucine aminopeptidase, lipase, pectate lyase, α-arabinofuranosidase, ceramidase,

chitin deacetylase, β-glucosidase, polygalacturonidase, trypsin, aspartyl proteinase, xyloglucanase, carboxypeptidase and α-amylase (*Fusarium graminearum*/*Humulus lupulus*); and mucin, transglutaminase and glucanase (*Phytophthora ramorum*/Oak) (Grenville-Briggs et al. 2005; Phalip et al. 2005; Meijer et al. 2006). Yajima and Kav (2006) cultured *Sclerotinia sclerotiorum* and identified four different fungal proteins viz. exopolygalacturonase, cellobiohydrolase-1-catalytic domain, acid protease and aspartyl proteinase using proteomic tools. In these studies, various proteins involved in defense, and stress responses, signal transduction, photosynthesis, electron transport and metabolism, have been identified.

Among the many proteins identified in plants during plant–fungus interactions are different classes of pathogenesis-related (PR) proteins such as chitinase, β-1,3 glucanase, peroxidases and thaumatin-like protein (*Oryza sativa*, *Triticum aestivum* and *Lycopersicon esculentum*). The PR proteins β-1,3 glucanase and peroxidases have also been identified in *Zea mays* and *Arabidopsis thaliana*, respectively. Several other proteins reported from plants are glutathione S-transferase, glyceraldehyde 3-phosphate dehydrogenase, fructose-bisphosphate aldolase, probenazole-induced protein, adenosine kinase, superoxide dismutase (SOD), glutamate dehydrogenase, thioredoxin, 20S proteasome β unit, chaperonin 60 β precursor, disease-resistance-response protein pi 49, receptor-like protein kinase, 14-3-3-like protein, etc. (Mehta et al. 2008).

Babich and Katam (2016) investigated molecular changes in the grape leaf during the process of berry development and tolerance to anthracnose. Proteins extracted from leaf of different cultivars were separated using 2D-PAGE and characterized using MS and later compared with *Vitis* database. They observed a total of 56 differentially expressed proteins, which are known to be involved in the processes of pathogen response, photosynthesis and metabolism. The tolerant grape cultivars showed more abundant proteins in comparison to susceptible cultivars.

Flajsman et al. (2016) did pioneering research using proteomic tools to identify proteins secreted in xylem sap following infection with *Verticillium nonalfalfae* spp. VnaPRX1.1277 of hop plants (*Humulus lupulus*). Three fungal proteins of *V. nonalfalfae* were found to be present in abundance, α-N-arabinofuranosidase (VnaAbf4.216), peroxidase (VnaPRX1.1277) and a hypothetical protein (VnaSSP4.2). These three proteins are the first ever secreted proteins which have been identified in xylem sap upon infection with *Verticillium* spp. They also reported that in planta expression of the two protein genes VnaPRX1.1277 and VnaSSP4.2 increase with the progress in colonization, thereby establishing their significance in fungal virulence. Also the deletion mutants of *V. nonalfalfae* for these two genes showed compromised pathogenicity, and hence it is imperative to consider VnaPRX1.1277 and VnaSSP4.2 as virulence factors necessary for colonization of hop plants by *V. nonalfalfae*.

During the last 10 years, comparative molecular profiles of compatible and incompatible plant–pathogen interactions have been studied. Demirci et al. (2016) analysed proteome profiles of interacting wheat and *Puccinia striiformis* f. sp. *tritici* (Pst). Proteins were isolated from infected and control samples and separated using 2D-LC system. The proteome of the two samples were compared. The differentially

expressed proteins were excised, eluted and identified with the help of nano-LC-ESI-MS/MS. Out of the total differentially expressed proteins identified, 62% belonged to the wheat database and 38% were Pst proteins. The subcellular localization and signal peptide motifs of these identified proteins were ascertained with the help of bioinformatics tools. All the identified wheat proteins were categorized in seven functional groups, viz. defense, stress responsive, gene expression, signal transduction, metabolism, electron transport and photosynthesis. Most of the identified proteins belonged to defense and stress-responsive groups. The defense group proteins included the antioxidant and detoxification proteins as dehydroascorbate reductase (DHAR), glutathione S-transferase (GST), superoxide dismutases (SODs), ascorbate peroxidase (APX), catalase (CAT), peroxidases (PX), peroxiredoxins (PRX), etc. About 64 proteins were identified from the pathogen *Puccinia striiformis* f. sp. *tritici.* Out of these, 30 proteins were categorized in five different functional groups, namely, structure, attack, metabolism, gene expression and signal transduction. No function could be assigned to the remaining 34 proteins, and thus they were considered hypothetical.

Larsen et al. (2016) established label-free proteomics to study the pathogenic interaction of *P. infestans* with three cultivars (Kuras, Bintje and Sarpo Mira) of potato plant. About 3248–3529 unique proteins were detected and identified from each cultivar of the potato plant. From the pathogen (*P. infestans)*, nearly 758 proteins were identified. The data set detailing the information about these proteins is available via ProteomeXchange, with the identifier PXD002767.

11.4 Apoplast Proteomics: An Emerging Field to Uncover Plant Pathogen Crosstalk

The extracellular matrix of the plant cell is called apoplast. The apoplastic fluid circulates in the intercellular spaces. The cell wall, the apoplast, the apoplastic fluid and the extracellular space outside the plasma membrane are the first compartmental venue in the plant body, which faces the pathogen challenge (Agrawal et al. 2010). It potentially perceives and transduces signals from the external environment to the symplast. The apoplastic space is the first physiological compartment for the interaction of plant and the pathogen, and the key processes that occur there determine whether a successful parasitism will be established or not (Doehlemann and Hemetsberger 2013). The apoplastic fluid constituents are nutrients, polysaccharides, secondary metabolites and secreted proteins. It facilitates intercellular communications and has significant role in regulation of growth, biotic and abiotic stress responses and cell wall maintenance (Ellis et al. 2007; Tseng et al. 2009). The mechanism of reactive oxygen species (ROS) accumulation and altered synthesis of extracellular protein takes place in apoplast as the first line of defense. A fine regulation of expression of apoplastic proteins is necessary for pathogen perception and for maintaining integrity of cell wall (Pechanova et al. 2010).

In the recent few years, proteomic tools have been harnessed to study the apoplast during pathogenesis process. Yang et al. (2015) reported the results of a survey

of leaf apoplastic proteome in the resistant and susceptible varieties of wheat in response to the pathogen *Zymoseptoria tritici*. This pathogen inhibits the apoplast of the wheat plant and is responsible for causing *Septoria tritici* blotch (STB) on the foliage. They demonstrated that resistance of plants to *Z. tritici* has correlation with responses at proteome level.

They concluded that factors such as carbohydrate metabolism, reinforcement of cell wall, and synthesis of PR proteins in apoplast are linked with disease resistance. The pathogen has to overcome all these armours of host defense responses in order to achieve successful colonization.

Delaunois et al. (2014) provided an insight to highlight the key molecules involved in plant–pathogen interaction using proteomic tools to study the apoplast. The apoplastic proteome under biotic stress still remains poorly characterized. Thus the future studies should aim at identification of apoplastic proteins during pathogen infection so that the mechanism of perception of pathogen stressors and regulation of stress could be understood.

Currently, apoplast proteomics is an emerging topic of research among scientists studying the complex phenomenon of plant–pathogen interaction. More light will be thrown upon this intricate relationship by studying the apoplast proteome in the coming years.

11.5 Conclusion

In recent years, the various gel- and non-gel-based protein separation methods coupled with advanced spectrometry techniques have evolved as the prominent tools for protein identification and characterization.

As the proteomic tools have evolved and become more sensitive, the number of proteins that can be observed, identified and characterized has increased. The high-volume protein information, when synergized with high-throughput genomic information, provides a window for protein complement to be characterized in silico. Thus the modern bioinformatics increase the efficacy of the proteomic studies immensely.

Numerous proteins have been identified so far both from host and the pathogen, and their specific roles are being explored. Since proteomics provides a rapid insight into the plant–pathogen intricacies, it is expected to be one of the imminent and integrative tools in biological research.

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Role of Pathogenesis-Related (PR) Proteins in Plant Defense Mechanism

12

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Abstract

Plant growth and development is often challenged by several abiotic and biotic stresses, such as drought, cold, salinity, wounding, heavy metals, and pathogen attacks, respectively. A plant responds to these threats by activating a cascade of genes, encoding different effectors, receptors, and signaling and protective molecules. Among all, the induction and accumulation of pathogenesis-related (PR) proteins in plants in response to these adverse conditions is very important as PR proteins are an indispensible component of innate immune responses in plants under biotic or abiotic stress conditions. The PR proteins protect the plants from further infection by not only accumulating locally in the infected and surrounding tissues but also in remote uninfected tissues. Induction of PRs has been reported from many plant species belonging to different families suggesting a general role for these proteins in adaptation to biotic or abiotic stress conditions. PR proteins are also involved in hypersensitive response (HR) or systemic acquired resistance (SAR) against infection. Thus, PR proteins have been defined as "proteins encoded by the host plant but induced only in pathological or related situations," the latter inferring situations of nonpathogenic origin. In this chapter, structure, biochemistry, source, regulation of gene expression, and role in defense mechanism of various pathogenesis-related proteins will be discussed.

Keywords

Abiotic stress · PR proteins · Stress response · Biotic stress · Pathogenesis

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12.1 Introduction

Plants, as sessile organisms, are often encountered by various abiotic and biotic stresses affecting their growth and agricultural yield. Plant stress tolerance and susceptibility are governed by a complex exchange of signals and responses collectively known by a general term, cellular stress response occurring under given environmental conditions in plants. So, the key difference between resistant and susceptible plants is the timely recognition of the invading pathogen or stress and the rapid and effective activation of host defense mechanisms.

Cellular stress response is a complex trait that happens due to the balanced coordination of physiological and biochemical alterations at the cellular and molecular level. These alterations could be the physical strengthening of the cell wall or through accumulation of various osmolytes, late embryogenesis abundant (LEA) and PR proteins. Physical strengthening of cell wall is often through lignification, suberization, and callose deposition. Cellular alterations mostly coupled with an efficient antioxidant system and prevent pathogen invasion by producing phytoalexins, phenolic compounds, and PR proteins. Various strategies acquired by plants during cellular stress responses serve the adaptive purpose of protecting a cell against unfavorable environmental conditions, both through short-term mechanisms that minimize acute damage to the overall cell's integrity and through long-term mechanisms which provide the cell a measure of pliancy against similar adverse conditions. For any organism sustainability, the cellular machinery must be activated in response to the various stresses, to ensure that the resources are used when required. Accordingly, plant cells have evolved to perceive different signals from their surroundings, to integrate them, and to respond by modulating the appropriate gene expression that may involve protein phosphorylation, ion fluxes, reactive oxygen species (ROS), and other singling events. A diverse array of plant protectants and defense genes get activated whose products include glutathione S-transferases (GST), peroxidases, proteinase inhibitors, cell wall proteins, hydrolytic enzymes (e.g., chitinases and β-1,3-glucanases), pathogenesis-related (PR) proteins, and phytoalexin biosynthetic enzymes, like phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) (Hammond-Kosack and Jones 1996). Among all, the synthesis and activation of pathogenesis-related (PR) proteins is very critical in response to any stress situation and/or invading pathogen.

During incompatible host-pathogen interactions, the plant's defensive responses restrict the damage caused by the pathogen. Subsequent infection by different types of pathogens is often limited by the defensive responses that is associated with a coordinated and integrated set of metabolic alterations. Further, various novel proteins are synthesized and induced which are collectively known as "pathogenesis-related proteins" (PRs). Pathogen-related proteins (PRs) have been defined as "proteins encoded by the host plant but induced only in pathological or related situations," the latter referring situations of nonpathogenic origin (Antoniw and Pierpoint 1978). PRs have not been identified because of their anti-pathogenic action, but solely because of their easily identification in infected plants. A number of PRs have been reportedly induced in many plant species belonging to various families suggesting a general protective role of PRs against biotic stress (Van Loon 1999). PRs not

only accumulate locally in the infected tissue but are also induced systemically. Thus, PRs are associated with the development of systemic acquired resistance (SAR) or hypersensitive response (HR) against further infection by pathogenic fungi, bacteria, and viruses. HR is characterized by necrotic lesions resulting from localized host cell death at the site of infection (Goodman and Novacky 1994). Moreover, plants respond to pathogen infection by activating defense responses in uninfected parts of the plant. As a result, the whole plant develops resistance to subsequent infections. This systemic acquired resistance (SAR) is a generally occurring phenomenon and often confers broad-based resistance to a variety of different pathogens (Ryals et al. 1996; Delaney 1997) instigating the defensive capacity of plants in response to necrotizing infections. Over the decades, a number of reports depicted the role of different classes of PR proteins during abiotic and biotic stresses and their defense responses in plants; however, the mechanism of action is sparsely described. In this chapter, we will be briefly discussing the biochemistry, source, regulation of gene expression, and mode of action of PR proteins in defense mechanisms.

12.2 PR Proteins: An Overview

PR proteins comprise a huge family of proteins ubiquitous in the plant kingdom. Plant PR proteins were first identified and reported in tobacco plants infected by tobacco mosaic virus (Van Loon and Van Kammen 1970). Later, these proteins were reported in many different plant species. Most plant PR proteins share the common biochemical properties of being acid soluble, low molecular weight, and protease resistance (Leubner-Metzger and Meins 1999; Neuhaus 1999). PR proteins have similar functions, but depending on their isoelectric points, they may be acidic or basic proteins. Most acidic PR proteins are secreted in the extracellular spaces, whereas basic PR proteins are predominantly found in the vacuole (Legrand et al. 1987; Niki et al. 1998) through the signal peptide at C-terminus. However, such localization cannot be generalized for all PR proteins. Though PRs are most abundant in the leaves, they are detected in almost all plant organs including leaves, stems, roots, and flowers. Usually, acidic PRs are upregulated by various signaling molecules like salicylic acid (Yalpani et al. 1991; Sinha et al. 2014) and reactive oxygen species (Chamnongpol et al. 1998), while basic PRs are upregulated by gaseous phytohormone ethylene and methyl jasmonate (Xu et al. 1994) during pathogen attack. Apart from various environmental factors, there are certain internal developmental factors too that trigger the synthesis of these PR proteins.

Based on molecular mass, isoelectric point, localization, and biological activity, the PR proteins have been categorized into 17 families (Table 12.1), including β-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidases, ribosome-inactivating proteins, thionins, nonspecific lipid transfer proteins, oxalate oxidase, and oxalate-oxidase-like proteins (Van Loon and Van Strien 1999) and numbered in the order in which they were discovered. When dealing with a stress-related sequence possibly related with PRs, it is necessary to gather information at both the nucleic acid and the protein level as homologies at the cDNA, or genomic level may be encountered without information on the expression of the encoded protein.

Table 12.1 Classification of pathogenesis-related proteins

Families	Properties	Example
PR-1	Antifungal	Tobacco PR-1a
PR-2	**β**-1,3-Glucanase	Tobacco PR-2
PR-3	Chitinase type I, II, IV, V, VI, VII	Tobacco P, Q
PR-4	Chitinase type I, II	Tobacco "R"
PR-5	Thaumatin-like	Tobacco S
PR-6	Proteinase inhibitor	Tomato inhibitor I
PR-7	Endoproteinase	Tomato P69
PR-8	Chitinase type III	Cucumber chitinase
PR-9	Peroxidase	Tobacco "lignin-forming peroxidase"
PR-10	Ribonuclease-like	Parsley "PR1"
PR-11	Chitinase, type I	Tobacco "class V" chitinase
PR-12	Defensin	Radish Rs-AFP3
PR-13	Thionin	Arabidopsis THI2.1
PR-14	Lipid transfer protein	Barley LTP4
PR-15	Oxalate oxidase	Barley OxOa (germin)
PR-16	Oxalate oxidase-like	Barley OxOLP
PR-17	Unknown	Tobacco PRp27

Though such sequences belong to the PR-type families, they are to be named PR-like proteins (PRLs) and cannot be considered to correspond to pathogen-induced PRs (Van Loon et al. 1994). Among these PRLs, chitinases and β-1,3-glucanases are two important hydrolytic enzymes that accumulate in many plant species after infection by different types of pathogens. These hydrolytic enzymes play the main role of defense reaction against fungal pathogen by degrading cell wall, because chitin and β-1,3-glucan are major structural components of the cell walls of many pathogenic fungi. Some of the tobacco PRs were characterized as chitinases and β-1,3-glucanases (Kauffmann et al. 1987) with potential antifungal activity suggested the group of PRs might be inhibiting pathogen growth and be responsible for the SAR. In spite of their common name, PR proteins show a great diversity in species specificity and in the mechanism of action and do not share any structural relationship among themselves.

PR proteins unveil diverse functions within the plant. Many PRs exhibit antifungal activity (Caruso et al. 1996) though few of the PR proteins also show antibacterial, insecticidal, nematicidal, and antiviral activity (Edreva 2005). PR proteins thus have a critical role in disease resistance, seed germination, and plant facilitation to adapt to the environmental stress.

12.2.1 Plant Chitinases

Chitinases (E.C. 3.2.1.14) are widely distributed across plant, animal, fungi, and bacteria kingdoms. These enzymes catalyze the cleavage of a bond between C1 and C4 of two consecutive N-acetyl-D-glucosamine monomers of chitin which is a

common component of fungal cell walls and of the exoskeleton of arthropods (Bartnicki-Garcia 1968). Usually the plant chitinases are endo-chitinases capable of degrading chitin as well as inhibit fungal growth (Schlumbaum et al. 1986; Broekaert et al. 1988). Chitinases are either localized in the vacuole (Class I) or outside the cell (Class III) (Neuhaus et al. 1996). Many reports strongly indicated that chitinases, together with β-1,3 glucanases, play critical role in the plant defense response against fungal pathogens (Abeles et al. 1971).

Plant chitinases have been classified into seven classes, class I through VII, based on their primary structures. Certain isoforms of chitinases are induced by particular elicitors, and only few isoforms have antifungal activities, while some isoforms have shown another role like antifreeze activity (Sela-Buurlage et al. 1993; Yeh et al. 2000). Class I chitinases have a cysteine-rich N-terminal chitin-binding domain (CBD) that is homologous to havein, a chitin-binding lectin from the rubber tree (Suarez et al. 2001). Class II chitinases are similar to class I but they lack the N-terminal CBD. Class III chitinases are unique in structure and belong to the PR-8 family and family 18 of glycosyl-hydrolases. Class III chitinases are more closely related to the bacterial chitinases and generally have lysozyme activity. Class IV, V, VI, and VII chitinases belong to the PR-3 family of proteins (Meins et al. 1994).

Various biotic and abiotic factors have been known to induce chitinases in the plant. Various studies reported the inhibitory effect of plant chitinases on fungal growth by demonstrating on the growth of chitin-containing fungi (Mauch et al. 1988a, b). Various studies showed that chitinase expression is induced against phytopathogen systems, and resistant varieties have stronger upregulation than susceptible varieties in the sugar beet (Nielsen et al. 1992), wheat (Anguelova-Merhar et al. 2001), and tomato (Lawrence et al. 2000). Transformation of chitinase genes was performed in tobacco (Brogue et al. 1991), grapevine (Yamamoto et al. 2000), rice (Datta et al. 2001), and peanut (Rohini and Rao 2001), and enhanced disease resistance has been achieved. Overexpression studies have been made with chitinase genes, alone or together with β-1,3-glucanase genes in a number of plant species, and in most cases, the resulting transgenic plants exhibited enhanced levels of fungal disease resistance or delayed symptom development as compared to the control plants (Zhu et al. 1994; Jach et al. 1995; Jongedijk et al. 1995). However, several studies showed that plants transformed with either chitinase or β-1,3-glucanase gene alone did not exhibit resistance to certain pathogens or showed less resistance. Plant chitinases alone are unable to effectively degrade harder chitin structures of fungi as they usually affect only the hyphal tip, but when coexpressed with β-1,3-glucanase, these two enzymes act synergistically against fungal pathogen.

12.2.2 Plant β-1,3-Glucanases

Plant β-1,3-glucanases belong to the PR-2 family of pathogenesis-related proteins and reportedly play an important role in plant defense responses to pathogen infection. These enzymes have been identified across plants, yeasts, actinomycetes, bacteria, fungi, insects, and fish (Pan et al. 1989). These enzymes catalyze the cleavage of the

β-1,3-glucosidic bonds in β-1,3-glucan (Simmons 1994) which is another major structural component of the cell walls of many pathogenic fungi (Adams 2004). β-1,3-Glucanases play an important role in plant defense and other physiological functions such as cell division and cell elongation (Fulcher et al. 1976), fruit ripening (Meins et al. 1992), pollen germination and tube growth (Meikle et al. 1991), fertilization (Ori et al. 1990), somatic embryogenesis (Helleboid et al. 2000), seed germination (Buchner et al. 2002), and flower formation (Akiyama et al. 2004). The role of plant β-1,3-glucanases as an important component of plant defense mechanisms against pathogens has been well documented (Legrand et al. 1987; Cordero et al. 1994). It has been postulated that β-1,3-glucanases hydrolyze fungal cell walls, which consequently causes the lysis of fungal cells when defending against fungi. On the pathogen encounter, β-1,3-glucanases also cause the formation of oligosaccharide elicitors, which elicit the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins (Klarzynski et al. 2000).

β-1,3-Glucanase genes have been reported from a wide range of plant species, and many studies have shown that the synthesis of β-1,3-glucanases is stimulated by pathogen infections (Alonso et al. 1995; Roulin et al. 1997) and can change during plant development (Wyatt et al. 1991). Different plant species may have different β-1,3-glucanase genes, and a single plant species may have various copies of β-1,3-glucanase genes. Plant β-1,3-glucanases were classified into two major classes I and II and two minor classes based on their amino acid sequence, structural properties, and cellular localizations (Beerhues and Kombrink 1994). Generally, β-1,3-glucanases are stress regulated, but a few β-1,3-glucanases are exclusively developmentally regulated and do not show a stress-related regulation (Bucciaglia and Smith 1994; Sharma 2013). β-1,3-Glucanases are usually expressed at low concentration in plants, but when plants are challenged by fungal, bacterial, or viral pathogens, β-1,3-glucanases enzyme accumulate dramatically (Castresana et al. 1990; Lusso and Kuc 1995). Class I β-1,3-glucanases and class I chitinases showed synergistic effect in pathogen defense. Class I β-1,3-glucanase accumulated only at the site of tobacco mosaic virus (TMV) infection in tobacco plants, while class II and III β-1,3-glucanases accumulated both at the site of infection and systemically (Vögeli-Lange et al. 1994; Livne et al. 1997). Many reports showed that the transcript levels of glucanases accumulated after infected with pathogens, such as barley infected by powdery mildew (Ignatius et al. 1994), maize infected with *Aspergillus flavus* (Lozovaya et al. 1998), pepper infected with *Xanthomonas campestris* pv. *vesicatoria* and *Phytophthora capsici* (Jung and Hwang 2000), wheat infected with *Fusarium graminearum* (Li et al. 2001), chickpea infected with *Ascochyta rabiei* (Pass.) Labr. (Hanselle and Barz 2001), and peach infected with *Monilinia fructicola* (Zemanek et al. 2002). β-1,3-Glucanases and other PR protein induction in the plant can also occur due to some elicitors, including fungal β-glucan, chitin, chitosan, glycoproteins, and N-acetylchito oligosaccharides (Chang et al. 1992; Kaku et al. 1997), or by other factors, for example, salicylic acid-induced accumulation of mRNAs of class II and III β-1,3-glucanases in wild-type tobacco plants (Ward et al. 1991), abscisic acid (ABA) in tobacco (Rezzonico et al. 1998),

and methyl jasmonate, ethylene, and gibberellin A3 in tomato seeds and leaves (Wu and Bradford 2003). Stress factors like wounding, drought, exposure to heavy metals, air pollutant ozone, and ultraviolet radiation can also upregulate β-1,3-glucanases in some plants (Thalmair et al. 1996; Fecht-Christoffers et al. 2003).

12.3 Role of PR Proteins in SAR and HR

PRs are most common in hypersensitive responses but appear to contribute to SAR also. An induced systemic resistance (ISR) can be induced by nonpathogenic rhizobacteria, considerably modified the relationships between necrotic lesion formation, PRs, and SAR. ISR induction with these rhizobacteria shows no symptoms in plants; however, this resistance is independent of the production of salicylic acid (SA) by the plant and is not associated with the accumulation of PRs (Pieterse et al. 1996; Van Loon et al. 1998). This indicates that plants can substantially enhance their defensive capacity against variety of pathogens in either SA-dependent or independent way (Pieterse and Van Loon 1999). SAR is mainly SA-dependent (Ryals et al. 1996) while ISR is SA-independent. Until now the mechanism involved in ISR has been unclarified. At least in *Arabidopsis*, similar to SAR, ISR depends on the functioning of the *npr1* gene, which in turn distinguishes ISR from the JA- and ethylene-dependent inducible defense response pathway effective against *Alternaria brassicicola*, which is independent of *npr1*. PRs are often associated with SAR, but not with ISR, which have led to hypothesize that PR accumulation is not a prerequisite for the induction of resistance, but they contribute to the protective state (Van Loon 1997). The JA- and ethylene-dependent pathway induced by, and effective against, *A. brassicicola* involves increase in SA, JA, and ethylene levels resulting in detection of PRs in the infected plants. The differential expression of various PRs determines the extent of the plant's response and its effectiveness to inhibit further infection. Recent report shows that SA-dependent expression of PR-1, PR-2, and PR-5 is required for increased protection against the biotrophic fungus *Peronospora parasitica* in *Arabidopsis*, whereas SA-independent but JA-dependent induction of PR-3 and PR-4 is associated with the induced resistance against the necrotrophic fungi *A. brassicicola* (Penninckx et al. 1996), *Botrytis cinerea* (Thomma et al. 1998), and *Fusarium oxysporum* f.sp. *matthiolae* (Bohlmann et al. 1998). These results suggest that PRs appear to contribute differentially to the induced resistance against different pathogens.

12.4 Signaling Involved in Pathogen-Induced Expression of PRs

The pathogen-activated PR gene expression plays a critical role in plant defense against pathogens. Regulation of PR gene expression has always been a highly active research area since the time PR proteins were discovered. However, the signaling behind the

pathogen-induced PR gene expression is still poorly understood in plants. This is partly due to the complexity of environmental stimuli and stimulation by phytohormones that can induce the expression of various PR genes (Brederode et al. 1991).

12.5 Signals and Putative Receptors Involved in PR Gene Expression

During plant-pathogen interactions, a number of molecules derived from pathogens can serve as elicitors for PR gene induction such as chitin fragments and glucans from fungal cell wall, extracellular glycoproteins/peptides from few fungal species, oligosaccharides and harpins from bacteria, and Avr proteins derived from bacterial and fungal pathogens (Boller and Felix 1996). Though a large number of signals are known to induce PR gene expression, no receptors have been unambiguously established for these signal molecules. For example, β-glucan elicitor (GE) is released from *Phytophthora sojae* cell wall by β-1,3-glucanase from soybean and reportedly induces phytoalexin biosynthesis (Darvill and Albersheim 1984). Recently, a GE-binding protein (GEBP) has been purified from soybean whose antiserum partially inhibited the binding of GE to soybean membrane proteins and reduced the phytoalexin accumulation elicited by GE. Another set of elicitors is the polypeptide encoded by pathogen avirulence *(avr)* genes. Any pathogen containing a particular *avr* gene is recognized by the corresponding resistance *(R)* gene of the host plant and activates a variety of defense responses in the host, including increased PR gene expression. In the past decade, a number of *R* genes and *avr* genes have been isolated. For example, an elicitor encoded by the *avr9* gene from *Cladosporium fulvum*, a fungal pathogen of tomato, rapidly activated the transcription of glucanase and chitinase genes in plants carrying the cognate *R* gene *Cf9* (Ashfield et al. 1994; Wubben et al. 1996). Another fungal elicitor, NIP1 protein from the barley pathogen *Rhynchosporium secalis* is known to activate PR genes (Rohe et al. 1995). The only evidence that an Avr protein directly interacts with an R gene product comes from the study of bacterial speck disease in tomato. Tomato plants having *R* gene *Pto*, encoding cytoplasmic kinase, are resistant to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* carrying the *avrPto* gene (Martin et al. 1993). The Pto and AvrPto proteins showed a highly specific association in yeast too (Scofield et al. 1996; Tang et al. 1996). The results confirmed Pto as the receptor for the AvrPto protein.

12.6 Different Pathways for PR Genes' Activation by Pathogens

Plants often exhibit increased production of reactive oxygen species (ROS), salicylic acid (SA), ethylene, and jasmonates upon infection by pathogens (Hammond-Kosack and Jones 1996; Yang et al. 1997). These molecules may serve as secondary signals to activate plant defense, and many of these reportedly work as inducers for PR gene expression. For example, SA induces acidic PR genes that are normally

activated during SAR, whereas ethylene and jasmonates are known to induce proteinase inhibitors, defensin, thionin, and basic PR proteins (Brederode et al. 1991; Ward et al. 1991; Epple et al. 1995; Donnell, et al. 1996; Penninckx et al. 1996). However, the involvement of secondary messengers in the PR gene induction is uncertain in majority of studies. Cross talks are often common between signaling pathways mediated by these secondary messengers. The use of signaling pathway mutants would be supportive in clarifying the roles of these secondary messengers in plant defense responses against pathogen attacks.

12.7 Functions and Relevance of PR Expression in Disease Resistance

In the last decades, proteinase, peroxidase, ribonuclease, and lysozyme activities were assigned to PR-7, PR-9, PR-10, and PR-8, respectively. Also, membrane-permeabilizing functions are characteristic of defensins (PR-12), thiols (PR-13), lipid transfer proteins (LTPs, PR-14), and of osmotins and thaumatin-like proteins (PR-5). Multiple enzymatic, structural, and receptor functions are reported in germins (PR-15) and germin-like proteins (PR-16) (Van Loon and Van Strien 1999; Bernier and Berna 2001; Selitrennikoff 2001; Park et al. 2004a, b). Besides this, some PRs also exhibited antibacterial, insecticidal, nematicidal, and antiviral action, though an important common feature of most PRs is their antifungal effect. Their hydrolytic, proteinase inhibitory, and membrane-permeabilizing ability made them toxic to pathogens. Thus, hydrolytic enzymes (β-1,3-glucanases, chitinases, and proteinases) can effectively weaken and decompose fungal cell walls, containing glucans, chitin, and proteins, while PR-8 can damage gram-positive bacteria due to lysozyme activity (Van Loon and Van Strien 1999; Selitrennikoff 2001). The defensive functions of PRs against pathogens can be attributed to a number of their ingenious properties; their constitutive expression in seeds and plant organs, high fungitoxicity of seed osmotins and thaumatin-like proteins (Vigers et al. 1992; Abad et al. 1996), their accumulation in plant cell wall appositions formed against pathogen invasions (Jeun 2000; Jeun and Buchenauer 2001). Inspite of all studies and reports, the defensive mechanism of PR function against pathogen attack is still unclear. The protective role of PRs is supported by following evidences:

(a) *Transcript accumulation of PRs in pathogen-tolerant and susceptible plants*. Recently, the differential responses of resistant/susceptible plants were reported in tomato plants, inoculated with *Cladosporium fulvum* (Wubben et al. 1996), *Phytophthora infestans*-infected potato (Tonón et al. 2002), *Venturia inaequalis*-inoculated apple (Poupard et al. 2003), *Pseudomonas syringae*-infected grapevine (Robert et al. 2001), *Xanthomonas campestris* pv. *vesicatoria*, and TMV-Po-infected hot pepper (Park et al. 2004a, b).
(b) *The plants with high natural disease resistance constitutively express PRs*. This correlation has been proved in many pathosystems, such as apple-*Venturia*

inaequalis (Gau et al. 2004), tomato-*Alternaria solani* (Lawrence et al. 2000), and potato-*Phytophthora infestans* (Vleeshouwers et al. 2000).

(c) *Overexpressing PRs in transgenic plants results in increased resistance to pathogens*. Tobacco overexpressing *PR1a* gene showed increased tolerance to *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al. 1993). Similarly, overexpression of thaumatin-like PR-5 in transgenic rice and orange plants showed increased tolerance to *Rhizoctonia solani* and *Phytophthora citrophthora*, respectively (Datta et al. 1999, Fagoaga et al. 2001); transgenic potato overexpressing PR-2 and PR-3 had improved resistance to *Phytophthora infestans* (Bachmann et al. 1998); transgenic carrot overexpressing PR-2 and PR-3 genes, coding for β-1,3-glucanase and chitinase, respectively, showed increased resistance to several fungal pathogens; and the transgenic tomato simultaneously expressing tobacco β-1,3-glucanase and chitinase genes had improved resistance to fungal pathogens (Melchers et al. 1998).

(d) *Accumulation of PRs in plants with locally or systemically induced resistance*. As discussed before, PRs are identified as markers of the systemic acquired resistance (SAR). SAR and the associated set of PRs are induced by different pathogens and various chemicals predominantly in a salicylic acid-dependent pathway. It is important to note that the direct role of PRs in disease resistance is being suggestive by their high expression in resistant or SAR-expressing plants, as well as transgenic resistant plants exhibiting high antimicrobial activity (Rauscher et al. 1999; Tonón et al. 2002; Anand et al. 2004).

12.8 Transcriptional Regulation of PR Gene Expression

The most active area in PR gene research is to study its transcriptional regulation. Several *cis*-regulatory elements mediating pathogen-induced PR gene expression have been identified and characterized through traditional promoter deletion analysis coupled with mutagenesis of putative regulatory elements, gain-of-function studies with synthetic promoters, and DNA-fingerprinting analysis (Yang et al. 1997; Rushton and Somssich 1998). Many of these elements are W-box (consensus TTGACC or TGAC-[N]x-GTCA), GCC-box (consensus AGCCGCC), MRE-like sequence (consensus A[A/C]C[A/T]A[A/C]C), G-box (consensus CACGTG), and SA-responsive element (SARE, with a consensus of TTCGACCTCC). Among all, the GCC-box and W-box have been extensively studied and have a wide role in PR gene regulation (Hart et al. 1993; Ohme-Takagi and Shinshi 1995). Defense responses mediated by ethylene are often associated with GCC-box and are known to confer ethylene-induced transcription of the tobacco *gln2* gene encoding a β-1,3-glucanase (Ohme-Takagi and Shinshi 1995). EREB clones (1–4) were identified in tobacco cDNA library with radiolabeled GCC-box as probe (Ohme-Takagi and Shinshi 1995). EREBP transcripts are induced by ethephon, a compound known to

release ethylene upon its degradation that suggests that ethylene further induces the expression of EREBP genes. The EREBP-1 gene was reportedly induced by *Pseudomonas* bacteria and SA, suggesting a role of this gene in plant defense (Zhou et al. 1997; Horvath et al. 1998). The direct correlation of the EREBP proteins with a disease-resistance pathway was confirmed by the study of the signaling pathway mediated by the tomato gene *Pto* (Zhou et al. 1997). Phosphorylation also plays an important role in the activation of PR gene expression during pathogen attacks (Raz and Fluhr 1993). Another highly conserved *cis*-element, W-box, is present in parsley *PR1-1* and *PR1-2* (both encoding the PR1 protein), tobacco *CHN50* (encoding a class I basic chitinase), asparagus *AoPR1* (encoding the PR10 protein), potato *PR-10a* (encoding the PR10 protein), and maize *PRms* (encoding the PR1 protein). Besides PRs, W-box is also present in the promoter of other pathogen inducible genes such as the potato glutathione S-transferase gene *prp1* and the grape phytoalexin synthesis gene *Vst1*, suggesting a broader role for this element in pathogen-induced gene expression (Rushton and Somssich 1998). Three parsley cDNAs encoding W-box-binding proteins were identified by using a south-western screening (Rushton et al. 1996). These proteins are termed WRKY family proteins and contain the consensus sequence, WRKYGQK. Since the in vivo function of the cloned transcription factors is still to be worked upon, so rigorous tests on transgenic plants with altered expression of the transcription factor genes are required to establish their roles in PR gene expression and defense responses. In addition, it is necessary to answer few questions. How different signals affect PR gene expression? Do different signals converge on the same transcription factor? Do these transcription factors interact? Answering these questions help us in better understanding of cross talks between different signaling pathways.

12.9 Conclusions

PR proteins and their homologues are generally responsible for the defense against various stresses including pathogen attacks, wounding, use of chemicals, and pollutants. However, many PR proteins (members of PR 1, 2, 3, 4, 5, 8, 10, and 14 families) have demonstrated allergenicity, but the allergenicity is also guided by several environmental factors like the use of chemical inducers in agriculture and environmental pollutants. Recent reports have documented their critical importance as preservative agents in food industry and for producing disease-resistant plants by genetic engineering. Various studies have revealed that transgenic plants overexpressing PR genes mediate host plant resistance to phytopathogenic fungi. Such genetically modified (GM) plants with enhanced expression of PR proteins may be associated with increased allergenicity and toxicity, thus raising a serious question for their commercial acceptability, so different strategies are adopted to monitor the transformed crops for their allergenicity.

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Antimicrobial Compounds and Their Role in Plant Defense

13

Anupama Razdan Tiku

Abstract

Plants are important nutrient source for several organisms like microbes, heterotrophic plants, insects as well as vertebrates. Even though they lack a proper defense mechanism like animals, still they have developed a mixture of chemicals which are mainly protein based and are used as a means of defense by detecting attacking organisms and preventing them from causing major damage. In order to protect themselves from these microbes like fungi, bacteria, etc., plant cells have developed the capability to identify attacking pathogens and use inducible defense mechanism by producing toxic chemicals or antimicrobial compounds in the form of pathogen-degrading enzymes and secondary metabolites involved with plant defense. Secondary metabolites generally are grouped into three major classes of chemicals, i.e. terpenoids, phenolic and alkaloids. Some of these antimicrobial compounds are constitutive in nature, i.e. they occur in biologically active forms in healthy plants, whereas other metabolites are inductive in nature. Glucosinolates and cyanogenic glycosides exist in inactive form and are activated as a response to attack by pathogen or tissue damage. These compounds are activated by release of plant enzymes at the time of breakdown of cells. Preformed antimicrobial compounds are termed as "phytoanticipin", while "phytoalexins" are those antimicrobial compounds which are synthesized (as a result of synthesis of enzymes) from precursors as a response to attack by pathogen. Preformed inhibitors are usually tissue specific and are mainly present in the outer layers of the cells of plant organs. These inhibitors are mostly successful against comprehensive range of probable pathogens, and specific virulent pathogens might circumvent the effect of these secondary metabolites by eluding them or by enduring or by detoxification. Most of these constitutive plant compounds show antifungal activity, e.g. phenols, phenolic

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glycoside, unsaturated lactones, sulphur compounds, saponins, etc. "Phytoalexins" are the most considered antimicrobial plant defense compounds. These compounds are pathogen specific and therefore more effective in plant defense mechanism. Transcriptional and translational activities in a plant are prerequisite for the production of phytoalexins. Examples of these antimicrobial phytoalexins are scopoletin, camalexin, glucosinolates, etc. This chapter will mainly discuss the role of both phytoanticipins and phytoalexins as plant defense antimicrobial compounds and also their use as "antibiotic potentiators" and virulence attenuators along with their role in crop protection/phytoprotection.

Keywords
Defense mechanism · Secondary metabolites · Phytoalexins · Phenolics · Toxins and alkaloids

13.1 Introduction

All plants growing in natural habitats are surrounded by a large number of antagonists including variety of microbes, nematodes, insects and herbivores and various kinds of abiotic environmental stress which are major sources of hindrance for crop yield. Plants have evolved with a wide variety of inherent and stimulative defense mechanisms to shield themselves from their potential enemies (Cowan 1999). Inherent defense includes many preformed obstructions including cell wall, bark and cuticular wax to protect the plant from attack of pathogens. Plants use stimulative defense mechanism including production of toxic chemicals, enzymes which degrade pathogens and cell suicide to protect themselves from attacking pathogens. Research on the role of these chemicals acting as plant defense compounds took off in the mid of the nineteenth century, e.g. Muller and Borger introduced phytoalexins based on their observation of *Phytophthora infestan* infection in potato tubers. Plant cells showed inhibition towards the pathogen by producing a chemical which reacted hypersensitively and was named as phytoalexin (Jeandet et al. 2014a, b; Jeandet 2015).

Plant compounds are identified as primary metabolites and secondary metabolites.

Primary metabolites (sugars, amino acids, nucleic acids) are involved in growth, development or reproduction, whereas secondary metabolites serve as toxic chemicals or defense-related proteins (Freeman and Beattie 2008). Plants produce diverse varieties of secondary metabolites using resultants of primary metabolisms including enzymes involved in various metabolic pathways and major biomolecules of the cell (Table 13.1) (Fig. 13.1). These are used to protect plants against invading microbial pathogens on account of their toxic nature. Some of them are as well involved in protection of plants from abiotic stress (such as UV-B exposure) and also used for communication between plants and other organisms (including attracting useful organisms like pollinators) or hostile interactions (such as restraint against pathogens and herbivores) (Schafer and Wink 2009).

Table 13.1 Plants containing antimicrobial compounds

Common name	Scientific name	Compound	Class	Activity
Allspice	*Pimenta dioica*	Eugenol	Essential oil	General
Aloe	*Aloe barbadensis*, *Aloe vera*	Latex	Complex mixture	*Corynebacterium*, *Salmonella*, *Streptococcus*, *S. aureus*
Apple	*Malus sylvestris*	Phloretin	Flavonoid derivative	General
Ashwagandha	*Withania somnifera*	Withaferin A	Lactone	Bacteria, fungi
Bael tree	*Aegle marmelos*	Essential oil	Terpenoid	Fungi
Barberry	*Berberis vulgaris*	Berberine	Alkaloid	Bacteria, protozoa
Basil	*Ocimum basilicum*	Essential oils	Terpenoids	*Salmonella*, bacteria
Bay	*Laurus nobilis*	Essential oils	Terpenoids	Bacteria, fungi
Betel pepper	*Piper betle*	Catechols, eugenol	Essential oils	General
Black pepper	*Piper nigrum*	Piperine	Alkaloid	Fungi, *Lactobacillus*, *Micrococcus*, *E. coli*, *E. faecalis*
Blueberry	*Vaccinium* spp.	Fructose	Monosaccharide	*E. coli*
Brazilian pepper tree	*Schinus terebinthifolius*	Terebinthine	Terpenoids	General
Buchu	*Barosma betulina*	Essential oil	Terpenoid	General
Burdock	*Arctium lappa*		Polyacetylene, tannins, terpenoids	Bacteria, fungi, viruses
Buttercup	*Ranunculus bulbosus*	Protoanemonin	Lactone	General
Caraway	*Carum carvi*		Coumarins	Bacteria, fungi, viruses
Cascara sagrada	*Rhamnus purshiana*	Tannins	Polyphenols	Viruses, bacteria, fungi
			Anthraquinone	
Cashew	*Anacardium*	Salicylic acids	Polyphenols	*P. acnes* bacteria, fungi
	Pulsatilla			
Ceylon cinnamon	*Cinnamomum verum*	Essential oils, others	Terpenoids, tannins	General
Chamomile	*Matricaria chamomilla*	Anthemic acid	Phenolic acid	*M. tuberculosis*, *S. typhimurium*, *S. aureus*, helminths

(continued)

Table 13.1 (continued)

Common name	Scientific name	Compound	Class	Activity
Chaparral	*Larrea tridentata*	Nordihydroguaiaretic acid	Lignan	Skin bacteria
Chilli peppers, paprika	*Capsicum annuum*	Capsaicin	Terpenoid	Bacteria
Clove	*Syzygium aromaticum*	Eugenol	Terpenoid	General
Coca	*Erythroxylum coca*	Cocaine	Alkaloid	Gramnegative and grampositive cocci
Cranberry	*Vaccinium* spp.	Fructose	Monosaccharide	Bacteria
		Other		
Dill	*Anethum graveolens*	Essential oil	Terpenoid	Bacteria
Eucalyptus	*Eucalyptus globulus*	Tannin	Polyphenol	Bacteria, viruses
Fava bean	*Vicia faba*	Fabatin	Thionin	Bacteria
Gamboge	*Garcinia hanburyi*		Resin	General
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxide	General
			Sulfated terpenoids	
Ginseng	*Panax notoginseng*		Saponins	*E. coli*, *Sporothrix schenckii*, *Staphylococcus*, *Trichophyton*
Glory lily	*Gloriosa superba*	Colchicine	Alkaloid	General
Goldenseal	*Hydrastis canadensis*	Berberine, hydrastine	Alkaloids	Bacteria, *Giardia duodenalis*, trypanosomes
				Plasmodia
Gotu kola	*Centella asiatica*	Asiaticoside	Terpenoid	*M. leprae*
Grapefruit peel	*Citrus paradisi*		Terpenoid	Fungi
Green tea	*Camellia sinensis*	Catechin	Flavonoid	General
				Shigella
				Vibrio
				S. mutans
				Viruses
Hemp	*Cannabis sativa*	βResercyclic acid	Organic acid	Bacteria and viruses
Henna	*Lawsonia inermis*	Gallic acid	Phenolic	*S. aureus*

Hops	*Humulus lupulus*	Lupulone, humulone	Phenolic acids	General
Horseradish	*Armoracia rusticana*		Terpenoids	General
(Japanese) herb	*Rabdosia trichocarpa*	Trichorabdal A	Terpene	*Helicobacter pylori*
Legume (West Africa)	*Millettia thonningii*	Alpinumisoflavone	Flavone	*Schistosoma*
Lemon balm	*Melissa officinalis*	Tannins	Polyphenols	Viruses
Lemon verbena	*Aloysia triphylla*	Essential oil	Terpenoid	Ascaris
Licorice	*Glycyrrhiza glabra*	Glabrol	Phenolic alcohol	*S. aureus*, *M. tuberculosis*
Mountain tobacco	*Arnica montana*	Helenalins	Lactones	General
Oak	*Quercus rubra*	Tannins	Polyphenols	General
		Quercetin (available commercially)	Flavonoid	
Olive oil	*Olea europaea*	Hexanal	Aldehyde	General
Onion	*Allium cepa*	Allicin	Sulfoxide	Bacteria, *Candida*
Oregon grape	*Mahonia aquifolium*	Berberine	Alkaloid	*Plasmodium*
				Trypanosomes, general
Pau d'arco	*Tabebuia*	Sesquiterpenes	Terpenoids	Fungi
Papaya	*Carica papaya*	Latex	Mix of terpenoids, organic acids, alkaloids	General
Pasqueflower	*Anemone pulsatilla*	Anemonins	Lactone	Bacteria
Peppermint	*Mentha piperita*	Menthol	Terpenoid	General
Periwinkle	*Vinca minor*	Reserpine	Alkaloid	General
Peyote	*Lophophora williamsii*	Mescaline	Alkaloid	General
Poppy	*Papaver somniferum*	Opium	Alkaloids and others	General
Purple prairie clover	*Petalostemum*	Petalostemumol	Flavonol	Bacteria, fungi
Quinine	*Cinchona* sp.	Quinine	Alkaloid	*Plasmodium* spp.

(continued)

Table 13.1 (continued)

Common name	Scientific name	Compound	Class	Activity
Rauvolfia, chandra	*Rauvolfia serpentina*	Reserpine	Alkaloid	General
Rosemary	*Rosmarinus officinalis*	Essential oil	Terpenoid	General
Sainfoin	*Onobrychis viciifolia*	Tannins	Polyphenols	Ruminal bacteria
Savory	Satureja montana	Carvacrol	Terpenoid	General
Senna	*Cassia angustifolia*	Rhein	Anthraquinone	*S. aureus*
Cabbage	*Brassica oleracea*	Quercetin	Flavonoids	General
Rape seed	*B. rapa*	Cyanidin	Hydroxycinnamic acid	
Nabicol	*B. napus*	p-coumaric acid		
		Ferulic acid		
Broad bean	*Vicia faba*	Quinolizidine	Alkaloids	Bacteria
		Piperidine	Amines	Fungi
		Pyridine		
		Indolizidine		
Wild barley	*Hordeum chilense*	Hydroxamic acid	Aglucones	Pests
	H. brevisubulatum			Pathogens
	H. bulbosum			
Grapes	*Vitis vinifera*	Gallic acid	Polyphenols	Generals
		Catechin		
Makoi	*Solanum nigrum*	Luteolin	Flavonoids	Bacteria
		Oleuropein glycoside	Alkaloids	Fungi
		Veremivirine		
		Myristic acid		

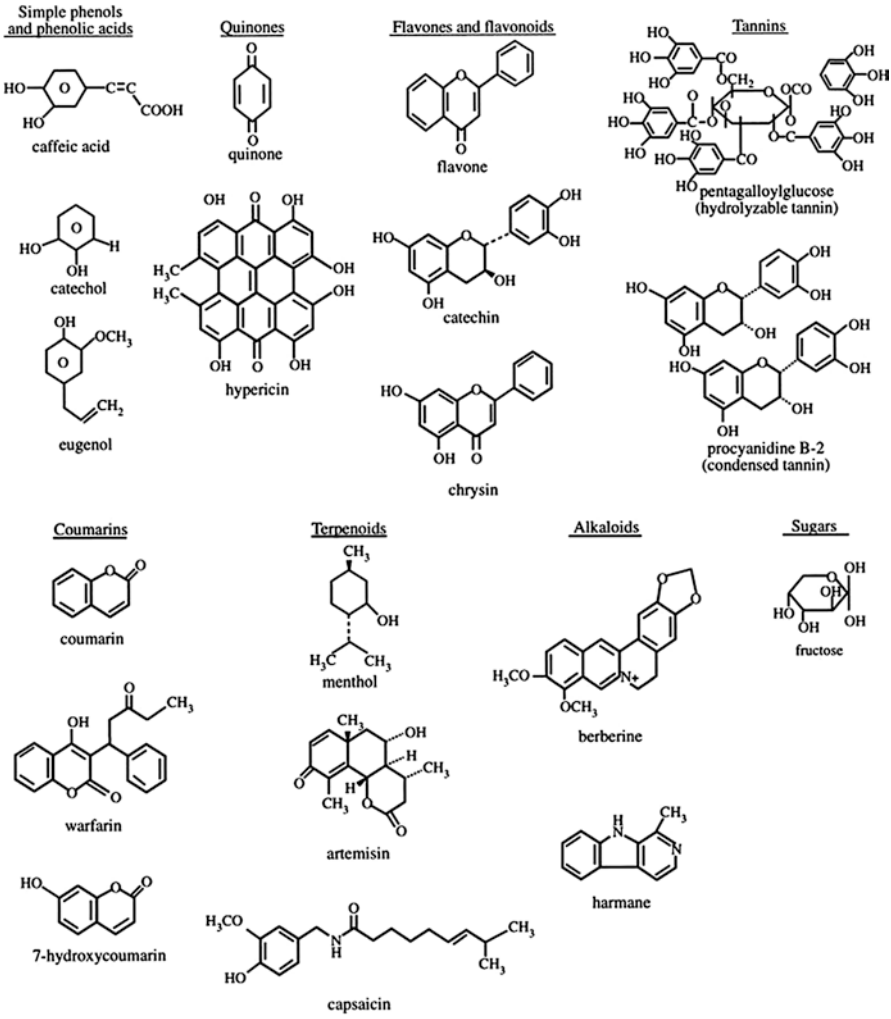


Fig. 13.1 Structures of common antimicrobial plant chemicals

The three major types of secondary metabolites are terpenes/terpenoids, phenolics and nitrogen- and sulphur-containing compounds produced mostly from common amino acids. Terpenes are formed from *5-C isopentanoid* units, which are toxic in nature and hence discourage many herbivores from eating the plants. Phenolics produced mainly from *shikimic* acid pathway are vital to protect the plants from invading microbial pathogens and fungi. Their defensive roles have been confirmed by in vitro experiments conducted by changing expression of secondary metabolites of plants using modern molecular methods (Mes et al. 2000; Van Etten et al. 2000). Studies have shown that around 200,000 secondary metabolites produced by plants are part of biochemical protective systems of plants, evolved over millions of years

during which the respective plants and their foes have existed together (Wink 1999). Due to high energy and nutrient requirements required for production and maintenance of secondary metabolites, plants normally wait till the time pathogens are detected to produce toxic chemicals/defense-related proteins. Hence, plant defense chemicals can be grouped into two types which are constitutive metabolites and induced metabolites. Constitutive metabolites are also known as "prohibitins" or phytoanticipins. Induced metabolites, also known as phytoalexins, are formed to protect the plant from infection involving synthesis of enzymes (complex molecules) from simple molecules (Van Etten et al. 1994; Grayer and Harborne 1994).

Phytoanticipins consume large amount of carbon and energy exhibiting fitness cost under natural conditions. They are accepted as the primary chemical defense that a pathogen has to overcome, whereas production of phytoalexin takes up to 2–3 days, as enzyme synthesis is required for converting their precursors into the desired defense protein (Hammond-Kosack and Jones 1996). Moreover the same type of secondary metabolites/toxic chemicals exists in plants belonging to the same species or taxonomically related species. We will discuss possible types/groups of secondary metabolites acting as antimicrobial compounds used in plant defense mechanism in this chapter.

13.2 Terpenes/Terpenoids

They exist in almost all the plants representing the biggest group of secondary metabolites. There are more than 22,000 compounds described as terpenes, and they are grouped together based on their common biosynthetic origin from glycolytic intermediates or acetyl-CoA (Mazid et al 2011). Hydrocarbon isoprene (C5H8) which is a volatile gas produced during photosynthesis can protect cell membrane from damage due to extreme temperature or light conditions. It is the simplest form of terpenoid. Terpenoids are classified on the basis of number of the isoprene units which are used in their construction, for example, monoterpenoids (two isoprene units), sesquiterpenoids (three isoprene units), diterpenoids (four isoprene units) and triterpenoids (six isoprene units). Terpenes are assumed to be intricate part of plant defense mechanism in the form of toxins and feeding restraint to many insects and herbivores (Gershenzon and Croteau 1991). They are also active against bacteria, fungi and protozoa, for example, pyrethrins, menthol, camphor, farnesol and artemisinins. Capsaicin present in chilli peppers has bactericidal properties. Below several examples will be discussed from five major subclasses of terpenes.

13.2.1 Monoterpenes (C_{10})

Monoterpenes along with its derived compounds are vital representatives of insect toxicity, for example, pyrethroids/pyrethrins (monoterpene esters) exist in the leaves and flowers of chrysanthemum species presenting robust insecticidal reactions (neurotoxin) towards insects like bees, beetles, wasps, moths, etc. They are key

elements of commercial insecticides. Monoterpenoids as well as sesquiterpenoids are the key elements of highly volatile compounds and essential oils, which are the reason for the fragrance of plants producing them. They are insect toxins and prevent bacterial and fungal infections in the plant. For example, mint plants (*Mentha* spp.) produce huge quantity of monoterpenes (menthone and menthol), formed and stored in glandular trichrome present in epidermal cells. In gymnosperms (conifers) like fir, lime and pine, monoterpenes collect in resin ducts of needles, twigs and trunk primarily in the form of alpha-pinene, beta-pinene, limonene and myrcene which are potent insect repellents effecting bark beetles and other serious pests of conifer species (Turlings et al. 1995).

13.2.2 Sesquiterpenes (C_{15})

Many sesquiterpenes have been reported till now, and the part they play in plant defense is mainly of anti-herbivore representatives belonging to Asteraceae family classified by a lactone ring with five members (a cyclic ester) having tough feed repelling properties affecting several insects and herbivores.

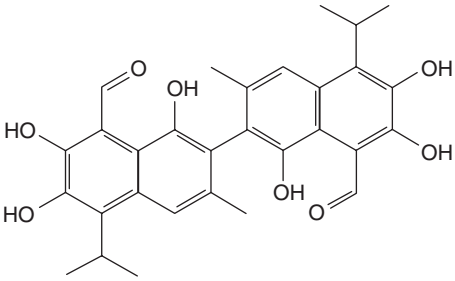
ABA is a well-known example of sesquiterpene playing important role as a plant hormone. In cotton four different sesquiterpenoid phytoalexins, desoxyhemigossypol, hemigossypol, desoxy-6-methoxygossypol and 6-methoxygossypol (Garas and Waiss 1986), help the plant to fight against fungus *Verticillium dahliae*. Resistant varieties of tobacco with *Phytophthora* had induction of sesquiterpenoid phytoalexins like capsidiol, rishitin, etc.

13.2.3 Diterpenes (C_{20})

Abietic acid, a significant diterpene, mostly exists in resin canals of tree trunks of legumes and pine trees. Toxic resin serves as chemical deterrent against the feeding insects which pierce these canals. Phorbol (an ester) is another example of diterpene which is present in members of Euphorbiaceae and proves to be a skin irritant as well as toxic for feeding herbivores. Gossypol (Fig. 13.2) which has tough antifungal and antibacterial characteristics is produced by cotton (*Gossypium hirsutum*) (Bennett and Wallsgrove 1994).

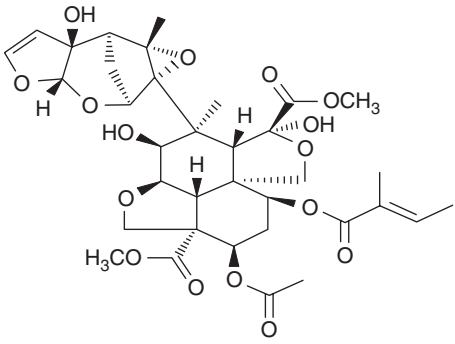
13.2.4 Triterpenes (C_{30})

Triterpene structures are quite similar to plant and animal steroidal hormones and sterols. The milkweeds generate numerous bitter-tasting glycosides, i.e. sterols which protect the plants against insects and herbivores. Limonoid is a bitter substance in citrus fruits and serves as anti-herbivore compounds present in family Rutaceae. Azadirachtin (Fig. 13.3), a complex limonoid, is present in *Azadirachta indica*, which restrains some insects from feeding on the plant and also protects the



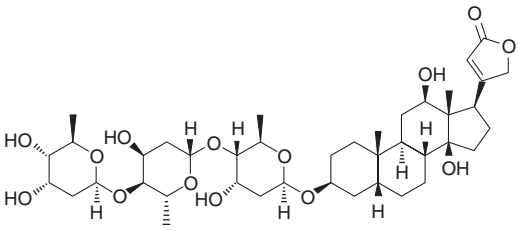
Gossypol

Fig. 13.2 Structure of diterpene gossypol



Azadirachtin

Fig. 13.3 Structure of triterpene azadirachtin



Digoxin

Fig. 13.4 Structure of triterpene digoxin

plant from microbial infections (Mordue and Blackwell 1993). Foxglove (*Digitalis purpurea*) also produces glycosides known as digitoxin and digoxin (Fig. 13.4).

13.2.5 Polyterpenes $(C_5)_n$

These are high molecular weight terpenes, for example, rubber has many polyterpenes showing antimicrobial properties. Rubber is found in long vessels known as laticifers made up of 1500–15,000 isopentenyl units having nearly all C-C double

bonds with cis (Z) configuration. Another example is gutta rubber which has its double bonds in trans (E) configuration (Eisner and Meinwald 1995). Few polyterpenes (tetraterpenes) effect growth, e.g. hormone gibberellic acid, and also contribute to red, yellow and orange pigments (carotenoids).

13.3 Saponins

Saponins exist in large concentration in healthy plants exhibiting strong antifungal activity. These molecules have been found to be determinants of plant resistance to fungal attack (Osbourn 1996). These compounds also exhibit other properties like piscicidal, insecticidal and molluscicidal. Saponins are triterpenoids with attached sugar group, i.e. glycosylated triterpenoids which exist in membranes of cell of several species of plants. Having detergent properties they disrupt the membranes of cells of attacking fungal pathogens. Saponins are classified into three major groups based on the types of aglycone which are triterpenoid, a steroid or a steroidal glycoalkaloid. Saponins exist in the form of *triterpenoid saponins* mostly in dicotyledonous plants and also in few monocots, while *steroid saponins* exist mostly in monocots, for example, plants belonging to family Agavaceae, Liliaceae, Dioscoreaceae, etc. Saponins in the form of *saponin digitoxin* mostly exist in dicots, for example, foxglove. *Avena* has both the types mentioned above (Price et al. 1987). Saponins in the form of steroidal glycoalkaloids exist mostly in Solanaceae family (potato, tomato, etc.) along with Liliaceae family. Saponins found in oats and tomato and their function in defense of plants against phytopathogenic fungi have been studied in detail (Osbourn 1996).

Some vital saponins involved in antimicrobial activity are avenacins and avenacosides (mainly present in oats and related species like *Arrhenatherum elatius*). Avenacins exist only in roots, while avenacosides exist in roots as well as shoots. Avenacosides which are inactive biologically are transformed into antifungal monodesmosidic saponin 26-desglucoavenacosides. Oats are able to resist root-infecting fungus because of the presence of triterpenoid avenacin saponins. *Gaeumannomyces graminis* var. *tritici* is not able to infect oat plant even though it wreaks havoc to wheat and barley plant because of the presence of avenacins (Fig. 13.5a).

One of the major saponin, α-tomatine (Fig. 13.5b), a monodesmosidic steroidal glycoalkaloid, exists in its biologically active form in a healthy plant. It safeguards the tomato plants from both *Verticillium albo-atrum* (Pegg and Woodward 1986) and vascular wilt fungi *Fusarium oxysporum* f.sp. *lycopersici* (Smith and Machardy 1982). Saponins prove to be toxic for fungi as a result of the ability of saponins to associate with sterols present in membrane leading to formation of pores (Price et al. 1987; Fenwick et al. 1992). Actions of saponins like α-tomatine are dependent upon pH, but some fungi render the saponins ineffective by altering the pH at the infection site. Plants have developed a mechanism to safeguard themselves from their own saponins by placing them in the vacuole or in other organelles, whose membranes can avoid lysis because of low or altered sterol composition.

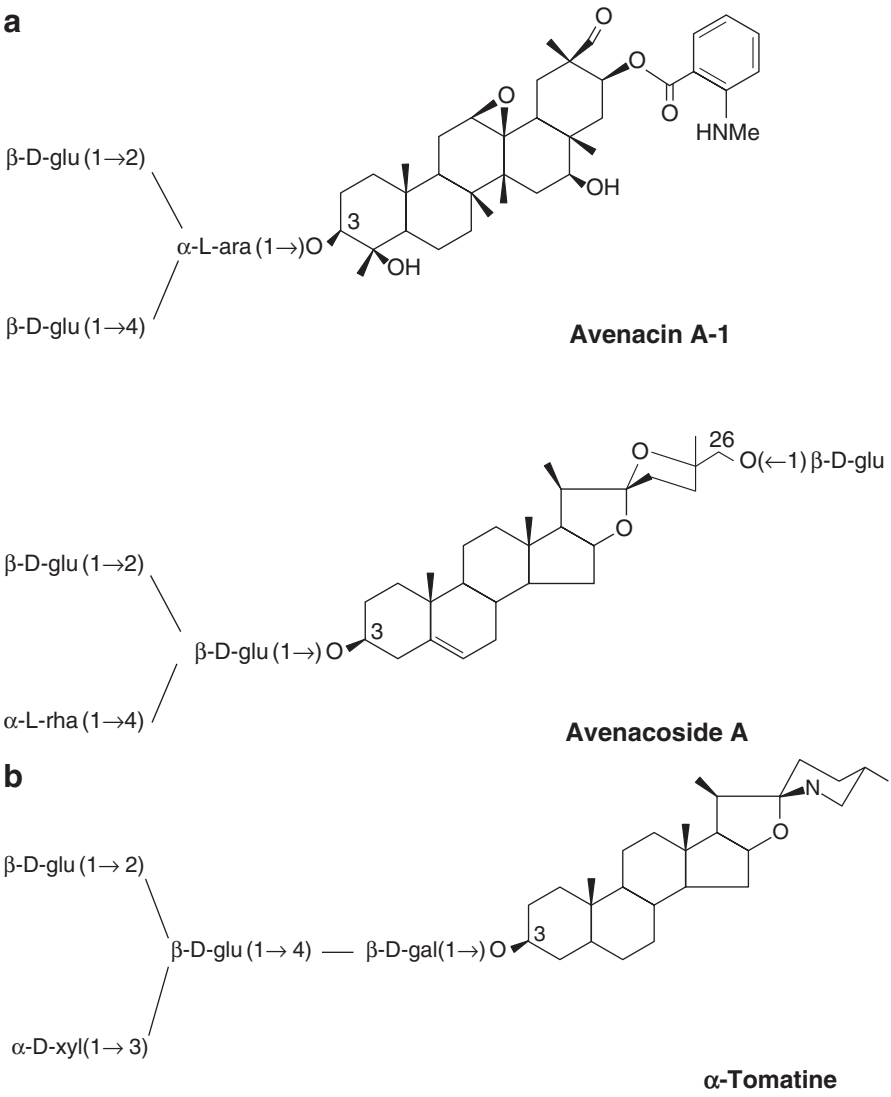
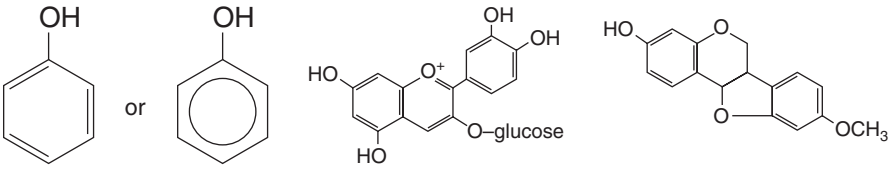


Fig. 13.5 (**a**) Structure of avenacin saponins present in oat (**b**) Structure of saponin present in tomato

13.4 Phenolics

Phenolics are a big class of secondary metabolites formed by plants to safeguard themselves against pathogens. They are created mostly via shikimic acid and malonic acid pathways in plants, comprising extensive variety of protective metabolites such as furanocoumarins, lignin, anthocyanins, flavonoids, tannins and



Phenol, the simplest phenolic compound

Cyanin glycoside, an anthocyanin

Medicarpin, a phytoalexin

Fig. 13.6 Structure of important phenolics

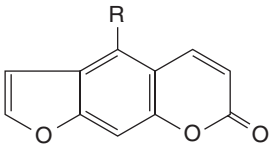
phytoalexins. These secondary products play a vital part in plant defense mechanism against threats like fungi, bacteria and nematodes as well as restraint herbivores from feeding the plant (Mazid et al. 2011). They contain phenol which is a hydroxyl functional group on an aromatic ring named phenol (Fig. 13.6).

13.4.1 Coumarin

Simple phenolic compounds made up of fused benzene and α-pyrone rings occur extensively in vascular plants. They protect various plants in many ways against threats from insects and fungi. They are mostly derived through shikimic acid pathway and are involved actively against wide range of microbes (Brooker et al. 2008). These cyclic compounds behave as natural pesticides for plants and represent a point from where the exploration of new derivatives which possess a range of enhanced antifungal activity can start. Derivatives of halogenated coumarin operate efficiently against fungal growth, for example, 7-hydroxylated simple coumarin works against *Orobanche cernua* preventing its penetration and germination in host vascular system. Some coumarins also show high defense activity against soilborne plant pathogenic fungi (Brooker et al. 2008). Hydroxycinnamic acid, related to coumarins, shows inhibition towards gram-positive bacteria. Phytoalexins which are hydroxylated derivatives of coumarins formed in carrots show antifungal activity.

13.4.2 Furanocoumarin

A wide variety of plants produce furanocoumarins which are phenolic compounds. Plants belonging to family of Apiaceae usually produce these compounds. These phenolic compounds become toxic only when activated by light (UV-A), and they get integrated into the pyrimidine bases of DNA of vertebrate and invertebrate herbivore causing rapid cell death due to blocked transcription and repair mechanism. Psoralen is a basic furanocoumarin used against fungi (Fig. 13.7).



Psoralen, furanocoumarin

Fig. 13.7 Structure of furanocoumarin psoralen

13.4.3 Lignin

Branched polymer of phenylpropanoid groups exists primarily in the secondary cell walls of plants. Three different types of alcohols which are coniferyl, coumaryl and sinapyl oxidize to free radicals (ROS) by a ubiquitous plant enzyme – peroxidase – which reacts randomly as well as simultaneously to produce lignin. The reactive proportions of these three monomeric units in lignin fluctuate among species, plant organs and even different layers of single cell wall (Lewis and Yamamoto 1990). These phenolic monomers are present hundred or thousand times in the structure of lignin and constitute the main component of wood. It is insoluble, rigid and virtually indigestible therefore serving as exceptional physical wall against attack by pathogens. The physical toughness and chemical durability of lignin causes it to be impossible for herbivores and insect pathogens to digest it. Lignification prevents the development of pathogens and microbes and is formed frequently during infection or wounding.

13.4.4 Flavonoids

Flavonoids, which are one of the biggest classes of plant phenolics, perform diverse roles from pigmentation to defense mechanism of plants. Flavones and flavonols are two main groups of flavonoids present in flowers. They protect the cells from harmful UV-B radiation by accumulating in epidermal layers of stems and plants (Lake et al. 2009). Anthocyanins as well as colourful water-soluble flavonoid pigments are formed by plants to protect their foliage from UV damage. They are hydroxylated phenolic substances existing as C_6–C_3 unit connected to an aromatic ring, produced by plants in response to microbial infection. Because of their capability to complex with extracellular and soluble proteins and bacterial cell wall proteins, they can act against extensive range of microorganisms. Lipophilic flavonoids, often pathogen specific in their toxicity, disrupt cellular structure, microbial membranes and pathogen metabolism. An example is catechin, a flavonoid occurring in leaves of oblong green tea. Tea leaves showing antimicrobial activity have mixture of catechin compounds working against various bacteria and microorganisms, for example, *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, etc. Phloretin existing in some varieties of apples displays action against many varieties of microorganisms. Galangin which

is produced from *Helichrysum aureonitens* (a perennial herb) is effective against extensive array of gram-positive bacteria as well as microbes and fungi.

13.4.5 Isoflavonoids

Isoflavonoids play a vital role in development of plant as well as defense mechanism. Their precursor is naringenin which is a flavanone intermediate, present in plants. They play a vital part in the formation of nitrogen-fixing nodules by symbiotic rhizobia and are secreted by legumes (Sreevidya et al. 2006). Phytoalexins are isoflavonoids, produced in response to pathogen attack having antibiotic and antifungal characteristics. These pathogen-specific, toxic compounds unsettle cellular structure and metabolism of pathogen, for example, medicarpin which is formed by alfalfa (*Medicago sativa*), rishitin also a toxin formed in tomatoes as well as potatoes (family Solanaceae) and camalexin formed by *Arabidopsis thaliana*.

13.4.6 Tannins

Tannins are group of polymeric phenolic substances which have the property of astringency which gives the ability to tan leather or precipitate gelatin from solution. They exist mostly in various plant parts like roots, bark, wood, fruits and leaves. Tannins are grouped into two types, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid (multiple aster of D-glucose), while flavonoid monomers are precursors of condensed one which are also known by the alias "proanthocyanidins". Condensed flavan derivatives in plant woody tissues and polymerization of quinone units are also known to form tannins. These phenolics are water soluble and mainly stored in vacuoles of cell. Tannins combine with salivary proteins and digestive enzymes of insects and herbivorous animals making the insect protein inactive. A herbivore or an insect having large intake of tannins does not gain weight and eventually dies. Tannins are capable of binding proteins, for example, protocatechuic and chlorogenic acids playing a vital role in disease resistance in certain plants. In onions smudge disease caused by *Colletotrichum circinans* (fungus) and growth of other fungi is restricted due to formation of these two tannins. Some tannin like chlorogenic acid is oxidized into effective quinones showing antifungal properties in case of some disease-resistant plants (Cowan 1999).

13.5 Nitrogen-Containing Secondary Metabolites

Nitrogen-containing secondary metabolites including alkaloids, cyanogenic glycosides and nonprotein amino acids are synthesized from common amino acids and are of significant interest due to their anti-herbivore defense role in plants.

13.5.1 Alkaloids

Alkaloids are the largest family of bitter-tasting nitrogenous compounds (Fig. 13.8) present in many vascular plants (20%) (Hegnauer 1988) mostly in herbaceous dicots and some gymnosperms and monocots. Pyrrolizidine alkaloids (Pas) are quite toxic and help in defense against infection caused by microbes and attack by herbivores. They are usually produced from few common amino acids like lysine, tyrosine, aspartic acid and tryptophan (Pearce et al. 1991). Cocoa, coffee and tea contain caffeine which is an alkaloid. These are toxic to both insects and fungi. Nicotine, an alkaloid, is formed by tobacco plant roots which is further transported to leaves of tobacco plant and is stored in vacuoles. Atropine, another neurotoxin and cardiac stimulant alkaloid, is formed by lethal nightshade plant (*Atropa belladonna*) and is highly toxic in large quantities. Capsaicin formed by members of genus *Capsicum* also has antimicrobial properties and works actively in plant defense mechanism. Their action involve effect on nervous system especially the chemical transmitters, membrane transport system, protein synthesis and miscellaneous enzyme activities (Creelman and Mullet 1997). Shikimic acid is a precursor for indole and its derivatives, amino acid tryptophan and its derivatives (psychedelic compounds, dimethyltryptamine), many alkaloids and other aromatic metabolites (Figs.13.9 and 13.10) which play an important part in resistance against microbes and fungi and nematodes.

13.5.2 Cyanogenic Glycosides

Hydrogen cyanide (HCN), a lethal chemical that halts cellular respiration in aerobic organisms, is produced by breaking down of cyanogenic glycosides (very deadly

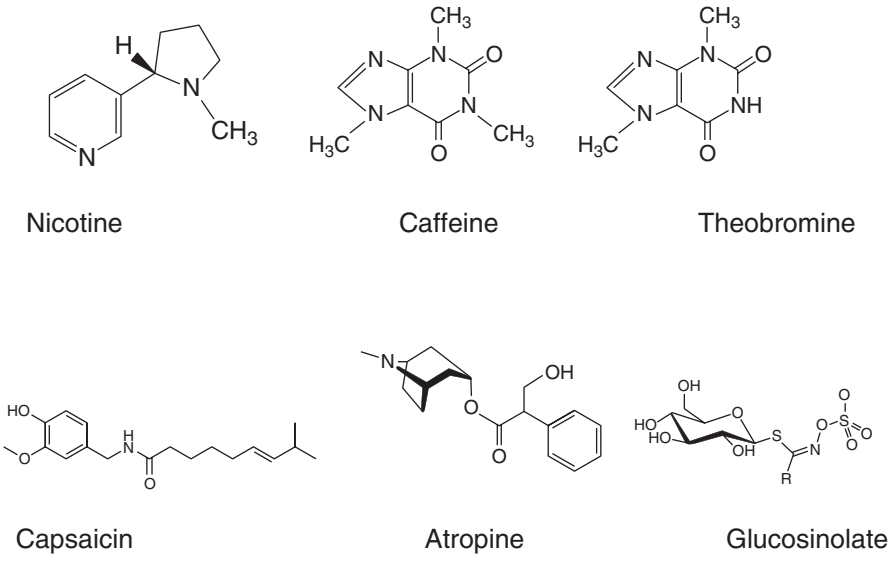
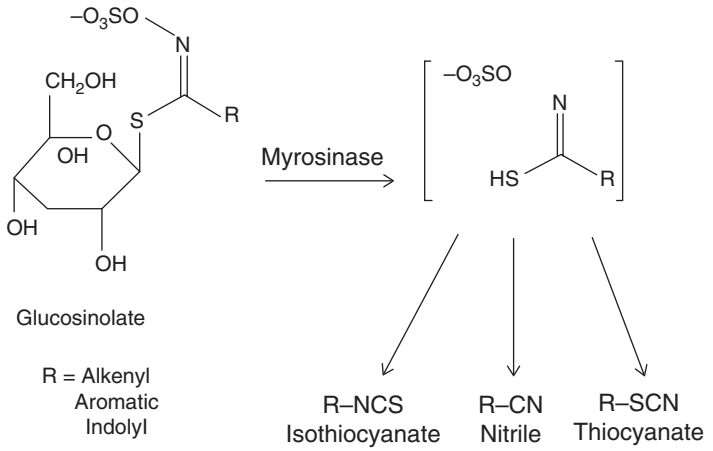


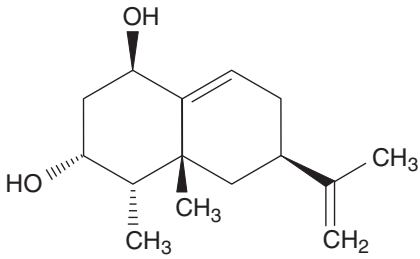
Fig. 13.8 Structure of common alkaloids



The structure of glucosinolates, and their myrosinase-catalysed breakdown.

Fig. 13.9 Structure of glucosinolates and their myrosinase - catalysed breakdown

Fig. 13.10 Structure of phytoalexin capsidiol



Capsidiol is a phytoalexin produced by certain plants in response to pathogenic attack

nitrogenous compounds). When the herbivores feed on the plants that produce cyanogenic glycosides, the plant tissue gets damaged which leads to mixing of enzyme (including glycosidases and hydroxynitrile lyases) and substrate which are stored in separate sections of the healthy plant, producing HCN. HCN binds itself to the Fe-containing heme group of cytochrome oxidase as well as other respiratory enzymes causing cellular respiration poisoning. Cyanogenic glycosides are mainly present in families Gramineae, Rosaceae and Leguminosae (Seigler 1991). Seeds of almond, apricot, cherries and peaches contain a common glycoside called amygdalin, whereas *Sorghum bicolor* contains dhurrin (Poulton 1990). Cassava has a long shelf life because of the presence of cyanogenic glycosides. Lima bean (*Phaseolus lunatus* L.) is a classic example of a plant which may be researched to understand defense mechanism in plants showing inducible indirect anti-herbivore properties achieved by producing VOC, i.e. volatile organic compounds (Ballhorn et al. 2009).

Among members of mustard family (Brassicaceae), mustard oil glycosides are present which when broken down by thioglucosidase enzymes produce cyanide gas. Cyanogenic glycosides are the best example where defenses are produced only in reaction to damage to plant tissue or attack by pathogen like "phytoalexins".

13.5.3 Nonprotein Amino Acids

Several plant species have rare amino acids known as nonprotein amino acids that do integrate into proteins but are also present as free forms and act as shielding defensive substance (Johnson et al. 1989). For example, azetidine-2-carboxylic acid and canavanine block the synthesis or uptake of protein amino acids. They are close analogs of arginine and proline. After consumption, canavanine (NPA) is detected by herbivore enzyme that sticks arginine to arginine tRNA molecule and gets assimilated into protein in place of arginine. Resulting protein is a nonutility one due to its faulty tertiary structure or disrupted catalytic site (Rosenthal 1991). Plants which produce nonprotein amino acids do not get harmed themselves due to its toxicity, but they gain defense against a wide array of pathogenic microbes, insects and herbivorous animals.

13.6 Sulphur Containing Secondary Metabolites

These metabolites directly or indirectly defend plants against pathogen microbes. Defensins, thionins, GSH, phytoalexins and GSL are the examples of sulphur containing secondary metabolites (Saito 2004; Grubb and Abel 2006; Halkier and Gershenzon 2006).

13.6.1 GSH

GSH is the main form of organic S which is present as soluble fraction in plants. GSH gets amassed in the plant after it is attacked by fungi. Main cellular sections of the plant can carry between 3 and 10 mM of GSH. GSH displays properties of an antioxidant as well as reducing agent in an effort to diminish the oxidative stress (Kang and Kim 2007).

GSH targets and detoxifies xenobiotic and cytotoxins in vacuoles. For synthesis of GSH and other phytochelatins involved in heavy metal detoxification, enzymes are produced inside specialized epidermal cells like trichomes.

13.6.2 GSL (Glucosinolates)

GSL increases the resistance of higher plants to parasites, predators as well as competitors. They are a group of N and S with low molecular mass and holding plant glycosides. The breakdown products which are released in the form of protective

volatile substances exhibit lethal or repelling effects (DeVos and Jander 2009), for example, allyl sulfoxides in *Allium* (Leustek and Saito 1999) and mustard oil glycosides in Cruciferae. Myrosinase catalyses the volatiles coming from GSL (Fig. 13.10) cleaving glucose from its bond with S atom. Resulting product (aglycone) reorganizes the loss of sulphate giving pungent as well as chemically reactive products, which includes isothiocyanates (R-N=C=S), thiocyanates and nitriles. These toxic and feed repelling products protect the plants from herbivores (Geu-Flores et al. 2009). It prevents various fungal diseases including light leaf spot (*Pyrenopeziza brassicae*), sclerotina stem rot (*Sclerotinia sclerotiorum*) and alternaria (*Alternaria brassicae*) from affecting the plants. The GSL become effective only when it comes in contact with myrosinase (a plant enzyme) which happens only if plant tissue gets damaged. (In healthy plants both enzymes and glucosinolate substrates are separated from each other due to compartmentalization.) The activity of enzymes involved in antioxidant defense mechanism may get affected by isothiocyanates, and defense of cell from DNA damage and GST activity may get affected because of detoxification from xenobiotic (Lipka et al. 2010). These three hydrolysis products are toxic for a wide array of fungi, and the breakdown products' nature depends upon the glucosinolate structure, myrosinase enzyme existing, the species of plant and variety of factors such as protein cofactors, temperature, metal ion concentrations and pH. Resistance of cabbage to *P. parasitica* and resistance of Indian mustard and oilseed rape to *L. maculans* have been associated with high glucosinolate levels. Breakdown products of glucosinolate also have effect on several nonpathogens of *Brassica*. These compounds are used as naturally available fungicides to regulate several pest harvest pathogens and cereal diseases related to fruits and vegetables (Mari et al. 1993).

13.6.3 Phytoalexin

Plants synthesize antimicrobial and often antioxidative elements de novo. These then accumulate quickly at infected areas affected by pathogen (bacteria or fungi) in order to limit the spread of invading microbes (Fig. 13.10). Organic phytoalexins produced by most of the plant families have a diverse chemistry, and mostly they are grouped under the classes including alkaloids, glycosteroids and terpenoids. The examples include isoflavonoids of Leguminosae and sesquiterpenoids of Solanaceae. S metabolites are only produced in Cruciferae plant family (Harborne 1999; Gross et al. 1994; Monde et al. 2000). The phytoalexins which are produced in the plants have a toxic effect on the attacking pathogens because they are responsible for rupturing the walls of the cell, delaying maturation, disrupting metabolism and preventing pathogen reproduction. The plant mutant which is incapable of producing phytoalexin experiences widespread presence of pathogens compared to wild plants which produce phytoalexin. Production of phytoalexins comes under systemic acquired resistance (SAR) or long-term resistance, which involves communication between the damaged tissue and the rest of the plant as well as usage and synthesis of plant hormones including salicylic acid, abscisic acid, ethylene and jasmonic acid. The entire mechanism involves genes that transcribe enzyme involved in synthesis of phytoalexins. Natural phenols such as isoflavonoids, polyphenols as well

as associated substances have very important part to play in defense of plant, for example, in *Vitis vinifera* (grape) trans-resveratrol (a phytoalexin) plays a role in plant defense against fungal pathogens including *Botrytis cinerea*, and another phytoalexin *delta*-viniferin is present in grapevine formed during fungal infection by *Plasmopara viticola*. Sakuranetin is a flavonoid type of phytoalexin found in *Polymnia fruticosa* and rice against the pathogen *Pyricularia oryzae.* 6-Methoxymellein (a dihydroisocoumarin) is synthesized in carrot slices by UV-c gene against the pathogen *Botrytis cinerea* (Kurosaki and Nishi 1983). Phytoalexin danielone exists in papaya fruit displaying extraordinary antifungal activity against *Colletotrichum gloeosporioides* (Echeverri et al. 1997). Stilbenes are a phytoalexin produced in *Eucalyptus sideroxylon* during pathogen attacks.

Allicin with the structure 3-hydroxy-5-methoxy-6-methyl-2-pentyl-4H-pyran-4-one is the first phytoalexin isolated from garlic (Illic et al. 2011). Allicin is another phytoalexin obtained from garlic. It is an organosulfur compound formed when alliinase enzyme converts alliin into allicin which also gives aroma to the fresh garlic. Allicin being an unstable compound quickly changes into a series of other sulphur-containing compounds such as diallyl disulphide having antibacterial, antifungal, antiviral or antiprotozoal activity. Other examples of associations of phytoalexin induction with the resistance in plants are displayed in Table 13.2. Production and function of phytoalexins for disease resistance can be restored or enhanced through genetic engineering techniques by introducing simple genetic constructs carrying phytoalexin synthesizing genes, for example, in grapevine, phytoalexin "resveratrol" is synthesized by stilbene synthase (STS) gene. STS genes (Vst1 and Vst2) from grapevine were transferred to tobacco through genetic transformation; and when the assays were performed in transformed plants, they showed higher resistance to *B. cinerea* (Hain et al. 1993). STS gene responsible for phytoalexin production was later transformed from different sources into many plant systems like *Arabidopsis*, papaya, tomato, wheat, barley, rice and alfalfa conferring resistance to various pathogens (Jeandet et al. 2013). In alfalfa, overexpression of enzyme isoflavone 7-O-methyltransferase played a critical part in biosynthesis of phytoalexin "maiackiain" which was associated with resistance of the plant to *Phoma medicaginis He and Dixon (2000).* Transformation of soya bean hairy root with both AhRS3 (peanut resveratrol synthase 3) and ROMT (resveratrol-0-methyltransferase) gene resulted in the resistance of transformed plant against *Rhizoctonia salami* (Zernova et al. 2014). Cytokinin overexpression in tobacco plant resulted in surge of resistance of plant against the pathogen *Phytophthora syringae* due to up-regulated synthesis of capsidiol and scopoletin, the two phytoalexins which we involved in the process (Grosskinsky et al. 2011). Similarly in the case of *Arabidopsis*, production of camalexin as well as resistance to *B. cinerea* was severally affected because of the mutation occurring in two MAP kinases (MPK3 and MPK6) (Ren et al. 2008). Some genes which function as phytoalexin biosynthesis regulators have also been identified, for example, overexpression of Rac protein in rice leads to accumulation of phytoalexin momilactone (Ono et al. 2001) and resistance of plant against bacterial blight. Overexpression of non-expressor of pathogenesis-related gene 1 induced the biosynthesis of phytoalexin gossypol (Parkhi et al. 2010).

Table 13.2 Phytoalexin induction and resistance to plant pathogens

Plant	Pathogen	Phytoalexin
Grapevine *{Vitis* spp.)	*Plasmopara viticola*	Viniferins
Grapevine *(Vitis* spp.)	*Botrytis cinerea*	Viniferins
Carnation *(Dianthus* spp.)	*Fusarium oxysporum*	Dianthalexin
		Methoxydianthramide S
Pea *(Pisum sativum)*	*Pseudomonas syringae*	Pisatin
Chickpea *(Cicer arietinum)*	*Ascochyta rabiei*	Medicarpin
		Maakiain
Citrus spp.	*Phytophthora citrophthora*	Scoparone
Oat *(Avena sativa)*	*Puccinia coronata*	Avenalumins

13.7 Proteins and Enzymes

Several plants and their seeds have protein that impedes pathogen and best enzymes after developing complexes that would block active sites or modify enzyme conformations. These proteins have small structure and mostly consist of amino acid cysteine. They include proteinase inhibitors, lectins, anhydrase inhibitors and defensins. Unlike simple chemicals like phenolics, alkaloids, terpenoids, etc. they need large amount of energy and resources of plant for their production. They are usually produced in significant concentration only after the attack by pest or pathogen. On activation these defensive protein and enzymes would effectively impede the growth of pathogens including fungi, bacteria as well as insect herbivores and nematodes.

13.7.1 Defensins

Small cysteine-rich protein displaying comprehensive antimicrobial activity was first isolated from endosperm of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*). They are best characterized in seeds but are also present in other plant tissues like leaves, pods, tubers, fruits, barks, etc. They display extensive array of biological activities and impede the spread of wide-ranging fungi and bacteria (Thomma et al. 2002). They also impede digestive protein in herbivores. The exact mechanism of their action is still under consideration; it looks like that they attack molecular targets in the pathogens' plasma membrane. They disrupt cellular ion balance by forming new membrane pores as well as impede pre-existing ion channels.

13.7.2 Digestive Enzyme Inhibitors

Digestive enzyme inhibitors inhibit the digestion and disrupt the nutrient absorption by herbivores. Alpha-amylase inhibitor proteins bind to amylase enzyme impeding digestion of starch in legumes. Glycoproteins and non-enzymatic proteins like

lectins bind to carbohydrates displaying various properties, for example, in invertebrates they cause lumping of blood cells and in insects they disrupt digestion (Peumans and Van-Damme 1995). Ricin combines lectin molecule with an N-glycoside hydrolase to produce a very powerful toxin in *Ricinus*. It impedes protein synthesis in animal cells.

13.7.3 Protease Inhibitors

Protease inhibitors impede digestive enzymes like chymotrypsin and trypsin. They are produced by plants as a defense mechanism to protect them from attack by herbivores. They have wide distribution but mainly seen in legumes, members of Solanaceae and grasses.

13.7.4 Hydrolytic Enzymes

When pathogens attack plants, some of the plants in order to defend themselves produce hydrolytic enzymes. These enzymes act upon pathogenic fungi by degrading their cell walls in the extracellular spaces where the enzymes get accumulated. For example, the degradation of chitin in cell wall of fungi is catalysed by chitinases. The degradation of glycosidic linkages in glucans existing in several oomycete (water moulds) cell walls is catalysed by glucanases. Another example of a hydrolytic enzyme with the ability to degrade cell walls of bacteria is lysozymes.

In this review we have discussed the utility of secondary metabolites occurring in plant in their defense system. We have tried our best to portray the role of different chemical compounds produced by plants itself against various plant pathogens. Several factors are involved in the interaction of a species of plants with its respective pest and non-pest and hence quite complex. Synthesis and accumulation of these compounds occur in tissues which are young and developing like leaves as well as in tissues of reproduction like seeds and flowers, thereby protecting young plant tissues. Some of the antimicrobial secondary metabolites are preformed, while others are induced by infection itself (phytoalexins, cyanogenic glycosides, glucosinolates, etc.). Organic farming can be made sustainable by identifying and properly using the natural chemical compounds to combat the pathogens, thereby eliminating or at least minimizing the use of fungicides in agriculture. Further research is required for development of natural pesticides. The genes required for producing these valuable defensive compounds can be isolated and then synthesized in bulk for the crop plants to be reengineered metabolically. This will make the plants more resistant towards threats posed by microbial pathogens, various herbivores and several environmental stresses.

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Explorations of Plant's Chemodiversity: Role of Nitrogen-Containing Secondary Metabolites in Plant Defense

14

Sanjay Kumar Singh

Abstract

In nature, plants are surrounded by a number of biotic and abiotic environmental stresses. Biotic ecosystems contain a wide variety of bacteria, viruses, fungi, nematodes, mites, insects, mammals, and other herbivorous animals, greatly responsible for heavy reduction in crop productivity. Henceforth, to cope up from these biotic stresses, the plant defense mechanism increasingly requires the availability of large numbers of phytochemicals. Chemodiversity in plants offers a valuable source; for example, nitrogen-containing secondary metabolites, previously regarded as waste products, are now recognized for their resistant activity against herbivores, pests, pathogens, and diseases. In this chapter, I have described the increasing role of nitrogen-containing secondary metabolites during plant defense. These metabolites impose their effects by acting as deterrence/antifeedant, toxicity, or precursors to physical defense systems. Many specialized herbivores and pathogens do not merely circumvent the deterrent or toxic effects of secondary metabolites but actually utilize these compounds as host recognition signals and/or nutrients. This is true for both cyanogenic glucosides and glucosinolates which are discussed in detail. Their biochemical and molecular mechanism of action is compared and contrasted.

Keywords

Secondary metabolites · Plant defense · Pathogen · Herbivores

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14.1 Introduction

The term chemodiversity, generally, leaves aside larger molecules, which involve in vital primary metabolic functions and form the majority of the organic body mass of living beings. Thus, small molecules that often have a defensive or offensive signaling function mainly contribute to the chemodiversity. Since the beginning, humans have utilized the plants, one of the most prolific sources of biochemical diversity, for its own benefits. Since ancient times, plants have provided mankind with cures for health problems and continue to be the most capable pool of bioactive chemicals for the development of modern drugs (Dias et al. 2012; Cragg and Newman 2013; Harvey et al. 2015). More than 20,000 natural molecules have been studied so far, and numerous have been used as novel anticancer, antibiotic, anti-inflammatory or anti-pain agents, etc. In the previous few decades, plants have turned into a critical source for the discovery of novel and unique pharmaceutical compounds (Cordell 2000; Farnsworth 1988; Newman et al. 2000). Plants are reported to have high chemodiversity including more than 21,000 alkaloids, 700 nonprotein amino acids (NPAAs), 200 cyanogenic glycosides (CGs) and glucosinolates, >20,000 terpenoids, >10,000 polyphenols, >1500 polyacetylenes and fatty acids, 750 polyketides, and 200 carbohydrates (Wink 2008, 2013; Theis and Lerdau 2003).

Approximately 450 million (M) years ago, plants began to inhabit the terrestrial earth during the mid-Ordovician period and over the subsequent 40 M years spread across the earth surface. The evolution of species-specific metabolic systems from core metabolic pathways of aquatic ancestors was one of the reasons behind the success of early land plants, as they were able to synthesize the structurally and functionally diverse chemicals to cope with frequent biotic and abiotic ecological pressures (Weng et al. 2012). Several of these chemicals, such as cuticular components and phenolic compounds, are universal in all land plants and, therefore, provide indispensable physical and chemical protection against desiccation and UV radiation (Fig. 14.1). Other classes of specialized metabolites, including those that contribute to plant-specific flavors, colors, and scents, frequently occur in a lineage-specific manner and play specialized roles for the host species in their natural habitat (Weng et al. 2012). Present knowledge of secondary metabolism and its evolution in the plant has been primarily driven by studying of angiosperms or flowering plants, ranging from well-studied model species, such as rice and *Arabidopsis* (Romeo 2004; D'Auria and Gershenzon 2005), to the reference species including medicinal plants with remarkable pharmaceutical properties, e.g., *Vinca minor*, *Catharanthus roseus*, and *Rauvolfia serpentina* (Facchini and De Luca 2008; De Luca et al. 2012; Patra et al. 2013). These studies revealed massive chemical diversity in flowering plants and provide deep insight on their widespread speciation and global domination over the last 170 M years following the Permian-Triassic extinction event (Wikström et al. 2001). The vast expansion of plant chemodiversity associated with secondary metabolites reflects the tremendous adaptability of land-dwelling plants. For example, plant hormones regulate various aspects of plant growth and development in response to environmental cues, whereas phenolic and waxy cuticles act as UV protectant and prevent excessive water loss. Plant polymers

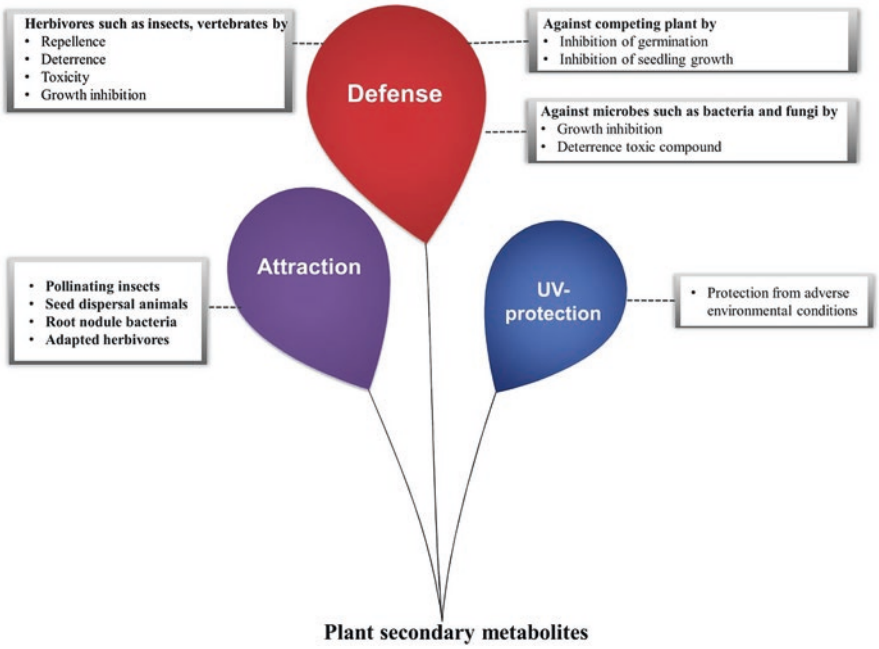

Fig. 14.1 Functional diversity of plant secondary metabolites

including lignin and sporopollenin provide mechanical support, gamete protection, and wound healing. New metabolic pathways continuously arose throughout terrestrial plant evolution, resulting in a contemporary collection of secondary metabolites. Therefore, some of these specialized metabolites are common across various taxonomic groups, while others were found in some limited species.

14.2 Secondary Metabolites Are Divided into Three Major Groups

On the basis of their chemical nature, plant secondary metabolites can be divided into three chemically distinct groups: terpenes, phenolics, and nitrogen-containing compounds.

14.2.1 Terpenes

Terpenes (also known as terpenoids) constitute the largest class of secondary metabolites. Plants and other natural sources are reported to produce more than 30,000 terpenoids (Bohlmann et al. 1998).

In plants, terpenes are biosynthesized in at least two different pathways. The main and well-studied biosynthetic route is known as the mevalonic acid (MA) pathway.

Table 14.1 Important molecules of terpenoids

Number of carbon	Name	Example
C5	Hemiterpene	Isoprene, prenol, isovaleric acid
C10	Monoterpene	Limonene, eucalyptol, pinene
C15	Sesquiterpene	ABA (abscisic acid)
C20	Diterpene	Gibberellin
C25	Sesterterpenes	Ophiobolin A, ceroplastol
C30	Triterpene	Brassinosteroids, squalene, lanosterol
C40	Tetraterpene	Carotenoids, lycopene
C>40	Polyterpenes	Ubiquinones, rubber, cytokonines, vitamin E

In the MA pathway, three molecules of acetyl-CoA are joined together in a stepwise manner to form MA. This key six-carbon intermediate then undergoes different chemical modifications like pyrophosphorylation and decarboxylation to produce isopentenyl diphosphate (IPP). Finally, IPP acts as a building block of terpenes. The second route of terpene biosynthesis is known as methylerythritol-4-phosphate (MEP) pathway, which operates in plastids (Tholl and Lee 2011; Lichtenthaler 1999). Glyceraldehyde-3-phosphate and two carbon atoms derived from pyruvate condense to form the five-carbon intermediate, 1-deoxy-d-xylulose 5-phosphate. The 1-deoxy-d-xylulose 5-phosphate further rearranged and reduced to MEP, which eventually converted into IPP.

Terpenes are the structurally diverse class of secondary metabolites from hemi- to polyterpenes (Table 14.1). All terpenes are originated from the union of five-carbon elements (also referred to as C5 units) that have the branched carbon skeleton of isopentane. The basic structural elements of terpenes are also known as isoprene, and, thus, terpenes are sometimes also called as isoprenoids. The terpenes can be classified in different groups on the basis of a number of C5 units they comprised of (Table 14.1). For instance, 10-carbon terpenes, which contain two C5 units, are called monoterpenes, while 15-carbon terpenes (three C5 units) are sesquiterpenes. In spite of structural similarities, terpenes can be synthesized in different compartments in the cell. For instance, nowadays it is believed that sesquiterpenes and triterpenes are synthesized through the cytosolic MA pathway, whereas mono-, di-, and tetraterpenes are derived from the chloroplastic MEP pathway (Thimmappa et al. 2014).

Terpenes have roles in both primary and secondary metabolism. Certain terpenes have been well studied for their functions in plant growth or development and therefore can be considered as primary rather than secondary metabolites. For instance, the gibberellins, an important group of phytohormones which are essential for numerous growth and developmental processes in plants including seed germination, leaf expansion, stem elongation, pollen maturation, trichome development, and the induction of flowering (Achard and Genschik 2009), are diterpenes. Brassinosteroids, also a class of plant hormones with growth-regulating functions such as activation of the cell cycle during seed germination (Zadvornova et al. 2005), control of cell cycle progression (González-García et al. 2011), and induction of exaggerated growth of hydroponically grown plants (Arteca and Arteca

2001), are derived from triterpenes. Terpenes are toxins and also act as a feeding deterrent to many herbivorous insects and mammals (Gershenzon and Croteau 1992). For instance, pyrethroids, a monoterpene ester reported from *Chrysanthemum* species, show remarkable insecticidal activity (Mori 2012). Monoterpenes accumulate in resin ducts found in the needles, twigs, and trunk of conifers, such as Douglas-fir, lodgepole pine, *Pinus contorta*, *Picea engelmannii*×*glauca*, and *Abies lasiocarpa*×*bifolia*, and are toxic to numerous insects, including bark beetles, a serious pest of conifer species throughout the planet (Trapp and Croteau 2001).

Essential oils, which lend a characteristic odor to their foliage, are mixtures of volatile monoterpenes and sesquiterpenes. Essential oils have been broadly used for bactericidal, virucidal, fungicidal, insecticidal, medicinal, and cosmetic applications (Isman 2000). Recently they are also used in pharmaceutical, sanitary, cosmetic, agricultural, and food industries (Holley and Patel 2005). *Mentha piperita*, *Citrus limon*, *Ocimum basilicum*, and *Salvia officinalis* are some well-known plants that contain essential oils. Essential oils are frequently found in glandular hairs and serve to repel the potential herbivores even before they take a trial bite. Caryophyllene, a sesquiterpene, is a common constituent of the essential oil of numerous plants including *Piper nigrum* and *Syzygium aromaticum*. Caryophyllene is known to possess anti-inflammatory, antimicrobial, anticarcinogenic, antibiotic, antioxidant, and local anesthetic properties (Legault et al. 2013; Kuwahata et al. 2012; Lee et al. 2005).

14.2.2 Phenolic Compounds

Plants produce a large variety of secondary metabolites that contain a phenol group: one or more hydroxyl functional groups on benzene rings (Randhir et al. 2004). These substances are classified as phenolic compounds or phenolics. The structures of these phenolics may range from simple phenolic molecule to complex high-molecular-weight polymer (Velderrain-Rodriguez et al. 2014). Phenolic compounds are found in nearly all the plant kingdom and located in nearly all plant parts. Main classes of phenolic compounds reported in higher plants are given in Table 14.2.

Shikimic acid and malonic acid are two basic pathways involve in the biosynthesis of phenolic compounds in plants. The shikimic acid pathway is involved in biosynthesis of most plant phenolics. Shikimic acid pathway converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway (PPP) into the three aromatic amino acids: phenylalanine, tyrosine, and tryptophan. Phenylalanine acts as a precursor of biosynthesis of most abundant classes of secondary phenolic compound in the plant.

Phenolic compounds play a vital role in growth and reproduction of plants, providing protection against pathogens and herbivores (Bravo 1998). Phenolic compounds are also involve in providing the color and sensory characteristics of fruits and vegetables (Alasalvar et al. 2001), in absorbing harmful ultraviolet (UV) radiation, and in reducing the growth of nearby competing plants. Phenolic compounds also have a wide range of physiological properties, such as antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, cardioprotective, and vasodilatory

Table 14.2 Main classes of phenolic compounds in higher plants

Classes and subclasses	Examples of specific compounds	Natural sources
Non-flavonoid compounds		
Phenolic acids	Hydroxybenzoic acids; hydroxycinnamic acids	*Macrotyloma uniflorum*
Benzoic acids	Gallic acid; protocatechuic acid	Quercus infectoria, *Hibiscus sabdariffa*, *Vitex agnus-castus*
	4-hydroxybenzoic acid	
Hydroxycinnamic acid	Coumaric acid; caffeic acid; ferulic acid; sinapic acid	*Arachis hypogaea*, *Eucalyptus globulus*, *Citrus limon*
Hydrolyzable tannins	Pentagalloylglucose	*Rhus chinensis*
Stilbenes	Resveratrol	*Fallopia japonica*
Lignans	Secoisolariciresinol; matairesinol; lariciresinol; pinoresinol	*Linum usitatissimum*, *Sesamum indicum*
Flavonoid compounds		
Condensed tannins or proanthocyanidins	Procyanidin, prodelphinidins	*Vitis vinifera*
Anthocyanidins	Pelargonidin; cyanidin; malvidin	*Geranium dissectum*, *Philodendron bipinnatifidum*
Flavanols	Catechins; gallocatechins	*Uncaria rhynchophylla*, *Camellia sinensis*
Flavanones	Naringenin; hesperetin	Citrus × paradisi, *Mentha aquatica*
Flavones	Apigenin; luteolin	*Petroselinum crispum*, *Apium graveolens*, *Ambrosia psilostachya*
Flavonols	Kaempferol; quercetin; myricetin	*Aloe vera*, *Coccinia grandis*
Isoflavones	Daidzein; genistein; glycitein	*Pueraria mirifica*

effects (Benavente-Garcia et al. 2000; Manach et al. 2005; Middleton et al. 2000; Puupponen-Pimiä et al. 2001; Samman et al. 2001).

Lignin is formed from three different phenylpropanoid alcohols, namely, coniferyl, coumaryl, and sinapyl. The physical toughness of lignin acts as a herbivore deterrent, while its chemical durability makes it relatively indigestible to herbivore and insect pathogens (Lattanzio et al. 2006; Rosenthal and Berenbaum 2012). The flavonoids, one of the largest classes of plant phenolics, are involved in pigmentation and defense (Treutter 2005). Tannins, a mainly constituent of woody plants, are general toxins that significantly reduce the growth and survivorship of many herbivores and also act as feeding repellents (Barbehenn and Peter Constabel 2011). Protocatechuic acid prevents smudge in onions, a disease caused by the fungus *Colletotrichum circinans*, and prevents spore germination and growth of other fungi as well (Kakkar and Bais 2014).

14.2.3 Nitrogen-Containing Compounds

A large number of plant secondary metabolites have nitrogen as part of their structure. They are synthesized from common amino acids. Nitrogen-containing secondary metabolites can be categorized into four categories: alkaloids, cyanogenic glycosides, glucosinolates, and nonprotein amino acids.

14.2.3.1 Alkaloids

Alkaloids are typically defined as plant-derived pharmacologically active basic compounds, which synthesized from amino acids and may contain one or more heterocyclic nitrogen atoms. The alkaloids are an extremely heterogeneous group of more than 15,000 nitrogen-containing secondary metabolites. The alkaloids include more than 150 families and found in around 20% of the vascular plant species. Alkaloids in plants are common in families of seed-bearing vascular plants or angiosperms, e.g., Magnoliaceae, Solanaceae, Papaveraceae, Leguminosae, Ranunculaceae, Rubiaceae, and Apocynaceae. The alkaloidal plant species may contain single or multiple alkaloids. For example, *Catharanthus roseus* contains 130 terpenoid indole alkaloids, including anticancerous vinblastine, and their synthesis can be regulated by multiple pathways (van Der Heijden et al. 2004; Patra et al. 2013). The alkaloids can accumulate in a different part of the plants including leaf, epidermal and hypodermal cells, bundle sheaths, and latex vessels. Alkaloids are usually synthesized from one of a few common amino acids, such as lysine, tyrosine, or tryptophan. However, the basic carbon skeleton of some alkaloids may contain a component derived from the terpene pathway also. Table 14.3 lists the major alkaloid types, their amino acid precursors, and natural plant sources. Alkaloids usually occur as salts of organic acids, such as acetic, malic, lactic, citric, and oxalic, in plants, while some basic alkaloids, like nicotine, also occur freely in nature (Ramawat et al. 2009). Very often, the alkaloids are biosynthesized in a particular plant organ but accumulate in another. For example, in tobacco, nicotine is synthesized in roots but is translocated to and stored in leaves (Shoji et al. 2000; Yazaki 2005; Morita et al. 2009). The alkaloids may be divided into three subclasses: proto-alkaloids, true alkaloids, and atypical alkaloids. Proto-alkaloids and true alkaloids are directly derived from amino acids, while atypical alkaloids are derived from sources other than amino acids, e.g., terpenoid-containing alkaloids.

14.2.3.1.1 Proto-alkaloids

These are nitrogen-containing alkaloids which originated from amino acids. Proto-alkaloids include mescaline, adrenaline, and ephedrine.

14.2.3.1.2 True Alkaloids

These alkaloids, generally, contain a heterocyclic ring with nitrogen, derived from amino acids and always basic in nature. These alkaloids are toxic and normally present in plants as salts of organic acids, e.g., nicotine, morphine, and codeine.

Table 14.3 Example of some true alkaloids and their natural sources

Alkaloid class	Example	Natural occurrence	Biosynthetic precursor
Pyrrolidine	Stachydrine, hygrine	*Erythroxylum coca*,	Aspartate
		Leonurus japonicus	
Piperidine	Coniine, piperine, solenopsin	*Piper nigrum*	Lysine
		Psilocaulon absimile	
		Petrosimonia monandra	
		Conium maculatum	
Tropane	Atropine, racemic, hyoscyamine	*Atropa belladonna*	Aspartate
		Hyoscyamus niger	
		Mandragora officinarum	
Isoquinoline	Papaverine, narcotine, berberine	*Papaver somniferum*	Tyrosine
		Argemone mexicana	
Quinolizidine	Lupinine	*Lupinus albus*	Lysine
Indole	Reserpine, ergatomine	*Ipomoea violacea*	Tryptophan
		Turbina corymbosa	
Pyrrolizidine	Heliotridine	*Adenostyles alliariae*	Aspartate
		Cordia myxa	

14.2.3.1.3 Atypical Alkaloids

These are alkaloid-like compounds that do not derive from amino acids. The atypical alkaloids include terpene-like alkaloids, steroid-like alkaloids, and purine-like alkaloids such as caffeine, theobromine, ephedrine, colchicine, erythromycin, and taxol. These are less commonly found in nature.

14.2.3.2 Cyanogenic Glycosides (CGs)

CGs are a group of nitrile-containing plant secondary metabolites that produce cyanide following their enzymatic breakdown. There are approximately 25 known CGs which occur in at least 2600 plant species, such as members of Fabaceae, Rosaceae, Leguminosae, Linaceae, and Compositae family, of which a number of species are used as food including apples, apricots, cherries, peaches, plums, quinces, cassava, peas, beans, barley, and sorghum (Eisler 1991; Haque and Bradbury 2002; Ganjewala et al. 2010; Vetter 2000). Chemically, CGs are glycosides of α-hydroxynitriles which are stored in cell vacuoles (Vetter 2000; Fleming 1999). The CG content in plant discourages feeding by insects and other herbivores. Most of the CGs are believed to be derived from L-valine, L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine, and cyclopentenyl-glycine, a nonprotein amino acid. In plants, CG biosynthesis occurs in three steps (Vetter 2000). In the first step, two successive N-hydroxylations of amino group of parent amino acid are catalyzed by an enzyme of cytochrome P450 family which, finally, converted into aldoxime. The second step includes conversion of aldoxime into cyanohydrin by another cytochrome P450 enzyme. In the final step, cyanohydrins get glycosylated by a soluble enzyme

UDP-glucosyltransferase. CGs play pivotal roles in organization of chemical defense system in plants and in plant-insect interactions (Zagrobelny et al. 2004).

14.2.3.3 Glucosinolates

Glucosinolates (also known as mustard oil glycosides) are the second class of glycoside after CGs. Glucosinolates are sulfur- and nitrogen-containing plant secondary metabolites common in the agriculturally important Brassicaceae family. Glucosinolates degrade to produce the compounds responsible for the smell and taste of vegetables such as cabbage, broccoli, and radishes, which act as toxin and herbivore repellents. More than 130 glucosinolates have been identified in plants (Radojčić Redovniković et al. 2008). The glucosinolate biosynthesis comprises three steps: amino acid chain elongation, conversion of the amino acid moiety to the glucosinolate core structure, and subsequent side chain modifications. The structural diversity of glucosinolates arises from side chain elongation of the amino acid precursors and from various secondary modifications including oxidation, desaturation, hydroxylation, methoxylation, sulfation, and glucosylation. Most glucosinolates in the member of the Brassicaceae are synthesized from methionine that is modified by the sequential addition of one to nine additional methylene groups to its side chain (Graser et al. 2000). Glucosinolates are stored in the intact plant discretely from the enzymes (myrosinase) that hydrolyze them, and they are brought into contact with the hydrolyzing enzymes only when the plant is crushed because of wounding and insect or pathogen attack. Loss of cellular integrity triggers the binary glucosinolate-myrosinase system and causes the generation of thioglucose, sulfate, and an unstable intermediate which spontaneously rearranges into several degradation products which can include nitriles, epithionitriles, isothiocyanates, oxazolidine-2-thiones, and thiocyanates (Radojčić Redovniković et al. 2008).

14.2.3.4 Nonprotein Amino Acids (NPAAs)

There are common 20 amino acids, also referred to as protein amino acids, which are incorporated into proteins by plants and animals. Nonetheless, several plants also contain unusual amino acids, called NPAA, that are not incorporated into proteins. Instead, these NPAAs are present in the free form and act as defensive molecules. Many NPAAs are very similar in structure to protein amino acids and, therefore, have similar properties. NPAAs can mimic the behavior of standard amino acids and, thus, can act as metabolic antagonists or inhibitors. For instance, canavanine and azetidine-2-carboxylic acid have structure much like that of arginine and proline, respectively. About 900 NPAAs have been isolated from plants. Of these, some 250 are found, particularly, within a small subset of plant families including the Hippocastanaceae, Leguminosae, Sapindaceae, Aceraceae, and Cucurbitaceae (Wink 2011).

14.2.4 Role of Nitrogen-Containing Secondary Metabolites in Plant Defense

Plants have a range of defense mechanisms, which occur soon after the pathogen attack that leads to the formation of a wide range of phytochemicals and by-products including nitrogen-containing secondary metabolites. These chemicals help the plant to respond to the incompatible interaction and finally help them to cope up with adverse conditions (Dixon 2001).

14.2.4.1 Alkaloids

Alkaloids are a diverse group of secondary metabolites with a variety of targets and biological activities including interference with neurotransmitters, disruption of DNA replication, and inhibition of protein synthesis (Mithöfer and Boland 2012). Alkaloids are produced by a large number of higher plant species and mostly involved in defense-related functions such as inhibition of competitors and herbivore deterrents (Roberts 2013). The inhibitory effects of alkaloids on glycosidase and trehalose metabolism deter herbivores, and the capability to quench singlet reactive oxygen confers protection against this toxic photosynthetic by-product (Mithöfer and Boland 2012; González-Lamothe et al. 2009). Alkaloids also act as phytoanticipins and phytoalexins and, naturally protect the plants from disease (González-Lamothe et al. 2009). The α-tomatine, for example, is a spirosolane-type alkaloid that occurs in tomato plants and possesses antimicrobial, antifungal, and anti-inflammatory activities (Friedman 2002; Chiu and Lin 2008; Ito et al. 2007; Morrow et al. 2004; Simons et al. 2006; Thorne et al. 1985). Several potently antibacterial alkaloids have been identified in the different classes of alkaloid including indole, indolizidine, isoquinoline, aaptamine, piperazine, quinoline, quinolone, aaptamine-indole, bisindole, and indole-quinoline in plants like *Zanthoxylum tetraspermum*, *Prosopis glandulosa*, *Clausena heptaphylla*, and *Teclea afzelii* (Maneerat et al. 2012; Chakraborty et al. 1995a, b; Samoylenko et al. 2009; Nissanka et al. 2001; Iwasa et al. 2001; Kuete et al. 2008; Wang et al. 2013).

Alkaloids have toxic and repellent effects on a wide range of generalist herbivores in order to reduce or prevent damage to plants (van Dam et al. 1995; Hartmann 1999; Hartmann and Ober 2000; Ober 2003). Sugar-mimic alkaloids act as inhibitors of several sugars and glycosidase-metabolizing enzymes leading to toxic effects on the insect. Morus species are a good example of plants that contain sugar-mimic alkaloids. Leaves exude of *Morus* species rich in sugar-mimic alkaloids, 1,4-dideox y-1,4-imino-d-arabinitol and 1-deoxynojirimycin, which are toxic to the *Samia ricini* (also known as eri silkworm), a generalist herbivore, but not to the domesticated silkworm, *Bombyx mori*, a mulberry specialist (Hirayama et al. 2007). Yasuda et al. (2002) reported 13 sugar-mimic alkaloids from the pods of *Angylocalyx pynaertii*, a member of Leguminosae (Yasuda et al. 2002). The nature of toxicity and target of plant alkaloid can be diversified but frequently involves in cell signaling disruption (Mithöfer and Boland 2012). Sanguinarine ((13-methyl[1,3] benzodioxolo[5,6-c]-1,3-dioxolo[4,5]phenanthridinium), a benzophenanthridine alkaloid, mainly found in the Papaveraceae family, which includes *Sanguinaria*

canadensis, *Argemone mexicana*, and *Chelidonium majus*, is shown to have antioxidant, antitumor, antibacterial, and anti-inflammatory properties (Chaturvedi et al. 1997). Sanguinarine is also reported to suppress cyclooxygenase, lipoxygenase, cholinesterase, Na^+/K^+-ATPase, cAMP- and Ca^{2+}-dependent protein kinase, NF-κB activation, nitric oxide synthase, and mitogen-activated protein kinase phosphatase-1 activities (Jeng et al. 2007; Vavrečková et al. 1996; Ulrichová et al. 1983; Seifen et al. 1979; Wang et al. 1997; Chaturvedi et al. 1997; Huh et al. 2006; Vogt et al. 2005). Sanguinarine inhibits choline acetyltransferase, an enzyme that catalyzes the biosynthesis of the neurotransmitter acetylcholine, and, finally affect neurotransmission. Nicotine, mostly found in leaves of *Nicotiana* species, binds to nicotinic acetylcholine receptors and blocks or displaces the endogenous neurotransmitters. Nicotine acts as either an agonist or antagonist targeting nicotinic acetylcholine receptors in insects, causing continual stimulation of the parasympathetic nervous system which finally leads to paralysis and death of insect (Dewey and Xie 2013).

Toxic effects of plant alkaloids on bacterial and fungal activities have been shown in a number of studies. Quinolizidine alkaloids (QAs) which frequently occur in members of Fabaceae family, like *Lupinus*, *Baptisia*, *Thermopsis*, *Genista*, *Cytisus*, *Echinosophora*, and *Sophora*, are involved in plant protection against insect pests (Philippi et al. 2015; Wang et al. 2000; Zhao et al. 1998). QAs extracted from *Lupinus angustifolius* and *Genista vuralii* have shown to have antibacterial properties (Erdemoglu et al. 2007, 2009). The antifungal properties of alkaloids also have been proved for several plant-associated fungi by bioassay experiments (Wippich and Wink 1985; Ma et al. 1999; Zhao et al. 1998; Zhou et al. 2003). The antifungal alkaloids are reported from different plants, such as *Corydalis incisa*, *Corydalis ambigua*, *Dictamnus dasycarpus*, and *Veratrum taliense*, which are reported to be effective against a wide range of phytopathogenic fungi including *Cladosporium cucumerinum*, *Erysiphe graminis*, *Cladosporium herbarum*, *Phytophthora capsici*, and *Rhizoctonia cerealis*.

14.2.4.1.1 Cyanogenic Glycosides

CGs can act as a defense molecule both against herbivory and phytopathogens. In general, an inverse correlation is frequently reported between the degree of herbivore pressure and the CG content in plant (Schappert and Shore 1999; Gleadow and Woodrow 2000; Ballhorn 2011). Dhurrin (4-hydroxymandelonitrile-β-d-glucoside) is a well-studied CG, reported to be present in several plant species including *Sorghum bicolor.* Dhurrin acts as an oviposition activator for the pests such as *Atherigona soccata* and *Chilo partellus* (Alborn et al. 1992). Efficient hydrolysis of dhurrin and, subsequent, release of cyanide are essential to deter insect herbivory in *Sorghum bicolor* (Krothapalli et al. 2013). Larvae of *Phyllotreta nemorum* eat 80% less tissue of the dhurrin-overproducing transgenic *Arabidopsis* plant compared to wild-type (Tattersall et al. 2001). The CG content, the rate of HCN release, and the susceptibility of the attacker to HCN are three main factors which determine the effectiveness of CGs against attackers (Ballhorn et al. 2005; Kadow et al. 2012). Many organisms, including humans, have mechanisms to detoxify and excrete HCN; therefore, HCN poisoning occurs only when the rate of detoxification is

lesser than the rate of intake. Depending on the insect species, CGs can act both as feeding deterrents or phagostimulants. For instance, CG acts as a feeding stimulant for *Spodoptera eridania* larvae as it prefers to graze on CG-containing plants, such as *Phaseolus lunatus*, and grows better when cyanide is present in their diet (Brattsten et al. 1983). In contrast, *Prunus dulcis* plants with a high concentration of CGs are resistant to larvae of *Capnodis tenebrionis* (Malagon and Garrido 1990). Ellsbury et al. (1992) studied the variation in feeding damage to *Trifolium repens* (white clover) by larvae of *Hypera postica* (alfalfa weevils) (Ellsbury et al. 1992). They found that larvae of *Hypera postica* preferred leaflets of *Trifolium repens* with less or no CG content. Although all CGs have a potential danger through the production of HCN, there are differences in the sensitivity of different animal species. CG content of *Prunus padus*, also known as bird cherry, triggers the anorexia, weakness, depression, stupor, circling, bruxism, excessive salivation, and tenesmus in herbivores which, finally, leads to death (Sargison et al. 1996). CGs are also reported to have the antifungal properties. For instance, CGs can inhibit the growth of some fungi, such as *Magnaporthe oryzae* (also known as blast fungus), in dose-dependent manner (Seo et al. 2011).

CGs can be harmful to human also. Different types of CGs may be found in various cyanogenic food plants, for example, taxiphyllin in bamboo shoots and linamarin and lotaustralin in cassava (Organization 2013). The tubers of cassava which is used as staple food in many tropical countries, such as the Pacific Island countries, Latin America, Africa, and regions of Asia, contain high levels of CGs. Although traditional tuber processing methods, such as grating, grinding, soaking, and drying, caused the removal or degradation of a major fraction of the CGs present in cassava tubers. However, partial paralysis of the limbs caused by chronic cyanide poisoning is still widespread in cassava-eating regions. Tropical ataxic neuropathy and konzo are some health-related issues that can be caused by continuous dietary exposure to CGs (Tylleskär et al. 1992; Ernesto et al. 2002; Oluwole et al. 2000).

14.2.4.1.2 Glucosinolates

Most of the glucosinolates in plants are involved in responses to external or environmental stimuli. Glucosinolates are also involved in communicating and activating a variety of information relating to plant defense against insects, bacteria, and fungi. Depending on developmental stage and environmental condition, glucosinolate pattern varies between species and ecotypes as well as between and within individual plants. Environmental conditions such as temperature and light (Hasegawa et al. 2000; Engelen-Eigles et al. 2006), changes in nutritional status (Kaur et al. 1990; Underhill et al. 1980), biotic (e.g., fungal infection and insect damage), and abiotic (e.g., wounding) (Halkier and Gershenzon 2006; del Carmen et al. 2013) stress can alter the glucosinolate profile significantly. A change of the glucosinolate profile by several environmental factors has supported the idea regarding possible roles of glucosinolates in the plant defense against insects, herbivores, and microbial pathogens.

Glucosinolates and their hydrolysis products evidently act as mediators in plant-insect interactions. Glucosinolates can function as general poison and deterrent for generalist insects. Glucosinolates in *Brassica* show growth inhibition or feeding

deterrence to a wide range of general herbivores such as birds, land slugs, and generalist insects (Giamoustaris and Mithen 1995, 1996). Martin and Müller (2007) found that *Sinapis alba* (white mustard) respond to *Athalia rosae* (turnip sawfly) damage by systematically accumulating higher levels of glucosinolates and, thus, apparently increasing their resistance (Martin and Müller 2007). An increase in short-chain aliphatic methylsulfinyl glucosinolates in *Arabidopsis thaliana* in response to both specialist and generalist phloem-feeding aphids is also known (Mewis et al. 2005). *Brassica napus* lines with higher glucosinolate content are also reported to have less damage in response to generalists such as pigeons and slugs (Giamoustaris and Mithen 1995). *Brassica juncea* with high glucosinolate concentrations is less prone to damage caused by both crucifer specialist, *Plutella xylostella*, and the generalist, *Spodoptera eridania* (Li et al. 2000). Moreover, insect herbivore feeding may substantially increase the levels of glucosinolates in plants. In *Arabidopsis*, comparison of glucosinolate accumulation and expression of glucosinolate biosynthetic genes in wild-type and mutant lines affected in defense signaling indicated that feeding of the aphid generalist *Myzus persicae (Sulzer)*, the aphid specialist *Brevicoryne brassicae* (L.), and the *Spodoptera exigua Hübner*, a lepidopteran generalist, can increase the accumulation of aliphatic glucosinolate content (Mewis et al. 2006). The plant also alters the nature of glucosinolates in affected area to deter the herbivores. For instance, *Myzus persicae* feeds on *Arabidopsis* and causes an overall decrease in glucosinolate content, but the production of 4-methox yindol-3-ylmethylglucosinolate is induced. This altered composition of glucosinolate, finally, acts as a deterrent for herbivores (Kim and Jander 2007).

The role of glucosinolates in defense against pathogens is not well studied like for herbivores. However, there are several reports indicating glucosinolate and its hydrolysis products can be toxic to bacteria and fungi (Smolinska et al. 2003; Mari et al. 2002; Li et al. 1999). *Brassica* crops are used as a break crop. The glucosinolates and their hydrolysis products secreted from *Brassica* canola and Indian mustard show inhibitory effects on soilborne fungal pathogen, *Gaeumannomyces graminis* var. *tritici*, which causes take-all of wheat (Angus et al. 1994). The 4-methylsulphinylbutyl isothiocyanate, a glucosinolate-derived isothiocyanates, is reported to have broad spectrum of antimicrobial activity. Growth of wide range of the fungi, such as *Alternaria brassicicola*, *Plectosphaerella cucumerina*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Peronospora parasitica*, and bacteria, like *Erwinia carotovora* and *Pseudomonas syringae*, is inhibited by the presence of 4-methylsulphinylbutyl isothiocyanate (Tierens et al. 2001). Also, tryptophan-derived indole glucosinolates are reported to enhance the resistance of *Arabidopsis thaliana* against fungi like *Plectosphaerella cucumerina* and *Phytophthora brassicae* (Sanchez-Vallet et al. 2010*;* Schlaeppi et al. 2010).

Additionally, exogenous treatment of phytohormones like jasmonic acid (JA) and salicylic acid (SA), key signal regulators of plant defenses, to the plant also alters the glucosinolate profile which, again, proves the role of glucosinolates in plant defense. Previous studies showed that exogenous JA application can induce the accumulation of indole glucosinolate content in white mustard and oilseed rape (Bodnaryk 1994; Doughty et al. 1995). In addition, SA application is also reported

to alter glucosinolate accumulation in oilseed rape (Kiddle et al. 1994). The hydrolysis products of glucosinolate have negative effects on vertebrates too. A diet highly rich in glucosinolates can cause the growth depression, poor palatability, decreased food efficiency, hypertrophy and hyperplasia of the thyroid, and liver lesions and necrosis in vertebrates (Anilakumar et al. 2006).

14.2.4.1.3 Nonprotein Amino Acids

NPAAs are commonly found in plants. NPAAs are present in widely consumed animal foods also. For instance, *Medicago sativa* is rich in canavanine, while *Lens culinaris*, a widely used edible pulse, contains homoarginine. In plants, NPAAs possess different roles including antiherbivory, antimicrobial, and allelochemical activity. The NPAA can protect the producer plants against stress, microorganisms, plants, insects, or higher animals including human (Bell 2003; McSweeney et al. 2008). NPAAs exert their toxicity in several ways. Some block the synthesis or uptake of protein amino acids, while others can be misincorporated into proteins and, finally, lead to production of nonfunctional proteins.

The protein-synthesizing machinery of plants that produce NPAAs can discriminate between protein and NPAAs, and, therefore, they are not susceptible to the toxicity of NPAAs. For instance, *Convallaria majalis* produces an analog of the protein amino acid L-proline known as L-azetidine-2-carboxylic acid. Although *Convallaria majalis* can differentiate the L-proline and L-azetidine-2-carboxylic acid, it can be easily misincorporated in proteins of *Vigna aureus*, which does not synthesize azetidine-2-carboxylic acid, and strongly inhibit the growth of germinating seedlings (Fowden 1963).

14.2.4.1.4 Aliphatic NPAAs

β-methylamino-L-alanine (BMAA) is a derivative of the alanine with a methylamino group on the side chain. BMAA is produced by the cyanobacteria in root nodules of cycads and has potent neurotoxic properties. BMAA is also accumulated in the seeds of cycads and causes amyotrophic lateral sclerosis/parkinsonism-dementia (ALS/P-D) (Steele and Guzman 1987; Ince and Codd 2005). ALS is a rare group of progressive neurological disorders that mainly involve the neurons responsible for controlling voluntary muscle movements such as chewing, walking, and breathing. Dencichine (β-N-oxalyl-l-α,β-diaminopropionic acid) is a hemostatic agent present in widely used traditional Chinese medicinal herbs, such as Panax species and *Lathyrus sativus*. Dencichine is a neuro-excitatory NPAA which causes the motor neuron disease, neurolathyrism, a condition with acute neurotoxic symptoms such as the inability to stand, neck stiffening, and head retraction (Campbell et al. 1993). Canavanine, an arginine analog, is synthesized in some leguminous plants (Bell et al. 1978) and plays a pivotal role in plant chemical defense against insects (Rosenthal 2001). Canavanine functions as an allelopathic chemical and inhibits plant growth (Nakajima et al. 2001). Incorporation of canavanine in place of arginine produces structurally aberrant proteins which exhibit altered protein conformation and impaired function in insects, such as *Manduca sexta* and *Heliothis virescens* (Rosenthal and Dahlman 1986; Berge et al. 1986). Animals fed on seeds

of canavanine-containing plants developed hematological and serological abnormalities and induce antibody-mediated autoimmune phenomena (Bell 2003). Indospicine is a hepatotoxic NPAA found in *Indigofera* plant species. It accumulates as the free amino acid in the tissues (like muscle) of grazing animals including the horse and acts as a competitive inhibitor of arginase and causes reproductive losses and severe to mild liver disease (Fletcher et al. 2015). Djenkolic acid commonly found in *Archidendron pauciflorum* causes djenkolism, an acute kidney malfunction (Bunawan et al. 2014; Bell 2003). L-methionine sulfoximine, seleno-cystathionine, selenomethionine, and dl-phosphinothricin are examples of other NPAAs of plant origin that are involved in plant defense (Bell 2003; Shaw et al. 1999; Schrauzer 2000; Kitajima and Chiba 2013; Tardito et al. 2012).

14.2.4.1.5 NPAAs with Aromatic Skeletons

Plants produce several NPAAs with aromatic skeletons, such as L-3,4-dihydroxyphenylalanine (L-DOPA) and m-tyrosine, that are involved in plant defense. L-DOPA is a compound with strong allelopathic activity. It is found in leaves and seeds of *Mucuna pruriens* (velvet bean) that has a nutritional quality similar to the soybean (Nishihara et al. 2005). L-DOPA acts as a precursor of many alkaloids, such as catecholamines and melanin, which are released into soils and inhibit the growth of nearby plants. L-DOPA is an important secondary metabolite for chemical defense against herbivores in plants (Huang et al. 2011; Van Alstyne et al. 2006). Plants with high L-DOPA content are less prone to attack of small mammals or insects (Rehr et al. 1973). It is also a key chemical involving in sclerotization and melanization of insects which finally affects the development and immunity of insects (Gallot et al. 2010; Andersen 2010). The L-DOPA acts as a herbicide and suppresses the growth of several weed species such as *Sinapis arvensis*, *Cirsium arvense*, *Papaver rhoeas*, and *Lamium amplexicaule* (Topal and Kocaçalişkan 2006). *m*-Tyrosine is an example of another NPAA with aromatic skeletons with phytotoxic properties. It is exuded from the roots of fine fescue grasses and inhibits the growth of a wide range of neighboring plant and, therefore, grants a competitive advantage to fescue grasses (Bertin et al. 2007; Huang et al. 2012). The toxicity of *m*-tyrosine is due to its misincorporation into cellular protein in place of protein amino acid phenylalanine (Gurer-Orhan et al. 2006; Klipcan et al. 2009). The *m*-tyrosine can also prevent the growth of bacteria including *Escherichia coli* and *Bacillus* species (Smith et al. 1964; Aronson and Wermus 1965).

14.2.4.1.6 NPAAs with Cyclic and Heterocyclic Skeletons

The 5-hydroxytryptophan (5-HTP) is found in the seeds of *Griffonia simplicifolia* and has been associated with the insecticidal properties (Janzen et al. 1977). Homoproline, a lysine-derived NPAA, is a critical regulator of systemic acquired resistance (SAR) and basal immunity to bacterial infection in plants including *Arabidopsis thaliana* and *Nicotiana tabacum* (Navarova et al. 2012; Vogel-Adghough et al. 2013). Homoproline signals the plants for effective biosynthesis of defense signal SA, accumulation of the phytoalexin camalexin, and expression of

defense-related genes. Mimosine and its derivatives (α-amino-β-(3-hydroxy-4-oxo-1,4-dihydropyridin-1-yl)-propanoic acid), found in a leguminous *Leucaena leucocephala* (Xuan et al. 2006), have a strong herbicidal impact on several plants namely *Brassica rapa* and *Phaseolus vulgaris* (Xuan et al. 2006, 2016). Mimosine has insecticidal (Ishaaya et al. 1991) properties also and can inhibit the growth of first-instar larvae of *Tribolium castaneum*. β-(Isoxazolin-5-on-2-yl)-alanine (BIA), found in *Pisum*, *Lens*, *Lathyrus*, and *Vicia* plant species (Lambein et al. 1990), is a potent growth inhibitor of several eukaryotic organisms, such as yeasts; unicellular green algae; phytopathogenic fungi, such as *Botrytis cinerea*, *Pythium ultimum*, and *Rhizoctonia solani*; and higher plants (Schenk et al. 1991).

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Plant Cell Wall: A Simple Physical Barrier or a Complex Defense Modulator – Exploring Its Dynamic Role at Plant-Fungus Interface

15

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Abstract

Plants are continuously threatened by many pathogens, among which fungal pathogen accounts for a measureable large quantity. Understanding of plant-fungal interaction is constantly coevolving along with the evolution of both the interacting partners. According to the previous scientific literature, many fungi are associated with a single host. Present chapter is focused at elaborating the role of plant cell wall in participating in the interaction. Host cell wall is the outermost barrier which any pathogen has to breach for successful invasion and establishment. Now the question is what is the role of host cell wall in regulating the pathogen's infiltration or restriction? Present study explains the structural dynamism of cell wall which is believed to have functional relevance and is dependent on the behavior of the infecting fungi. Moreover, cell wall is also known to elicit immune signals that brings about transcriptional reprogramming and helps in mounting defense against attacking fungi. Additionally, cell wall-mediated responses trigger expressions of many antimicrobials which are regulated by hormone signaling pathways. This study also sheds light on the impact of plant-fungal association on tritrophic interactions with other beneficial and pathogenic biotic components. But the role of host cell wall while dealing with multiple interacting partners is still elusive. Thus, the knowledge of cell wall glycobiology is expected to proceed further based on researches conducted at natural microenvironments.

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15.1 Introduction: Cell Wall Versus Immunity

The tug-of-war between plants and pathogens is endless. In order to defend oneself, both the interacting partners constantly opt for appropriate counter-reactive measures that are temporally and spatially regulated (Jones and Dangl 2006). But is this dynamic interplay between plants and its invading pathogens completely understood? This is undoubtedly a million dollar question with a number of answers that are also coevolving with the up gradation of understanding of the plant-pathogen interaction chemistry.

However, there are few conceptions that have received unanimous scientific endorsements. The pathogens, being aggressive in nature, are believed to make the initial attempt of overpowering their host. The pathogen's selection of host may or may not be specific. But, pathogens usually possess some conserved pathogen-associated molecular patterns or microbe-associated molecular patterns (PAMPs/MAMPs) which are readily recognized by the pattern recognition receptors (PRRs) of the hosts. The PAMPs/MAMPs and PRR recognition event leads to activation of downstream defense reactions termed as pattern-triggered immunity (PTI) (Tsuda and Katagiri 2010). Sudden imbalance of ionic concentrations, increase in calcium ionic influxes, activation of MAP (mitogen-associated protein) kinases, phosphorylation of some targeted proteins, and secretion of antimicrobial compounds all leading to cell wall reinforcement of host at the site of attempted penetration are said to be the features of PTI (Buchanan et al. 2015). Additionally, pathogen invasion induces the generation of some host components referred to as danger-associated molecular patterns (DAMPs) such as callose, glucans, fructans, etc. which are recognized as nonself and trigger similar set of downstream signals known as danger-triggered immunity (DTI) (Boller and Felix 2009). Both PTI and DTI provide basal resistance that non-specifically restrict a large number of pathogens at the site of penetration. But, a few diplomatic invaders evade the restrictions imposed by PTI/DTI and secrete specific effector proteins which lead to effector-triggered susceptibility (ETS). As counter-defensive measure, hosts secrete effector-specific R proteins (resistance proteins) that directly or in combination with decoy/guardees interact with pathogen effectors and activate effector-triggered immunity (ETI). The features of ETI not only overlap with those of PTI but, being of much greater amplitude, compensate for the shortfalls of the effect of PTI (Thomma et al. 2011). However, the pathogen has to infringe through the initial physical barrier of host cell wall in order to successfully establish itself within the host (Malinovsky et al. 2014). Thus it is logical to comment that cell wall has roles in allowing or restricting the invader. Present study shall explore the role of plant cell wall at plant-fungus interface.

15.2 Structural Complexity Versus Functional Relevance

Cell wall is the initial physical obstacle that a pathogen has to break for successful establishment within the host. Thus, the structural complexity undoubtedly regulates the function of the cell wall against phytopathogenic fungi. Occasionally the cell wall is covered with a protective layer called cuticle that the fungi breaks through by secreting cell wall-degrading enzymes which also act as important virulence factors (Nuhse 2012). The load-bearing cellulose microfibrils are known to provide the structural integrity of the primary cell wall. They are synthesized by multimeric complexes made up of cellulose synthase catalytic subunits (CESAs) that are well characterized in *Arabidopsis thaliana* (Endler and Persson 2011). CESAs comprise of two subfamilies that synthesize cellulose in both primary (CESA 1, 3, 6) and secondary (CESA 4, 7, 8) cell walls with some crossovers that has still not been characterized (Endler and Persson 2011). However, perturbation of cell wall integrity due to fungal invasion which is diplomatic in case of biotrophic attack and destructive in case of necrotrophic attack, is often compensated by increased lignifications and downstream resistance responses offering the fact that structural cohesiveness of the outermost barrier is always under the alert surveillance of plant defense armory (Caño-Delgado et al. 2003; Hernández-Blanco et al. 2007). The structural integrity is also maintained by the hemicelluloses that are composed of beta 1–4-linked backbone of mannose, glucose, or xylose. They help in reinforcing the interactions with both cellulose and lignin (Endler and Persson 2011). Among several hemicelluloses, xylans are found to be predominant in secondary cell walls where the beta 1–4 xylose residues are often substituted by arabinosyl residues that are esterified with ferulic acid groups. These ferulate esters aid the cross-linking of xylans with lignins ultimately imparting enhanced structural rigidity (Harris and Stone 2008). Xylans are degraded by fungal xylanases and recognized as potential PAMPS by host PRRs in *Lycopersicum esculentum* (Ron and Avni 2004). In addition to feruloylation, xylans can also undergo acetylation and methylation that are specifically targeted by some fungal xylan degrading enzymes. Degraded xylan molecules perceived as DAMPS are known to trigger downstream defense signals (Pogorelko et al. 2013).

Pectin, a hetero complex polysaccharide, serves as the matrix of primary cell wall. Pectic backbones are comprised of either homogalacturonan (HGA) or rhamnogalacturonan. Among these, HGA appears to be more relevant in defense signaling since they undergo specific time-dependent chemical substitutions like methyl esterification and de-esterification at C6 position and acetylation at C2–C3 positions. Such chemical modifications provide a fine tune balance to the host plant according to its prevailing local requirements by forming calcium-mediated cross-linked gels and helping in cell adhesion (Cabrera et al. 2008). HGA is cleaved by fungal polygalacturonases (PG) into oligogalacturonide (OG) fragments that either aid pathogenic entry or act as nonself DAMPs and impart resistance during compatible and incompatible interaction, respectively (Galletti et al. 2009). Interestingly, studies on endogenous pectin methyl esterases (PMEs) showed that they hold dual roles in degrading host HGA into OGs during fungal penetration and also trigger

host resistance response by aiding the formation of gel structures to bolster damaged cell walls (Bethke et al. 2014). Such counterintuitive reports support the fact that apart from giving a structural integrity, the role of pectin in defense is quite dubious and still demands extensive case-specific experimentation to come to generalized conceptualizations. Lignin is the prime phenolic polymer of secondary cell walls that increases mechanical strength and water impermeability (Albersheim et al. 2010). Lignin not only provides structural firmness but also actively regulates defense responses by regulating phenylpropanoid pathway and the production of antimicrobial phytoalexins, stilbenes, coumarins, and flavonoids. Besides the key stress hormone that modulates defense, salicylic acid (SA) is also produced from the same biosynthetic pathway that produces lignin polymers (Lozovaya et al. 2007). But, the random incorporation of lignin monolignols to form complete lignin polymers, whether has any ecological implication to meet the protective demands of the host against attacking fungi, is still not clearly understood.

15.3 Plant-Fungus Interface

Immediately after contact the interaction between plant and fungus starts that largely depends on the topology, chemical composition, and molecular features of the contact areas of both the associated partners.

15.3.1 Fungal Penetration Strategy

15.3.1.1 Spore Properties

Spore texture, composition, and molecular properties influence the attachment, adhesion, and penetration of the pathogenic fungus within the plant. On the other side, most of the plants are coated with protective waxes that affect fungal attachment, adhesion, and invasion. Biotrophic fungi of barley *Blumeria graminis* perceive signals from the host surface and direct the growth of germ tube. Germination takes place only after the spore becomes proximate with the host surface stratum (Nielsen et al. 2000). Spore attachment of necrotrophic pathogen of wheat *Stagonospora nodorum* takes place immediately after 30 s and depends on conidial and host surface-released glycoproteins (Newey et al. 2007). In case of hemibiotrophic pathogen of bean *Colletotrichum lindemuthianum*, the spore outer surface consists of fibrillar porous layer abundant in carbohydrates that are required for attachment to hydrophobic layers and sense hard textured surface needed for appressoria formation (Rawlings et al. 2007). Besides in blast fungi *Magnaporthe grisea*, the fungal-released proteins of extracellular matrix (ECM) play a crucial factor in spore attachment, germ tube, and appressoria formation (Inoue et al. 2007). Additionally, the conidial cell wall composition specially beta 1–3 glucan content is also important for pathogenicity as reported in *Alternaria brassicicola* (Joubert et al. 2011) suggesting that cell wall integrity is central to protection of fungal pathogens against host antimicrobial compounds.

15.3.1.2 Mode of Penetration

Following attachment, the germination of the fungal spore and penetration within the host interior appear to be a hallmark achievement for the successful fungus which occurs in different ways. Few fungi develop specialized structures that are capable of piercing the plant cuticular layer and cell wall of epidermal cells, while most of the others rely on their secreted cell wall-degrading enzymes (CWDE) that digest the host cell wall and facilitate their invasion (Łaźniewska et al. 2012). In a handful of cases, involvement of both specialized structures and CWDE occurs. In case of *Magnaporthe grisea*, it develops highly melanized dome-shaped appressoria that exerts tremendous physical force due to high turgor pressure and forces into the host epidermal cells (Choi et al. 2011). *Rhizotonia solani* forms complex appressorial structures known as infection cushions (Pannecoucque and Höfte 2009). CWDE of fungi comprises of xylanases, exogalacturonases, pectin methylesterases, endoglucanases, and polysaccharide deacetylases (Carapito et al. 2008). However the biotrophic fungi and necrotrophic fungi differ strategically in using their CWDEs. The biotrophs employ CWDE for loosening the cell wall of the host and allowing the fungi stealthily, while necrotrophs apply brute force and engage their CWDEs in totally digesting the cell wall of the host (Spanu and Kämper 2010). The function of CWDEs is largely dependent on the pH of the host cell sap (Niture and Pant 2007). Again some fungi like *Fusarium oxysporum* f.sp. *ciceri* and *Puccinia striiformis* f.sp. *tritici* rely on the natural openings such as breaches within root hairs, stomata, etc. of the host for entry (Gupta et al. 2009; Moldenhauer et al. 2006).

15.4 Role of Cell Wall in Elicitation

Previous researchers have well documented the role of cell wall in elicitation during fungal infection (Malinovsky et al. 2014). The following section shall brief how the elicitation mediated by cell wall changes trigger the defense signaling in the infected host plant.

15.4.1 Role of Cell Surface Chemistry and Topology

Hydrophobicity of host cell surface is undoubtedly perceived by most pathogenic fungi for successful attachment which is followed by germination of infecting structures and establishment of the pathogen within the host. Now the cell wall's hydrophobic nature is largely imparted by the presence of the protective layer cuticle. Cuticle is composed of polyester cutin or cutan and epi and intracuticular waxes. Besides triterpenoids and phenyl propanoids are also present (Nawrath 2006). The structure and chemical composition of cuticle differ according to the microenvironment and the nature of plant-fungal interaction as studied in *Arabidopsis thaliana*-fungi interaction (Kurdyukov et al. 2006). On the contrary, different fungal pathogen has developed different breaching mechanism which is capable of using the phylloplane cuticle topology and chemistry for self-sustenance and pathogenicity. Even then, cuticle is known to hold dual roles where in one hand they serve as protective

layer and prevent fungal invasion, and on the other hand, their altered structure helps in release of some antimicrobials that lead to resistance against the attacking fungi (Mang et al. 2009).

Cell wall-associated trichomes also have prominent roles in plant-pathogen recognition. Similar to cuticle they also have twofold function but of disparate nature. They are known to trap fungal spores and secrete specialized exudates rich in secondary metabolites having antifungal activity (Nonomura et al. 2009). Besides, studies on *Nicotiana tabacum* have revealed the expression of several pathogenesis-related proteins (PR5 and PR14) and lipid transfer protein (LTP) in leaf trichomes during *Peronospora tabacina* attack (Harada et al. 2010). Conversely, the spatial organization and topology of trichomes are sensed by some group of fungi like *Fusarium graminearum* and *Colletotrichum acutatum* infecting strawberry and *Arabidopsis thaliana*, respectively (Salazar et al. 2007; Skadsen and Hohn 2004). Moreover, the high density of trichomes increase the humidity of the host surface resulting in colonization of many pathogenic fungi that mark specific weak areas in and around the trichomes as probable entry gates (Calo et al. 2006).

15.4.2 Role of Wall-Degrading Enzyme Inhibitors

Fungal CWDEs are important molecules which they categorically use to promote their growth and establishment *in planta* (Łaźniewska et al. 2012). In opposition, host plants also activate immune responses that restrict the devastations caused by fungal CWDEs. Degradation of the primary component of cell wall pectin, the homogalacturonan by polygalacturonases (PGs), results in release of fragments of oligogalacturonides (OGs) that are important DAMP molecules. Studies on *Arabidopsis thaliana* during *Botrytis cinerea* attack revealed enhanced expression of defense proteins like PAD3 (phytoalexin deficient 3) and PGIP (polygalacturonase-inhibiting protein) due to release of OGs from infected cell wall of host (Galletti et al. 2009). Additionally there exist many other CWDE inhibitors of infected hosts that have been reviewed by Lagaert et al. (2009). Pepper pectin methylesterase inhibitor protein (CaPME11) exhibits antifungal activity against *Fusarium oxysporum* f.sp. *matthiole*, *Alternaria brassicicola*, *and Botrytis cinerea* (An et al. 2008). Ongoing researches on fungal enzyme verses host inhibitor projects a constant evolution of both the interacting cognate proteins that are subjected to dynamic ecological selection pressure (Beliën et al. 2007).

15.4.3 Host Cell Wall Reinforcement Strategies

The most common counter response of the infected host against attempted penetration of the fungal pathogen is to reinforce its cell wall and prevent further fungal ingress. Reinforcement is achieved by deposition of a number of compounds such as callose, phenolics, lignin, cellulose, pectin, suberin lipids, hydroxyproline-rich glycoproteins (HRGPs), and peroxidases (Schmelzer 2002). The nature and chemical

constituent of the deposition is dependent on a particular pathosystem and is temporally regulated. For example, *Septoria tritici* infecting wheat induces callose deposition, whereas accumulation of syringly rich lignins is found in wheat during *Puccinia graminis* f.sp. *tritici* attack (Shetty et al. 2009; Menden et al. 2007). Callose (beta 1–3 glucan) deposition is one of the most important reinforcement molecule deployed by the host. It leads to papillae formation at the site of attempted penetration and restricts a wide range of fungi. The key enzyme of callose synthesis is callose synthase PMR4. PMR4 is known to regulate salicylic acid and PR1 protein expression responses via NPR1 expression in *Arabidopsis thaliana* (Dong et al. 2008). Interestingly, in *Cicer-Fusarium oxysporum* f.sp. *ciceri* interaction, the degradation of callose was found to be linked to enhanced susceptibility indicating that callose deposition and/or degradation is often governed by the invading fungus also that diplomatically reprograms the host machinery for its self-sustenance (Gupta et al. 2010).

Lignins are also known to form cell wall apposition and provide penetration resistance in wheat during powdery mildew attack (Bhuiyan et al. 2009). Lignin content is also regulated by phenyl propanoid pathway and jasmonic acid (Taheri and Tarighi 2010). HRGPs are also important cell wall strengthening compounds that are released in the apoplast in the form of monomers that undergo cross-linking with the help of H_2O_2 and class III peroxidases, thus forming thick-walled network of extensin. These extensins not only serve as anchor for host cell wall lignification but also cause agglutination of the infecting fungi (Almagro et al. 2009) (Fig. 15.1).

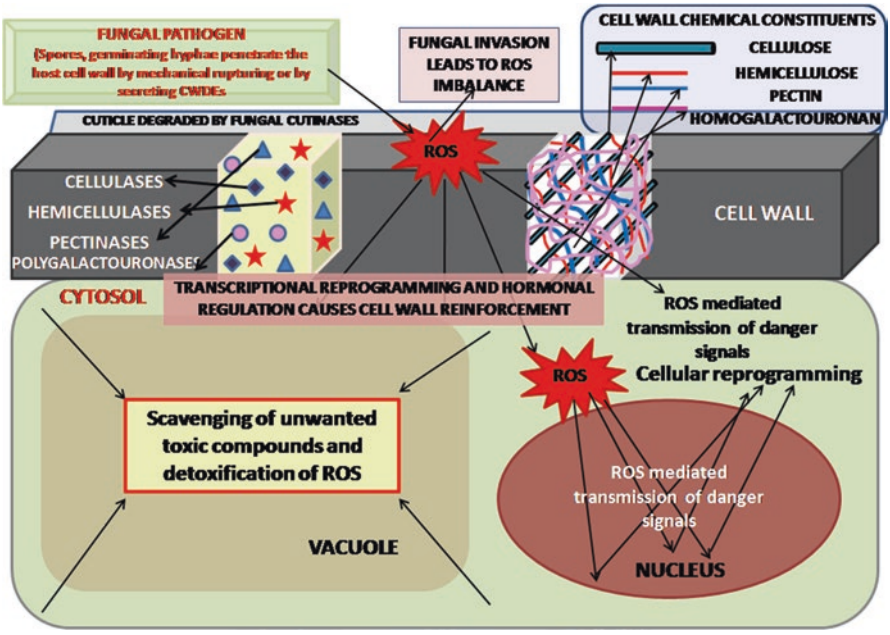

Fig. 15.1 Schematic diagram representing the role of cell wall in regulating plant defense against pathogenic fungi

15.4.4 Role of Peptide Hormones

Peptide hormones are a class of new entrants in the list of plant hormones. Till date few peptide hormones have been identified that are known to regulate diverse roles starting from controlling cell differentiation and growth to inducing defense signaling (Ryan et al. 2002). In the present section, light will be shed on the role of systemin and rapid alkalinization factor (RALF) in controlling defense in particular. Systemin was initially identified in tomato leaves as a result of systemic wound response during *Manduca sexta* pest infestation (Pearce et al. 1991). Follow-up researches have identified many systemins from other solanaceous as well as non-solanaceous member (Ryan et al. 2002). But, their structure and sequence homology greatly differ across different plant species suggesting a need to look through as, why nature has retained the functional conservation of systemin, in spite of its structural dissimilarity across diverse species. Wounding is a response that is deeply associated with the architectural change in the cell wall. Fungal penetration definitely induces wounding, although there has been no report of plant systemins associated with fungal penetration till date, which demands extensive research ahead on this field.

RALFs were reported from tobacco known to cause rapid alkalinization of the medium (Pearce et al. 2001). This medium alkalinization is reported to induce MAP kinases in vitro in tobacco and tomato cell cultures (Pearce et al. 2001). Systemins also induce medium alkalinity, but RALFs are identified to be larger components having signaling role other that regulating defense (Ryan et al. 2002). Although RALF has been reported to have growth and development regulatory roles in tobacco, its exact role in regulating defense is still not proven. Interestingly, in *Cicer-Fusarium oxysporum* f.sp. *ciceri* case study, RALF was reported to be induced in resistant plants during fungal invasion predicting its role in somehow adversely affecting the fungal spread and establishment within the incompatible plant (Gupta et al. 2009). However, the exact mode of action of this novel molecule needs to be elaborated.

15.5 Cell Wall Influencing Transcriptional Reprogramming and Hormonal Regulation

The role of cell wall in transcriptional reprogramming during fungal attack is indeed very important. Although it is well implied but not very clearly correlated in previous studies, that the cell wall being the external and first barrier to be encountered by the attacking fungi surely transmits a sense of danger to its interior parts. Transmission of the danger alarms leads to the activation of cell wall fortification strategies and expression of antimicrobials all of which are controlled by the transcriptional reprogramming (Malinovsky et al. 2014). Callose deposition is coupled with late PTI responses. Besides, attempted penetration of fungal pathogen causes an obvious imbalance in cellular ionic concentrations thus culminating in generation and accumulation of reactive oxygen species (ROS) at the site of invasion (Kobayashi et al.

2007). This ROS accumulation causes downstream transcriptional upregulation leading to calcium spiking, MAP kinase, and calcium-dependent protein kinase expression (CDPK), which are influenced by the changes in cell wall architecture and integrity (Boudko 2012; Boudsocq et al. 2010). Among the genes related with cell wall defense, penetration genes (*PEN*) are most widely characterized. *PEN1* contains a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) domain and encodes membrane-associated syntaxin SYP121/PEN1. It is essential for papillae formation and helps in delivering the cell wall reinforcement material to the appropriate site of attack (Assaad et al. 2004). *PEN2* gene encodes glycoside hydrolase that participates in peroxidase-mediated antifungal production, while *PEN3* gene-encoding ABC (ATP-binding cassette) transporters deliver the compound to the necessary site of attempted penetration (Stein et al. 2006).

Although indirect, the role of cell wall in modulating hormonal expression during fungal attack is also noteworthy. Many fungi like *Fusarium* sp. rely on natural opening like stomata or breaches adjoining root hairs for entering the host. Thus, in order to prevent fungal entry, the host guard cell wall modifies accordingly, decreases the osmotic potential, and initiates abscisic acid (ABA)-mediated stomatal closure (Ton et al. 2009). Besides, callose synthase that affects the deposition of callose is known to trigger NPR1-mediated SA response in *Arabidopsis thaliana* (Dong et al. 2008). Similarly lignins that are products of phenyl propanoid pathway are regulated by jasmonic acid pathway (Taheri and Tarighi 2010).

15.6 Plant-Fungus Interaction and Its Impact on Other Biotic Interactions at Different Trophic Levels: Is the Role of Host Cell Wall Defined During Multiple Interactions?

Fungus is one of the most commonly occurring plant-associated organisms with a considerable diversity comprising 120,000 described species. According to Hawksworth's (1991) extrapolation, there are 1.5 million fungal species on earth, most of which consume plant matter. The ratio of fungi to plant species is 5:1 considering approximately 300,000 plant species on earth. Thus, it is likely that a plant is associated with more than five fungi (Arnold et al. 2007; Jumpponen and Jones 2010). With such expansive species diversity, it is understandable that plant-fungal interactions are dynamic and ecologically complex (Southworth 2012).

It is presumed that all plants in the ecosystem are symbiotic with fungal endophytes that reside in plant tissues (Petrini 1996). These symbiotic association aids the host plant in gaining fitness benefits such as conferring biotic and abiotic stress tolerance and inducing growth and development. For example, class 2 fungal endophytes play a crucial role in plant adaptation to various abiotic stresses such as drought, salinity, and temperature (Redman et al. 2001; Márquez et al. 2007; Rodriguez et al. 2009, 2008). However, in the present section, we confine ourselves to fungi-mediated biotic interactions only. From a plant's perspective, fungi can mediate both beneficial and harmful interactions. In the following section, we shall discuss few of such naturally occurring tritrophic interactions mediated by fungi (Fig. 15.2).

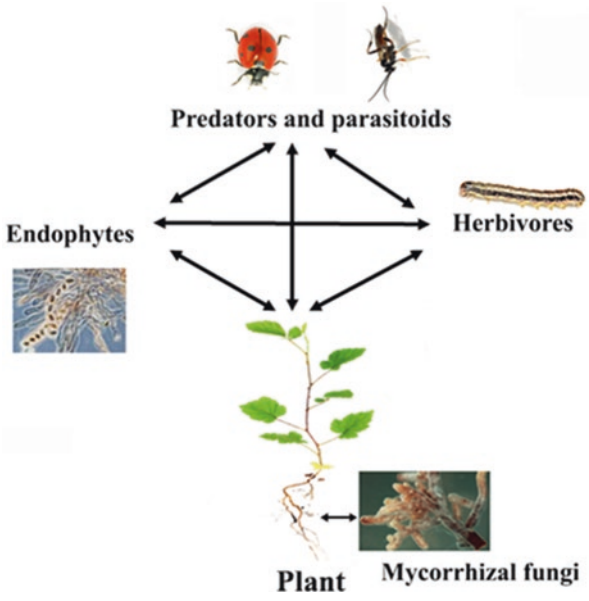

Fig. 15.2 Simplified pictorial representation of interaction dynamics of plant and its associated organisms including insects, fungus (harmful + beneficial), and parasite/predators (Model adapted from Lamit and Gehring 2012)

15.6.1 Fungi as Mediators of Symbiotic Trophic Associations

Plants endorse different mutualistic associations where they provide nutritional or structural resources to their partners and obtain essential components related to their growth. Otherwise, there is a tremendous competition of utilizable carbon sources in the soil among soil organisms. Plants often produce excess photosynthate in presence of adequate nitrogen and release some part of it into the rhizosphere (Hikosaka 2005). These nutritional conditions provide opportunity for symbiosis between plants and fungi; plants require nitrogen, and fungi need accessible carbon, which they are unable to scavenge efficiently from soil (Smith et al. 2009). There are several examples of plant-fungus symbiotic interactions. Many of these fungal symbiotic partners, including mycorrhizal fungi, are obligate in nature and are therefore unable to survive without a plant host. In this symbiotic association, fungus receives carbon, in form of plant-derived sugar, and in return, they provide their host with the limiting soil nutrients such as nitrogen and/or phosphate. Several gene expression and transporter-staining experiments provide evidence for such mutual interactions (Fellbaum et al. 2012; Jones et al. 2009; Balestrini and Lanfranco 2006; Karandashov and Bucher 2005; Guether et al. 2009).

Metarhizium is an insect pathogenic, root colonizing fungus. They are not obligate biotrophs like mycorrhizal fungi. Their survival also depends on the reciprocal nutrient exchange with a host plant, whereby the fungus receives carbon in exchange for the insect-derived nitrogen (Hajek and St. Leger 1994; Behie et al. 2017; Behie and Bidochka 2014). They infect soilborne insects and transfer the insect-derived nitrogen to the host plants via fungal hyphae for the plant-derived photosynthate (Behie et al. 2017). This serves as a classic example of a secondary interaction

between plant and insect mediated by fungus where insects become the prey and plants are provided with insect-derived nitrogen by the entomopathogenic fungus.

Occasionally, fungus benefits the host plant through cross-compartment signaling in both directions such as shoot to root and vice versa. For instance, aboveground inoculation with an incompatible race of the hemibiotrophic fungus *Fusarium oxysporum* (Foc race 1) on Cavendish banana enhances resistance of the host plant to a compatible strain of fungus (Foc race 4) when the strain is subsequently inoculated to the roots (Wu et al. 2013). The enhanced resistance, shoot to root SAR (systemic acquired resistance), is associated with the higher levels of salicylic acid (SA) and other defense-related genes including PR-1 (pathogenesis-related protein). Conversely, the hemibiotrophic fungus, *Colletotricum graminicola*, strongly suppresses the same pathogen growth when it is inoculated in the leaves after 6 days of preinoculation of roots in maize plants. Transcriptional studies confirmed the significant increase in the expression of ABA (abscisic acid), SA, and other genes associated with the biosynthesis of benzoxazinoids (DIMBOA) and PR proteins until 4 dpi (days post inoculation) (Balmer et al. 2013) indicating an active root to shoot SAR. Moreover, Leath et al. (Leath and Byers 1977) reported reduced pea aphid densities on the forage legumes infected by *Fusarium* in roots.

Interestingly, plant-fungal interaction can also modulate herbivore population by attracting the natural enemies. Plant volatile compounds facilitate the host searching by the natural enemies (Turlings et al. 1991a, b; Kessler and Baldwin 2001). The plant-emitted chemical signals may originate from various sources like the host plant, herbivore-damaged plant, fungus-infected plants, etc.. For example, infection by the fungus *Alternaria brassicae* on *Brassica rapa* (mustard) seedlings induces release of volatile cues from glucosinolate degradation (Doughty et al. 1996). The white mold fungus, *Sclerotium rolfsii*, infected *Arachis hypogaea* (groundnut) plant releases E-4, 8-dimethyl-1, 3, 7-nonatrine, methyl salicylate, etc. (Cardoza et al. 2002). Interestingly, some herbivores find ways to escape natural enemies by evolving mechanisms to reduce volatile cues from the damaged plants. Hence, in addition to the host plants, the parasitoids feeding upon herbivores need to depend on other sources for volatile cues to locate their prey from faraway. Fungus-infected plants not only provide valuable cues to herbivores for host finding and oviposition, but they also emit volatiles that are highly attractive to parasitoids. Studies even revealed that there is a difference between the volatile emission profiles from the host plant after compatible or incompatible interactions with the fungal pathogens (Huang et al. 2003). Moreover, pathogen in combination with herbivory shows a different emission profile from the host (Cardoza and Tumlinson 2006), which may serve as a valuable cue for the natural enemies such as parasitoid wasps like *Cotesia marginiventris*. For instance, the volatile blend produced by beet armyworm (BAW) and white mold-infected groundnut plant contains methyl salicylate and fungus-produced 3-octanone, in addition to all the volatiles produced from the healthy groundnut plant alone (Cardoza et al. 2002). This infected groundnut plant exposed to BAW plants is more attractive to *C. marginiventris* (Cardoza et al. 2003) compare to healthy plants.

15.6.2 Fungi as Inducers of Pathogenic Trophic Associations

There are a number of pathogenic fungi that induce or facilitate other harmful interactions with plants (i.e., promote egg laying of herbivores) in addition to the damage they cause. In other words, when a pathogen attacks, the plant's response to those attacks affects the oviposition preference of different herbivores. There are many reports of higher oviposition of herbivores on pathogen-infected host plants in different pathosystems. *Helicoverpa armigera* (Hübner) adult females were reported to deposit more eggs on tomato leaves inoculated with root fungal endophyte, *Acremonium strictum* (Jallow et al. 2008). Similarly, stem-boring weevil, *Apiononopordi* (Coleoptera: Apionidae), prefers *Puccinia punctiformis* (rust fungus, Uredinales)-infected shoots of its host gray-green perennial herb, *Cirsium arvense* (Asteraceae) (Friedli and Bacher 2001). Abreha et al. (2015) showed that susceptible potato cultivar after fungus infection is preferred by the generalist moth, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). Females were found to lay more eggs on the *Phytophthora infestans* inoculated potato plant over uninoculated ones indicating a preference of *Spodoptera* females for diseased plants over healthy plants. There was no significant difference on the larval performance after feeding on the infested leaves. However, white mold fungus, *Sclerotium rolfsii*-infected peanut plants are more preferred by BAW, *Spodoptera exigua*. BAW grows better on the mold-infected plants due to higher level of soluble sugars and lower levels of soluble phenolics (Cardoza et al. 2003). *Phytophthora plurivora* (root pathogen) enhances the performance of gypsy moth (*Lymantria dispar*). However, herbivores do not always prefer to lay eggs on leaves infected with pathogens. For example, BAW avoided oviposition on powdery mildewed (*Podosphaera pannosa*) leaves of *Rosa chinensis* (Yang et al. 2013), leaf beetle *Phaedon cochleariae* does not prefer to oviposit on *Alternaria brassicae*-infested chinese cabbage (Rostas and Hilker 2002), and *Cassida rubiginosa*, leaf-feeding beetle, prefers laying eggs on healthy plants over *Phoma destructiva*-inoculated *Cirsium arvense* (Kruess 2002).

There is another interesting dimension in the plant-fungus-insect interaction where insect-fungus mutualistic relationships help them in successful colonization in host plant, for example, bark beetles (Coleoptera: Curculionidae, Scolytinae) ectosymbioses with fungi (Harrington 2005; Six 2012) for successful colonization on the host plant (Fig. 15.3), to be more specific, the western pine beetle (*Dendroctonus brevicomis LeConte*) symbiosis with two symbiotic fungal partners, *Entomocorticium* sp. (Basidiomycota) and *Cerato cystiopsis brevicomi* (Ascomycota) (Paine and Birch 1983; Hsiau and Harrington 1997). The two fungi carried in a prothoracic mycangium were found only in female beetles. During tree colonization, females inoculate the tree with their symbiotic fungi and oviposit in the tree's phloem layer. Larvae feed and grow initially on a combination of fungi and phloem. However, in the second instar, larvae start feeding on the nutrient-poor bark (Miller and Keen 1960), which is hypothesized to be mediated by the symbiotic fungi. This transition is crucial for larval survival, as they require both bark and phloem in the diet for development (Valiev et al. 2009). This is an example of

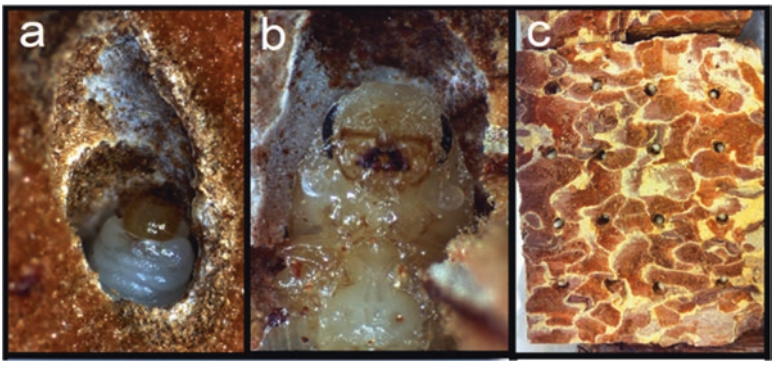

Fig. 15.3 Plant-fungus-insect tritrophic interaction. (**a**) Western pine beetle feed on symbiotic fungi during early phase of larval development on ponderosa pine bark. Fungi are visible as white mass in larval tunnel. (**b**) Fungal spores line the pupal chamber for incorporation into the beetle mycangia after metamorphosis. (**c**) Pseudo-chambers created by Bracewell et al. showed one pupa in each chamber (Adapted with permission from Bracewell and Six 2015)

symbiosis between insect and fungus since the fungi gain transportation and entry to the host trees by insects while, in return, the fungi provide nutritional benefit to the growing larvae.

Thus, plant-fungal-herbivore interactions are highly variable with no broad generalizations at present. However, these ecologically significant findings demonstrate the impact of phytopathogen (i.e., fungi)-induced alteration in plant chemistry (including volatile emission profile) influencing the plant-insect interactions, not only at the second trophic level but also the third level.

But, in order to mediate such tritrophic interactions, the plant cell wall definitely has a specific role in allowing different combinations of these multiple agents (symbiotic/pathogenic fungi along with beneficial insect/harmful herbivore) within itself. How the host cell wall perceives and channelizes the entire signaling that regulates the entry or exit of multiple organisms simultaneously or in sequence within the resident host is still a gross mystery. But, since penetration and invasion are a critical event for establishment of a symbiont or pathogen, the role of host cell wall is fundamental, which needs to be extensively studied that too under natural ecological microenvironment.

15.7 Challenges of Cell Wall Biology Ahead

According to the thumb rule of nature, cell wall is a universal barrier, which is sufficient to restrict a wide range of fungal pathogens. But what still remains unanswered are:

1. How does the cell wall of a particular host restrain so many wide range of fungal pathogens that constantly try to threaten it?

2. The PAMP/DAMP versus PRR recognition is primarily cell membrane centric and under transcriptional regulation. How does cell wall specifically function during PTI/ETI signaling?
3. It is believed that the initial fungal penetration is manifested within host interior by changes in ROS level. Is the same signal transmitted systemically to distant parts? What roles do the distant cell walls have? Do they also have any memory that helps in priming?
4. How is the chemical glycobiology of cell wall regulated according to functional demands more importantly during gradual invasion and establishment of the fungal pathogen? Does the role of host cell wall become redundant after pathogen establishes itself within the host?
5. How does the cell wall of a host cater to multiple invaders?

Apart from the above, there probably lie many more questions in scientific minds that are likely to receive satisfactory answers as the study of cell wall biology proceeds. On the contrary, many more queries will also come up in the near future that shall pave the path of new and novel researches ahead.

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