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FUNCTIONAL GENOMIC APPROACHES FOR DECIPHERING PLANT- VIRUS INTERACTIONS

by

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ABBREVIATIONS

°C	Degree centigrade
2D-PAGE	Two dimensional-Polyacrylamide gel electrophoresis
ABA	Abscisic acid
ACC	1-aminocyclopropane-1- carboxylate
ACMV	*African cassava mosaic virus*
AGO	Argonaute proteins
ARB-TMV	TMV-expressing plants after resistance break
ARFs	Auxin regulation factors
AtMC1	Metacaspases
AtNOA1	NO-associated protein 1
ATP	Adenosine tri phosphate
AtpC	ATP synthase γ-subunit C
AUX /IAA	Auxin/ Indole acetic acid
AVR	Avirulence factors
AzA	Azealic acid
BLAST	Basic alignment search tool
BRB-TMV	TMV-expressing plants before resistance break
CaMV	*Cauliflower mosaic virus*
CC	Coiled-coil domains
cDNA	Complementary DNA
cGMP	Cyclic guanidine monophosphate
CHS	Chalcone synthase
CMTs	Chromatin Methyl Transferases
CMV	*Cucumber mosaic virus*
CO	CONSTANS
COI1	Coronatine insensitive1
CP12	12 kDa chloroplast protein
Cq	Quantification cycle
CymRSV	*Cymbidium ringspot tombusvirus*
DA	Abietane diterpenoid dehydroabietinal
DAB	3,3'-diaminobenzidine
DAS-ELISA	Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay
DCL	Dicer-like enzymes
DIR1	Defective in induced resistance 1
DRM2	Domains rearranged methyl transferase 2
ds	Double stranded

dsRNA	Double stranded RNA
EDS1	Enhanced disease susceptibility 1
eEF1A	Eukaryotic elongation factors 1A
eIF4E	Eukaryotic translation initiation factor 4E
EIN1	Ethylene insensitive 1
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ERF1	Ethylene response factor1
ERS1	Ethylene response sensor 1 and 2
EST	Expressed sequence tags
ET	Ethylene
ETI	Effector triggered immunity
ETR1	Ethylene response 1
Fd	Ferridoxin
FHV	*Flock house virus*
FNR	Ferredoxin NADPH oxidoreductase
FT	FLOWERING LOCUS
G3P	Glycerol-3-phosphate
GA	Gibberellic acid
GABA	γ-aminobutyric acid
h	Hour
H_2O_2	Hydrogen peroxide
HATs	Histone acetyl-transferases
hc	Hetero chromatin
HEN1	HUA ENHANCER1
hpRNAs	Hairpin RNAs
HR	Hypersensitive reaction
HSP70h	Hsp70 homologue protein
HSPs	Heat shock proteins
IAA26	Indole acetic acid 26
IC	Isochorismate pathway
IR	Inverted repeat transcripts
IRES	Internal ribosome entry sites
IV	*Influenza virus*
JA	Jasmonic acid
JAR1	Jasmonate resistant 1
JIN1	Jasmonate insensitive1
kDa	Kilo Dalton
KTF1	Kow-domain containing transcription factor 1

LCD	La-type winged helix-turn-helix motif
LHC II	Light harvesting complexes
LMMs	Lesion mimic mutants
LMV	*Lettuce mosaic virus*
lnc RNAs	Long non-coding RNAs
LOL1	LSD-one-Like 1
LRR	Leucin-rich repeat domain
lsd1	Lesions stimulating disease resistance response 1
lsiRNAs	Long siRNAs
MAMP/PAMP	Microbe/pathogen associated molecular pattern
MAPKs	Mitogen-activated protein kinase
MeSA	Methylsalicyclate
miRNA	Micro-RNA
mM	Milli Molar
MP	Movement protein
MTI/PTI	MAMP/ PAMP triggered immunity
nat-siRNAs	Natural antisense siRNAs
NB-LRR	Nucleotide binding leucin rich repeat
NBS	Nucleotide binding sites
NBT	Nitro blue tetrazolium
NDR1	Non-race specific disease resistance
ng	Nano gram
NGS	Next generation sequencing
NLS	N-terminal nuclear localization signal
NO	Nitric oxide
$NO2^-$	Nitrile
NOS	Nitric oxide synthase
NPR1	Non-expressor of PR genes1
NR	Nitrate reductase
nt	Nucleotides
NtROS1	Repressor of silencing1
$O2^-$	Superoxide
OEC	Oxygen evolving complex
$ONOO^-$	Peroxynitile molecule
OPPP	Oxidative pentose phosphate pathway
ORFs	Open reading frames
PAD4	Phytoalexin deficient 4
PAL	Phenylalanine ammonia lyase
PAP85	Vicilin like seed storage protein

PAPK	Plasmodesmatal-associated protein kinases
PAR	Photo assimilation related
PAZ	Piwi/Argonaute/Zwille
PBS	Phosphate buffered saline
PCD	Programmed cell death reaction
PCR	Polymerase chain reaction
PD	Plasmodesmata
PK	Protein kinase domain
PME	Pectin Methyl Esterase
Pol II	RNA polymerase-II
pol IV	Polymerase IV
pol V	Polymerase V
PR	Pathogenesis-related protein
pre	Precursor
pri	Primary
PRK	Phosphoribulose kinase
PRRs	Pathogen recognition receptors
PS II and PS I	Photosystems II and I
PsaN	PS I reaction center subunit
PTGS	Post-transcriptional gene silencing
PVX	*Potato virus X*
PVY	*Potato virus Y*
RT-qPCR	Quantitative real-time PCR
ra-siRNAs	Repeat- associated siRNAs
Rboh	Respiratory burst oxidase homologue
RBCL	Ribulose-bisphosphate carboxylase large subunit (RBCL)
RCA	Rubisco activase
RdDM	RNA dependent DNA-methylation
RDR	RNA dependent RNA polymerase
RDV	*Rice dwarf virus*
R-genes	Resistance genes
rgs-CaM	Regulator of gene silencing calmodulin
RIN4	RPM1-interacting protein 4
RIPs	Ribosomal inactivating proteins
RISC	RNA-induced silencing complexes
RITS	RNA-induced transcriptional silencing
RLKs	Receptor-like kinases
RLPs	Receptor like proteins
RNA Pol II	RNA polymerase-II enzyme

RNAi	RNA interference
ROS	Reactive oxygen species
R-proteins	Resistance proteins
RPS2	Resistance to pseudomonas syringae 2
SA	Salicyclic acid
SABP2	Salicyclic acid binding protein 2
SAR	Systemic acquired resistance
SEL	Size exclusion limit
Sid2	SA induction-deficient2
siRNAs	Small interfering RNAs
SPMMV	*Sweet potato mild mottle virus*
ss	Single stranded
TAS	Transacting siRNA genes
tasiRNAs	Transacting siRNAs
TCA	Tricarboxylic acid cycle
TGBp	Triple-gene block proteins
TGS	Transcriptional gene silencing
TM	Trans-membrane domains
TMV	*Tobacco Mosaic Virus*
TMVi	TMV infected
TOM1	Tobamovirus multiplication 1
ToMV	*Tomato Mosaic Virus*
TrAp	Transcriptional activator protein
TRV	*Tobacco rattle virus*
TYLCV	*Tomato yellow leaf curl virus*
TYMV	*Turnip yellow mosaic virus*
µg	Microgram
VIGS	Virus induced gene silencing
VSRs	Viral suppressors of RNA silencing
wt	Wild type

ABSTRACT

Plant-virus interactions are very complex in nature and lead to disease and symptom formation by causing various physiological, metabolic and developmental changes in the host plants. These interactions are mainly the outcomes of viral hijacking of host components to complete their infection cycles and of host defensive responses to restrict the viral infections. Viral genomes contain only a small number of genes often encoding for multifunctional proteins, and all are essential in establishing a viral infection. Thus, it is important to understand the specific roles of individual viral genes and their contribution to the viral life cycles. Among the most important viral proteins are the suppressors of RNA silencing (VSRs). These proteins function to suppress host defenses mediated by RNA silencing and can also serve in other functions, e.g. in viral movement, transactivation of host genes, virus replication and protein processing. Thus these proteins are likely to have a significant impact on host physiology and metabolism. In the present study, I have examined the plant-virus interactions and the effects of three different VSRs on host physiology and gene expression levels by microarray analysis of transgenic plants that express these VSR genes. I also studied the gene expression changes related to the expression of the whole genome of *Tobacco mosaic virus* (TMV) in transgenic tobacco plants.

Expression of the VSR genes in the transgenic tobacco plants causes significant changes in the gene expression profiles. HC-Pro gene derived from the *Potyvirus Y* (PVY) causes alteration of 748 and 332 transcripts, AC2 gene derived from the *African cassava mosaic virus* (ACMV) causes alteration of 1118 and 251transcripts, and P25 gene derived from the *Potyvirus X (PVX)* causes alterations of 1355 and 64 transcripts in leaves and flowers, respectively. All three VSRs cause similar up-regulation in defense, hormonally regulated and different stress-related genes and down-regulation in the photosynthesis and starch metabolism related genes. They also induce alterations that are specific to each viral VSR. The phenotype and transcriptome alterations of the HC-Pro expressing transgenic plants are similar to those observed in some Potyvirus-infected plants. The plants show increased protein degradation, which may be due to the HC-Pro cysteine endopeptidase and thioredoxin activities. The AC2-expressing transgenic plants show a similar phenotype and gene expression pattern as HC-Pro-expressing plants, but also alter pathways related to jasmonic acid, ethylene and retrograde signaling. In the P25 expressing transgenic plants, high numbers of genes (total of 1355) were up-regulated in the leaves, compared to a very low number of down-regulated genes (total of 5). Despite of strong induction of the transcripts, only mild growth reduction and no other distinct phenotype was observed in these plants.

As an example of whole virus interactions with its host, I also studied gene expression changes caused by *Tobacco mosaic virus* (TMV) in tobacco host in three different conditions, i.e. in transgenic plants that are first resistant to the virus, and then become susceptible to it and in wild type plants naturally infected with this virus. The microarray analysis revealed up and down-regulation of 1362 and 1422 transcripts in the TMV resistant young transgenic plants, and up and down-regulation of a total of 1150 and 1200 transcripts, respectively, in the older plants, after the resistance break. Natural TMV infections in wild type plants caused up-regulation of 550 transcripts and down-regulation of 480 transcripts. 124 up-regulated and 29 down-regulated transcripts were commonly altered between young and old TMV transgenic plants, and only 6 up-regulated and none of the down-regulated transcripts were commonly altered in all three plants. During the resistant stage, the strong down-regulation in translation-related transcripts (total of 750 genes) was observed. Additionally, transcripts related to the hormones, protein degradation and defense pathways, cell division and stress were distinctly altered. All these alterations may contribute to the TMV resistance in the young transgenic plants, and the resistance may also be related to RNA silencing, despite of the low viral abundance and lack of viral siRNAs or TMV methylation activity in the plants.

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1. INTRODUCTION

Phytopathogens cause damages to crop plants and cause billions of dollars in losses every year across the world. Different pathogens use plants only for nutrients (necrotrophs) or as living hosts to complete their life cycles (biotrophs), or for both nutrients and as living host (hemibiotrophs). Bacteria and fungi can exhibit both necrotrophic and biotrophic pathogenesis, whereas viruses are totally biotrophic pathogens. Viruses can replicate only intracellularly and they can move and spread via the plasmodesmata and vascular system to cause systemic infection in their susceptible hosts. (Irrelevant section deleted)

Plants have also effective multilayered defense mechanisms to prevent pathogen entry and infection process. The first lines of defense are constitutive and composed of preformed physical or chemical barriers, which provide non–specific protection against a wide range of pathogens. Different structural barriers like waxy layers, cutin, suberin, robust cell walls and their associated enzymes, and secondary metabolites like terpenoids and phenolic and alkaline compounds provide defense against a large number of pathogens (Chassot et al., 2007; Chassot et al., 2008; Huckelhoven, 2007; Mysore and Ryu, 2004; Thordal-Christensen, 2003). In order to invade their host plants, the pathogens need to be able to evade or break through any existing plant defense mechanisms. Therefore, they can employ a variety of methods to break these constitutive barriers. (Irrelevant section deleted)

Successful pathogens that evade the constitutive defense mechanisms are confronted with the second line of defenses i.e. the induced defenses composed of two branches of interconnected mechanisms (Dodds and Rathjen, 2010; Jones and Dangl, 2006). The first mechanism known as MAMP/PAMP triggered immunity (MTI/PTI) recognizes a variety of microbe/pathogen associated molecular pattern structures (MAMPs/ PAMPs) by the surface-based receptors known as pathogen recognition receptors (PRRs). The PAMPs consist of a variety of pathogen components like flagella, chitins, peptidoglycans, peptides and elongation factors EF-Tu, lipopolysaccharides and quorum sensing factors (Boller and Felix, 2009; Monaghan and Zipfel, 2012). Interestingly, these PAMPs are continuously under selective pressure to change and avoid the recognition by the PTI/ MTI mechanism. Thus they frequently develop modified key residues or epitopic regions (Cai et al., 2011; McCann et al., 2012). The PTI mechanism consists of two types of PRRs i.e. of membrane based transmembrane receptor-like kinases (tRLKs) and receptor like proteins (RLPs). Nearly 610 RLKs type PRRs and 57 RLPs have been identified in plants so far (Lehti-Shiu et al., 2009; Wang et al., 2008). The tRLKs have an extracellular leucin-rich repeat domain (LRR), transmembrane domain and intracellular serine/threonine kinase domain, whereas

RLPs comprise of only extracellular lysine and transmembrane domain without any intracellular domain (Akira et al., 2006). Several studies indicate that the LRR-domain containing PRRs (LRR-PRRs) interact with the peptide PAMPs (Lee et al., 2009; Takai et al., 2008) and the Lysine-domain containing PRRs (LysM-PRRs) recognize the peptidoglycan PAMPs (Iizasa et al., 2010; Kaku et al., 2006). Upon activation by PAMPs, the PRR initially exhibit alkalization, followed by activation of mitogen-activated protein kinase (MAPKs), reactive oxygen species (ROS) formation, callose deposition in the plasmodesmata region in the cell walls, and production of other defense-related and pathogenesis-related proteins (PR) and defensins. However, certain pathogens can evade this PTI-mediated defense mechanism by producing different effector molecules. Most studied bacterial effector molecules are secreted through Type III secretion systems, and are involved in creating suitable environment to the bacterial pathogens to adopt to the plants such as nutrients supply or suppression of the PTI defense (Badel et al., 2002; Jones and Dangl, 2006).

Plants have also developed resistance genes (R-genes), which recognize the pathogen effector molecules and provide further resistance by effector triggered immunity (ETI). The ETI is considered as an amplified version of PTI, and upon activation, it leads to a cascade of defense responses like hypersensitive cell death (HR), systemic acquired resistance (SAR), and activation of defense proteins, ion fluxes and salicylic acid production (Boller and He, 2009; Dodds and Rathjen, 2010; Jones and Dangl, 2006).

Also another evolutionarily conserved defense mechanism i.e. RNA silencing is present in plants, and efficiently provides resistance against viral pathogens. It is also involved in the regulation of numerous plant genes, maintenance of genome integrity and proper development in plants (Baulcombe, 2004; Brodersen and Voinnet, 2006; Incarbone and Dunoyer, 2013; Meins et al., 2005; Voinnet, 2001; Wang et al., 2012). This versatile mechanism is induced by different double stranded RNA (dsRNA) molecules that are formed, for instance, by replicating viral RNAs (Baulcombe, 2004). Thus this mechanism is induced by a pathogen "elicitor" in a similar way as the R-genes in plants but producing a defense reaction that is specific against the inducing sequence. However, the defense mechanism itself is generic, and induced by the dsRNA produced by any replicating virus. Plant viruses produce specialized proteins known as viral suppressors of RNA silencing (VSRs) to counteract the RNA silencing mechanism. These VSRs can interfere with RNA silencing mechanism at different steps to evade the viral RNA degradation. At the same time they may cause disturbances in the micro-RNA (miRNA) pathways, thereby inducing various developmental defects, different metabolic and regulatory pathway impairment, and all-in-all, provoking very complex plant-virus interactions in the infected host (Baulcombe, 2004; Incarbone and Dunoyer, 2013; Kasschau et

al., 2003; Lu et al., 2004; Roth et al., 2004; Shimura and Pantaleo, 2011; Vance and Vaucheret, 2001).

Altogether, these antiviral plant defense mechanisms evoke complex intercellular signaling events in plants, ranging from the phytoalexins production to hormonal cross talk, defense gene induction, SAR, small RNA-mediated resistance and disturbance of the developmental and signalling pathways.

This dissertation focuses on the gene expression patterns related to the complex plant-virus interactions involving the antiviral defense mechanisms like ETI and RNA silencing, and counter defense mediated by the VRS factors. First, I analyse a simplified situation, where single viral VRS genes are expressed in transgenic tobacco plants to see how these alone affect the gene expression profile of the host. The full complex interactions between a viral genome and host plant is analysed by using transgenic tobacco plants that express the whole infectious genome of *Tobacco mosaic virus* (TMV). Although, TMV typically has a compatible reaction in tobacco host, these plants are first resistant to the endogenously expressed virus, and become susceptible to it only at a later stage. The gene expression profiles are compared to the wild type tobacco plants that are naturally infected with TMV.

1.1 Plant resistance against pathogens

The recognition mechanisms between plants and pathogens are typically specific for each plant-microbe combination. Still, after recognition stage, the defense reactions and signaling pathways can be very similar to the different types of pathogens. The current understanding of these pathways is described here.

1.1.1 Structure and classification of the resistance proteins

Resistance proteins (R-proteins) are the second line of defense present in plants. Primarily, these proteins provide the resistance against different pathogens by recognizing the effector molecules, but some of them are involved also in other functions like down-stream signaling of resistance, cytokinin pathway and photomorhogenesis process (Faigon-Soverna et al., 2006; Gabriels et al., 2007; Igari et al., 2008). R-proteins contain a variety of conserved structural motifs such as nucleotide binding sites (NBS), LRR, Toll-interleukin-1 receptors (TIR) that have similarities with toll like receptor in drosophila and mammalian interleukin-1 receptor, coiled-coil domains (CC), trans-membrane domains (TM) and protein kinase domains (PK) (Glowacki et al., 2011). Based on these domain structures, R-proteins are categorized mainly to four classes i.e. the NBS-LRR type, receptor-like kinases (RLKs), LRR-TM and TM-CC R-proteins.

The widely distributed NBS-LRR class is further divided into the TIR-NBS-LRR and CC-NBS-LRR sub-classes based on the N-terminal domain of the NB-LRR protein (Liu et al., 2007). Interestingly, the distributions of these sub-classes are varied in different plant species. For example, about 60 of the R-proteins in *Arabidopsis* and in *Brassica rapa* are of the TIR-NBS-LRR type and about 30 are of the CC-NBS-LRR type, whereas rice genome codes for more than 500 CC-NBS-LRR type R-genes (Bai et al., 2002; Mun et al., 2009; Zhou et al., 2004). This indicates that the monocots have more of CC- type R-genes, and the dicots more of the TIR- type R-genes (Meyers et al., 2003; Mun et al., 2009; Zhou et al., 2004).

The domains present in R-proteins have specific functions: NBS domain has the NTP-hydrolyzing activity that starts the molecular signal transduction after R-protein activation, and LRR domains are predicted to be involved in protein-protein interactions (i.e. Effector recognition) whereas both CC- and TIR- domains are involved in defense signaling and effector recognition (Burch-Smith et al., 2007; Ellis et al., 2000). Evolutionary studies prove the constant pressure on the R-genes to keep up and combat with the adaptable pathogens that constantly change strategies. Duplications, i.e. tandem and segmental factors, mutations and LRR structural differences appear to be involved in the allelic diversification of the R-genes and in defense against a variety of pathogens (Joshi and Nayak, 2012).

1.1.2 Effector recognition by R-proteins and their activation

The R-gene mediated resistance is triggered by specific avirulence factors produced by the pathogens. For instance, the N-gene of tobacco and the helicase domain of the replicase protein of TMV, the resistance gene Tm-2(2) and the movement protein of *Tomato Mosaic Virus* (ToMV) are examples of R-proteins, and their corresponding triggering effector molecules (Luderer and Joosten, 2001; Padgett et al., 1997; Weber et al., 1993) Three models have been proposed to explain the effector recognition mechanisms by the R-proteins, known as direct, indirect and combined bait and switch-type interactions (Dodds and Rathjen, 2010; van der Hoorn and Kamoun, 2008) (Figure 1). The direct recognition mechanism involves physical interaction between the effector molecules and the R-proteins, as indicated by studies of different chimeric viruses, and by using Yeast Two-Hybrid (YTH) and Bifluorescence confocal microscopy (BiFC). In indirect effector recognition, interaction between the effector molecules and R-proteins can be mediated by the accessory molecules known as guardee (the guard hypothesis) or decoy molecules (the decoy model). An example of the guard hypothesis is illustrated by the Arabidopsis RPM1-interacting protein 4 (RIN4 protein, guardee), which is guarded by the NB-LRR proteins RPM1 and Resistance to Pseudomonas Syringae 2 (RPS2). Any modification or degradation of this RIN4 protein by the *Pseudomonas syringae* protease effectors AvrB

and AvrRpm1 or AvrRpt2 leads to the RPM1 or RPS2 R-protein activation (Mackey et al., 2002; Mackey et al., 2003). However, guard hypothesis does not prove yet the RIN4 protein as an effector target, and it also faces some evolutionary constraints. To circumvent these problems, the decoy model is proposed to explain indirect recognition of an accessory protein that is produced by duplication of the effector target gene. This duplication of target gene is exemplified by tomato Pto-family kinase that is involved in complex formation with tomato R-protein Prf. Pto kinase shares similarity with kinase domains of FLS2 and CERK1, which are targets of the effector molecules AvrPto and AvrPtoB of *Pseudomonas syringae* (Dodds and Rathjen, 2010; Mucyn et al., 2006; van der Hoorn and Kamoun, 2008). However, decoy model fails to explain the role of the accessory molecule other than the effector perception. For instance, Pto kinase interaction with effector molecules disrupts the Pto interaction surface that leads to trans-phosphorylation events, which are necessary for the signalling process (Ntoukakis et al., 2013). Later bait and switch-model was proposed to explain the role of the accessory molecule. In this model the effector molecule first interacts with the accessory protein (bait) that is engaged with R-protein and causes subsequent R-protein activation (switch) by ATP nucleotide binding and alteration of LRR/ARC2 domains interface (Collier and Moffett, 2009) (Figure 1).

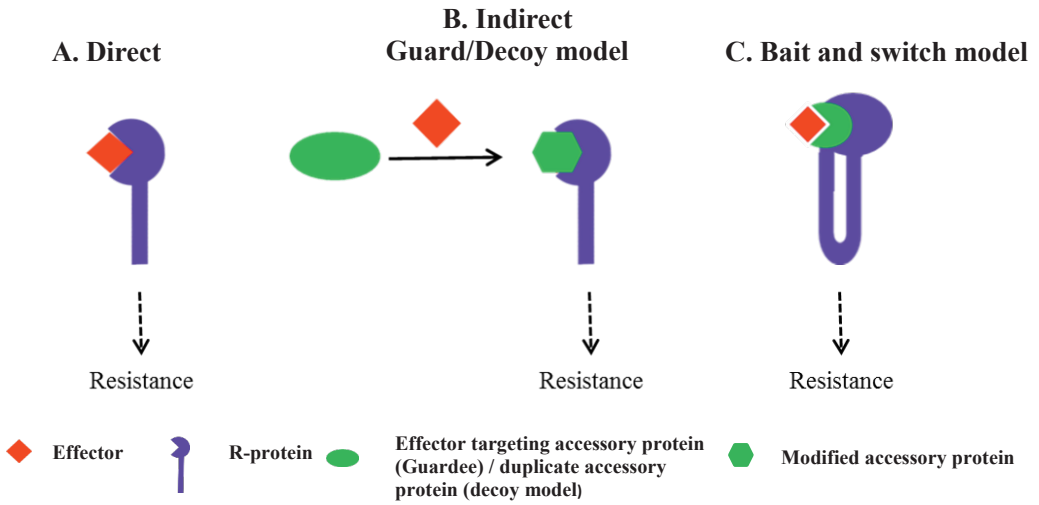

Figure 1. Three different models explaining the recognition between the host R-protein and the pathogen effector. (A) In Direct model, receptor-ligand binding type physical interaction occurs between effector molecule and R-proteins and leads to resistance. **(B)** In Indirect model, effector molecule targets and modifies the host accessory protein (Guard model) or its duplicate (Decoy model) and the modified protein is recognized by the R-protein and leads to resistance. **(C)** In Bait and switch model, the accessory protein (bait) associated with R-protein is first targeted by effector and subsequently activates R-protein (switch) i.e. leads to resistance. (Adapted and modified from the Dodds et al., 2010, Nat Rev Genet 11:539-548)

Activation mechanisms of R-genes are still unknown, but presently some models are available to explain them. Takken et al. (2009) proposed a model, which states that in the pathogen absence, the R-proteins are in an ADP-bound "OFF" state, i.e. autoinhibited by the multi-domain intramolecular interaction and stabilized by the LRR domain (Takken and Tameling, 2009). The R-proteins NB-domain and its ARC sub-domains appear to play the key role in this autoinhibitory process (Rairdan and Moffett, 2006). During the effector recognition, two conformational changes occur in the R-protein domains. An open conformation is formed by changing the interface between the N-terminal part of the LRR domain and ARC2 sub-domain followed by the nucleotide exchange (ADP to ATP conversion). This provides further conformational changes between the central NB-ARC, the N-terminal TIR/CC and C-terminal LRR domains. This results in the "ON" state where R-proteins can initiate defense signaling. Once the ATP hydrolysis occurs R-proteins rearrange back to the ADP-bound autoinhibited "OFF" state (Takken and Tameling, 2009).

1.1.3 Signaling and defense responses

The down-stream signalling events of R-genes are less known. The TIR- and CC-type R-protein functions are known to be mediated by the Enhanced disease susceptibility 1 (EDS1) pathway and Non-race specific disease resistance (NDR1) pathway, respectively (Day et al., 2006; Wiermer et al., 2005) (Figure 2). The EDS1 is an important signalling molecule acting together with its partner Phytoalexin deficient 4 (PAD4) in plants and provides the resistance to the biotrophic and hemi-biotrophic pathogens. The EDS1 is homologous to the eukaryotic lipase molecule and the null *eds1* mutants in Arabidopsis have lost the (ability to exhibit) resistance even in the presence of autoactivation mutations in TIR-NBS-LRR proteins (Falk et al., 1999; Wiermer et al., 2005). The NDR1 mediated signalling pathway is activated by the guard proteins RPM1 and RPS2 in the resistance reaction mediated by the RIN4 protein interaction with the *P. syringae* effector molecule AvrRpt2 (Day et al., 2006). *NDR1* mutants are unable to generate the ROS accumulation, and have defects in the salicylic acid (SA) accumulation and SAR induction (Shapiro and Zhang, 2001).

R-protein response comprises the HR and SAR defense responses. Likewise with the PTI mechanism, ion flux changes, kinase cascade activation, ROS and Nitric oxide (NO) production, defense hormones induction and transcriptional changes occur in the early stages of ETI, which subsequently lead to the HR and activation of SAR in plants (Pontier et al., 1998). The early signaling activities are mediated by a variety of molecules like calcium, NO and ROS, which could amplify the physiological response through the transcriptional and metabolic changes (Figure 2).

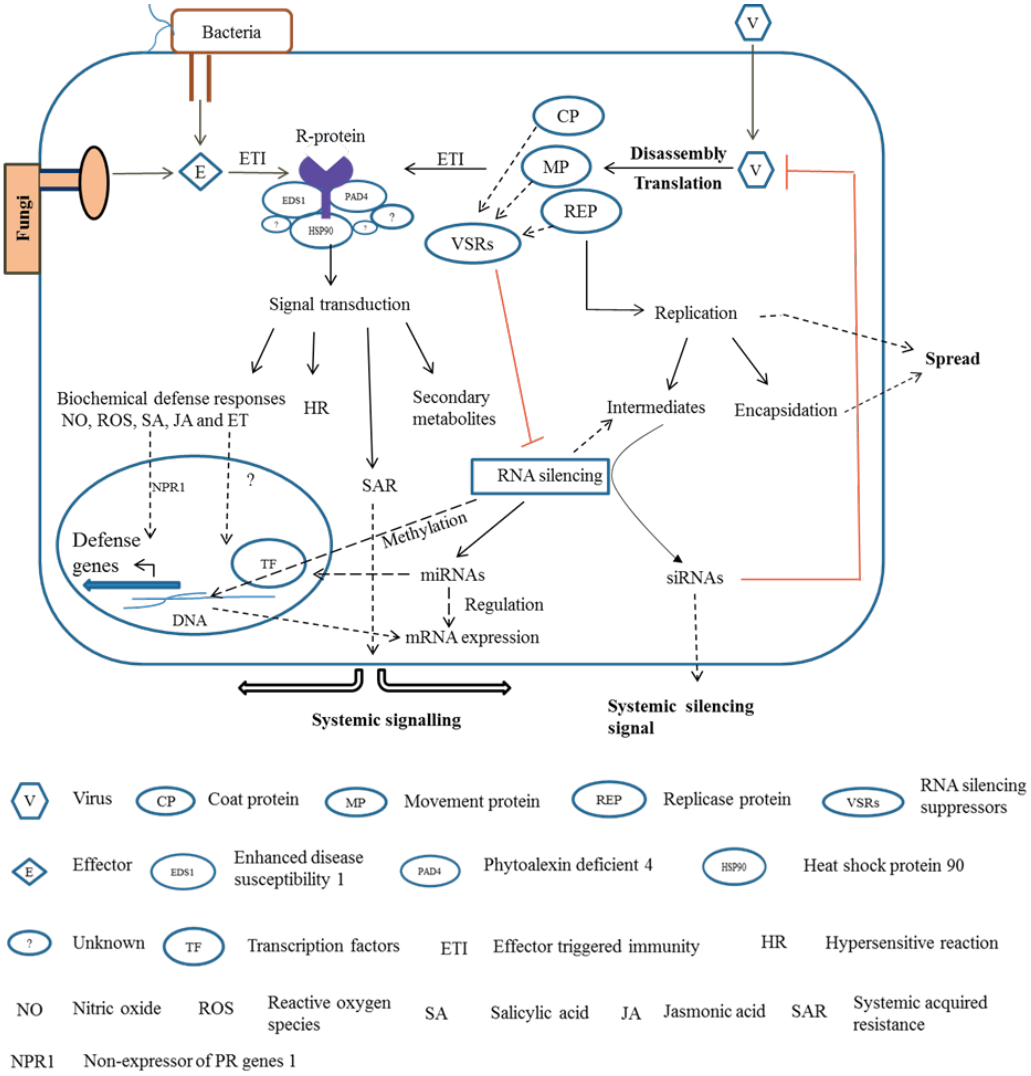

Figure 2. Brief overview of R-gene signalling and RNA silencing events and their interconnections during the virus infection process (Adapted and modified from the http://www.helsinki.fi/ppvir/research/mol_bio_anal_poty/ website, Manadi K et al., 2013; 25:1489-1505)

1.1.3.1 Calcium

In the ion fluxes, calcium is the important mediator that acts as a secondary messenger. It carries the perceived information to be translated to specific biological response by changing the cytosolic free calcium concentration (Dodd et al., 2010). Calcium signal maintains the different forms of signatures for different stimuli like light, temperature, drought and stress. Interestingly, these signatures are different even for the same stimulators (Lecourieux et al., 2002; Lecourieux et al., 2006). In plants, three types of calcium channels mediate the calcium signalling, i.e. the nucleotide gate channels, glutamate like receptors and two pore channels (Ma and Berkowitz, 2011; Michard et al., 2011; Pottosin et al., 2009). During the

avirulent pathogen infection calcium shows biphasic accumulation, so that the first pulse is a common response, and the second one is specific to the avirulent pathogen (Grant et al., 2000b). In the early events, the external calcium influx occurs from the apoplastic region to the cytoplasm, whereas in the intense phase the influx comes from the internal organelles such as endoplasmic reticulum (ER), vacuoles, mitochondria and chloroplasts (Garcia-Brugger et al., 2006; McAinsh and Pittman, 2009). Calcium flux is also needed for the generation of hydrogen peroxide that participates in the oxidative burst, which indicates the calcium involvement in the other early events like ROS generation (Neill et al., 2002)

1.1.3.2 ROS

R-gene recognition of avirulent pathogens elicits the oxidative burst in the insusceptible plants, which is operated by the ROS, including superoxide (O_2^-) and hydrogen peroxides (H_2O_2) molecules. Similar to the calcium biphasic accumulation, ROS accumulation against avirulent pathogens also includes two phases i.e. the common or transient and the more intense phase (Grant and Loake, 2000; Lamb and Dixon, 1997). The ROS is mainly generated apoplastically by NADPH oxidases, cell wall peroxidases and polyamine oxidases, and intracellularly by the plastids and mitochondria, as byproducts of the photosynthesis, respiration and photorespiration mechanisms (Bolwell et al., 2002; Brown et al., 1998; Grant et al., 2000a; Wojtaszek, 1997; Yoda et al., 2006). The NADPH oxidases in Arabidopsis are encoded by ten respiratory burst oxidase homologue (*Rboh*) genes that code for 300 N-terminal amino acids with two calcium binding EF-hand motifs. Double knock-out mutants of *RbohD* and *Rbohf* plants exhibit compromised HR-responses indicating that these genes are involved in the plant defense and stress signaling responses (Torres et al., 2002; Torres and Dangl, 2005).

Plants maintain the equilibrium of ROS molecule accumulation and scavenging activities purposely for cell needs. Different antioxidant mechanism act as ROS molecule scavengers during the oxidative stress condition in the cell, whereas in other cases such as defense response, plant uses ROS molecules as important defense signals, and also in the strengthening of the cell walls via cross linking of glycoproteins (Bradley et al., 1992; Levine et al., 1994). Indeed, the ROS levels in plants can determine the fate of the cell survival. For instance, if the ROS are induced to low level the protective antioxidant molecules are induced and allow the cell survival, but if ROS are induced to medium and high levels, the cell undergoes oxidative stress and programmed cell death (HR-PCD) reaction (Karuppanapandian et al., 2011).

1.1.3.3 NO

NO also plays a key role in the plant disease resistance mediated by the R-genes. NO is a lipophilic gaseous molecule at atmospheric conditions, which reacts with the oxygen and its radicles and forms the peroxynitrile molecule ($ONOO^-$), which is very destructive

(Bonfoco et al., 1995). Besides its defense role, NO plays a role in biological functions in plants such as promotion of seed germination, floral transition and mediation of stomatal movements (Wilson et al., 2008). It conducts most of these functions either via its oxidative or antioxidative properties, which depend on NO concentrations and environmental conditions (Beligni and Lamattina, 1999; Leshem and Haramaty, 1996). As in mammals, NO signalling in plants also operates through the cyclic guanidine monophosphate (cGMP) as a downstream signal molecule by inducing the PR1 and Phenylalanine ammonia lyase (PAL) defense genes (Durner et al., 1998).

The NO in the plants might be produced enzymatically by Nitric oxide synthase (NOS) and nitrate reductase (NR) enzymes, and might also be produced by the chemical reduction of nitrile (NO_2^-). However, in plants, no direct homologues of *NOS* genes have been identified, but some recent studies provide proof of closely related homologues of animal *NOS* in the unicellular algae *Ostreococcus tauri* (Foresi et al., 2010). Guo et al. 2003 also reported the gene *AtNOS1* in Arabidopsis exhibiting NOS activity. It has some similarity with snail *NOS*-gene but not with mammalian NOS enzyme (Guo et al., 2003). However, recombinant AtNOS1 does not exhibit any NOS activity and it is thought to play an indirect role in NO production, thus it has been renamed also as NO-associated protein 1 (AtNOA1) (Crawford et al., 2006; Zemojtel et al., 2006). The second probable route for NO generation in plants can be operated by the NR enzyme that convert the nitrate to NO and N_2O molecules in a NADP-(H) dependent manner. Arabidopsis encodes for two NR genes, namely *NIA1* and *NIA2*. The double mutant plant of these genes exhibits NO production suppression and is defective in stomatal closing response to the abscisic acid (ABA) (Desikan et al., 2002). Also, several other non-enzymatic routes such as carotenoids and light, and SA and H_2O_2 involvement in the NO production or accumulation are reported in plants (Gaupels et al., 2008; Wojtaszek, 2000).

These changes, i.e. the calcium, ROS and NO production are followed by the induction of defense hormones in the plants. SA, ethylene (ET) and jasmonic acid (JA) are considered as important defense hormones in plants, which act specifically, synergistically and also antagonistically to provide the defense against a variety of pathogens (biotrophic, hemi-biotrophic and necrotrophic) (Koornneef and Pieterse, 2008).

1.1.3.4 Hormones

SA provides the resistance mainly to the biotrophic and hemi-biotrophic pathogens. Besides this, SA is also involved in plant development, metabolism, growth and signalling interactions (Vlot et al., 2009). Chemically SA is an ortho-hydroxy-benzoic acid molecule, and originates from chorismate, from the shkimate pathway in the chloroplasts. From the chorismate, SA can be produced in two ways i.e. by the Isochorismate pathway (IC) or by the PAL pathway. During pathogen infections, the SA appears to be produced by the former pathway (Chen

et al., 2009; Wildermuth et al., 2001). Various genes like *EDS1, SA induction-deficient2 (Sid2), EDS4, EDS5* and *PAD4* are acting as upstream SA signaling components. Both EDS1 and PAD4 encode for putative lipase molecules and their mutants are unable to accumulate SA, and defective in oxidative burst and HR-PCD reaction (Feys et al., 2001; Jirage et al., 1999; Parker et al., 1996). The downstream SA signalling is mainly operated through Non-expressor of pathogenesis related genes1 (NPR1), which controls the expression of 90% of SA-dependent genes (Wang et al., 2006). NPR1 is reported to play a cofactor role in the SAR response in the plants by transiently interacting with TGA transcription factors at their DNA-unbound fraction (Jakoby et al., 2002; Johnson et al., 2008).

JA plays a major role in defense against insects, wounding and necrotrophic pathogens along with other functions such as root and tuber formation, seed germination and opening of stomata (Wasternack, 2007). It resembles structurally prostaglandins in the mammals. JA signalling consists mainly of three genes i.e. coronatine insensitive1 (*COI1*), jasmonate resistant 1(*JAR1*) and jasmonate insensitive1 (*JIN1*). *COI1* mediates the JA response by encoding an F-box protein, JAR is involved in the synthesis of bioactive JA molecule by conjugation of isoleucine molecule to JA and the *JIN* encodes the transcription factor that activates the JA responsive genes (Lorenzo et al., 2004; Staswick and Tiryaki, 2004; Xie et al., 1998).

During the R-gene activation, the gaseous hormone ethylene is released to induce the defense responses, or to act synergistically with other defense hormones to induce the defense responses. It is well known for its functions such as senescence, fruit ripening, and abscission in plants (van Loon et al., 2006). Ethylene signaling in Arabidopsis is operated by five kinds of molecules, namely ethylene response 1 and 2 (ETR1, ETR2) ethylene response sensor 1 and 2 (ERS1, ERS2) and ethylene insensitive 1 (EIN1). It can be synthesized by a three step process from methionine precursor using 1-aminocyclopropane-1- carboxylate (ACC) synthase, ACC oxidase and ATP as the main players. Different pathogens use their effector molecules to targets the ethylene defense mechanism to invade the plants (Kim et al., 2013).

1.1.4 HR response

HR has been first identified and described as a rapid and localized cell death by Stakman in 1915. HR phenotype usually occurs as brown colored lesion on and around the infected dead cells, the spots ranging from one cell to many. Some of the morphological HR symptoms in different pathosystems contain membrane blebbing, nucleus condensation, and production of apoptotic body like structures, DNA fragmentation, mitochondrial swelling and cell shrinkage (Pontier et al., 1998). Studies of Etalo et al. 2013 indicate that the HR response is an energy driven process that obtains most of its energy from the hydrolysis of sugars, ubiquitin-mediated protein degradation and lipid catabolism. Also,

accumulation of secondary metabolites like hydroxycinnamic acid amides and WRKY transcription factors are observed (Etalo et al., 2013).

HR is considered as a form of PCD and its regulation is very important to the plant survival and defense responses (Pontier et al., 1998). The candidate genes or important players involved in this regulation has been elucidated from various studies by using the lesion mimic mutants (LMMs) that exhibit HR-like phenotype. LMM plants are either initiation mutants, which exhibit the regulated PCD or propagation mutants, which exhibit uncontrolled PCD (Lorrain et al., 2003). Lesions stimulating disease resistance response 1 (*lsd1*) mutant is an example of the uncontrolled cell death mutant. In these plants, abnormal superoxide accumulation is observed during the onset of PCD and its spread to adjacent cells, which indicates a role of superoxide in cell death spreading (Jabs et al., 1996). The LSD1 shows interaction with other proteins such as transcription factors Atbzip10, LSD-one-Like 1 (LOL1) and metacaspases (AtMC1), which are key players in the PCD reaction (Coll et al., 2010; Coll et al., 2011; Epple et al., 2003; Kaminaka et al., 2006).

1.1.5 SAR

The SA produced during the HR response induces SAR in the infected plant. This SAR provides resistance to unchallenged tissues by mounting non-specific long lasting broad range defense responses (Durrant and Dong, 2004). Endogenously accumulated SA induces the SAR response with the help of NPR1 (Cao et al., 1997), and the stability of SAR is maintained by NPR1 paralogs called NPR3 and NPR4 (Fu et al., 2012). In high SA concentrations, the NPR1-NPR3 interaction occurs in the nucleus, which results in the degradation of NPR1 protein and induces the PCD and ETI responses. Simultaneously, in the neighboring cells, low or intermediate levels of SA provoke the NPR1-NPR4 disruption, which leads to accumulation of the NPR1 protein in the nucleus and causes the induction of SAR (Fu and Dong, 2013).

SAR induction, i.e. the elicited defense response in the distal parts of the plants is maintained by the mobile signal from the infection site. So far, several different molecules have been proposed for the SAR mobile signals, but their contributions are still debated. In earlier studies, the signal was proposed to be SA, due to its presence in the phloem sap upon pathogen infection. However, in grafting experiment SA has been proven dispensable for SAR, but needed for mobile signal to induce SAR in distant tissues (Metraux et al., 1990; Vernooij et al., 1994). Later, two other molecules, i.e. methyl salicylate (MeSA) and JA compounds have been proposed as SAR mobile signals, based on the grafting and mutant studies: Salicylic acid binding protein 2 (SABP2) converts the inactive MeSA to active SA, and induction of SAR was failed in the SABP2- mutants. Also, failure of JA-related mutants *opr3* and *jin1* failed to induce SAR, and conversely, exogenous JA application induced SAR (Park et al., 2007; Truman et al., 2007). However, Attaran et al.

2009 provided evidence that neither MeSA nor JA are essential for the induction of SAR (Attaran et al., 2009). Subsequently, petiole exudate analyses have provided more putative SAR mobile signal molecules, such as Azealic acid (AzA), Glycerol -3-phosphate (G3P) and Abietane diterpenoid dehydroabietinal (DA) (Chanda et al., 2011; Chaturvedi et al., 2012; Jung et al., 2009). Interestingly, all these three molecules need Defective in induced resistance 1 (DIR1) lipid transfer protein for their functions, despite their accumulation time after avirulent pathogen infection (Dempsey and Klessig, 2012).

1.2 RNA silencing

RNA silencing is a conserved and widespread antiviral defense mechanism present in various eukaryotic organisms. As its name suggests, RNA silencing mechanism controls the RNA levels and their expression activities by sequence-specific RNA degradation, or by translation inhibition mechanism (Baulcombe, 2004; Brodersen et al., 2008; Voinnet, 2001). It can target both viral and endogenous messenger RNAs. Similarly, it can also operate at transcriptional levels by inducing epigenetic modification (methylation) of DNA (Brodersen and Voinnet, 2006). RNA silencing was discovered in plants in the 1990's via observations of two plant scientists, C. Napoli and V. Krol. They noticed that a transgene reduced expression of its homologous endogene in transgenic petunia plants in experiments that aimed to enhance the color forming pigments. The phenomenon became known as "co-suppression" (Galun, 2005; Napoli et al., 1990; van der Krol et al., 1990). Later, other pioneering studies in plants for instance by JA. Lindbo, D. Baulcombe, PM.Waterhouse and K. Bohmert and in several other organisms have provided more details about the RNA silencing mechanism, and in recent years this mechanism has been thoroughly studied in multitude of eukaryotic species (Brodersen and Voinnet, 2006; Eamens et al., 2008; Incarbone and Dunoyer, 2013). RNA silencing mechanism is called with different names in different organisms. In fungi, it is named as "quelling" (Fulci and Macino, 2007) and RNA interference "RNAi" in animals (Hannon, 2002). In plants it is called as "post-transcriptional gene silencing" (PTGS) or co-suppression (Vaucheret et al., 2001), transcriptional gene silencing (TGS) if it is directed to DNA (Vaucheret and Fagard, 2001), and also "virus induced gene silencing" (VIGS), if it is induced by virus (Baulcombe, 1999; Lu et al., 2003; Ratcliff et al., 1999).

Diverse RNA silencing pathways are known to operate in plants to control the exogenously and endogenously originated RNA targets, with different operating molecules and target-depended outcomes (Tang et al., 2003) (Figure 3). Despite of this, RNA silencing mechanisms share some common components in all organisms (Baulcombe, 2004). They are initially triggered by the dsRNA molecules. Next the Dicer or Dicer-like enzymes (DCL) processes the trigger molecules to produce short RNA duplexes. The dsRNA methylating HUA ENHANCER1 (HEN1) enzyme further methylates the DCL-produced duplex molecules at the 3'-OH group. Finally, Argonaute proteins (AGO) form

the RNA-induced silencing complexes (RISC) or RNA-induced transcriptional silencing complexes (RITS) with one of the strands of the methylated short RNA molecules i.e. miRNAs or small interfering RNAs (siRNAs) derived from the cleaved duplex dsRNA. In plants the RISC complex targets usually the fully complementary mRNA molecules and cleaves them at the complementary site. Alternatively, the partial complementarity leads to mRNA translational inhibition (Baulcombe, 2004; Brodersen and Voinnet, 2006; Chen, 2004; Shimura and Pantaleo, 2011; Tang et al., 2003).

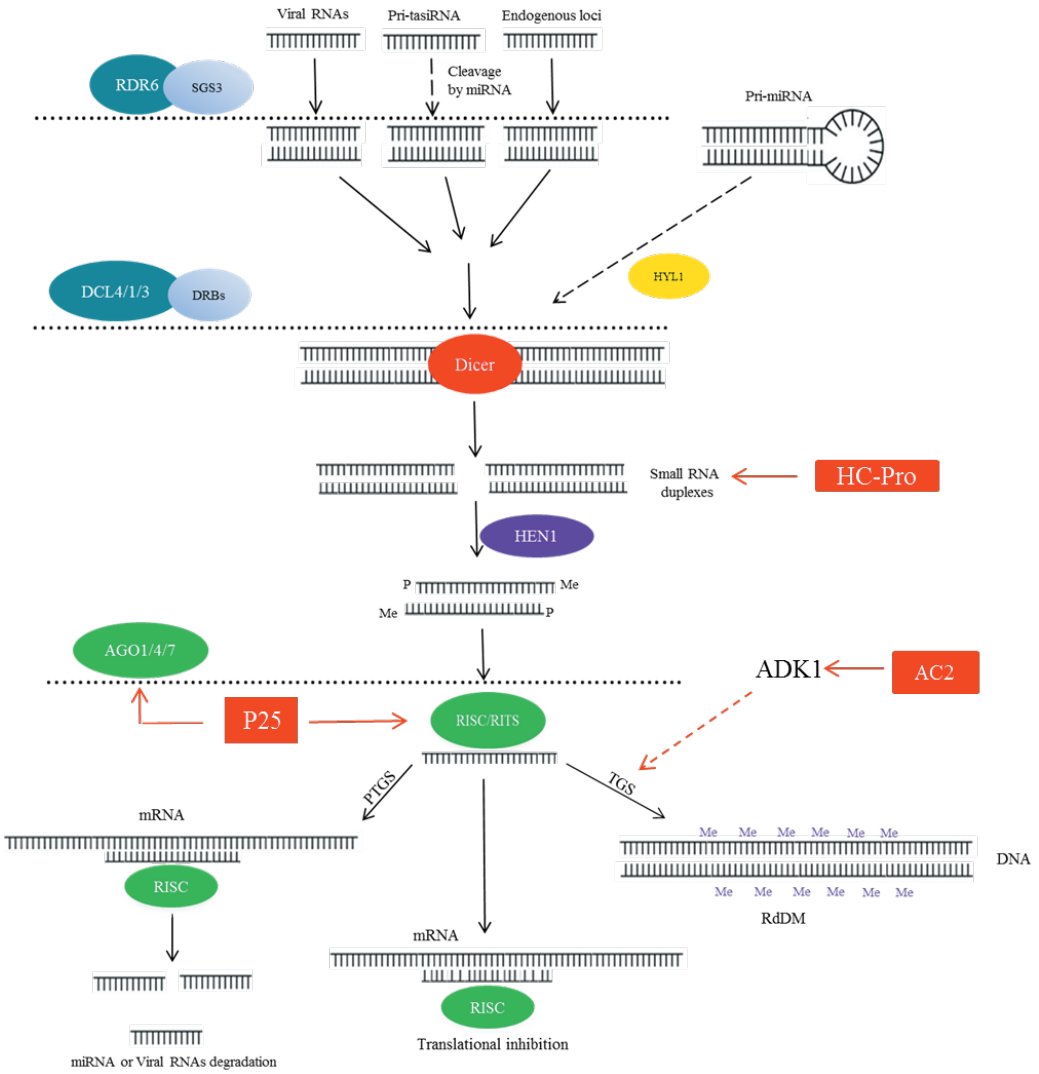

Figure 3. Brief overview of different RNA silencing pathways and their common machinery in plants, and of the putative interactions of different VSRs with the pathways. Various VRS proteins can target most of the steps of the pathways, but we show here only those that are analyzed in this work (adapted from Waterhouse et al., 2003; Shimura and Pantaleo, 2011; Qu et al., 2003; Chiu et al., 2010)

1.2.1 RNA silencing machinery

1.2.1.1 Inducer: The double stranded RNA (dsRNA) and its formation

The dsRNA molecules such as replicative intermediates of the viral RNAs, inverted repeat transcripts (IR) and bidirectionally transcribed transgenes serve as potent RNA silencing inducers (Tenllado and Diaz-Ruiz, 2001; van der Krol et al., 1990; Waterhouse et al., 1998). There are several possibilities for viral dsRNA formation in plants (Ding and Voinnet, 2007; Shimura and Pantaleo, 2011). Studies of Donaire et al. 2008 on *Tobacco rattle virus* (TRV) siRNA formation, revealed that viral siRNAs are derived from both positive and negative polarities of the viral genome, and depend on the host RNA dependent RNA polymerase (RDR) enzyme activities to form the complementary dsRNA duplex (Donaire et al., 2008). Bi-polar transcription of ssDNA viruses also results in the formation of the dsRNA structures and trigger PTGS in plants (Chellappan et al., 2004). Many experiments also show disparity in the accumulation of positive and negative strand specific siRNAs, where the former accumulates more than the latter suggesting that the internal base pairing in the viral ssRNA genomes is also a likely way to form the dsRNA structures (Donaire et al., 2009; Szittya et al., 2002; Szittya et al., 2010). For instance, studies on *Cymbidium ringspot tombusvirus* (CymRSV) genomes designate the presence of multiple specific regions or hot spots in their structures to form the local duplexes for the Dicer cleavage (Molnar et al., 2005).

Furthermore, the dsRNA production from aberrant or abnormal RNAs is documented in several experiments. The aberrant or abnormal RNAs accumulated at high levels, and RNAs lacking either the 5'methyl cap or 3' poly-A tail can promote the dsRNA formation (Han and Grierson, 2002; Metzlaff et al., 2000; van Eldik et al., 1998). These abnormal RNAs act as templates for the RDR6 enzyme, which copies them to produce the dsRNA (Luo and Chen, 2007). Thus, RNA silencing also serves for suppression of deadenylated or dysfunctional ssRNA and contributes in the RNA quality control mechanism (Moreno et al., 2013).

1.2.1.2 Enzymes

Several enzymes, RNA binding proteins and their complexes are required to execute the RNA silencing mechanism in plants. The enzymes participating in RNA silencing pathways include the DNA dependent RNA polymerases, RNA dependent RNA polymerases, RNase III-type endonucleases, RNA methyl transferases and RNA exonuclease, argonaute proteins, effector complexes and other auxiliary proteins. These proteins perform different functions in the RNA silencing pathways ranging from the small RNA biogenesis, their modifications, mRNA regulation and also epigenetic control mediated by the RNA dependent DNA-methylation (RdDM) complexes.

I. DNA dependent RNA polymerases

In RNA silencing, DNA dependent RNA polymerases like RNA polymerase-II (Pol II), -IV (Pol IV) and –V (Pol V) play important roles, the first one by the transcription of miRNA (MIR) genes and the other ones by transcribing the heterochromatin sequences, for the production of the endogenous siRNAs and epigenetic silencing via RdDM mechanism (He et al., 2011; Lee et al., 2004; Xie et al., 2005). Also, the Pol II is involved in RdDM at intergenic low copy number loci by producing the scaffold RNAs that recruit AGO4/siRNAs to the homologous region and lead to its methylation (Zheng et al., 2009). Both Pol IV and Pol V have some structural similarities with Pol II, and to each other, but show differences in their large subunits NRPD1, NRPE1 and NRPB1 (Ream et al., 2009).

The Pol IV enzyme accounts for the production of 90% of siRNAs in plants (Zhang et al., 2007), and produces them mainly from the transposable elements, repeats and pericentromeric regions (Herr et al., 2005; Pontier et al., 2005). The Pol IV produced transcripts are converted to dsRNA by RDR, processed to 24nt siRNAs and directed to the DNA as AGO4-siRNA complexes (Chinnusamy and Zhu, 2009). Pol V is known to produce the long non-coding RNAs (lnc RNAs), which interact with the AGO4-siRNA complex and guide them to specific genomic loci (Wierzbicki et al., 2009). Consequently, AGO4 recruits suppressor of Ty insertion 5 - like (SPT5-like) protein and guides Domains rearranged methyl transferase 2 (DRM2) in the DNA methylation process (Rowley et al., 2011; Wierzbicki et al., 2012).

II. RNA dependent RNA polymerase (RDR)

In both Arabidopsis and rice six different types of RDR are found and separated into two clades based on their structural motifs. RDR1, RDR2 and RDR6 share C-terminal DLDGD motif, whereas the RDR3a, RDR3b and RDR3c have typical DFDGD motif with unknown function (Wassenegger and Krczal, 2006). Interestingly, the recent study by Verelan et al. 2013 found that the Ty1 and Ty3 resistance gene alleles encode putative RDR-enzymes that share similar DFDGD motif as RDR3a, RDR3b and RDR3c, and provide the resistance against *Tomato leaf curl virus* in *Nicotiana. attenuata* (Pandey et al., 2008; Verlaan et al., 2013). This suggests that the silencing pathway component also plays a role as pathogen effector recognition in ETI-type resistance.

RDR1, RDR2 and RDR6 enzymes are mainly involved in the production of siRNAs against the different invading RNA sequences (Diaz-Pendon et al., 2007; Jiang et al., 2012; Vaistij and Jones, 2009). Particularly, RDR6 is involved in the production of dsRNA from transgenes, and from any other aberrant RNAs, and in the PTGS pathway, whereas RDR2 is mainly involved in transcriptional silencing of transposons by producing the 24nt siRNAs (Dalmay et al., 2000; Donaire et al., 2008; Haag et al., 2012; Harmoko et al., 2013; Mourrain et al., 2000). RDR1 provides the resistance to certain viruses via a

SA-mediated defense. It also suppresses the RDR6-mediated PTGS defense i.e. it acts as antagonistic to RDR6 activity (Ying et al., 2010).

The recognition mechanism and preferences by the different RDRs upon the ssRNA molecules to copy them into dsRNA molecules is still unknown. As was said earlier, several studies indicate that abnormal RNAs or aberrant RNAs, e.g. mRNA without 5'cap structures or 3' poly-A tail induce the RNA silencing process (Luo and Chen, 2007). These abnormal RNAs become the templates for plants RDRs and are thus copied into dsRNA. However, biochemical studies indicate that at least RDR6 does not show any distinctiveness for the normal and abnormal RNA i.e. with or without cap or poly-A tail (Curaba and Chen, 2008).

III. RNase III type endonucleases

As in all eukaryotes, in plants the dsRNA is targeted by the DCL enzymes that have RNase III type endonuclease activity and produce small 21–24 dsRNA molecule duplexes (Liu et al., 2009). Previous model claimed that dicers can select their cleavage part only from the 3'overhang terminus of the dsRNA by measuring the distance, but the recent finding shows that both 5'- and 3'- ends are recognized by the 5'- end 3'- end pockets in human dicer (Park et al., 2011). Arabidopsis genome encodes four different DCL enzymes (DCL1-DCL4), which have different functions, ranging from miRNA based gene regulation to antiviral immunity (Baulcombe, 2004; Liu et al., 2009; Rogers and Chen, 2012). The four plant DCLs appear to have undergone gene duplication after divergence from the animals (Margis et al., 2006). Structurally, dicers comprise six types of domains, and among them the N-terminal PAZ domain, RNAse III and dsRNA binding (dsRBD) domains are mainly involved in the dsRNA processing to small RNA duplexes (Macrae et al., 2006). The human dicer has the 65Å sized region between the PAZ and RNase III domains that act as a molecular ruler and as a structural basis for formation of RNA products in distinct sizes (21-24nt) (Lau et al., 2012; Macrae et al., 2006).

DCL1 mainly participates in the miRNA biogenesis, but is also involved in some endogenous natural antisense siRNAs (nat-siRNAs) and long siRNAs (lsiRNAs) production (Katiyar-Agarwal et al., 2007; Kurihara and Watanabe, 2004; Wu, 2012; Zhang et al., 2012). Both DCL4 and DCL2 play crucial role in the antiviral activity in plants by producing 21nt and 22nt duplexes respectively, and DCL3 mainly participates in the formation of heterochromatin siRNAs to mediate the DNA methylation (Bouche et al., 2006; Chan et al., 2005; Deleris et al., 2006).

Some of the DCL proteins exhibit functional redundancy in plants. The DCL1 null mutants exhibit embryo lethality in the Arabidopsis indicating that DCL1 function (processing of pre-miRNA to miRNA duplex) does not overlap with the functions of

the other dicers (Henderson et al., 2006a). Instead, the DCL3 mutants show functional redundancy in epigenetic regulation mechanism. They produce repetitive associated siRNAs (ra-siRNAs), which actively participate in DNA methylation at specific loci in the DNA (Chan et al., 2005). This mechanism is not disrupted much in the DCL3 mutants as it is compensated by the DCL2 and DCL4 proteins that produce 22-23nt and 21nt ra-siRNAs respectively, to maintain the methylated DNA (Gasciolli et al., 2005). The main function of DCL4 is production of the 21nt siRNAs from viral RNAs i.e. the antiviral silencing. Studies also indicate that there is compensation for DCL4 by DCL2, which produces the 22nt siRNAs if the former is blocked by viral activity (Bouche et al., 2006).

IV. RNA methyl transferase

The small dsRNAs produced by the dicing activity are stabilized by the 2'-O-methylation at their 3'termini, a process mediated by the HEN1 enzyme and its homologous enzymes in the plants (Yang et al., 2006; Yu et al., 2005). HEN1 consists of two putative dsRBD domains separated by a La-type winged helix-turn-helix motif (LCD) at the N-terminus, and a Rossman fold methyltransferase region at C-terminal domain in its structure. Both dsRBD1 and dsRBD2 are involved in the small RNA substrate recognition, whereas the duplex length is measured by the methyltransferase domain. According to the phylogenic studies, HEN1 catalytic domain is related to the small molecule methyl transferases rather than 2'-OH methyl transferases (Huang et al., 2009; Tkaczuk et al., 2006). The plants lacking the HEN1 gene (*HEN1* mutants) exhibit reduced methylation, abundance and diversity in size of miRNAs. These mutants also show uridylation at 3'- ends of the small RNAs by having an extended oligonucleotide tail with 1 to 5 uridine residues (Li et al., 2005). In vitro, HEN1 enzyme preferably methylate the 21-24 small RNA duplexes i.e. both miRNA and siRNA with 2nt 3'overhangs (Yang et al., 2006).

According to the kinetic studies, HEN1 enzyme is catalytically efficient and does not need any supplementary proteins to perform the catalytic activity. It methylates the small RNA duplex in a successive manner i.e. via hemimethylated state (Vilkaitis et al., 2010). Moreover, the recent kinetic studies with different unmethylated, hemimethylated and doubly methylated substrates reveal that HEN1 preferably binds to the un- and hemi-methylated small RNA duplexes, and that the successive methylation of these duplex happens in a non-distributive manner (Plotnikova et al., 2013).

V. Argonautes (AGO)

AGO proteins are involved mainly in the formation of the RISC or RITS complexes together with either miRNA or siRNA molecules that target them to the complementary RNA or DNA sequences, respectively. 10 AGO proteins have been identified in

Arabidopsis and 16 in rice so far. In Arabidopsis, these proteins are classified into three major clades constituted of AGO1, 5 and 10, AGO2, 3 and 7 and AGO4, 6, 8 and 9 (Mallory and Vaucheret, 2010; Vaucheret, 2008). AGO proteins are structurally composed of four important domains such as N-terminal, PAZ, MID and PIWI domains. Among them, MID and PAZ domains are involved in the small RNA binding, where 5' phosphate is bound by the former and 3'-OH by the latter domains, respectively (Ma et al., 2004; Ma et al., 2005). PIWI domain is involved in the interaction with other cellular components like RNA Pol IV subunit NRPD1b. It contains the Asp-Asp-His active site that exhibits the slicing activity (Vaucheret, 2008).

AGO proteins show different preference for different miRNAs, based on their dicer origin and molecular sizes (Mallory and Vaucheret, 2010). Knock-down mutations of AGO1 protein show malformed phenotypes and are hypersusceptible to virus infections. In addition they exhibit reduced accumulation of miRNAs and increased accumulation of their targets, indicating a major role in RNA silencing process (Vaucheret, 2008). AGO1 is localized in both nucleus and cytoplasm, and it mainly incorporates the small RNA molecules generated by the DCL1 and DCL4 enzymes. Thus, it executes the miRNA and viral-siRNA (i.e. antiviral silencing) mediated regulation in plants (Baumberger and Baulcombe, 2005; Vaucheret et al., 2004; Zhang et al., 2006). Mutants of the AGO10 protein, also known as *ZWILLE* or *PINHEAD,* exhibit similar phenotype as the AGO1 mutants. Thus, AGO1 and AGO10 exhibit some functional redundancy. Double mutants of these genes are embryo lethal, which indicate their importance in the plant development process (Lynn et al., 1999). Additionally, the AGO10 protein is proposed to be a miRNA locker i.e. it specifically interacts with the miRNA 165/166 family to make them unavailable to the activated AGO1 protein (Manavella et al., 2011). AGO4 is involved in the transcriptional gene silencing process and other AGO proteins like AGO7 are involved in the tasiRNAs function (Vaucheret, 2008).

1.2.1.3 Operating molecules

I. Small RNAs

RNA silencing is based mainly on two important classes of small RNA molecules i.e. siRNAs and miRNAs. These two classes are mainly differentiated by their origin, where miRNAs are of endogenous origin, and siRNAs are both of exogenous and endogenous origin in nature (Chen, 2009). Axtell et al. 2013 further categorized the endogenously originated small RNA populations into hairpin RNAs (hpRNAs) and siRNAs, whose precursors are single stranded (ssRNA) and dsRNAs, respectively. Similarly, in secondary classification, the hpRNAs are classified into precisely processed miRNAs and imprecisely processed other hpRNAs. The endogenously originated siRNAs are classified into heterochromatic, secondary and nat-siRNAs (Axtell, 2013).

miRNAs are a class of small regulatory RNA molecules (20-24nt size), which negatively regulate mostly transcription factors and other complimentary genes by mRNA cleavage or translation suppression processes (Baulcombe, 2004; Reinhart et al., 2002). To date, 25141 mature miRNAs have been identified in different organisms. In plants, a total of 5401mature miRNA have been identified in both dicots and monocots (miRbase v19 release august 2012; www.mirbase.org) by using a variety of approaches like genetic screening, direct cloning and bioinformatics analysis. The plant miRNA genes identified so far have their own transcriptional units and are transcribed in a normal way by the Pol II enzyme to produce primary miRNAs (pri-miRNAs) (Xie et al., 2005). MiRNA gene regulation is not known well. However, experimental evidence indicates that miRNA gene expression occurs in a tissue-specific manner (Chen, 2004; Wienholds et al., 2005). Evolutionarily plant miRNAs are less conserved than animal miRNAs, and exhibit conservation in mature miRNA regions rather than the precursor-miRNAs (pre-miRNAs), as in animals (Reinhart et al., 2002; Zhang et al., 2006; Zhang et al., 2005).

The miRNAs perform very crucial functions in plants. They are spatially and temporally regulated in accordance with the vital cell functions like cell differentiation, cell division progression, and occur also in the vascular bundles to provide coordination in the organogenesis and development (Valoczi et al., 2006). These developmental functions have been explained using mutations of miRNA biogenesis components like *dcl1-9*, *ago1, hen1, hyl1* and *hasty* i.e. in genes which are very essential to the miRNAs production and their export from nucleus to the cytoplasm, and to the regulation of the complementary mRNAs. All these mutants result in abnormal phenotypes like distortion in leaves, flowers and inflorescences, and in reduced fertility, which all indicate the essentiality of miRNAs in the plants (Chen, 2004; Henderson et al., 2006b; Lu and Fedoroff, 2000; Schauer et al., 2002; Vaucheret et al., 2004). In addition to this, overexpression of miRNAs in the transgenic plants also causes severe developmental disturbances, such as flower and leaf malformations, indicating connection between hormonal balance and miRNAs in plants (Ru et al., 2006). Several plant viruses disrupt RNA silencing components and indirectly effect the accumulation of miRNAs in the infected plants, which results in the abnormal phenotypes of virus infected plants (Amin et al., 2011; Kasschau et al., 2003; Naqvi et al., 2010). The miRNAs exhibit also functional redundancy by having overlapping functions between the same family members, but this can be reversed by the mutations in the target sites of the complementary mRNAs (Jones-Rhoades et al., 2006).

Like miRNAs, siRNAs are small RNA molecules classified into 21-22nt and 24-25nt sizes. They are involved in the control of exogenous viral RNAs, endogenous transposons, DNA-methylation and systemic silencing in plants (Hamilton et al., 2002). As was said earlier, viral siRNAs may be produced either from the specific ds-regions forming hot spots in their genomic structure (Molnar et al., 2005; Szittya et al., 2010). Recent studies

have also revealed different kinds of endogenously originated siRNAs, which are varied in their origin and also in their size. They are the transacting siRNAs (tasiRNAs) derived from the non-coding RNA transcripts, similarly to miRNAs, and mediate specific target mRNA regulation (Vazquez et al., 2004). In Arabidopsis, eight tasiRNA genes (TAS) have been identified so far by deep sequencing and computational approaches (Howell et al., 2007). The tasiRNAs are mainly involved in the regulation of auxin regulation factors (ARFs), and their biogenesis is mediated by the miR390, and RNA silencing pathway (Allen et al., 2005; Yoshikawa et al., 2005). The second types of endogenous siRNAs are nat-siRNAs, derived from the pairing of the complementary sense and antisense transcripts. For example, two SRO5 and P5CDH transcripts in the Arabidopsis partially overlap with each other and form dsRNA that is subsequently processed to 24nt siRNAs, to provide salt tolerance (Borsani et al., 2005). The ra-siRNAs are still another class of endogenous siRNAs involved in maintenance of the methylation of specific repetitive and retro-elemental DNAs (Chan et al., 2005; Onodera et al., 2005).

The 21-24nt sized single stranded siRNA molecules are involved in the systemic spreading of the silencing, orchestrated by production of secondary siRNAs by an amplified chain reaction (Molnar et al., 2011). In this process, the primary siRNAs produced from the dsRNA target the complementary mRNA molecule for cleavage. The cleavage products are used as templates to produce more dsRNAs via RDR activity that are subsequently cleaved into secondary siRNA molecules. This transitivity phenomenon is seen more in the connection with transgenes than endogenes (Vazquez and Hohn, 2012; Voinnet, 2008).

1.3 Viral suppressors of RNA silencing

Plant viruses have evolved to produce different VSRs to counteract the major defense mechanism mediated by RNA silencing in plants (Qu and Morris, 2005; Roth et al., 2004; Vance and Vaucheret, 2001). In some cases, VSRs are also known as pathogenicity factors of the viruses indicating their critical role in spreading and infectivity of viruses in plants (Brigneti et al., 1998). It appears that most if not all plant viruses and also some animal viruses produce some kind of VSR factors. Surprisingly, VSRs have different structures and their functional strategies are also very varied between different virus families and sometimes even within in the virus families (Burgyan, 2008; Diaz-Pendon and Ding, 2008; Voinnet et al., 1999; Voinnet et al., 1999). Besides their suppression activity, VSRs are involved in multiple other functions such as viral movement, replication transmission and function as transcriptional activators or proteases (Ballut et al., 2005; Plisson et al., 2003; Voinnet et al., 2000). Thus, VSRs have become valuable tools in studying both the RNA silencing mechanisms and plant-virus interactions.

VSRs employ different strategies to paralyze the RNA silencing mechanism (Figure 3). These strategies range from the sequestration of small RNA molecules to blocking the DCL or AGO proteins, and causing RNA silencing disturbances. Thus, they also interfere with host's endogenous RNA silencing pathways.

Many VSRs target the small RNA molecules. For instance, P19 encoded by tombusviruses, one of the most studied VSR sequesters the siRNA duplex by measuring their length and firmly binding them in a sequence independent manner, thereby making them unavailable for downstream RNA silencing mechanism (Vargason et al., 2003; Ye et al., 2003). Some VSRs target the components of RNA silencing pathways and inactivate them, or degrade them by the protein degradation mechanism. For instance, the 2b protein of *Cucumber mosaic virus* (CMV) interacts with siRNA loaded PAZ domain of the AGO1 protein, and inhibits the silencing activity (Zhang et al., 2006). Also, P25 VSR encoded by *Potato virus X* (PVX) degrades the AGO1 protein by proteasomal degradation pathway (Chiu et al., 2010). Other VSR such as *Sweet potato mild mottle virus* (SPMMV) P1 targets and suppresses RISC activity through binding the AGO1 protein by its N-terminal WG/GW motifs (Giner et al., 2010). Recent studies also indicate that transient expression of several viral suppressors increase the miR168 levels, which is a regulator of the AGO1 component of RNA silencing (Varallyay et al., 2010; Varallyay and Havelda, 2013).

1.3.1 HC-Pro of *Potato virus Y* (PVY)

HC-Pro is a one of the multifunctional viral proteinases derived from the single polyprotein precursor encoded by the *Potato virus Y* (PVY), of family *Potyviridae*. Potyviruses contain filamentous particles and a single stranded RNA genome, which possess 5'-VPg protein and 3' poly-A tail. HC-Pro is encoded as the second protein in the polyprotein precursor adjacent to the P1 protein (Urcuqui-Inchima et al., 2001). It seems to be involved in all important events in virus life cycle, ranging from transmission, multiplication and cell-to-cell movement, and to RNA silencing suppression (Anandalakshmi et al., 1998; Anandalakshmi et al., 2000; Atreya et al., 1992; Carrington et al., 1996; Jin et al., 2007a). HC-Pro consists of three domains i.e. N-terminal, central and C-terminal domains in its structure, with approximately 100, 200 and 150 amino acids, respectively. The conserved N-terminal KITC domains and the C-terminal PTK motifs are involved in the aphid transmission, whereas the several different domains are involved in the RNA silencing suppression (Plisson et al., 2003; Varrelmann et al., 2007). The latter activity seems to be mediated by the non-specific binding of the protein to the siRNA and miRNA molecules and sequestering them, a function mediated by the FRNK box located in the central part in its structure, at least in the PVY HC-Pro protein (Shiboleth et al., 2007). Furthermore, the HC-Pro interacts with the different host proteins involved in the defense response. It interacts with the 20S proteasome subunits in Arabidopsis, chloroplast division regulator

NtMinD in tobacco, and also with eukaryotic translation initiation factor 4E (eIF4E) by its 4E binding motif (Ala-Poikela et al., 2011; Ballut et al., 2005; Jin et al., 2007; Blanc et al., 1997). Also, it shows interaction with regulator of gene silencing calmodulin (rgs-CaM) protein, which acts as negative regulator of PTGS (Anandalakshmi et al., 2000). Recent genome tiling microarray studies indicate that HC-Pro needs the host ethylene inducible transcription factors RAV2 to block the RNA silencing mechanism (Endres et al., 2010; Paper I).

1.3.2 AC2 of *African cassava mosaic virus* (ACMV)

African cassava mosaic virus (ACMV) belongs to the bipartite *Geminiviridae* family, with two ssDNA molecules as its genome. DNA-A consists of six open reading frames (ORFs), which are arranged in different directions, i.e. in virion sense and in complementary sense. AC2 VSR is encoded by the complementary sense strand of DNA-A molecule (Vanitharani et al., 2005). It is a multifunctional transcriptional activator protein (TrAp), known to be involved in the transactivation of viral and host proteins, suppression of RNA silencing, and reduced genome wide cytosine methylation in plants (Buchmann et al., 2009; Sunter and Bisaro, 1992; Trinks et al., 2005). Structurally, AC2 VSR comprises three important domains i.e. N-terminal nuclear localization signal (NLS), middle zinc finger and C-terminal acidic domains. Both zinc finger and C-terminal acidic domains are involved in the AC2 transactivation activity (Bisaro, 2006). Its expression in the plants causes inactivation of host adenosine kinase enzyme (interaction is involved in suppression of TGS) and primary immune response factor SNF1, suggesting that AC2 may operate its RNA silencing suppressor activity via suppression of these proteins (Hao et al., 2003; Wang et al., 2003).

1.3.3 P25 of *Potato virus X*

PVX is a single stranded RNA genome containing virus, which belongs to the *Flexiviridae* family. It has been widely used for studying the R-gene mediated resistance and gene silencing mechanisms in plants. PVX genome consists of five ORFs; the first ORF encodes for the viral replicase, next three central region ORFs encode for partially overlapping triple-gene block proteins (TGBp), and the final ORF encodes for the viral coat protein (Verchot-Lubicz et al., 2007). In TGB block, P25 is the largest and multifunctional protein, with the RNA silencing suppression and plasmodesmatal (PD) dilation activities, the latter function being mediated by the ATPase activity of the GKS and DEY motifs in its N-terminal region (Angell et al., 1996; Howard et al., 2004; Lu et al., 2003; Morozov et al., 1999; Voinnet et al., 2000). Also, other studies indicate that this P25 mediated viral cell-to-cell movement depends on the suppression of RNA silencing (Bayne et al., 2005). P25 over-expression in transgenic tobacco plants induces only very

little or any changes in morphology, although it reduces carbohydrate content and the photosynthetic rate (Paper III; Kobayashi et al., 2004). There is still uncertainty in how the P25 VSR interacts with the components of the RNA silencing machinery. Previous studies have suggested that P25 interacts with the RDR6 enzyme (Schwach et al., 2005; Verchot-Lubicz et al., 2007). Recently, Chiu et al. 2010 reported interaction of PVX-P25 with the AGO1 protein, and consequent targeting of the AGO1 into proteasomal degradation, indicating that P25 VSR may affect the RNA silencing mechanism via this mechanism. Also, P25 protein has been shown to interact with AGO1-4 proteins but not with AGO5 and AGO9 proteins (Chiu et al., 2010).

1.4 Viral interactions with the host

Viruses are obligate intracellular parasites, most of them with very limited genetic size and coding capacity. Thus they heavily depend on the host components to sustain and complete their life cycle in the hosts (Gergerich and Dolja, 2006). Based on the virus adaptability or ability to infect the host, two distinct types of plant-virus interactions have been described, known as compatible and incompatible plant-virus interactions. In incompatible interactions plants are able to evade the virus infection by preformed defenses by evoking complex defense responses through R-genes, or the plants lack components needed for viral infection process. In contrast, compatible reactions are favorable to the virus so that it can infect and utilize the host components to successfully complete its life cycle (Lodha and Basak, 2012; Walton, 1997). In both of these interactions, massive reprogramming in host gene expression is observed (Brigneti et al., 1998; Golem and Culver, 2003; Gongora-Castillo et al., 2012; Kobayashi et al., 2001; Lu et al., 2012; Whitham et al., 2003). Often this is associated in the plants with viral symptoms like chlorosis, vein clearing, ring spots, striking and general stress and stunting. These gene expression alterations could be a mixture of host defensive responses and reprogramming to stop the present and future virus infection, and of the virus induced activation and hijacking of the important host factors to complete their life cycle. In other words, this complex interplay constitutes the plant defense and virus counter defense responses, and the alterations programmed by the virus to promote its life cycle. These together lead to the disease development in the plants (Culver and Padmanabhan, 2007; Pallas and Garcia, 2011; Wang et al., 2012).

1.4.1 *Tobacco mosaic virus* (TMV)

The infectious TMV or its constituent genes have served as model systems to investigate the plant-virus interactions and to dissect the details of viral replication, movement, host resistance and physiological alterations (Bhat et al., 2013; Golem and Culver, 2003;

Hwang et al., 2013; Ishibashi et al., 2010; Wright et al., 2007). TMV belongs to the single stranded RNA viruses and its genome encodes for four proteins, i.e. the 126 kDa replicase, and the overlapping 183 kDa replicase, the 30 kDa movement protein (MP) and the 17.5 kDa coat proteins (CP). Both 126 kDa and 183 kDa proteins are translated from the viral genomic RNAs directly but the movement protein and coat protein are translated each from its own 3'co-terminal sub-genomic RNA. Like other positive strand RNA viruses, TMV replication proceeds via the synthesis of complementary negative strand, and from this template, progeny of the viral positive strands and the sub-genomic mRNAs are efficiently synthesized (Liu and Nelson, 2013). Both 183 kDa and 126 kDa replicases are important for the viral replication process. These proteins contain the helicase and methyl transferase domains in their structures, and the former, being the read-through product of the latter, contains also the RdRp at its C-terminal end (Ishibashi et al., 2010). Mutational studies have revealed that the 126 kDa alone is not sufficient for TMV replication. Also deletion of the 126 kDa amber stop codon leads to loss of infectivity of the virus. However, if the amber codon is replaced by the tyrosine codon, weak virus infectivity is restored (Ishikawa et al., 1986). TMV forms the membrane bound viral replication complex, a function mediated by the replication proteins along with movement protein, viral RNA and several host proteins (Liu and Nelson, 2013). Host factors such as tobamovirus multiplication 1 and 2 (TOM1 and TOM2), elongation factors 1A and B (eEF1A and B), eIF3, NAC domain transcription factor, host chloroplast proteins and vicilin like seed storage protein (PAP85) are known to be involved in the TMV infection process (Bhat et al., 2013; Chen et al., 2013; Ishibashi et al., 2010; Wang et al., 2009b).

The expression of TMV MP in transgenic plants and other micro- injection experiments have shown that it functions to significantly increase the PD size exclusion limit (SEL) (Deom et al., 1990; Wolf et al., 1989). TMV MP associates with the TMV RNA in a non-sequence specific manner and forms an extended MP-RNA complex. These complexes associate with the host cytoskeleton system and gate the PD (Citovsky, 1999). The TMV MP amino acid residues of 3-5 and 195-213 are essential for PD localization and 126-224 amino acid residues are responsible for increasing the PD SEL (Citovsky et al., 1992; Ding et al., 1992). The TMV movement protein phosphorylation also plays regulatory role in the movement of the virus (Waigmann et al., 2000). The MP has several interaction partners in the host cells to facilitate the movement process. It is known to interact with Pectin Methyl Esterase (PME), calreticulin, RIO serine protein kinase and some PD-associated protein kinases (PAPK) (Chen et al., 2005; Dorokhov et al., 2006; Lee et al., 2005).

1.5 Studies of the plant-virus interactions

The plant-virus interaction studies can be conducted by a variety of techniques ranging from limited analysis of specific genes in infected tissues, to expression of the single viral genes in transgenic plants and to whole transcriptome profiling of infected hosts. Expression level of alterations of a single or limited sets of genes can be observed e.g. by the *in situ* hybridization or quantitative real-time PCR (RT-qPCR) technique, which can be targeted to any specific host gene in any tissues. The recent revolution in various molecular techniques, such as sequencing that provide the complete genome sequences of target organisms, and accurate synthesis of probes has led to the development of highly sophisticated large scale gene expression analysis methods, e.g. microarrays (Di Carli et al., 2012; Wan et al., 2002).

Microarray technology has become a sophisticated technology in addressing the study of thousands of genes in response to a single treatment (Slonim and Yanai, 2009). In the typical microarray techniques such as oligonucleotide arrays and complementary DNA (cDNA) arrays, the cDNA molecules derived from total mRNA sample by reverse transcription process are labeled with fluorescent dyes, and hybridized to the large number of representing DNA probes on microarray plate (Alba et al., 2004; Maruyama et al., 2014). After hybridization, the microarray plate is scanned for fluorescent emission wavelengths with laser device and the signal strength for each hybridization spot indicates the amount of corresponding polynucleotides in the sample. This technique, using two types of fluorescent dyes that have different emission spectra enables hybridization of two comparative samples in a single array (Aharoni and Vorst, 2002; Maruyama et al., 2014; Slonim and Yanai, 2009).

Microarray techniques are widely used to elucidate the plant-virus interactions. Several virus infection microarray studies have indicated the complex but variable host response to the different viruses, with respective to the source tissue, post infection time, strain of virus and the host (Whitham et al., 2006). The comparative microarray studies of the related or un-related viruses reveal the common and specific responses between different host-virus combinations (Dardick, 2007; Whitham et al., 2003). Similarly, the transgenic plants expressing the whole viruses or specific viral genes reveal their specific effects on the gene expression (Papers I-IV). Some of the studies, such as microarray studies of compatible and incompatible virus infections, reveal the common and specific host response during their interaction with particular viruses (Postnikova and Nemchinov, 2012).

1.5.1 Common responses

Different viruses exhibit various pattern of interactions with host components and symptom development, indicating that they change the host regulatory systems in different ways (Senthil et al., 2005). Together with these specific responses, it is very important to know what kind of common responses are induced in plants by different viruses to understand the basic line of plant-virus interactions. This is achieved by comparative microarray experiments between different virus infections at controlled experimental conditions. Elegant microarray studies from the different research groups reveal some common responses between different virus infections (Postnikova and Nemchinov, 2012; Rodrigo et al., 2012; Senthil et al., 2005; Whitham et al., 2003). Some of the common responses observed in different virus infections belong to the biotic stress, abiotic stress, apoptosis, protein folding, and developmental process, and changes in physiological and metabolic processes such as photosynthesis, lipid, starch and alcohol metabolisms (Figure 4) (Elena et al., 2011).

Some of these common changes caused by different virus infections and their related gene expression, as observed in microarray analysis experiments, are discussed briefly in the following sections.

1.5.1.1 Stress responses

It is documented by several studies that both abiotic and biotic stress responses are interlinked in enhancing the resistance and defense response to the plants. This connection is maintained by signaling molecules and hormonal cross talks (Atkinson and Urwin, 2012). Hence, biotic stresses like viral infections also induce the abiotic stress responses. These abiotic stress responses include e.g. wound inducing proteins, salt responsive proteins, metal binding proteins such as copper binding proteins, heat shock-70, 83 and 23.6 type proteins and dehydration related proteins like dehydrins (Babu et al., 2008; Dardick, 2007; Whitham et al., 2003; Whitham et al., 2006). The biotic responses include the expression of several defense related genes, WRKY transcription factors, cell wall modulating enzymes and secondary metabolites. All these responses may play a significant role in the plant defense and in maintaining the viability of the infected plant. Specific features of these responses are discussed below.

I. Biotic stress responses

PR proteins represent an important class defense proteins induced during the biotic stress responses. These proteins accumulate differently in the compatible and incompatible plant-virus interactions, where in the former interactions they accumulate to lower or basal levels and in the later interaction to high levels (Whitham et al., 2006). Also, WRKY transcription factors accumulate commonly during the virus infections (Rodrigo et al.,

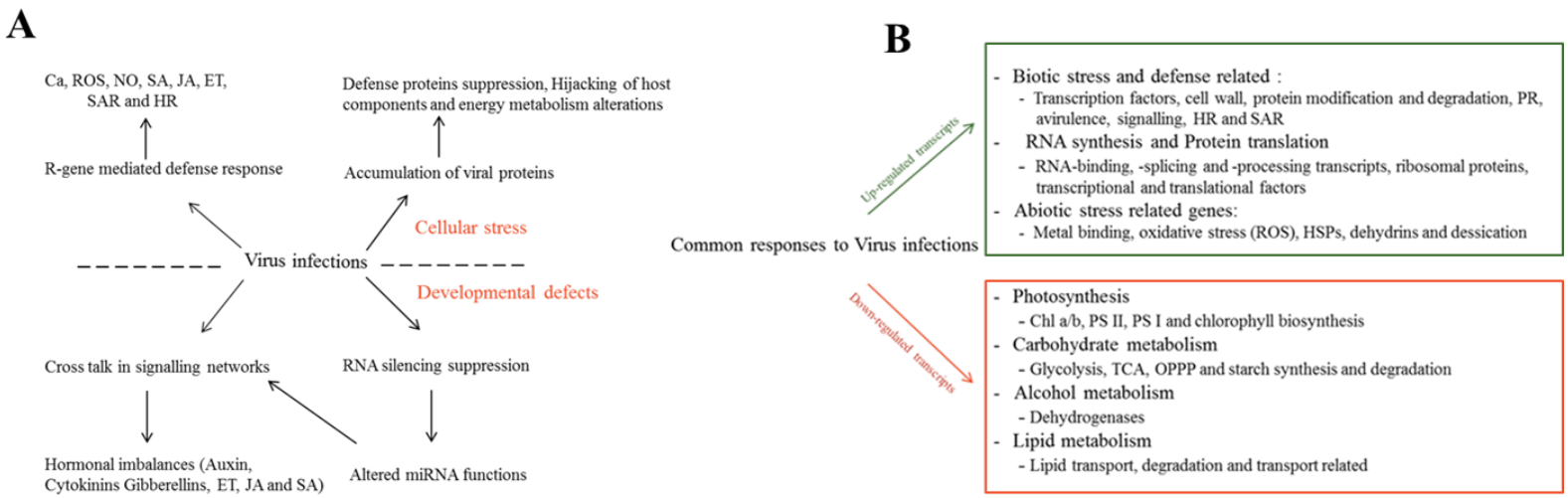

Figure 4. Schematic representation of different physiological, developmental and metabolic changes commonly occurring during viral infection in plants (biotic stress). A. disturbances of different regulatory pathways caused by various virus infections. B. Some of the most commonly altered transcripts, which are up- and down-regulated during different viral infections (marked in red and green, respectively) related to different functional groups (adapted from the Whitham et al., 2006; Elena et al., 2011)

2012; Senthil et al., 2005; Whitham et al., 2003). WRKY transcription factors contain the C-terminal zinc finger motif and N-terminal conserved DNA binding motif, and work in controlling the PR proteins (Pandey and Somssich, 2009). Recent finding of capsicum WRKY d (caWRKYd) in the TMV-P0-inoculated hot pepper plants indicate the role of this protein in other defense response such as HR and PCD, and PR protein induction (Huh et al., 2012; Kim et al., 2007). Also, cell wall-related transcripts that include cell wall synthesizing enzymes, arabinogalactan protein, extensin, and proline-rich and glycine-rich proteins are commonly affected during the plant virus infections (Babu et al., 2008; Whitham et al., 2003). For instance, in *Rice dwarf virus* (RDV) infected rice plants, majority of the altered transcripts are related to the cell wall associated functions (Shimizu et al., 2007).

II. Abiotic stress responses

During the viral infection process, host cellular remodeling causes destabilization in the cellular protein machinery, and misfolded proteins are corrected through the Heat shock proteins (HSPs). Also, these proteins are stress-related proteins, participate in the host defense process like HR, and accumulate commonly in the different virus infections (Aranda et al., 1999; Kanzaki et al., 2003; Whitham et al., 2003). Newly infected host cells show high accumulation of HSPs on mRNA and protein levels i.e. spatial and temporal regulation of HSP proteins occurs with plant virus infection (Aparicio et al., 2005; Aranda et al., 1996). Importance of the HSPs in plant infection process is further indicated by a closterovirus that encodes the hsp70 homologue proteins (HSP70h) in their genome, which is used for the virion assembly process and movement purposes (Agranovsky et al., 1998; Satyanarayana et al., 2000).

1.5.1.2 Physiological and metabolic changes

Virus infections cause imbalances in the host physiology and metabolism. Most of the commonly observed changes belong to the photosynthetic down-regulation and polysaccharide metabolisms. Additionally, various alterations relate to the alcohol metabolism, lipid metabolism and developmental signaling mechanisms (Elena et al., 2011).

Plant chloroplasts are the main centers for various metabolic pathways and effected easily by biotic stress response such as virus infections. Reduction of photosynthesis is the one of the most commonly observed phenomenon during different virus infections (Lehto et al., 2003; Pompe-novak et al., 2001; Rahoutei et al., 2001; Reinero and Beachy, 1989). Many of the transcripts related to the photosystems II and I (PS II and PS I), light harvesting complexes (LHC II) and oxygen evolving complex (OEC) related genes are commonly altered in several virus infections, as indicated by microarray analysis

(Bilgin et al., 2010; Itaya et al., 2002; Pompe-Novak et al., 2006). This down-regulation is manifested as different forms of symptoms such as chlorosis, vein clearing, mosaic and vein yellowing, and this down-regulation might be related to the host defensive processes against the assaulting viruses. For instance, TMV 126 kDa replicase protein interacts with tobacco 33K subunit of OEC and silencing of this host gene enhances TMV virus replication in plants (Abbink et al., 2002). Similarly, recent studies identified the chloroplast proteins such as γ-subunit (AtpC) and Rubisco activase (RCA) associated with the TMV replicase protein in the replication complexes, and AtpC and RCA silenced leaves exhibit enhanced TMV accumulation and pathogenicity (Bhat et al., 2013). Plant defense response by the photosynthetic proteins is further evidenced by the notable up-regulation of the Ferredoxin NADPH oxidoreductase (FNR) and ferridoxin (fd) related genes in biotic stresses, which are known to be involved in the plant defense responses (Bilgin et al., 2010; Huang et al., 2007).

The down-regulation of photosynthesis causes also the down-regulation the carbohydrate metabolism in plants. Several genes belonging to the Calvin cycle and oxidative pentose phosphate pathway (OPPP), such as phosphoribulose kinase (PRK), sepudoheptulose-1, 7 bisphophstase, RCA, transketolase, transaldolase and ribulose-5-isomerase are commonly down-regulated in the biotic stresses (Bilgin et al., 2010). Also, Handford et al .2007 showed that starch accumulation defects cause the increased symptom severity in the virus infected Arabidopsis plants (Handford and Carr, 2007).

Additionally, studies of Bazzini et al. 2011 revealed the different accumulation of metabolites during the TMV virus infections at biphasic level (Bazzini et al., 2011). These changes are related to common alterations in metabolic gene expression, such as synthesis of phenolics, lipids, tricarboxylic acid cycle (TCA) proteins and anti-oxidants, occurring in different virus infections (Elena et al., 2011). Three main groups of metabolites related to the ascorbate-, fatty acid- and phenolic compounds are mainly altered. Specifically, alterations in dehydroascorbate, gulonate and 2-oxo-gulonate of ascorbate metabolites, in palmitate, myristate, pelargonate and stearate of fatty acids metabolites, and in benzoate of phenolic compounds are observed during TMV infection. Also, some other metabolites related to the gamma-amino butyric acid (GABA), sugars of ribose, rhamnose and sorbose, and TCA cycle intermediates of malate, citrate and isocitrate are significantly up-regulated during the virus infections (Bazzini et al., 2011).

1.5.2 Developmental responses

The disease stage, including both pathogen induced interference and plant defense reactions, are often interlinked with developmental regulation and responses of the plant. These interactions can be mediated e.g. by hormonal or RNA silencing pathways

1.5.2.1 Hormonal changes

Plant hormones such as auxin, gibberellins, cytokinins, ethylene, and abscisic acid are known to play crucial roles in the plant growth and development (Gray, 2004). Different studies indicate that plant viruses exploit these hormones to their own benefits. For instance, TMV replicase protein interaction with auxin/ Indole acetic acid (AUX /IAA) related protein PAP1/IAA26 plays a key role in the enhancement of virus infection (Padmanabhan et al., 2008). Interestingly, this same interaction also induces the disease symptoms in plants. This is evidenced by the reduced symptoms of TMV with mutations in replicase (helicase domain) AUX/IAA interacting region although these mutants do not cause any impairment in the virus replication and spread. Moreover, the PAP1 protein silenced transgenic plants also exhibit the similar symptoms as wild type TMV infection (Padmanabhan et al., 2005). Similarly, RDV P2 protein interacts with host ent-kaurene oxidase enzyme that participates in the gibberellic acid (GA) synthesis. Mutants of this enzyme also show symptoms similar to RDV infection. This interaction also might play role in the phytoalexins biosynthesis reduction that helps the RDV replication process (Zhu et al., 2005). Furthermore, P6 protein of *Cauliflower mosaic virus* (CaMV) causes chlorosis and stunting symptoms in transgenic plants by interacting with ethylene signalling pathway (Geri et al., 2004). This interaction might be linked to the recent finding of P6 protein activity as a pathogenicity effector that suppresses the host SA-dependent defense responses by modulating NPR1 protein (Love et al., 2012).

1.5.2.2 RNA silencing suppression related effects

RNA silencing mechanism has been shown to have many connections with different pathways in plants ranging from defense to development (Figure 2). Especially, these pathways are interconnected by the small RNA molecules. Any disturbances that cause small RNA or RNA processing or RNA silencing pathways impairment may lead to overwhelming effects in the plants. This is evidenced by many virus-infected and VSR-expressing transgenic plants, which exhibit phenotypes like RNA silencing component mutants (Brigneti et al., 1998; Jay et al., 2011; Kasschau et al., 2003; Siddiqui et al., 2008). As was said earlier, VSRs are the important viral constituent proteins which perform multiple tasks related to the virus infection process. These VSR proteins do suppress the host defense mechanism, and mediate the movement and accumulation of the virus in plants. Some VSRs also disturb the host's intercellular trafficking by altering the PD SEL and permeability (Baulcombe, 2004; Li and Ding, 2006; Roth et al., 2004). These events directly affect the allocation of different cellular resources-carbohydrates, proteins, transcription factors, small RNA molecules-accumulation, and also the cell signaling events. These cause severe disturbances in physiology and development, and induce disease symptoms in the infected host plants (Chapman et al., 2004; Chellappan et al., 2005; Siddiqui et al., 2008; Silhavy and Burgyan, 2004).

Viruses interact and change the accumulation pattern of the small RNAs i.e. miRNAs and siRNAs by the positively charged structural domains of their VSRs proteins (Lakatos et al., 2006). Several studies show that VSRs effect the miRNA accumulation. For instance, transgenic plants expressing different VSRs such as P1/HC-Pro, P19, P21 and AC4 VSRs accumulate different miRNA* and mature miRNAs abnormally and thereby inhibit the miRNA guided cleavage (Chapman et al., 2004; Chellappan et al., 2005; Dunoyer et al., 2004). These interferences are manifested in various viral symptoms, for instance in the petunia plants infected with CMV virus, which loses its natural red star look due to the accumulation of the of chalcone synthase (CHS) siRNAs gene that is naturally silenced (Koseki et al., 2005).

In addition, viral VSRs also affect host defense responses by interacting with host defense constituents. The CMV 2b acts against the SA accumulation and SA mediated defense responses (Ji and Ding, 2001). Recent studies also indicate that the hosts R-genes are regulated by the miRNAs and siRNAs activity in the plants. Thus, VSRs have the possibility to disturb the important R-gene mediated defense (Li et al., 2012; Zhai et al., 2011). This is further supported by the species that have rapidly evolving R-genes, such as spruce, which have most of their 21 nt long siRNAs (about 90%) generated from the degradation of the NB-LRR R gene transcripts (Kallman et al., 2013).

2. AIMS OF THIS STUDY

In this study, I have aimed to elucidate the host plant transcriptome alterations caused by three isolated VSRs (HC-Pro form PVY, AC2 from ACMV and P25 from PVX), when expressed as transgenes in tobacco host. Furthermore, three distinct responses caused by TMV upon the transcriptome of its tobacco host were analysed, by using TMV-expressing transgenic tobacco plants, which initially show atypical resistance to the virus but later turn susceptible to the virus. These responses were compared to that caused by natural TMV infection in tobacco.

Specific aims of this study included:

1. Observe and analyse changes in the phenotype and growth habit of the transgenic plants

2. Analyze the effects of these transgenes on the transgenic tobacco plants transcriptome profiles by using Agilent microarrays.

3. Analyze in qualitative scale, using two dimensional-Polyacrylamide gel electrophoresis (2D PAGE), the changes caused by these transgenes in the plant protein profiles.

In some cases also the DNA methylation profiles of these transgenic plants was analysed by using methylation sensitive restriction digestions and differential PCR.

From these results, I deduce some of the molecular mechanisms, which are affected by these viral VSR proteins, indicating that they are also affected by the RNA silencing machinery, and are part of regulatory functions and interconnected regulatory networks in plants. From our TMV-expressing transgenic plants, I observe that the compatible TMV-tobacco reaction is compromised in the young plants. From the array results, I aim to identify gene expression alterations that are associated with resistant stage of these plants.

3. METHODOLOGICAL ASPECTS

Detailed methods are described within the enclosed papers I-IV. The overview of the methodology is described in the following sections.

3.1 Plant material and growth conditions

Plant materials used in paper I, II and III were the *N. tabacum* plants transformed with the different VSRs genes i.e. with HC-Pro from PVY, AC2 from ACMV and P25 from PVX, each expressed under the constitutive CaMV 35S promoter. All of these transgenic plants have been previously produced and characterized in our laboratory (Siddiqui et al., 2008). In the paper IV, *N.tabacum* plants transformed with the full length infectious TMV genome, also expressed under the constitutive expression of CaMV 35S promoter were used (Siddiqui et al., 2007). Both wild type and pBin 61vector transformed *N.tabacum* plants were used as controls. Plants used in this study were grown in normal growth conditions, with 150 μmol photons m^{-2} s^{-1}, at 60% RH, with a 16 h / 8 h light / dark cycle, at 22°C.

3.2 Plant material collection time

In the paper I, II and III the leaf samples (whole third leaf from the top) were collected from both wild type, and vector-transformed control plants and from VSRs (HC-Pro, AC2 and P25) expressing transgenic tobacco plants at 5–7 weeks after germination. Flower bud samples were also collected from the same plants, just prior to the bud opening. In the paper IV, the samples were collected at different time intervals from both the control wild type plants and TMV transgenic tobacco plants. Leaf samples were collected at 6 weeks (Before Resistance Break, BRB), 8 weeks (After Resistance Break, ARB) and 9 weeks (8 week wild type tobacco plants Infected with TMV, TMVi) after germination. Third leaf from the apex (about half of the mature leaf size) was always collected. All the leaf and flower samples were directly frozen in liquid nitrogen and used further for RNA extractions. For each of the studies, three individual biological replicate plants were used.

3.3 RNA extraction, microarray procedure and data handling

All the plant materials used in this study (Papers I-IV) were subjected to the same RNA extraction, cDNA synthesis, labeling, microarray analysis and further data handling procedures. First, the total RNA extraction, purification, DNaseI I treatment, RNA concentration, and the cDNA synthesis and labeling were done according to the respective reagent manufacturer's recommendations, as described in Papers I-IV. A total

of 700 ng of purified total RNA was used to produce the Cy3 labeled cDNAs. The quality of both total RNA and of the labeled cDNA was checked for any degradation. The Cy3 labeled cDNA concentration was measured and about 1.6 µg of Cy3 labeled cDNA was hybridized onto the Agilent's 4 × 44 K tobacco chip according to the manufacturer's recommendations, and as described in Papers I-IV.

Agilent chips were processed and scanned using manufacture's designated solutions, protocols and equipment, as described in the Papers I-IV. The numeric data was produced using Agilent Feature Extraction software version 10.5.1.1. Grid: 021113_D_F_20080801; Protocol: GE1_105_Dec08; QC Metric Set: GE1_QCMT_Dec08.

Resulted microarray raw numerical data was analyzed by the Chipster program (center of scientific calculating (CSC), Finland) that is based on R Project for Statistical Computing program (Kallio et al., 2011). The intensity values were normalized for both the control and transgenic plants samples together. It was done for three biological replicates by using the median signal values, median background values and "quantile" parameter. A background offset value (50) was added to prevent negative values during normalization. Subsequently, the normalized data was subjected to student t-test for the statistical significance with p value threshold level < 0.05. Two fold altered up- or down-regulated transcripts were considered as differentially expressed genes. All the array results were deposited in the array express database with their corresponding accession numbers stated in the publications (Papers I-IV).

3.4 Reannotation of differentially regulated genes in microarray data

The Agilent 4x44k microarray probe information provided by the manufacture was very limited and based on expressed sequence tags (EST) and cDNA sequences. This problem was mitigated by adapting most of the probe information from the Mapman website http://mapman.gabipd.org/web/guest/mapman-annotationexperts that maintains the publically available Agilent 4x44k tobacco probe information. Together with this, we also used other websites such as JCVI http://plantta.jcvi.org/ and BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi to obtain the relevant probe information. The probe functional grouping was done according to the information provided by the mapman.gabipd.org website and with some additional manual adjustments.

3.5 RT-qPCR

The RT-qPCR method was used to verify the microarray results according to the MIQE guidelines (Bustin et al., 2009). 1 µg of purified total RNA was used for the cDNA

synthesis using the Revert Aid reverse transcriptase enzyme by following the manufacture recommendations (Product # EPO451, Fermentas) as described in the Papers I-IV. In all studies (Papers I-IV), three biological replicate samples from both control and transgenic plants were used and for each sample 3-4 technical replicates were used to minimize the pipetting errors. The Bio-Rad iQ5 machine was used to perform the RT-qPCR and the results were calculated by employing the quantification cycle (Cq) method (delta delta Cq). Primer specificity was tested by checking the single peak in the DNA melting curves. The standard error of mean was calculated from the three biological replicates.

3.6 Proteomic analysis

Protein samples were prepared both from the controls and transgenic plants by using TRIsure-reagent (Bio line, UK) according to manufactures recommendations with a slightly modified protocol (TRIzol, Invitrogen Inc. USA). Lowry method was used to measure the protein concentration. 250 µg of the selected protein samples from both control and transgenic plants were first separated by using Bio-Rad laboratories 7 cm IPG strips, pH 3-10, and next in 15% SDS-PAGE large gels in Protean II apparatus (Bio-Rad) according to the manufacturer's guidelines. The separated gels were fixed and stained with Coomassie blue and some of them further with silver to see the low abundant protein spots using Page Blue and PageSilver staining kits, respectively (Fermentas), according to manufacturer's recommendations, and as described in Papers I-IV.

3.7 LC-ESI-MS/MS mass spectrometry analysis

From the HC-Pro Page Blue stained gels, four distinctly altered protein spots were selected and digested with trypsin enzyme, and analyzed by mass-spectrometry using Qstar i (Applied Bio systems/ MDS Sciex) coupled with a CapLC HPLC machine (Waters), as described in Paper I. Mascot program (v2.2.6) UniProt (release 2010_9) was used to search for the peptide sequences.

3.8 Photosynthesis, starch and anthocyanin measurements

1.0 g of leaf samples from both control and transgenic plants were extracted using thylakoid isolation buffer (0.3 M sorbitol, 50 mM Hepes/KOH pH 7.4, 5 mM MgCl2, 1 mM EDTA and 1% BSA). The thylakoid suspension was filtered with Mira cloth and 2 mls was pelleted by centrifugation, as described in Papers I-IV. The pellet was again suspended in 100 µl of O_2-electrode measuring buffer and the Clark type O_2-electrode was used to measure the oxygen evolution. 0.5 mM 2,6-dichloro-p-benzoquinone

(DCBQ) was used as electron donor. Chlorophyll concentration was measured according to the procedure stated in Porra et al. (1989)

In paper II, three 8 mm leaf discs from wild type and of AC2 expressing transgenic tobacco plants were taken to measure the anthocyanin concentration according to procedure stated by Neff and Chory, (1998). In the paper I and II the starch amount was measured by using total starch assay kit (Megazyme) according to the manufactures guidelines.

3.9 Analysis of DNA methylation

In the paper II, Genomic DNA was extracted from both control and AC2 transgenic tobacco plants by using the Nucleospin plant II kit (Macherye-Nagel, Germany) according to the manufactures recommendations. *BamHI* or *BamHI* and *HpaII* restriction enzymes were used to cut the genomic DNA and the digested samples were amplified by using Taq polymerase with 2 mM $MgCl_2$ and specific primers, and run on agarose gel.

3.10 Analysis of ROS production, oxidative stress and HR responses

Hydrogen peroxide and superoxide anions were identified by reacting with 3,3'-diaminobenzidine (DAB) and Nitroblue tetrazolium (NBT) stains, respectively, as described in Papers II and III. Whole third leaf from the top was collected from both control and transgenic plants and placed in the 20 ml of DAB or NBT solution in small Petri dishes. Leaves were incubated in the dark overnight and then treated with several changes of 96% ethanol until all the chlorophyll was removed. Then leaves were photographed.

Six to seven week old wild type and P25 expressing transgenic plants were treated with freshly grown *Pseudomonas syringae* pv. tomato DC3000 bacterial cells suspended in 10mM $MgCl_2$ solution, as described in Paper III. The bacterial solution (about 50 µl/spot) was gently infiltrated into intracellular space of leaves (about 50 µl/spot) by using a syringe. The infiltrated leaf samples were photographed after a week.

3.11 ELISA

Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) was performed to measure the concentrations of different viruses (PVY, PVX, PVA and TMV) according to the manufacturer's guidelines (Bioreba, Reinach, Switzerland) with a slightly modified protocol. 1:10000 diluted commercial polyclonal, alkaline

phosphatases conjugated antibodies (Bioreba, Reinach, Switzerland) were used to detect the viruses. P-Nitrophenyl phosphate substrate was used to develop the ELISA reactions and the developed reactions were measured at 405nm absorbance by using the ELISA plate reader, as described in Paper IV. 100 ng of purified virions were used as internal standards for the corresponding viruses.

3.12 *In situ* labeling of the TMV coat protein

The samples of TMV coat protein *in situ* labeling were prepared according to the procedure stated in Siddiqui et al. 2011. 7µm shoot apical domain sections of both control and TMV expressing transgenic plants, prepared at 7 weeks after germination (just before resistance break) were initially incubated in phosphate buffered saline (PBS) containing 4% bovine serum albumin, and then in TMV- specific alkaline phosphatase-conjugated polyclonal antibodies (dilution 1:50), washed, and stained with freshly prepared fuchsin substrate solution, as described in Paper IV. The samples were examined and photographed by using the Leitz, Laborlux S light microscope (Leica Microsystems AG) at 40× and 100× magnifications.

4. RESULTS

4.1 Transgenic plants as a tool to study the interactions of individual viral genes with the host cell

Viruses have small genomes that encode only for a few proteins and depend fully on the host plant to complete their infectious life cycle (Palukaitis et al., 2008). All of the viral genes are essential in establishing the viral infection process. Thus, it is very important to understand the specific role of individual viral genes and their contribution to the viral infection process, e.g. to develop viral resistant plants. This aim has been approached by producing the various transgenic plants that express different viral genes individually, or the whole viral genome. These help in envisaging the roles of viral genome constituents in the host defense suppression and physiological changes. In this study I have elucidated the plant-virus interactions in relation to gene expressional changes occurring in transgenic *N.tabacum* plants, which express different viral VSR genes or the whole infectious genome of TMV under the control of CaMV 35S promoter. These plants were earlier produced and initially characterized in our laboratory (Siddiqui et al., 2007; Siddiqui et al., 2008).

At first, I used microarrays to analyse the transcriptomes of the three transgenic plant lines, expressing different viral suppressors, which differ from each other in their structure and other contributions to the viral life-cycle. Also, the proteomes and various physiological parameters of these plants were analysed. In the papers I, II and III, I investigated the effects of the multifunctional HC-Pro protein derived from PVY, the transcriptional activator AC2 protein derived from ACMV, and the P25 movement protein from PVX on transgenic tobacco plants. In the second part, I investigated the gene expression alterations occurring in transgenic tobacco plants, which express the whole infectious TMV genome (Paper IV), both during the young TMV-resistant stage, and in older plants, after the plants have become infected with TMV. For comparison, also the transcriptomes of wt TMV-infected tobacco plants were investigated. With this transgenic material we have produced a system where the viral inoculum is introduced to the plant at a very early stage i.e. into the embryo. This artificial inoculation system allows the plant to mount novel type of resistance response, which lasts active through its young growth stages. This reaction is very different from the natural compatible TMV-tobacco interactions but it reveals the ultimate potential of the plant to control the virus throughout its vegetative stages. Although these transgenic plants constitute artificial systems in studying the viral studies, they are very helpful in expressing viral genes or whole virus in every cell of the expressed plants.

4.2 The effects of HC-Pro VSR on transgenic tobacco plants

Expression of HC-Pro VSR in transgenic tobacco plants causes severe reduction in growth and malformation of the leaves and flowers, and the HC-Pro expression levels are correlated to the phenotype severity (Siddiqui et al., 2008). The transgenic plants have short stems and display a bushy-like appearance. Flowering time was delayed by 2 to 3 months in these plants compared to the control plants, and also the flowers were malformed, with petals being frequently converted to stamens (Figure 1, Paper I). In about one out of four flowers, the anther filaments were converted to petals. Control plants transformed with the pBin61 vector showed phenotype similar to non-transformed wild type plants. In order to deduce the HC-Pro VSR effects, we did microarray analysis of leaves and flower samples of the wild type and of pBin61-vector transformed control plants, and of HC-Pro VSR expressing transgenic tobacco plants, with wild type plants and pBin61 vector-transformed control plants showing essentially the same expression pattern.

The microarray data analyses indicate that total of 748 and 332 transcripts were altered in the leaves and flowers, respectively (Table1, Paper I). Most of the transcriptional changes pertained to defense, various stresses, proteases and proteasomal degradation. Also, we analyzed the whole proteomic profiles of these plants, which show strong alteration compared to the control plants.

4.2.1 HC-Pro induces defense and stress gene expressions

Both in the leaves and flower samples of HC-Pro expressing transgenic plants, clear up-regulation of the defense hormones such as ethylene and jasmonic acid related transcripts was observed (Table 3, Paper I). Of special interest was the ethylene response factor1 (ERF1) accumulating to 5 times higher in leaf tissue and two times higher in flower samples of HC-Pro transgenic plants, as compared to the wt control plants. ERF1 integrates the ethylene and defense related transcripts. Similarly, several transcription factors such as WIZZ, JA 2 and ERF3 were induced in these plants. In the flower samples several ERF1 induced transcripts such as Avr9/cf9 were enhanced. Additionally, several negative regulators of ERF4 and JA-responsive defense genes were altered in the HC-Pro transgenic plants. Interestingly, most of these changes in the gene expression were similar to those observed in whole virus infected plants (Agudelo-Romero et al., 2008; Ascencio-Ibanez et al., 2008; Marathe et al., 2004). Various abiotic stress-related transcripts were also altered significantly in the HC-Pro transgenic plants, although the plants were grown in normal non-stressing conditions. Most of them were related to the cold, salt and dehydration stresses with transcript levels ranging from 2 to 4 fold, as compared to the wt control plants grown in the same conditions.

4.2.2 Alterations in the proteasomal proteins and protein profiles of the HC-Pro transgenic plants

HC-Pro is a multifunctional protein which exhibits cysteine-type endopeptidase and thioredoxin activities, and it functions in processing of the viral polyprotein (Carrington and Herndon, 1992). It may also contain other proteolytic activity as several proteasome-related transcripts, like ubiquitin ligases were induced in the HC-Pro transgenic plants. Similarly, several other protease inhibitors such as trypsin and metallocarboxy peptidase inhibitors type II were induced in these plants (Paper I). These alterations could also affect the protein profile of the plants. For this, we performed whole proteome 2D-gel analysis and also mass spectrometry analyses of few selected protein gel spots. The HC-Pro protein profiles showed strong alterations compared to the wild type plants (Figure 4, Paper I). Many of the proteins spots were either strongly induced or reduced. The LC ESI MS/MS mass spectrometry analysis of four of the altered spots indicated that they were related to the photosynthesis (Paper I). These identified up- or down-regulated proteins such as Ribulose-bisphosphate carboxylase large subunit (RBCL), oxygen evolving enhancer protein 3 (OEE3), 20 kDa CYP2 and 12 kDa chloroplast protein (CP12) were correlating with the microarray transcript accumulation data (Table 8, Paper I). In general, the large number of altered protein spots reflected the numerous alterations in the transcriptome, although individual transcript/protein correlations were not identified.

4.2.3 Flowering time delay and flower malformations in HC-Pro transgenic plants

In correlation with the phenotypic changes, expression of HC-Pro in transgenic plants altered several transcripts related to the flower development (Paper I). These changes pertained to the down-regulation of CYCLING OF DOF FACTOR 1, which might relate also to the later flowering by regulation of the FLOWERING LOCUS (FT) gene via induction of CONSTANS (CO) gene. Additionally, several genes related to the blue light receptor FKF1, GIGANTEA and PLPB proteins were up-regulated. Of special interest is the up-regulation of EARLY flowering 4 that is involved in the regulation of circadian clock and causes late flowering phenotype in Arabidopsis plants (McWatters et al., 2007). Thus, up-regulation of these transcripts in these plants may cause the late flowering phenotype. Also, transcripts related to the AP2 transcription factors such as ERF1 and RAV2 were induced both in leaves and flower samples of the HC-Pro transgenic plants, indicating that these may be related to the malformed phenotypes observed in the flowers.

4.2.4 Photosynthetic activity and starch accumulation are reduced in the HC-Pro transgenic plants

HC-Pro expression induces many of the energy production related transcripts in the transgenic plants. These up-regulated transcripts include the ATP synthetases, carbon

assimilation (RBCL) and starch degrading alpha-amylases and alpha-glucan water dikinase. Instead, chloroplast precursor phyteone synthase and PS II 22 kDa components, and many of the starch synthesis and glycolysis related transcripts were down-regulated (Table 8, Paper I).

To check the alterations of photosynthesis and carbohydrate related genes at the physiological level, we conducted oxygen evolution measurements. The measurements indicate that HC-Pro expressing transgenic plants show decreased photosynthetic activity compared to the control plants (Figure 3, Paper I). The clear down-regulation of starch levels was also visually observed during the thylakoid preparation in these plants.

4.3 The effect of Geminiviral AC2 VSR on transgenic tobacco plants

AC2 VSR is a transcriptional activator protein, which causes transactivation of many host genes, RNA silencing suppression and reduction in host methylation activity. Its expression causes the stubby growth and occasional malformation in the veins and leaves of the young transgenic plants. Interestingly, these malformations disappear as the plants grow and leaves become fully expanded. In older plants, the leaves become narrower and paler compared to the wild type plants. Root neck and root systems are less developed than in wild type plants. Flowers buds are often twisted around and the petals are often grown together, giving often a rectangular shape to the flowers (Figure 1, Paper II).

In this study, we used the microarray analysis to investigate specific effects induced by the AC2 transgene on the tobacco transcriptome. The analysis was done from both leaf and flower samples of the transgenic plants and the control plants. Also, we compare the results with those obtained from the analysis of the HC-Pro expressing plants (Paper II).

The microarray analysis indicated that 1118 and 251 transcripts were differentially accumulating in the leaves and flower samples, respectively (Table 2, Paper II). Many of the transcripts related to the stress, cell wall modifications and signalling were up-regulated, and transcription, translation and photosynthesis-related transcripts were down-regulated. Specifically, Jasmonate and ethylene biosynthesis transcripts and their related genes, and retrograde signaling-related transcripts were altered. Comparison of the microarray data obtained from the AC2 and HC-Pro expressing plants indicated that these two VSRs were exhibiting clearly distinct transcript expression pattern (Figure 2, Paper II).

4.3.1 Defense responses

AC2 VSR expression causes clear oxidative stress in the young transgenic tobacco plants. This stress was shown by specific visible HR-like symptoms in the young

transgenic plants, but not in the older plants, compared to the wild type plants at the same age. In corresponding to this, many SAR, ROS scavenging and respiratory burst oxidase related transcripts were altered in young AC2 expressing transgenic plants. To check the ROS accumulation levels we conducted oxidative stress experiments (Figure 3, Paper II). These experiments indicated increased levels of ROS accumulation in young leaves, where oxidative stress transcripts were also induced higher, but not in the older leaves.

Different defense transcripts pertaining to the JA-mediated regulatory pathways were also induced in these plants, including transcripts NtJAZ1 and NtJAZ3, JA-responsive genes and JA-signaling cascade, which were induced at 2-28 fold levels. Also, some of the ethylene biosynthesis transcripts were induced in these plants (Table 3, Paper II).

4.3.2 Translation factor- and ribosomal protein- transcripts were down-regulated

Many translation factors and ribosomal transcripts were strongly down-regulated in the AC2 VSR expressing transgenic plants. 52 translation-related transcripts including cytosolic 60S, and 40S, and organellar (chlorolplastic and mitochondrial) 50S, 30S and 23S ribosomal subunits were down-regulated (Table 5 and Figure 6, Paper II). These changes corresponded with the 30% decrease of total protein content per fresh weight, as compared to the wild type tobacco plants.

As the total protein content of the plants was clearly altered, we performed the 2D-gel analysis experiments to visualize the whole proteome. AC2 VSR expression clearly decreases the protein levels compared to the wild type plants (Figure 7, Paper II). Interestingly, these protein profiles exhibit some similar patterns with the protein profiles of the HC-Pro expressing transgenic plants, although the transcript-profiles of these plants were clearly distinct.

4.3.3 DNA methylation related transcripts were altered

Alterations in transcripts related to the RNA silencing-mediated DNA methylation process were observed in AC2 transgenic plants (Table 6, Paper II). Both KTF1 and AGO5 were up-regulated, where the former is known to involve in the RdDM process. Also, the AGO5 can effect methylation of the CG motifs rather than C residues in CNG or CNN contexts. Negative regulator of RNA silencing i.e. repressor of silencing1 (NtROS1) was up-regulated in the AC2 transgenic plants. Additionally, two Chromatin Methyl Transferases (CMTs) involved in *de novo* methylation process showed down-regulated expression.

To check the DNA methylation levels we conducted PCR- amplication experiments for some of genomic regions in contribution with three methylation sensitive restriction enzymes. The results showed increased methylation in the ERF1 and AP24 coding regions but not in the 18S RNA (Figure 8, Paper II).

Previous studies have indicated that AC2 VSR functions through suppression of S-adenosyl methylase (SAM) enzyme in the host plants (Buchmann et al., 2009; Wang et al., 2003). However, we did not find any SAM alteration in our microarray data although we found histone transcripts alterations. Additionally, transcripts related to histone acetyl-transferases (HATs) that are involved in chromatin opening were up-regulated. Cell cycle related transcripts were down-regulated in AC2 expressing transgenic tobacco plants (Paper II).

4.3.4 Photosynthetic activity was reduced in the AC2 transgenic plants

AC2 expression in the transgenic tobacco plants causes severe down-regulation in the photosynthesis related transcripts (Table 4, Paper II). Many of the transcripts related to chlorophyll biosynthesis like porphobilinogen deaminase, phyteone synthetase and chlorophyll synthase were down-regulated in these plants. Also, transcripts related to the chlorophyll fatty acid synthesis, PS II, PS I, primary carbon metabolism and chloroplast ribosomal protein were mainly down-regulated.

To check the chlorophyll content in AC2 transgenic plants, we measured the pigment content in these plants and compared them to wt plants at same age. The measurements indicate that both chlorophyll a and b were reduced. Also, we performed oxygen evolution to check photosynthetic activity. The measurements clearly indicate the significant decrease in photosynthetic activity in the AC2 expressing transgenic plants compared to the wt plants (Figure 4, Paper II). The starch amount was also reduced in the AC2 transgenic plants.

4.4 Effects of the P25 VSR from PVX virus in tobacco plants

In this study, I also analysed the effects of another RNA virus-derived VSR transgene, i.e. P25 isolated from the PVX genome. P25 is a multifunctional protein involved in the RNA silencing suppression activity and also in the viral movement process (Angell et al., 1996; Bayne et al., 2005). Here, I conducted the microarray analysis for both leaf and flower samples to study its effects on the transgenic tobacco plants, and also to compare these results with other analysed VSRs effects i.e. of HC-Pro and AC2 from PVY and ACMV, respectively (Paper III).

Phenotypically these transgenic plants were similar to the wild type plants through their growing stages, but occasionally the lower leaves of the plants turned yellow, differing from the same age wild type plants. However, the growth of the plants was about 10% smaller than wild type plants (Figure 1, Paper III). Still microarray analysis indicated massive transcriptome changes in these plants with most of them occurring in the leaf samples and only very few in the flower samples. Total of 1350 transcripts were up-regulated and only 5 transcripts were down-regulated in the leaf samples of these plants. With more relaxed statistical criteria, about 325 transcripts could be identified also as down-regulated in the leaf samples. In the flower samples, 51 and 13 transcripts were up and down-regulated, respectively. Altered transcripts were mainly related to the various stresses, protein translation, metabolic networks, transcriptional regulation and signaling pathways in the leaf samples (Figure 2 and 3, Paper III).

4.4.1 Stress related transcripts were induced

P25 expression in transgenic plants induces various transcripts related to both biotic and abiotic stresses (Figure 4, Paper III). A total of 138 stress-related transcripts were altered in the leaf samples, including PR transcripts, which were induced up to thousand fold. Also, various disease resistance genes including AVR9/Cf-9, chitinases, hairpin elicited proteins and SAR and HR-related genes were up-regulated in these plants, as were transcripts for biosynthesis of secondary metabolites such as phytoalexins, flavonoids and terpenoids. These were up-regulated up to 200-fold levels.

Various abiotic-stress-related transcripts were also altered in these plants, although plants were grown under normal conditions. Metal binding proteins, oxidative stress, dehydration, desiccation and heat shock proteins were induced up to 77 fold levels.

To check the effects of the various biotic stress-related transcripts in P25 transgenic plants, I conducted HR and oxidative stress tests. When infiltrated with *Pseudomonas syringae* DC 3000, P25 expressing transgenic plants showed enhanced HR response within 6 days after inoculation as compared to no reaction in the wild type plants at same age (Figure 7, Paper III). Surprisingly, despite induction of various oxidative stress transcripts, these plants did not show any increased oxidative stress as compared to the wild type plants.

4.4.2 Protein synthesis and modification, and metabolic networks were altered

P25 expression also significantly increased the accumulation of nine transcripts related to the translational machinery. Also, a total of 110 of the altered transcripts were related to the protein degradation mechanism, pertaining e.g. to ubiquitin proteases,

endopeptidases, carboxypeptidases, hydrolayses and AAA-type ATPases. Additionally, different sugar and amino acid metabolism related (total of 133), mitochondrial electron transfer related and photoassimilation related (PAR) transcripts were strongly induced in these plants (Paper III).

Fresh weight protein measurements were conducted (data not shown) to check how the transcription level alterations correlated to the protein content of the leaves. Interestingly, despite of strong induction in the transcripts, these plants show nearly equal amounts of soluble protein compared to the wild type plants. Also, the 2D-PAGE gel analysis indicated that the proteins profiles were about same as in the wild type plants (Figure 5, Paper III).

4.4.3 Commonly altered genes in P25, HC-Pro and AC2 VSR expressing transgenic plants

The data of the up-regulated transcripts in leaves of the P25 transgenic plants was compared to previously obtained data of up-regulated transcripts in the HC-Pro and AC2 VSR expressing plants, with total 464 and 843 transcripts, respectively (Papers I and II). Interestingly, these VSR-expressing plants showed distinct gene expression alterations, with only 141 transcripts being commonly enhanced in all of them (Figure 8, paper III). Transcripts related to various defense reactions including ethylene production, ethylene transcription factors, MAP kinases, SAR and WRKY transcription factors, cell wall modifications, oxidative stress transcripts like glutathione S-transferases, cytochrome p450 and secondary metabolites were some of the commonly altered transcripts in all these VSRs expressing transgenic plants. Also, altered functional group comparisons in these three VSRs related microarray data revealed the commonalities in defense, stress, cell wall, photosynthesis and carbohydrate metabolism related groups (Figure 5)

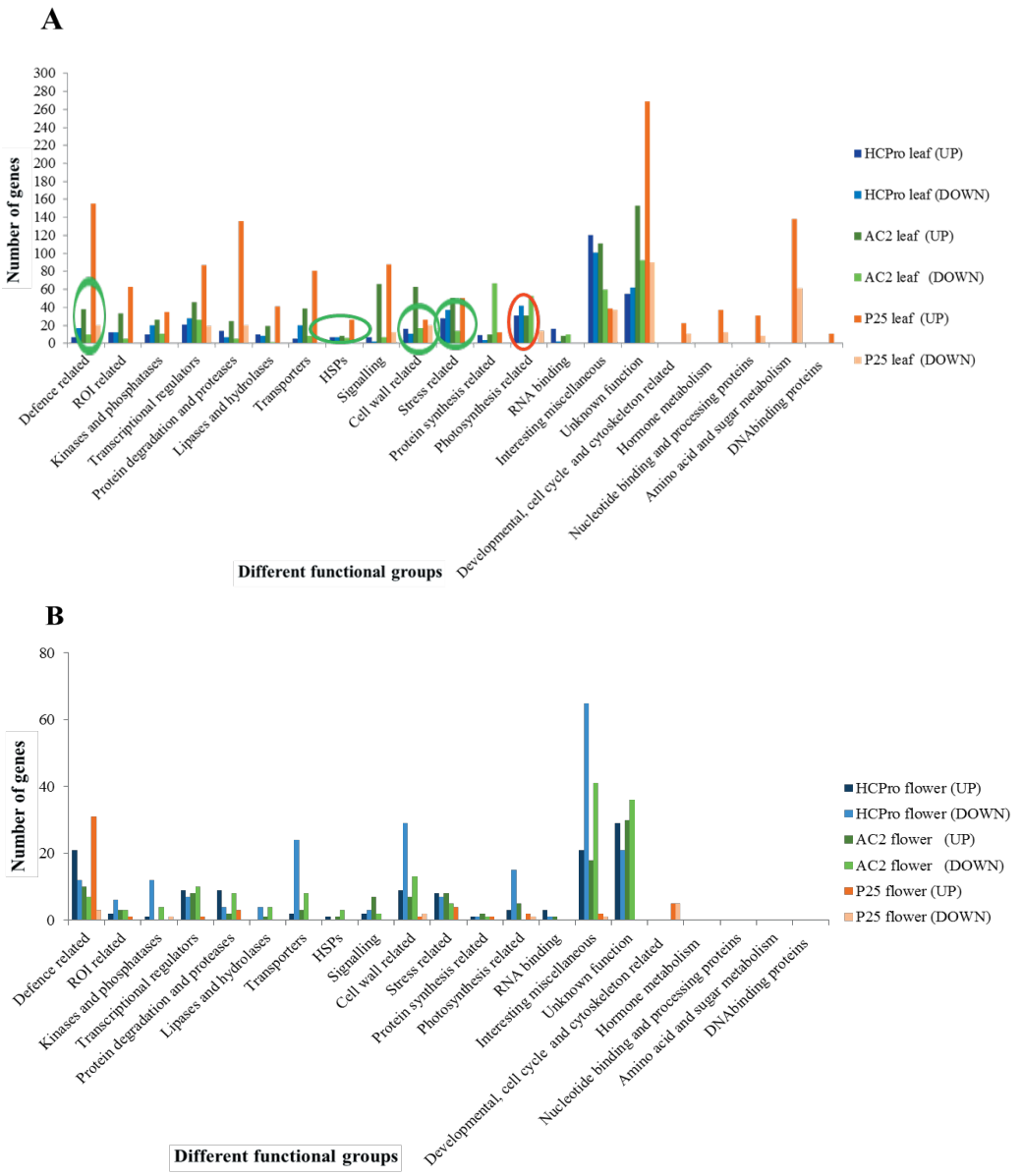

Figure 5. Alteration of transcripts related to different functional groups in the A: leaves, and B: flowers, of the three VSRs (HC-Pro from PVY, AC2 from ACMV and P25 from PVX) expressing transgenic tobacco lines. For the most part, these VSR cause specific gene expression alterations. The marked functional groups (green: up-regulated and red: down-regulated) are some of the commonly altered functional groups in the leaf samples of these plants.

4.4.4 Photosynthetic activity was reduced in the P25 expressing plants

Because of normal phenotype and down-regulation of phosyntheis related genes (transcripts observed in the more relaxed statistical criteria, data not shown), I

conducted oxygen evolution measurements to check the photosynthetic activity in the P25 transgenic plants. The measurements revealed 20% decrease in the photosynthetic activity compared to the wt plants (Figure 6, Paper III).

4.5 Transcriptomic alterations in the whole TMV genome expressing transgenic plants, and their role in the resistance against exogenous and endogenous TMV

The interest for this study was based on the whole TMV genome expressing transgenic tobacco plants expressing atypical resistance to TMV (Siddiqui et al., 2007). These plants exhibited two different stages during their development i.e. complete resistance against TMV up to 6-7 weeks after germination and resistance break stage at 7-8 weeks after germination. (Figure 1, Paper IV). During the resistant stage (Before resistance break, BRB), the transgenic plants did not show any viral symptoms and did not become infected from the endogenous viral sequence, nor could they be externally infected with TMV. No viral RNAs nor viral siRNAs or transgene methylation were detected in these plants i.e. no RNA silencing activity was observed. After resistance break (ARB), strong TMV RNA accumulation, corresponding with viral siRNAs and transgene methylation was detected (Siddiqui et al., 2007). Also, these plants developed typical TMV symptoms, correlating with the TMV RNA accumulation levels. These symptoms first appeared in the upper leaves (at apex) and progressed towards to the lower leaves.

To elucidate the resistome in these young BRB-TMV transgenic plants we conducted the microarray studies for BRB-TMV plants and compared them with the ARB-TMV transgenic plants and TMVi plants (Figure 6).

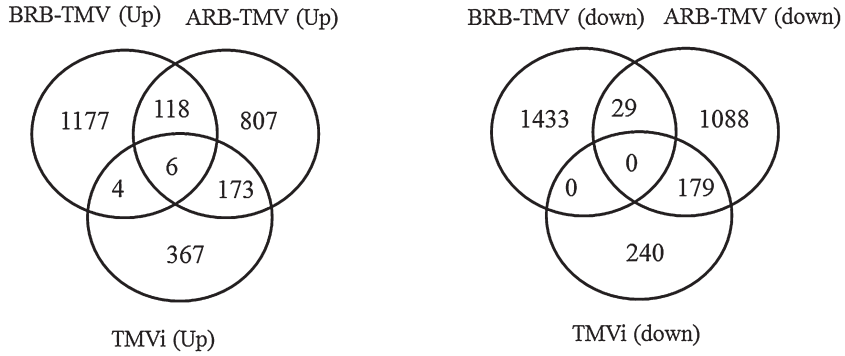

Figure 6. Venn diagram representing the numbers of specific and commonly altered up-and down-regulated transcripts in the TMV-expressing transgenic plants (BRB-TMV and ARB-TMV) and in TMVi plants

The microarray analysis indicated that 1362 and 1422 transcripts were up- and down regulated in the TMV resistant stage plants (BRB-TMV) as compared to the control plants (Figure 2, Paper IV). Transcripts related to defense and stresses were mostly up-regulated and those related to translation, hormones and development were mainly down-regulated.

4.5.1 Translation machinery was shut down in resistance stage of TMV transgenic plants

Protein synthesis machinery was strongly reduced in the resistant BRB-TMV transgenic plants as compared to the ARB-TMV and TMVi plants, where the protein translation related transcripts showed either normal or increased expression pattern (Supplementary Table 5 and Figure 3, Paper IV). The largest functional group of the down-regulated transcripts (750 out of 1422) in the BRB-TMV transgenic plants was that of the protein synthesis mechanism. Particularly, many of the cytosolic 60S (total of 391) and 40S (total of 222) ribosomal subunit transcripts were down regulated. Also, about 33 transcripts related to the translation-initiation and elongation factors were down-regulated. Similarly, about 150 protein degradation and processing-related transcripts were up-regulated. On the other hand, in ARB-TMV transgenic plants, protein translation-related transcripts were less affected, as only 120 transcripts were down-regulated and 135 up-regulated in these plants. Most of the down-regulated transcripts in these plants belonged to the proteases, peptidase, and ubiquitin-mediated protein degradation pathways, while up-regulated transcripts were related to the amino acid biosynthesis and protein degradation pathways, including proteases and ubiquitin proteasomal pathway transcripts. However, in TMVi plants, most of the protein translation-related transcripts were up-regulated (197). Interestingly, most of these up-regulated transcripts belong to 60S ribosomes (114) and 40S ribosomal subunits (55), many of which were strongly down-regulated in the BRB-TMV transgenic plants. Also, proteasomal degradation pathways-related transcripts were up-regulated in these plants

4.5.2 Multiple stress related transcripts were strongly induced in TMV transgenic plants but only few in TMV infected plants

The natural interaction between TMV and tobacco is known to be compatible in nature, and in this type of interactions, excessive amount of defense responses are typically not observed. However, the TMV expressing transgenic plants exhibit strong induction of defense responses compared to the infected wild type tobacco plants (Supplementary Tables 7 and 8, Paper IV). In BRB-TMV transgenic plants a total of 347 defense related transcripts were altered. Among them, 174 were up-regulated and 73 were down-regulated, with most of them pertaining to the defense responsive proteins such as elicitor inducible proteins,

disease resistance proteins, chitinases and heat shock proteins. Interestingly, 13 SAR and 28 HR related transcripts were induced in these plants, although plants did not show any HR.

In ARB-TMV transgenic plants, about same amount of defense related transcripts (192) were altered and interestingly, some SAR and HR transcripts were down-regulated, in contrast to the BRB-TMV transgenic plants. Most of the up-regulated transcripts belonged to PR-proteins, heat shock proteins, protease inhibitors and various defense proteins such as thaumatin, thionins and germins. TMV viral coat protein accumulated to very high levels in these plants, indicating high viral replication that is completely absent in BRB-TMV transgenic plants.

In TMVi plants, few stress related transcripts (total of 61) were altered as compared to healthy wt plants. Only 21 transcripts pertaining to heat shock proteins, chitinases, PR-proteins and senescence associated proteins were up-regulated in these plants, while remaining 40 transcripts were down-regulated including several heat shock proteins, wound induced proteins, glutathione-S-transferase and peroxidases. TMV viral coat protein was expressed highly in these plants as it was the in ARB-TMV transgenic plants.

4.5.3 Cell division

Concerning the cell division and cell organization transcripts, few numbers of transcripts related to b-type cyclins (16) and tubulins (13) were mainly down-regulated in the BRB-TMV transgenic plants. Instead, cell organization-related (11), tubulins (13) and other myosin related transcripts were up-regulated in these plants (Supplementary Tables 13 and14, Paper IV).

In the ARB-TMV transgenic plants, the same functional group transcripts (total of 36) were down-regulated. These transcripts were belonging to the tubulins, ankyrin repeat proteins and kinesin. Also, 17 transcripts were up-regulated in these plants including mainly cell division inhibiting proteins, motor proteins and cell cycle check point control proteins. Of special interest is the cell division check point control protein RAD9A, which was induced to 138 fold levels and is completely absent in the BRB-TMV transgenic plants (Supplementary Tables 13 and14, Paper IV).

In TMVi plants very few (5) transcripts of cell division related transcripts were altered, but interestingly the same strong accumulation of cell division check point control protein RAD9A (138 fold) was observed (Supplementary Tables 13 and14, Paper IV).

4.5.4 Hormones and development

Hormones are known to play important roles in symptom development in the plants. Hormones and various development-related transcripts were also differentially expressed

between the TMV-transgenic and TMVi plants (Supplementary Tables 9 and10, Paper IV). Of special interest is the strong induction in the BRB-TMV plants of 20 auxin-repressed and Auxin-responsive genes, and their down-regulation in the ARB-TMV and TMVi plants. Also, about 60 transcripts related to ethylene, abscisic acid, senescence and development associated genes were up-regulated specifically in the BRB-TMV transgenic plants.

In ARB-TMV transgenic plants, a total of 59 hormone and development-related transcripts were up-regulated. Most of them were pertained to ethylene, jasmonic acid and development- related embryo-specific proteins. In TMVi plants a total of 16 transcripts were down-regulated including auxin repressed genes, gibberellin oxidase, jasmonic acid and senescence-related genes.

4.5.5 Photosynthesis is strongly reduced in TMV accumulating plants but milder in resistance plants

Differential photosynthetic gene expression was observed in the young resistant plants compared to the old resistance break and TMVi plants (Supplementary Tables 11 and12, Paper IV). Very small numbers of photosynthesis related transcripts (total of 13) including RCA, plastocyanin and tetrapyrrole synthesis were down-regulated in the BRB-TMV plants. However, in ARB-TMV total of 239, and in TMVi plants total of 55 photosynthesis related transcripts were down-regulated. These transcripts are mainly related to the chlorophyll synthesis, chlorophyll binding, PS II, PS I, OEC and some of the starch metabolism.

I also conducted oxygen evolution measurements to check the photosynthetic activity differences in these plants (BRB, ARB and TMVi). The measurements indicated strong reduction of photosynthetic activity in the ARB-TMV and TMVi plants, but milder in the BRB-TMV plants during the resistant stages (Figure 7, Paper IV).

5. DISCUSSION

Microarrays have brought revolution in the field of plant-pathogen interactions. From the last decade, several microarray experiments related to the plant-virus interactions have been published. However, most of them pertained to the natural virus infections. In present work, I am presenting the foremost and specific VSR effects on the transgenic plants transcriptome alterations by conducting the functional genomic studies for different VSRs- expressing transgenic plants. A special benefit of these studies is that they are done in tobacco, a natural host of the viruses PVY and PVX and a non-host to ACMV, as compared to multiple studies in the model plant Arabidopsis. Comparisons of these VSR gene expression data provide some commonalities as well as specific transcriptome alterations in these transgenic plants.

As was said earlier, the VSRs interfere with the RNA silencing pathways in the host plants. These pathways are important in the plants, as they regulate the variety of mRNA molecules expression, and also are main connectors between the variety of physiological, metabolically and defense related pathways (MacLean et al., 2010). Expression of the VSRs affects many of these silencing-regulated transcripts in the transgenic tobacco plants, as presented in the Papers I, II and III. We also tested the whole proteomic profile of these VSR expressing transgenic plants and conducted various physiological experiments to validate the transcriptomic alterations.

5.1 VSRs induce similar transcriptional alterations as whole virus infections

Many virus infections cause significant changes in the expression profiles of the infected hosts (Babu et al., 2008; Lu et al., 2012; Pierce and Rey, 2013; Senthil et al., 2005; Whitham et al., 2003). Many of these expressional changes were also found in the transgenic plants expressing single viral VSRs genes indicating that these single genes can cause the whole virus like-effects in the plants (Papers I-III). Additionally, many of these transcripts are regulated by the RNA-silencing pathway, suggesting that VSRs might be one of the main causes of symptom development in the natural virus infections. Several other studies indicate that also many other factors such as hormonal interactions and physiological changes are involved in the symptoms development (Handford and Carr, 2007; Padmanabhan et al., 2008). VSRs are multifunctional in nature and known to target the central transcriptional nodes that are involved in controlling of the plant gene regulatory networks (Jones-Rhoades et al., 2006; MacLean et al., 2010). Alterations of several transcription factors which are regulatory nodes such as WRKY, ERF1 in defense, Kow-domain containing transcription factor 1(KTF1), ASYMMETRIC LEAVES -2 like,

NAC domains in development and AP2 transcription factors in flowering alterations, in the VSR expressing transgenic plants indicates that VSRs play a main role in disturbing the host physiology through all these regulatory routes (Papers I-III).

VSR-expressing transgenic plants exhibit phenotypes which may or may not be related with symptoms of natural viral infections. Severe phenotypes such as malformation in the flowers, and changes in the leaf shapes in HC-Pro and AC2 VSR expressing transgenic plants indicate silencing-related alterations in the developmental regulation (Chapman et al., 2004; Siddiqui et al., 2008). These effects, which are not usually observed in natural infections, may be due to the expression of the VSR-transgenes also in the meristematic and differentiating tissues of the plants.

Together all these changes in plant defense responses, developmental alterations, physiological and metabolic changes and the changes in the gene expression profiles indicate that VSRs are main pathogenicity causing agents in plants.

5.2 Common responses induced by the three VSRs on the host transcriptomes

Comparison of the transcriptomic microarray data obtained from the three VSRs-expressing tobacco lines indicated that some gene expressional changes are commonly induced in these three VSR expressing plants. Most of these are related to the physiological, stress and metabolic changes (Figure 5).

5.2.1 Stress

Stress responses are very commonly observed during most of the virus infections. These responses comprise biotic stress-related genes, and consequent interlinked induction of abiotic stress related genes and their combinations (Babu et al., 2008; Lu et al., 2012; Pierce and Rey, 2013; Senthil et al., 2005; Whitham et al., 2003). Many of the same stress-related transcripts were altered in all our analysed VSR expressing transgenic plants, including several transcription factors, and genes related to defence such as WRKY, PR, RAV, ERF1, JA, SAR, avirulence factors (AVR) and SA (Papers I-III). Plant stress signalling is very complex and interconnected in nature, and this whole complexity was observed in the transcriptome of VSR expressing transgenic plants, indicating that these defense networks are connected to RNA silencing (Fujita et al., 2006).

Among the commonly regulated stress transcripts were also cell wall modifications. These are important alterations that occur during viral infections, and are involved in the inhibition of the viral movement process (Babu et al., 2008; Shimizu et al., 2007).

Specifically, expansins, extensin and cell wall synthesizing enzyme related transcripts were either up-or down-regulated in the analysed plants. These are involved in the cell wall loosening and strengthening processes. This might indicate that the cell walls are the target for struggle between pathogen invasion (VSR) and the host defense.

HSPs are known to be expressed during the cellular stresses (Sullivan and Pipas, 2001). The HSPs also show different expression pattern during the virus infection processes (Aparicio et al., 2005). Some viruses e.g. tobamoviruses induce the HSPs during their infection (Whitham et al., 2003), and several studies indicate that HSPs are involved in promoting viral infection processes. Many of these transcript alterations in our VSR expressing transgenic plants might indicate that VSRs directly or indirectly alter the HSPs accumulation, and thus promote virus infections (Gorovits et al., 2013; Hafren et al., 2010; Wang et al., 2009a).

5.2.2 Photosynthesis

Plant chloroplasts are main centers for various physiological functions. Many stresses cause the imbalances in their environment and reduce photosynthetic activity. Viral-encoded VSRs may also interact with host chloroplast proteins and reduce their accumulation levels (Cheng et al., 2008; Jin et al., 2007b; Shi et al., 2007). Studies on several viral infections or other biotic stresses clearly indicate the strong down-regulation of the variety of photosynthetic components such as photosystems, light harvesting complexes and electron transport molecules. As a common response, most of these transcripts were also altered in the three VSR-expressing transgenic tobacco lines with strong down-regulation also on physiological level (Papers I-III).

Several studies indicate that photosynthetic proteins are related to the plant susceptibility and to the defense responses. For instance, in *Turnip yellow mosaic virus* (TYMV) infections, the chloroplasts become swollen and clumped together and involved in the formation of chloroplast membrane vesicles. Down-regulation of the 33 kDa protein of PSII complex enhances the TMV accumulation, and silencing of the AtpC and RCA proteins greatly enhances the TMV pathogenicity (Abbink et al., 2002; Bhat et al., 2013; Prod'homme et al., 2001). Similar type of down-regulation of photosynthesis and its related proteins in our transgenic plants may indicate that VSRs disturb the photosynthesis process to assist the viral infection process. Also, comparison of the microarray data of the photosynthesis related transcripts in TMV infected plants (Paper IV) vs VSRs-expressing plants indicates that the VSRs induce similar changes as the whole virus infections.

Reduction in photosynthesis may reduce the energy supply for the defense response. However, according to one hypothesis, the reduced photosynthesis produces fewer

nutrients to the replicating virus, and at the same time most of the plant energy is used in defense reaction as top priority (Bolton, 2009). Likewise, down-regulated photosynthetic genes in the VSR-expressing plants may indicate common internodes of photosynthetic/energy metabolism and plant defense responses. For instance, most of the light-harvesting chlorophyll a/b binding proteins were down-regulated in all our tested plants, and thus caused reduced response to the abscisic acid dependent stomatal movement, possibly leading to reduced ABA signaling that again interlinks with host defense responses (Cao et al., 2011; Xu et al., 2012).

5.3 Specific effects of different VSRs

5.3.1 HC-Pro

HC-Pro is known to be a strong viral VSR factor, and recent studies indicate that it blocks the RNA silencing mechanism by ET- inducible RAV2 transcription factors (Endres et al., 2010). The strong up-regulation of RAV transcription and ethylene related transcripts in these plants may relate to the reduced RNA silencing activity (Paper I). HC-Pro expressing transgenic plants significantly accumulate development and leaf morphology related miRNAs (Soitamo et al, manuscript in preparation), which may indicate the reduced RNA silencing activity in these plants. Additionally, PME1 is strongly up-regulated in these plants, and as it is known to be involved in the viral RNA degradation and in the viral movement process (Chen et al., 2000), this may indicate that HC-Pro provides the viral movement function by altering the PME1 gene expression (Paper I). As it was stated earlier, HC-Pro interacts with several host components such as the rgs-CaM, which act as a negative regulator of silencing mechanism (Anandalakshmi et al., 2000). Alteration of signalling-related transcripts in HC-Pro expressing plant indicates that it causes imbalances in the signalling processes to favor the viral infection. HC-Pro is a multifunctional protein that exhibits the cysteine endopeptidase and thioredoxin activities (Urcuqui-Inchima et al., 2001). Effects related to these activities are also seen in the HC-Pro expressing transgenic plants. Alteration of the photosynthesis and several sugar metabolisms related transcripts in these plants might be due to the endopeptidase activities of HC-Pro (Paper I). Additionally, an HC-Pro protein of *Lettuce mosaic virus* (LMV) interacts with 20S proteasomal complexes and slightly elevates their activities (Ballut et al., 2005) thus affecting protein turnover in the infected plants. This may affect indirectly also other regulatory processes. Protein profile alterations and clear up-regulation of protease inhibitors (Paper I) might indicate that HC-Pro may increase the proteolytic activity by interacting with proteasomal complexes in these plants.

5.3.2 AC2

AC2 VSR is known to mediate RNA silencing suppression by the transactivation of the host endogenous silencing repressor proteins (Trinks et al., 2005). Negative regulators of gene silencing proteins like repressor of silencing1 (NtROS1) is up-regulated in AC2-expressing plants, which may contribute the reduced RNA silencing activity (Paper II). In addition, increased accumulation of development and leaf morphology related miRNAs compared to the control plants indicates the disturbed RNA silencing process in these plants (Soitamo et al, Manuscript in preparation). The incompatible interactions between the plants and viruses involves strong induction of defense responses such as HR, defense hormones and defense signalling networks (Elvira et al., 2008). Expression of AC2 in the non-host tobacco also causes strong induction of defense response, beginning from their early growth stages (Paper II). These include HR like phenotype and strong up-regulation of JA and ET related transcripts. Also the strong down-regulation of ribosomal gene transcripts may indicate incompatible relationship, similar to the one observed in the resistant stages of the TMV-expressing tobacco plants (Paper IV). Also, induction of Ribosome inactivating proteins (RIPs) such as DNA-glycosylases may cause the inhibition of the ribosomal gene expression in these plants (Kaur et al., 2011). Increase of DNA methylation in the AC2 VSR expressing non-host tobacco plants is clearly opposite to its known effects in host plants i.e. reduced methylation and histone synthesis (Paper II) (Buchmann et al., 2009). This may indicate that interacting partners of host components are very important in virus infections.

5.3.3 P25

P25 is a multifunctional protein and causes significant changes with up-regulation of 1350 transcripts in the transgenic plants (Paper III). Many of these changes may be caused by its interaction with the AGO1 protein and its degradation (Chiu et al., 2010). The large number of transcripts might be up-regulated due to the absence of AGO1 activity. Interestingly, the strong up-regulation of these multiple mRNAs did not cause any phenotypic changes, which might indicate existence of a very strong buffering activity in the plants gene expression. The up-regulation of rgs-CaM transcript in P25 transgenic plants (Paper III) indicates that P25 may reduce the RNA silencing via activation of RNA silencing negative regulators (Anandalakshmi et al., 2000).

5.4 Whole virus interactions

In the paper IV, I conducted a microarray analysis of a specific case of transgenic tobacco plants expressing whole TMV genome. These plants contain an artificial inoculation system that constitutively expresses the infectious TMV genome, starting

from the germinating embryo stages. This causes very strong resistance reaction in the young TMV transgenic plants, but later, this resistance is broken and the same plants start showing accumulation of TMV RNAs and infection phenotypes. Interestingly the resistance is totally specific to the TMV, and does not affect other inoculated viruses such as PVY and PVA. Rather, these external inoculations with other viruses break also the TMV resistance break (Paper IV).

5.4.1 TMV resistome

Naturally infecting TMV has a compatible interaction with its tobacco host. This means that the virus can easily infect and successfully complete its life cycle without mounting strong defense responses in the host (Baebler et al., 2011; Elvira et al., 2008; Wang et al., 2009b). The strong induction of defense genes and various biotic and abiotic stress-related transcripts in TMV-expressing transgenic plants seems to indicate that during the young TMV resistant stage, the plants exhibit the incompatible reaction to the TMV virus. This view is further strengthened by the low induction of defense or stress-related transcripts in the TMV infected wt tobacco plants. In *Plum Pox virus* infections, down-regulation of ribosomal genes is observed in less susceptible Arabidopsis plants but not in more susceptible *N.benthamiana* plants (Babu et al., 2008; Dardick, 2007). Expression of the non-functional alleles nn of the TMV N resistance gene in the resistant stages, but not after resistant break or in virus-infected wt plants, also provides evidence of incompatible reaction during the in the resistance stage of the young transgenic plants (Paper IV).

To complete their life cycles in the host plants, viruses take use of the host's translation systems. To do this they employ a variety of mechanisms ranging from hijacking the translational factors to ribosomal recruitment mediated for instance by internal ribosome entry sites (IRES) and translational enhancer sequences in the non-coding terminal sequences of their genomes (Dreher and Miller, 2006; Gallie, 2002). Strong down-regulation of various ribosomal proteins and RNAs seems to indicate that the resistant young plants limit the availability of ribosomes to the protein synthesis to stop the viral life cycle (Paper IV). These plants showed also reduced total amounts of proteins (2D-picture). Also, plants are known to have increased level of RIPs, which can inactivate the ribosomal gene induction and instaneously stop the protein synthesis in the virus infected cells (Kaur et al., 2011). Thus, it is possible that some RIP proteins are activated in the young transgenic plants and cause TMV resistance.

During the resistant stage, auxin repressor genes were strongly up-regulated. These proteins are involved in the suppression of cell elongation, and thus arrest the growth in the plants. These genes are suppressed by increased endogenous auxin levels (Lee et al.,

2013; Tatematsu et al., 2005). Interestingly, auxin treatments are known to induce the ribosomal genes, and thus lead to increased proteins synthesis (Gantt and Key, 1985). Hence, the strong up-regulation of auxin repressor or dormancy associated genes at resistant stages versus and their down-regulation during normal TMV infections (Paper IV) may indicate that these genes serve in the young resistant plants to reduce the ribosomal gene expression, and to reduce protein synthesis.

Several protein translation initiation factors, and elongation factors, i.e. eIF4F, eEF1B and eEF1A are needed for TMV replication complex (Gallie, 2002; Hwang et al., 2013; Yamaji et al., 2006). All of these were down-regulated during the young resistant stages, and up-regulated in normal infections (Paper IV), and their unavailability could also have restricted the viral accumulation in the young transgenic plants.

Down-regulation of several transcripts A and B type cyclins in the resistant stage of the TMV transgenic plants suggests that cell division is halted in these tissues correlating with the strongly reduced growth (Bloom and Cross, 2007; Morgan, 1995). After the resistance break, cancerous type of cell division was indicated both in transgenic plants as well as in the TMV infected wt plants, by the abnormal expression of RAD9a protein, normally expressed during the DNA damaged conditions (Paper IV) (Lieberman et al., 2011; Zhu et al., 2008). This indicates that transgenic plants restricted cell division during their resistant stages. After resistance break, this restriction disappeared correlating with the strong accumulation of TMV. This abnormal accumulation of RAd9A and its role during the TMV infection is still to be elucidated

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Plant-virus interactions are very complex in nature, and comprise of variety of developmental, physical and metabolic changes. Viruses have only a few genes, but many of these encode for multifunctional proteins. All of these genes are very important in establishing the viral infection process. Transgenic plants expressing individual viral genes or whole virus genomes are effectively used to elucidate the roles of these genes in plant-virus interactions. Viral suppressor molecules suppress the RNA silencing-based host defense mechanisms and contribute to the symptom development in plants. Based on the results obtained by analyzing the transcriptomes of transgenic plant expressing different individual viral genes or whole infectious virus, I concluded that;

- VSRs cause very distinctive gene expression pattern alterations and some of these changes are similar to the alterations caused by whole viral infections.
- Expression of these different VSR genes cause common alterations related to the stress, defense, photosynthesis and starch metabolism.
- HC-Pro expression causes increased proteolytic activity.
- AC2 expression reduces translational activity by suppressing the ribosomal genes.
- P25 causes strong gene expression alterations in an organs specific manner, but still the plant phenotype remains normal
- Strong shutdown of translation –related genes is found in the young TMV-expressing plants, which are resistance against TMV.
- Cell division, hormones and stress related transcripts are differently altered in the different growth stages of the TMV-expressing plants.
- The transgenically expressed TMV genome causes a major defense response, while the natural TMV infection in wild type plants causes only a minimal induction of defense response genes.

The transgenic plants expressing different VSRs are valuable tools to uncover plant-virus interactions. New methods i.e. deep Next Generation Sequencing (NGS) are needed to reveal the very low expressed small RNAs such as miRNAs in the plants. In this aspect, I would like to study particularly the small RNA transcriptome in the context of AGO interacting VSRs such as P25 and 2b of CMV. VSRs are known to affect the

transcriptional factor networks by miRNAs; here I would like to map the transcriptional factor alterations and their interconnections to host physiology.

Ribosomal genes expression is mainly effected during the TMV resistance in the young transgenic plants, and I would like to study what are the causes of their down-regulation and related mechanisms. Cell division factor (RAd9A) seems to play key role in the breaking of the resistance against TMV transgene and external virus infections, and I would like to study particularly the role of this protein in these mechanisms and in TMV accumulation.

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