Defense Pathways in Arabidopsis Restricting Compatible Tobamoviruses during Infection

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Table of content

List of abbreviations

ABA	Abscisic Acid
ACC	1-Amino-Cyclopropane-Carboxylate
AGO	ARGONAUTE
BAK1	BRI1 associated kinase 1
BCTV	*Beet curly top virus*
BR	brassinosteroids
CaMV	*Cauliflower mosaic virus*
CDC48b	Cell Division Cycle 48b
CHL1	MAGNESIUM PROTOPORPHYRIN CHELATASE SUBUNIT I
CK	cytokinins
CLR	C-type lectin receptors
CMV	*Cucumber mosaic virus*
CP	coat protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
crRNA	CRISPR-derived RNA
DAMP	damage/danger-associated molecular patterns
DCL	DICER-like endonucleases
dpi	days post inoculation
DRB	dsRNA BINDING PROTEIN
DRD3	DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 3
dsRNA	double stranded RNA
DSTM1	Delayed Systemic Tobamovirus Movement 1
ER	endoplasmic reticulum
ET	Ethylene
FLS2	FLAGELLIN-SENSITIVE 2
GA	gibberilins
GFP	green fluorescent protein
HEN1	HUA-ENHANCER 1
HRT	HYPERSENSITIVE RESPONSE TO TCV
JA	Jasmonic Acid
JAX1	JACALIN-TYPE LECTION REQUIRED FOR POTEXVIRUS RESISTANCE 1
JAZ	JASMONATE ZIM DOMAIN
LOX	LIPOXYGENASE
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
miRNA	microRNA
MP	movement protein
natsiRNA	natural small interference RNA
NLR	NOD-like receptor
NRDP1a	NUCLEAR RNA POLYMERASE D 1a
NSP	nuclear shuttle protein
OG	oligogalacturonides
ORMV	*Oilseed rape mosaic virus*
PAMP	pathogen-associated-molecular-patterns
PD	plasmodesmata
PDF	PLANT DEFENSIN
PDLP5	PLASMODESMATA-LOCATED PROTEIN 5

PEPR	Pep-Receptor
PLMVd	*Peach latent mosaic viroid*
POLIV	DNA-dependent-RNA-polymerase IV complex
POLV	DNA-dependent-RNA-polymerase V complex
PR	PATHOGENESIS-RELATED PROTEIN
PRR	pattern-recognition-receptors
PTGS	Post-Transcriptional Gene Silencing
PTI	Pattern Triggered Immunity
PVX	*Potato virus X*
RCY1	RESISTANT TO CMV (Y)
RdDM	RNA-Directed DNA methylation
RDR	RNA-dependent-RNA-polymerases
RDV	*Rice dwarf virus*
RISC	RNA-Induced Silencing Complex
RLK	receptor-like kinases
RLM-RACE	RNA-Ligase Mediated-Rapid Amplification of cDNA Ends
RLP	receptor-like protein
RLR	RIG-1-like receptors
RMV	*Ribgrass mosaic virus*
ROS	reactive oxygen species
RTM1	RESTRICTIVE TEV MOVEMENT 1
SA	Salicylic Acid
SDE5	SILENCING DEFECTIVE 5
SGS3	SUPPRESSOR OF GENE SILENCING 3
Sha	Shahdara
SPSCV	*Sweet potato chlorotic stunt virus*
siRNA	small interfering RNA
sRNA	small RNA
ssRNA	single stranded RNA
tasiRNA	trans-acting small interference RNAs
TCV	*Turnip crinkle virus*
TGS	Transcriptional Gene Silencing
TLR	Toll-like receptors
TMV	*Tobacco mosaic virus*
TOM	TOBAMOVIRUS MULTIPLICATION
ToMV	*Tomato mosaic virus*
TRV	*Tobacco rattle virus*
TVCV	*Turnip vein clearing virus*
VIGS	Virus Induced Gene Silencing
VRC	viral replication centers
vsm1	Virus Systemic Movement 1
VSP	VEGETATIVE STORAGE PROTEIN
VSR	viral silencing suppressors
vsRNAs	viral RNAs
WAK1	Wall-Associated-Kinase1
XRN4	EXONUCLEASE 4
Y-sat	*Y satellite RNA*
WT	Wild type

Summary

Virus infected plants often develop strong disease symptoms including leaf deformation, chlorosis, necrosis and growth inhibition. In agriculture systems virus infection can lead to severe yield losses and a better understanding of plant defenses against viruses is therefore desirable in order for develop new strategies against diseases in crops caused by viruses. Specific resistance to viruses has been studied intensively in the past but host tolerance and recovery have received little attention.

Symptomatic virus infections can persist throughout the life of the host. However, in some cases a recovery from symptoms can be observed. Recovery has been associated with host anti-viral RNA-silencing targeting viral nucleic acids for destructing or inactivation. However, it is well established that compatible viruses suppress RNA-silencing in order to establish and maintain an infection and the exact role of RNA-silencing in onset and maintenance of recovery is therefore unclear. To address this question a "recovery-system" for the tobamovirus *Oilseed rape mosaic virus* (ORMV) was set up in the model plant *Arabidopsis thaliana* and characterized (chapter 2). Through the use of Arabidopsis mutants we show that specific RNA-silencing pathways are essential for recovery, included some known to be involved in non-autonomous RNA-silencing. Furthermore, mutants with increased RNA-silencing capacity did recover earlier than wild type plants, suggesting that oscillations in RNA-silencing activity could be involved in the onset of recovery.

RNA-silencing is an important anti-viral defense mechanism but also defense pathways regulated by hormones are induced during compatible virus infections. The changes in gene expression observed upon compatible virus infections are similar to those observed for infection with other biotic plant pathogens, but the importance of virus-induced defense responses is not fully understood. Non-viral plant pathogens predominately live in the apoplast and the presence of pathogen-derived "non-self" molecules is sensed through receptors in the plasma membrane, a mechanism referred to as Pattern-Triggered-Immunity (PTI). It is unclear if intercellular pathogens, such as viruses, can induce defense responses in plants through PTI and if PTI is involved in plant defense against viruses. In this thesis we show that mutants of BAK1; a regulator of many receptors involved in PTI, are hypersuceptible to several RNA viruses (chapter 3). Furthermore crude extracts from virus-infected plants contain compounds that can elicit PTI-responses (chapter 3). Taken together this indicates that virus infections induce PTI through an unidentified, likely plant-derived compound.

Studies of compatible virus infections have focused on plant-virus interactions that lead to disease symptom formation. However, virus infections can progress almost or completely symptomless referred to as tolerance. Mechanisms controlling tolerance to viruses in plants have not been described until now. Infections of Arabidopsis with tobamovirus *Tobacco mosaic virus* (TMV) progress almost symptomless in most ecotypes. Characterization of TMV infections in tolerant and symptomatic Arabidopsis ecotypes revealed that symptom formation is associated with accelerated viral movement and induction of defense responses (chapter 4). Furthermore is symptom formation independent of RNA-silencing and Salicylic Acid (SA) signaling (chapter 4).

1. General Introduction

1.1 Viruses as plant pathogens

Photosynthetic organisms have the unique ability to produce oxygen and to convert solar energy into complex biological molecules, making photosynthetic organisms the cornerstone of life on earth. In particular energy rich carbon compounds of higher plants are essential for terrestrial life. However, both in nature and particularly in agriculture systems plants are attacked by a wide range of pathogens. Plant pathogens include organisms such as bacteria, fungi, oomycetes, viruses and viroids. Viruses and viroids hold a unique place among the plant pathogens as they can only replicate inside host cells (Hull 2009). The close interaction between host and virus makes targeted disease control by pesticides difficult. Management of viral disease in the field normally focuses on eradication of insect vectors spreading the virus (Hooks and Fereres 2006) and the use of virus-free seeds and rootstock (Panattoni et al. 2013). New viral diseases can arise and spread very rapidly and viruses represent a threat to global food security (Strange and Scott 2005). Understanding the mechanisms that determine the development and spread of viral disease is therefore of importance.

1.1.1 Plant virus life cycle

Viral particles, virions, of plant viruses are of low complexity compared to other plant pathogens. Virions consist of the viral genome segment(s) of either single stranded RNA (ssRNA) or double stranded RNA (dsRNA) or single stranded DNA encapsidated by viral coat protein units. The architecture of virions ranges from simple rod-shaped virions as that of tobamoviruses and tobraviruses to complex spherical virions as found among caulimoviruses and comoviruses. Membrane enveloped plant viruses are rare and only observed for tospoviruses and rhabdoviruses (Hull 2009). The majority of viruses infecting plants have RNA-encoded genomes, which is different from invertebrates, prokaryotes and fungi where DNA viruses are predominant (Hull 2009). Once inside the plant cell virions undergo disassembly and the host translation system is hijacked for the production of viral proteins (Thivierge et al. 2005). The replication of the viral genome by virus-encoded replicase(s) takes place in viral replication centers (VRC) often in close proximity of host membrane structures (den Boon and Ahlquist 2010). To spread from the originally infected plant cell into adjacent cells viruses use intercellular channels in the plant cell wall called plasmodesmata (PD). This movement is actively supported by virus-encoded movement proteins. Viruses are known to cause structural changes of the PD to facilitate their own cell-to-cell movement (Niehl and Heinlein 2011). Systemic movement of viruses into other plant organs predominately occurs through the phloem following the source-to-sink pattern. The general mechanism behind phloem loading and un-loading of viral particles is not known. Some viruses are limited to the phloem, suggesting that un-loading may be an active process possibly involving the suppression of host defenses or close interaction with host factors (Kehr and Buhtz 2008; Harries and Ding 2011; Hipper et al. 2013). Plant viruses neither enter nor exit their host by themselves. Their spread to new hosts depends on mechanical

transmission, or on insect or nematode vectors. Some viruses are also spread via pollen or seeds (Hull 2009).

1.1.2 Disease symptom formation during virus infection

The outcome of a primary viral infection relies on host factors and viral subversion of host anti-viral defenses. The plant host may be either resistant or susceptible to a specific virus. Host resistance includes non-host resistance or active suppression of the virus through innate resistance (Maule et al. 2007; Palukaitis and Carr 2008). Non-host resistance is not well understood with the exception of potyviruses, where incompatibility with host elongation factors lead to non-host resistance (Nieto et al. 2011). Mechanisms of innate resistance to viruses include recognition of viral proteins or interactions between viral and host proteins leading to a hypersensitive response or to extreme resistance, which does not lead to cell death but inhibits the virus otherwise, as an example through direct interference with viral replication (Maule et al. 2007; Palukaitis and Carr 2008). If the host allows viral replication and movement, the interaction is regarded as compatible; hosts that allow viral infection without displaying disease symptoms are referred to as tolerant (Little et al. 2010). In some host-virus combinations the emergence of symptoms is robust while in other cases the development of disease symptoms is influenced by the plant growth condition (Martin et al. 1997). Genetic variation in both host and virus plays an important role in the emergence of viral disease symptoms. As an example, tolerance to a specific virus can often be observed in single ecotypes in studies with multiple ecotypes of the model plant Arabidopsis (Lee et al. 1994; Dardick et al. 2000; Park et al. 2002). Once established, symptomatic infection may also change to a non-symptomatic state, where newly developed tissue appears symptom-free. This phenomenon is known as “recovery” and highlights the dynamic nature of compatible plant-virus interactions. While mechanisms of plant resistance to virus have received much attention in the past, mechanisms controlling the outcome of compatible interactions are less well understood. In the last decade it has become clear that viral suppression of the plant’s RNA-silencing machinery is crucial for the establishment of infection (Ding and Voinnet 2007) and likely also for the development of disease symptoms (Pallas and Garcia 2011), but the exact role of RNA-silencing in host-range, disease severity and recovery still remains to be established.

1.2 The role of RNA-silencing in viral defense

1.2.1 RNA-silencing pathways in plants

RNA-silencing was first described in transgenic petunia plants where the attempt to over-express a plant gene by introduction of a transgenic copy led to the down-regulation of both the transgene and the endogenous gene (Napoli et al. 1990; van der Krol et al. 1990). A role for RNA-silencing in viral defense was established a few years later (Ratcliff et al. 1997), followed by reports on the role of RNA-silencing in the regulation of endogenous gene expression especially during development (Reinhart et al. 2002; Palatnik et al. 2003). The hallmark of RNA-silencing is the occurrence of 21-24 nt long small RNAs (sRNA) targeting complementary RNA for degradation (Post-Transcriptional Gene Silencing, PTGS) or DNA for

methylation (Transcriptional Gene Silencing, TGS). Although the PTGS and TGS pathways share similarities only few common components have been described.

1.2.2 Post transcriptional gene silencing

PTGS is a regulation of mRNA transcript abundance through degradation or by inhibiting translation mediated through sRNAs complementary to the mRNA sequence. The initiator of PTGS is dsRNA, which is processed in to sRNAs. Endogenous dsRNA templates for sRNA production include structured ssRNA, overlapping transcripts or dsRNA produced from processed RNA. Structured ssRNA are processed into microRNAs (miRNA) (Reinhart et al. 2002), while sRNA originating from overlapping transcripts and processed RNA is referred to as natural small interference RNA (natsiRNA) and trans-acting small interference RNAs (tasiRNA), respectively (Peragine et al. 2004; Vazquez et al. 2004; Borsani et al. 2005; Katiyar-Agarwal et al. 2006). Viral sRNAs (vsRNA) may originate from structured ssRNA or dsRNA. Components of the sRNA biogenesis pathways have been isolated through forward genetic screens in the model plant *Arabidopsis thaliana*, but the components are believed to be conserved across the plant kingdom (Nakasugi et al. 2013). The processing of dsRNA to sRNAs involves complexes containing DICER-like (DCL) endonucleases and dsRNA BINDING PROTEINS (DRB) (Fig. 1.1). DCL1 predominately process 21-24 nt miRNAs, while DCL4 is involved in the production of 21 nt tasiRNA and vsRNA of RNA viruses. DCL2 produces 22 nt sRNAs but the biological importance of DCL2 remains to be established (Vazquez et al. 2010). However, DCL2 may partly replace DCL4 in *dcl4*-mutant backgrounds (Deleris et al. 2006). DCL3 is needed for the production of 24 nt sRNA for TGS (see below). The different DCLs show high dsRNA template specificity but the molecular mechanism for template recognition is not known. In the case of vsRNA the processing DCL may be determined by sub-cellular location of the RNA, as vsRNAs from nuclear DNA viruses are predominantly 24 nt in length and DCL3-dependent while vsRNAs from cytosolic RNA viruses are 21 nt long and DCL2/4-dependent (Blevins et al. 2006; Deleris et al. 2006). However, the DCL4-processing may not take place in the cytosol since several independent sub-cellular localization studies have shown that all DCLs localize to the nucleus (Xie et al. 2004; Hiraguri et al. 2005; Kumakura et al. 2009; Hoffer et al. 2011). The RNA of viruses replicating in the cytosol may thus be transported to the nucleus for dicing, perhaps through the mRNA export factor SILENCING DEFECTIVE 5 (SDE5) (Hernandez-Pinzon et al. 2007). All classes of sRNAs are methylated by HUA-ENHANCER 1 (HEN1) (Yu et al. 2005) and loaded into ARGONAUTE (AGO) proteins (Fig. 1.1). AGO proteins associate with the RNA-Induced Silencing Complex (RISC) that use the AGO-loaded sRNA to guide cleavage or translational inhibition of complementary mRNA (Palatnik et al. 2003; Brodersen et al. 2008) (Fig.1.1). Arabidopsis encodes 10 AGO proteins of which AGO1 is the main player in PTGS but also AGO7 and AGO2 play a role (Mallory and Vaucheret 2010). RISC cleavage products are either further degraded or may serve as template for RNA-DEPENDENT-RNA-POLYMERASES (RDR) producing new dsRNA (Willmann et al. 2011) (Fig. 1.1). Arabidopsis encodes 6 RDR proteins which can be divided into two sub-groups, RDRα (RDR1,2,6) and RDRγ (RDR 3,4,5). Only RDRα proteins are known to be involved in silencing while the function of the RDRγ proteins

is still unknown (Willmann et al. 2011). Among the Arabidopsis RDR proteins, RDR1 and RDR6 represent the main actors in anti-viral defense. However, mutations in RDR1, RDR6 or both do not always result in increased viral titers (Mourrain et al. 2000; Diaz-Pendon et al. 2007; Qi et al. 2009; Wang et al. 2010). RDR6 activity depends on SUPPRESSOR OF GENE SILENCING 3 (SGS3), a protein of unknown function (Mourrain et al. 2000). sRNAs produced from RDR-dependent dsRNA are referred to as secondary siRNAs and commonly observed for PTGS of viruses or transgenes but endogenous protein-coding mRNA targeted by miRNAs seldom gives rise to secondary sRNAs (Vazquez et al. 2010). However, some protein-coding mRNAs serve as RDR-template after miRNA-guided cleavage (Si-Ammour et al. 2011; Shivaprasad et al. 2012) and hereby produce secondary sRNAs enhancing the down-regulation of the targeted transcript and other mRNAs with sequence similarity. Furthermore, several miRNA-cleaved non-coding RNA transcripts serve as RDR-template for the production of secondary sRNAs, known as tasiRNAs. These tasiRNAs regulate the abundance of other protein-coding mRNAs (Peragine et al. 2004; Vazquez et al. 2004).

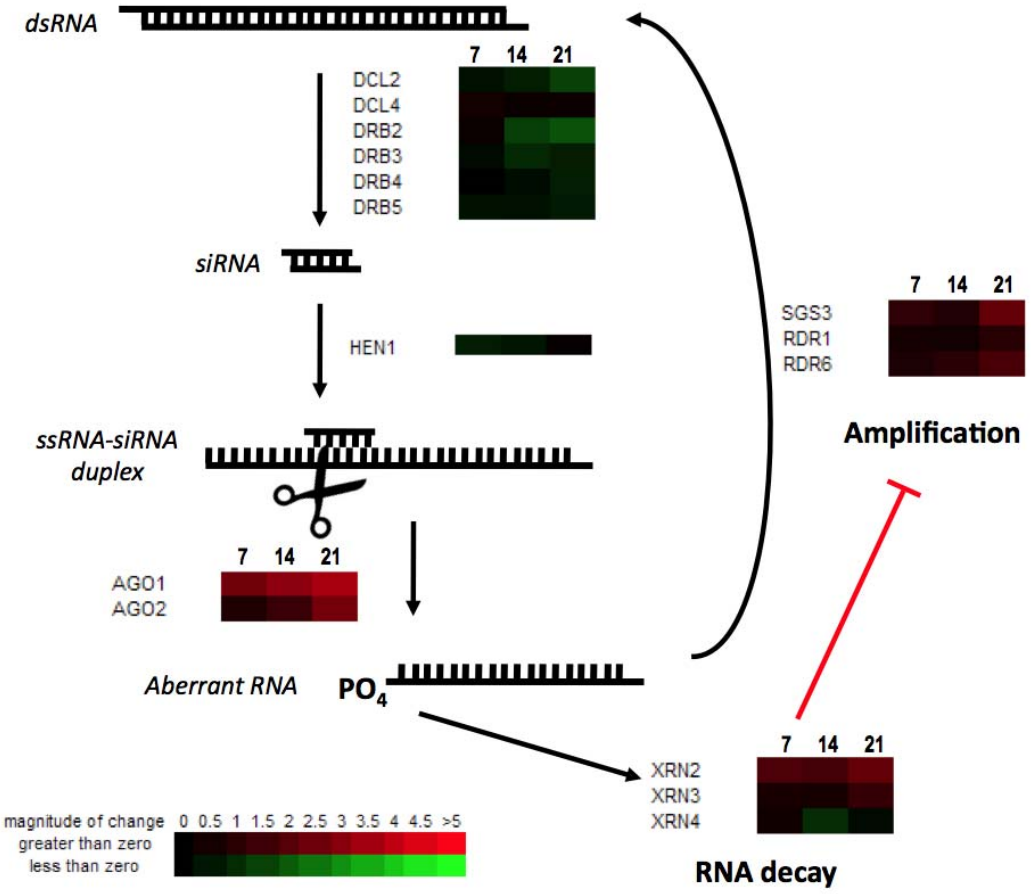

Figure 1.1 PTGS RNA silencing pathway components in Arabidopsis involved in viral defense. Viral dsRNA is processed to vsRNA by DCL2 or DCL4 in complexes with specific dsRNA-binding proteins (DRB). vsRNAs are methylated by HEN1 and loaded into AGO1 or AGO2-containing RISC-complexes to target complementary for cleavage or translational inhibition. Aberrant RNA may act as a template for RDR1 or RDR6 in complex with SGS3 to produce dsRNA which again is cleaved by DCL4 to give rise to a population of secondary vsRNAs. Aberrant RNA may also be degraded by exoribonucleases (XRN). Studies indicate that RDR-mediated amplification and XRN-dependent RNA decay compete for the same RNA templates and thus influence each other. Inserted heat maps depict the level of transcripts for RNA-silencing pathway genes during ORMV infection in Arabidopsis at 7, 14 and 21 dpi (Hu et al. 2011).

The criterions for production of secondary sRNAs are not understood but structural features of the miRNA duplex may play a role (Manavella et al. 2012). Genetic screens for mutants with enhanced silencing of transgenes led to the finding that mutations which reduce RNA decay cause an acceleration of RDR-dependent silencing (Gazzani et al. 2004; Vogel et al. 2011; Thran et al. 2012). This suggests that at least in mutant backgrounds competition between RDR-dependent dsRNA production and RNA degradation for aberrant RNA templates exists (Fig. 1.1).

1.2.3 Transcriptional gene silencing

Transcriptional gene silencing (TGS), or more specifically RNA-directed DNA Methylation (RdDM), acts through sRNA-guided DNA methylation of coding and non-coding genes, transposons and transgenes. The biogenesis of sRNAs involved in TGS is initiated by the production of dsRNA by the plant-specific DNA-dependent-RNA-polymerase IV complex (POLIV) together with RDR2. The dsRNA is further processed into 24 nt siRNAs by DCL3, CLASSY1 and HEN1. These sRNAs then guide the sequence-specific DNA methylation through AGO4, 6 or 9 together with the DNA-dependent-RNA-polymerase V complex (POLV) (He et al. 2011; Saze et al. 2012). Although PTGS and TGS are mechanistically similar, the two pathways involve their own sets of specific proteins, with the exception of the shared HEN1, which is needed for both pathways. TGS can also guide the methylation of viral DNA, for example the DNA of geminiviruses (Raja et al. 2008; Zhang et al. 2011). Until now there have been no publications reporting the direct involvement of TGS in defense against RNA viruses.

1.2.4 The role of non-cell-autonomous RNA-silencing in signaling

Small RNAs can move cell-to-cell and long distance and hereby modulate gene expression in neighboring cells or systemic tissue (Brosnan and Voinnet 2011). The mobile nature of sRNAs has been suggested to represent the greatest threat of RNA-silencing against viruses, as vsRNA may move ahead of infection and immunize naïve cells (Ding and Voinnet 2007). In PTGS systems, transgene derived sRNAs can move and induce silencing over a distance of 10-12 cells (Dunoyer et al. 2005; Smith et al. 2007). Their movement most likely occurs in the form of duplexes (Dunoyer et al. 2010b).Transgene derived sRNA may also move long distance via the phloem and trigger silencing in the recipient tissue (Voinnet and Baulcombe 1997; Voinnet et al. 1998; Brosnan et al. 2007). In addition to transgene derived sRNAs also endogenous miRNAs (Pant et al. 2008) and nat-siRNAs (Dunoyer et al. 2010a; Molnar et al. 2010) can move. Silencing triggered by sRNAs moving long distance are associated with TGS (Brosnan et al. 2007; Molnar et al. 2010), while sRNAs moving cell-to-cell depend on the PTGS pathway (Dunoyer et al. 2005; Dunoyer et al. 2007). Interestingly, the perception of mobile sRNA in recipient cells from transgenes silenced by PTGS also depends on POLIV and RDR2, proteins otherwise associated with TGS (Brosnan et al. 2007; Dunoyer et al. 2007; Smith et al. 2007).

1.2.5 Viral silencing suppressors

The potency of RNA-silencing targeting and degrading viral RNA as a anti-viral defense mechanism is supported by the fact that plant viruses encode at least one protein

suppressing RNA-silencing, called viral silencing suppressors (VSR). VSRs have likely evolved independently since VSRs from different viruses target different steps of the PTGS or TGS pathways (Burgyan and Havelda 2011) (table 1.1). VSRs interfering with the production and stability of sRNAs include P38 (*Turnip crinkle virus*, TCV), P14 (*Pothos latent aureusvirus*), P6 (*Cauliflower mosaic virus*, CaMV) and RNase III (*Sweet potato chlorotic stunt virus*, SPSCV). Both P38 and P6 have been shown to target the DCL4-dependet production of sRNAs (Deleris et al. 2006; Haas et al. 2008). Overexpression of P38 in Arabidopsis resulted in the loss of DCL4-dependent sRNAs and a shift from 21 nt DCL4-dependent sRNAs to DCL2-dependeent 22 nt sRNA. Based on these observations the authors suggested that P38 inhibits DCL4 activity, although the molecular basis of this inhibition is not known and may be indirect (Deleris et al. 2006). P6 reduces DCL4-activity by binding the DCL4-interacting protein DRB4 (Haas et al. 2008). Another mechanism to affect sRNA levels was shown for the SPSCV protein RNaseIII, which inactivates sRNAs by cleavage (Cuellar et al. 2009). Yet another mode of action of VSRs is sequestering of sRNAs, which often results in accumulation of sRNAs during viral infection (table 1.1) (Alvarado and Scholthof 2009; Burgyan and Havelda 2011). A well-characterized example is P19 encoded by tombusviruses, which binds siRNA duplexes in a size-specific manner (Silhavy et al. 2002; Lakatos et al. 2004). Another example is the potyviral Hc-Pro proteins, which bind sRNA duplexes through a FRNK-motif (Shiboleth et al. 2007). The accumulating sRNAs are in some cases non-methylated, suggesting that some VSRs, including Hc-Pro and 126k of tobamoviruses, sequester sRNAs in advance of HEN1-methylation, inhibit HEN1 activity or de-methylate sRNAs. However, no molecular data on HEN1-interference have been presented until now (Burgyan and Havelda 2011). VSRs may also target the production of secondary sRNA. The two unrelated VSRs V2 from *Tomato yellow leaf curl virus* and P2 from *Rice stripe virus* have both been suggested to inhibit the production of secondary sRNAs by binding the RDR6-interacting protein SGS3 (Glick et al. 2008; Du et al. 2011). Also processes downstream of sRNA production are targeted by VSRs. For example, polerovirus VSR P0, cucumovirus VSR 2b, ipomovirus P1 and carmovirus P38 all target AGO1, but the molecular mechanisms are different. The P0 protein contains an F-box domain that targets AGO1 for degradation by ubiquitination and thereby prevents the assembly of the RISC complex (Csorba et al. 2010). The VSR 2b, in contrast, binds to the PAZ-domain of AGO1 and thus blocks siRNA-guided cleavage of target RNA (Zhang et al. 2006). Another mode of action is used by P1 and P38. Both proteins contain a WG/GW motif, which mimics the WG/GW motif of endogenous AGO1-interacting proteins. By binding to AGO1 using this motif, P1 and P38 inhibit the loading of sRNAs into RISC (Azevedo et al. 2010; Giner et al. 2010). Several VSRs from DNA viruses have been reported to interfere with DNA methylation. The Rep protein from several geminivirus can stimulate the transcriptional down-regulation of DNA-methylases and hereby decrease DNA-methylation (Rodriguez-Negrete et al. 2013). Another strategy to suppress DNA-methylation is by inactivating enzymes in the active methyl cycle. The AL2 protein of *Beet curly top virus* (BCTV) and other related geminiviruses inactivates a adenosine kinase (Burgyan and Havelda 2011), while βC1 from the beta-satellite of *Tomato yellow leaf curl China virus* interacts with a S-adenosyl homocysteine hydrolase (Yang et al.

2011). In addition to the above-mentioned VSRs, numerous other VSRs with yet unknown function have been reported, e.g. VSRs of ampelovirus, closterovirus, flexivirus, hordeivirus, luteovirus, pecluvirus, phytoreovirus, potexvirus, tobravirus and vitivirus (Lu et al. 2004; Cao et al. 2005; Zhou et al. 2006; Alvarado and Scholthof 2009; Chiu et al. 2010; Burgyan and Havelda 2011; Gouveia and Nolasco 2012; Liu et al. 2012; Renovell et al. 2012).

Since viral proteins are often multifunctional, VSRs are not easily studied by the use of virus deletion mutants. However, a standard silencing suppression assay for VSR activity is the "patch test", which involves the transient expression of the VSR or VSR candidate protein in *Nicotiana benthamiana* leaves in the presence of a silencing reporter. Infiltration of agrobacteria carrying a 35S:GFP binary expression plasmid (leading to the expression of *Acquoria victoria* green fluorescent protein (GFP) under the control of the strong 35S promoter of CaMV) into leaves of the GFP-transgenic *N.benthamiana* line (16c) trigger both local and systemic silencing of GFP (Voinnet and Baulcombe 1997; Ruiz et al. 1998) while co-infiltration of agrobacteria carrying the 35S:GFP binary expression plasmid with a agrobacteria strain carrying a 35S:VSR binary expression plasmid blocks silencing (Silhavy et al. 2002). Indeed, many of the known VSRs have been identified using this method. The conservation of the RNA-silencing pathway is illustrated by the fact that VSRs even from plant viruses that do not infect *N.benthamiana* or VSRs of animal viruses can still suppress silencing in the patch test using *N.benthamiana* (Jing et al. 2011). A drawback of the patch test is that potential synergies between viral proteins, which would occur during natural infection, may be overlooked.

Table 1.1. Examples of viral silencing suppressor proteins and their molecular function

	Name	Virus	Genus	Function	Cit.
ssRNA	P14	*Pothos latent aureusvirus*	Aureusvirus	inhibits DCL activity	a
	p23	*Citrus tristeza virus*	Closterovirus	destabilizes sRNA	c
	Rnase III	*Sweet potato chlorotic stunt virus*	Crinivirus	cleavage of sRNAs	b
	P1/Hc-Pro	eg. *Turnip mosaic virus*	Potyvirus	sequestering of sRNAs, interference with methylation	a, b
	p21	*Beet yellows virus*	Closterovirus	sequestering of sRNAs, interference with methylation	a, b
	p126	*Tobacco mosaic virus*	Tobamovirus	sequestering of sRNAs, interference with methylation	b
	p19	eg. *Carnation Italian ringspot virus*	Tombusvirus	sequestering of sRNAs	a, b
	NS3	*Rice hoja blanca virus*	Tenuivirus	sequestering of sRNAs	a
	p10	*Grapevine virus A*	Vitivirus	sequestering/degradation of sRNAs	d
	P38	*Turnip crinkle virus*	Carmovirus	blocks AGO1 loading and DCL activity	a, b
	P1	*Sweet potato mild mottle ipomovirus*	Ipomovirus	blocks AGO1 loading	a, b
	P0	*eg. Beet western yellows virus*	Polerovirus	target AGO1 for degradation	b
	2b	*Cucumber mosaic virus*	Cucumovirus	blocks AGO1 cleavage, sequestering of sRNAs	a
	p25	*Potato virus X*	Potexvirus	target AGO1 for degradation	e
	p2	*Rice stripe virus*	Tenuivirus	binds SGS3	f
	16k	*Tobacco rattle virus*	Tobravirus	unknown	a
	p15	*Peanut clump virus*	Pecluvirus	unknown	a
	?b		Hordeivirus	unknown	a
	P0	*Wheat yellow dwarf virus*	Luteovirus	unknown	g
	P6	*Barley yellow dwarf virus*	Luteovirus	unknown	g
	p19.7	*Grapevine leaf roll associated virus 3*	Ampelovirus	unknown, p21-like	h
	P40	*Citrus leaf blotch virus*	Flexivirus	unknown	i
dsRNA	p10	*Rice dwarf phytoreovirus*	Phytoreovirus	unknown	j
DNA	P6	*Cauliflower mosaic virus*	Caulimovirus	binds DRB4 and reduces DCL4 activity	a
	Rep	*Tomato yellow leaf curl virus*	Begomovirus	transcriptional down-regulation of DNA methylases	k
	V2	*Tomato yellow leaf curl virus*	Begomovirus	binds SGS3, binds dsRNA	b
	L2	*Beet curly top virus*	Curtovirus	inactivates adenosine kinase, blocks DNA methylation	b
	βC1	*Tomato yellow leaf curl China virus B*	beta-satellite	interacts with S-adenosyl homocysteine hydrolase	l

a- Alavardo & Scholthof (2009), b –Burgyan & Havelda (2011), c- Lu et al. (2004), d- Zhou et al. (2006), e-Chiu et al. (2010), f – Du et al. (2011), g – Liu et al. (2012), h- Gouveia et al. (2012), i- Renowell et a.l (2012), j- Cao et al. (2005), k –Rodriguez-Negrete et al. (2013), l – Yang et al. (2011)

1.3 Hormone signaling during compatible virus infections

1.3.1 Plant hormone signaling in defense

Hormones are small molecules involved in plant development and in abiotic and biotic stress signaling. Two main hormone signaling pathways are known to modulate responses to biotic stresses depending on the life-style of the pathogen. Biotrophic pathogens that depend on living host tissue for their propagation activate the Salicylic Acid (SA) pathway, while necrotrophic pathogens that induce tissue death and insect wounding activate the Jasmonic Acid (JA) pathway (Fig. 1.2) (Pieterse et al. 2009; Robert-Seilaniantz et al. 2011). While necrotrophic pathogens activate JA pathways in combination with the gaseous hormone Ethylene (ET), insects often activate the JA pathway as well as the Abscisic Acid (ABA) pathway (Pieterse et al. 2009). In addition, other hormones such as cytokinins (CK), gibberilins (GA) and auxin may fine-tune defense responses. Upon recognition of the pathogen the appropriate pathway is activated often by transcriptional induction of the respective hormone biosynthesis genes. The activation leads to a series of downstream responses that are closely tailored to the attacking pathogen and include the production of reactive oxygen species (ROS), cell wall modifications, deterrent chemical compounds such as nicotine or glucosinolates, or anti-microbial proteins. A hallmark of activated SA-signaling is the transcriptional up-regulation of PATHOGENESIS-RELATED PROTEINS (PR) e.g. PR1, PR2 or PR5 (Pieterse et al. 2012). However, these genes may also be induced during abiotic stresses such as drought (Liu et al. 2013). A marker for activation of the JA/ET pathway is the up-regulation of PLANT DEFENSIN (PDF), while JA/ABA up-regulates VEGETATIVE STORAGE PROTEIN 2 (VSP2) and the family of JASMONATE ZIM DOMAIN (JAZ) proteins. Over the last years the presence of a complex regulative cross-talk between the SA and JA pathways has been established. As a rule of thumb it has been recognized that SA and JA pathways act antagonistically. However, exceptions are known (Fig. 1.2) (Pieterse et al. 2012).

1.3.2 Hormone pathways induced by compatible virus infection

As biotrophic pathogens, viruses are commonly linked to SA-mediated defense and SA is known to repress viral accumulation when applied exogenously in several different plant species (Singh et al. 2004; Lewsey and Carr 2009). However, the role of hormone signaling during compatible virus-plant interactions is still not well understood (Carr et al. 2010). SA-levels have been reported to increase during viral infection (Jameson and Clarke 2002; Krecic-Stres et al. 2005; Niehl et al. 2006; Lewsey et al. 2010; Miozzi et al. 2011) and PR-genes are often induced by virus infection (Fig. 1.2) (Whitham et al. 2003; Love et al. 2005; Ascencio-Ibanez et al. 2008; Hanssen et al. 2011; Hu et al. 2011; Lu et al. 2012; Mandadi and Scholthof 2012). It should be noted that tobacco plants over-expressing the bacterial gene *nahG*, which encodes the SA-degrading enzyme SALICYLATE HYDROXYLASE, have enhanced susceptibility to *Plum pox virus* (Alamillo et al. 2006). On the other hand most Arabidopsis SA mutants do not show an increased susceptibility to either RNA or DNA viruses (Huang et al. 2005; Love et al. 2005) and over-expression of PR2 in Arabidopsis did not affect accumulation of the tobamovirus *Turnip vein clearing virus* (Zavaliev et al. 2013). Contra dictionary to this the Arabidopsis *cpr1* mutant, in which several PR genes is induced (Bowling

et al. 1994) is more resistant to DNA viruses (Love et al. 2007; Ascencio-Ibanez et al. 2008). The mechanism of elicitation of SA-production during viral infection and the exact mechanism by which SA-defense acts on viral pathogens are not known. However, a possible mechanism linking viral movement and SA-defense has been proposed. PLASMODESMATA-LOCATED PROTEIN 5 (PDLP5) decreases PD permeability in a SA-dependent manner by inducing callose depositions at PD (Lee et al. 2011). It should be noted that it has not been experimentally shown that PLDP5-depedent callose depositions affect viral movement.

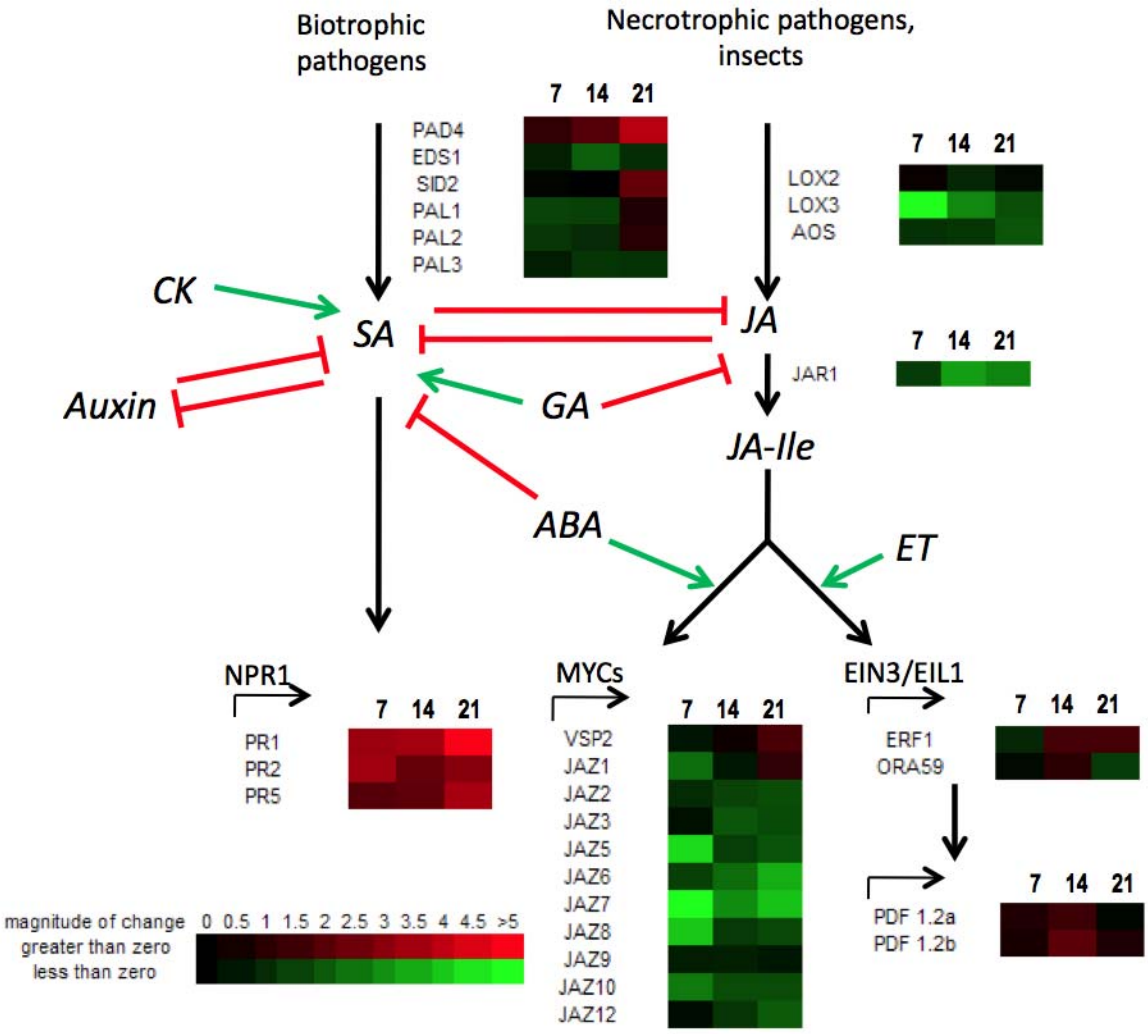

Figure 1.2 Hormone crosstalk in pathogen defense in Arabidopsis. SA and JA are the two main hormones playing a role during pathogen attack. Biotrophic pathogens trigger the accumulation of SA which causes several downstream responses including the induction of genes encoding pathogenesis-related (PR) protein through activation of NON-EXPRESSOR OF PR 1 (NPR1). Necrotrophic pathogens cause activation of the JA pathway, which induces the production of JA-Ile and the accumulation of ET. Together, JA and ET induce the expression of PDFs through several transcription factors. Insects trigger the JA/ABA pathway which among other responses induces the expression of VSP2 and JAZ genes. An antagonistic effect between the SA- and the JA-pathway is often observed. GA and CK support the SA-pathway while auxin and ABA may repress it. Inserted heat maps depict selected gene expression profiles during ORMV infection in Arabidopsis at 7, 14 and 21 dpi (Hu et al. 2011). Hormones are shown in italics; ABA – abscisic acid, CK-cytokinin, ET-ethylene, GA-gibberelin, JA- Jasmonic acid, JA-Ile – JA-isoleucine, SA- salicylic acid. Adapted from Pieterse et al. (2012).

1.3.3 Cross-talk between RNA-silencing and SA-signaling

The observations that ectopically applied SA and virus infections induce RDR1 transcript levels in tobacco, *N.benthamiana* and Arabidopsis led to the hypothesis that the anti-viral effect of SA may act through enhanced silencing (Xie et al. 2001; Yu et al. 2003; Yang et al. 2004). Although these observations suggested a mechanistic link between two important anti-viral defense mechanisms, more recent studies in Arabidopsis using a RDR1 promoter driving the expression of GUS:GFP construct could not verify the induction of RDR1 by SA (Xu et al. 2013). An independent experiment on RDR1 gene expression in Arabidopsis showed that a four to six fold up-regulation in gene expression of RDR1 could be measured after SA-treatment but only during the first six hours after treatment (Hunter et al. 2013). Also wounding and JA induced RDR1 gene expression in this set-up (Hunter et al. 2013). Moreover, SA-mediated resistance of Arabidopsis to *Cucumber mosaic virus* (CMV) and *Tobacco mosaic virus* (TMV) was shown to be independent of DCL2, 3 or 4, which all act up-stream of RDR1 (Lewsey and Carr 2009). Therefore, further work is needed to understand the molecular mechanism and significance of the SA-dependent induction of RDR1 gene expression in the context of virus infection.

1.3.4 Manipulation of hormone signaling pathways by viral proteins

Several viral proteins directly modulate hormone signaling. The C2 protein of geminiviruses, for example, actively represses the JA-pathway in Arabidopsis by interfering with the COP9 signalosome. JA-treatment decreases the accumulation of the geminivirus BCTV and the production of disease symptoms in infected plants (Lozano-Duran et al. 2011). However, although down-regulation of the COP9 signalosome in tomato caused reduced resistance to herbivorous *Manduca sexta* larvae and to the necrotrophic fungus *Botrytis cinerea* , the susceptibility to TMV was not altered (Hind et al. 2011). How BCTV benefits from a suppression of JA-signaling is not known, but down-regulation of the JA-signaling pathway would aid the feeding of insect vectors and hereby enhance the spread of insect transmitted viruses (Fig. 1.2) (Zhang et al. 2012). In addition to the geminivirus C2 protein, other viral proteins have also been shown to directly modulate hormone signaling. Another example is the P6 protein of CaMV, which upon overexpression in Arabidopsis suppresses SA-signaling and enhances JA-signaling (Love et al. 2012). However, the reverse pattern is observed for the CMV protein 2b (Lewsey et al. 2010). The 126k protein of TMV interacts with the defense-related NAC-transcription factor ATAF2 and studies indicate that ATAF2 is degraded during TMV infection. Moreover, overexpression of ATAF2 inhibits TMV accumulation and enhances PDF1.2, PR1 and PR2 transcription (Wang et al. 2009). Viral proteins may also interfere with the hormones GA and auxin, both best known for their role in growth and development. The P2 protein of phytoreovirus *Rice dwarf virus* (RDV) interacts with *ent*-kaurene oxidases that act during GA biosynthesis in rice. This interaction could explain the lower GA levels and disease phenotypes observed in rice infected with RDV (Zhu et al. 2005). The TMV 126k protein interacts with auxin signaling repressor Aux/IAA proteins and thereby alters auxin-mediated gene regulation and promotes disease development (Padmanabhan et al. 2005; Padmanabhan et al. 2006; Padmanabhan et al. 2008). The targeting of different

hormone pathways by different viruses and viral proteins suggests the existence of several, potentially overlapping, hormone-dependent plant defenses and viral counter-defense strategies that ultimately influence the outcome of specific host-virus interactions. It appears likely that viruses interfere with hormone signaling to manipulate plant cell physiology and thus increase the compatibility of the plant to the viral pathogen (Padmanabhan et al. 2005; Padmanabhan et al. 2008).

1.4 Innate defense against viruses in other organisms

RNA-silencing is a conserved anti-viral defense mechanism among eukaryotes (Ding 2010) and the RNA-silencing pathways of plants, fungi and nematodes are mechanistically very similar, while RNA-silencing in flies and mammals differ slightly. However, also other host innate anti-viral defense mechanisms have been described.

1.4.1 Viral defense in nematodes

Very little is known about anti-viral defense in nematodes due to the lack of viruses naturally infecting nematodes. However, with the recent discovery of nodaviruses infecting nematode model organism *Caenorhabditis elegans* this is likely to change (Felix et al. 2011). RNA-silencing deficient *C.elegans* mutants had higher viral accumulation (Felix et al. 2011) which supports previous studies identifying RNA-silencing as anti-viral in nematodes using transgenic *C.elegans* expressing viral amplicons of the fly-infecting nodavirus *Flock house virus* (Ding and Lu 2011).

1.4.2 Viral defense in insects and mammals

vsRNAs have been detected in both insects and mammals infected with viruses, suggesting that RNA-silencing act as an anti-viral defense mechanism also in these organisms. In the fly model Drosophila AGO2 and DICER2 are directly involved in targeting viral RNA and *ago2* and *dcr2* mutants are hypersensitive to viruses (Karlikow et al. 2013). Direct RNA-silencing of viruses seems less important in mammals (Ding 2010). In contrast, several DNA viruses infecting mammals have been shown to encode miRNAs targeting host mRNA, and viral miRNAs therefore enhance viral accumulation in a RNA-silencing depending manner (Cullen 2013). The minor role of RNA-silencing in defense against viruses in animals compared to plants can be explained by the existence of other innate anti-viral defense mechanisms in animals.

In animals viral components are recognized as “non-self” or as Pathogen-Associated Molecular Patterns (PAMP) by pattern recognition receptors (PRR), a mechanism referred to as Pattern-Triggered Immunity (PTI). Receptors with ss/ dsRNA as ligands include Toll-like receptors (TLR), RIG-1-like receptors (RLR) and NOD-like receptor (NLR) (Jensen and Thomsen 2012; Xu and Cherry 2013). C-type lectin receptors (CLR) recognize the presence of viruses through sensing of highly glycosylated viral proteins and other yet unidentified ligands (Osorio and Sousa 2011). In flies perception of viral PAMPs leads to transcriptional up-regulation of anti-viral genes (Xu and Cherry 2013). In animals recognition of viral dsRNA by a PRR activates several inflammatory response genes including interferons, leading to anti-viral responses and apoptosis (Karpala et al. 2005). It is well established that plants also

employ PRRs for the recognition of "non-self" molecules derived from bacteria, fungi and oomycete (Boller and Felix 2009). However, viral dsRNA are not believed to be sensed as "non-self" in plants likely due to the high abundance of endogenous dsRNA. Despite this, two plant encoded C-type lectin proteins, RESTRICTIVE TEV MOVEMENT 1(RTM1) and JACALIN-TYPE LECTIN REQUIRED FOR POTEXVIRUS RESISTANCE 1 (JAX1) have been identified to cause resistance to *Tobacco etch virus* and potexviruses, respectively (Chisholm et al. 2000; Yamaji et al. 2012). The resistance is in both cases specific and the molecular mechanisms behind RTM1- and JAX1-mediated resistance are not conserved. In the case of JAX1 the resistance was shown to be independent of hormone signaling (Yamaji et al. 2012).

1.4.3 Viral defense in bacteria and archaea

In bacteria and archaea an adaptive anti-viral system with resemblance to RNA-silencing known as Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) was recently described. During infection, 20-50 nt stretches of bacteriophage or plasmid DNA are incorporated into the CRISPR loci between unique spacers. In healthy cells CRISPR loci are transcribed and processed into shorter CRISPR-derived RNA (crRNA) transcripts, which together with the Cascade complex survey the cell for foreign nucleic acids with sequence similarity. Nucleotides complementary to crRNAs are degraded upon recognition (Waters and Storz 2009; Wiedenheft et al. 2012). CRISPR loci contain information of phages previously encountered by the cell or its ancestors with the most recent encounter at the 5’ end of the loci.

1.5 Tobamoviruses

Tobacco mosaic virus (TMV) of the genus tobamovirus was the first virus described and has become the best-studied plant RNA virus (Scholthof et al. 2011). The genus tobamoviruses is divided into three sub-groups according to viral genome organization and host range. TMV belongs to the Solanaceae-infecting subgroup 1 while crucifer-infecting tobamoviruses, incl. *Oilseed rape mosaic virus* (ORMV), cluster in subgroup 3 (Melcher 2003). The rodshaped tobamovirus virions consist of an about 6300 nt long +ssRNA genome encapsidated by a helical array of coat protein (CP). The virions are stable for many years in soil or water and believed to be transmitted mechanically rather than through insects, seeds and pollen (Hull 2009). The tobamoviral genome encodes two replicase proteins, 126k and 183k, a movement protein (MP) and a CP. The genomic RNA, which has a leaky stop codon terminating the 126k protein, produces both 126k and the translational read-through product 183k. MP and CP are translated from specific, co-terminal sub-genomic RNAs that are transcribed from the viral RNA during replication with the help of specific subgenomic promoters (Grdzelishvili et al. 2000; Dorokhov et al. 2006). Replication of the viral RNA takes place in VRCs associated with the endoplasmic reticulum (ER)-membrane and likely anchored to the ER by MP and sheltered from the cytosol by the CP (Asurmendi et al. 2004; Tilsner et al. 2009). The host proteins TOBAMOVIRUS MULTIPLICATION (TOM) 1, 2a and 3 are needed for the formation of VRCs (Ishibashi et al. 2010). The 126k acts as a strong silencing suppressor with sRNA binding activity (Csorba et al. 2007) and both host and viral 21 nt sRNAs accumulate during tobamovirus infection (Csorba et al. 2007; Vogler et al. 2007). The accumulating sRNAs appear to be non-methylated and it has been speculated that sRNA sequestration by 126k may compete with HEN1 for access or that 126k may de-methylate the sRNAs (Vogler et al. 2007; Burgyan and Havelda 2011). Intra- and intercellular movement of the viral RNA is independent of CP. Intracellular movement involves the trafficking of viral RNA:MP complexes, potentially early forms of VRCs (Kawakami et al. 2004), along the ER-membrane with the support and coordination of the actin and microtubule cytoskeleton (Niehl et al. 2013). Intercellular movement takes place through PD. To facilitate the intercellular transport of viral RNA, the MP increases the size-exclusion limit of PD through a process likely involving the recruitment of glucanases for the degradation of callose deposited at the PD neck region (Epel 2009).

Although the CP is dispensable for the establishment of virus infection and cell-to-cell movement it is required for long-distance transport in the phloem. The molecular mechanisms behind loading and un-loading of tobamoviruses to and from the phloem are not known, and it is possible that the CP is required for long-distance movement due to its function as a structural element of the virion.

Tobamoviruses are model viruses in plant virology and due to the large body of knowledge gathered over the years our understanding of their life cycle and interactions with the host is quite advanced (Scholthof et al. 2011). Plant defenses against tobamovirus infection have been studied and several genes conferring resistance to tobamoviruses have been cloned and described (Marathe et al. 2002; Lanfermeijer et al. 2003; Lanfermeijer et al. 2005; Ishibashi et al. 2007). During compatible infections tobamoviruses are, as all viruses in

plants, targets of RNA-silencing and strong silencing suppression is directly linked to high viral fitness (Kubota et al. 2003; Vogler et al. 2007). Apart from RNA-silencing also auxin signaling has been shown to modulate tobamovirus infection severity in Arabidopsis (Padmanabhan et al. 2005; Padmanabhan et al. 2008).

1.6 Aim of this thesis

Compatible virus-plant interactions are highly dynamic and responsive to environmental and developmental cues. The viral pathogen modulates host defense mechanisms to establish and maintain an infection. It is known that RNA-silencing represents such a plant defense mechanism that must be overcome by the virus. Defense related hormone pathways are also activated upon viral infection but their role in viral defense is more unclear. Despite recent advances in our understanding of anti-viral plant defenses, core questions such as why some infections are symptomatic while other infections progress symptomless and why some symptomatic infections regress to a non-symptomatic stage (recovery) are still unanswered. In this thesis the roles of several known and potential anti-viral defense mechanisms are investigated for their contribution in controlling viral accumulation and viral disease symptoms during compatible tobamovirus infection in the model plant Arabidopsis.

To address the role of RNA-silencing in recovery, the symptom-inducing tobamovirus ORMV was used as a model. The observation that Arabidopsis infected with ORMV initially show strong symptoms but recovered from symptoms at later infection stages, made it possible to study the role of individual components of the RNA-silencing pathway in recovery (chapter 2).

PTI plays an important role in viral defense in animals but so far no evidence for PTI as an anti-viral defense mechanism in plants has been provided. However, the importance of PTI in resistance against non-viral pathogens is well established in plants. A potential role of PTI in restricting compatible viruses was investigated in mutants of Arabidopsis with reduced PTI-capacity (chapter 3).

Symptomless virus infections are often observed but it remains unknown how the tolerant host plant controls the viral pathogen during such infections. Therefore, the involvement of RNA-silencing and hormone signaling during compatible non-symptomatic TMV infection in Arabidopsis was investigated (chapter 4).

2 Natural Recovery from RNA Viral Disease in Arabidopsis depends on both Post Transcriptional Gene Silencing and Transcriptional Gene Silencing Pathway Components

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2.1 Abstract

Virus-infected plants that initially show disease symptoms may start to develop new symptom-free leaves, a phenomenon known as recovery. Studies have indirectly linked recovery to anti-viral RNA-silencing, but the proteins involved remain unknown. Here we present a system to study recovery from RNA viral disease in *Arabidopsis thaliana.* Upon infection with the tobamovirus *Oilseed rape mosaic virus* (ORMV) Arabidopsis plants initially develop severe symptoms, but at late infection stages non-symptomatic leaves appear. Recovered leaves still contain virus but viral silencing suppression activity is absent. To identify pathways contributing to recovery, we infected different silencing-deficient Arabidopsis mutants with ORMV. As anticipated, mutant plants impaired in 21 nt siRNA-mediated post-transcriptional gene silencing (PTGS) (*rdr6, dcl2, dcl4, ago1, hen1, sgs3*) showed delayed or no recovery from symptoms. Some mutants impaired in transcriptional gene silencing (TGS) did not recover but this phenotype is independent of down-stream DNA-methylation. Interestingly, mutants of 5'-3' exoribonuclease XRN4 (*xrn4-3*, *ein5*), a known endogenous RNA-silencing suppressor, recovered faster than wild type (WT). Together these observations emphasize the importance of RNA-silencing not only in maintenance but also in the onset of recovery from viral disease in plants.

2.2 Introduction

Viral infection in plants can have several outcomes ranging from host death to complete immunity and clearance of virus from the host. Although compatible virus infections are often accompanied by strong developmental symptoms for some virus-host combinations a recovery from symptoms may be observed. Recovery was first described in the nineteen twenties for ringspot disease caused by a nepovirus (Wingard 1928). Recent work has reported recovery phenotypes for natural infections of both RNA viruses (Ratcliff et al. 1997; Ratcliff et al. 1999; Xin and Ding 2003; Jovel et al. 2007; Rajakaruna et al. 2007; Siddiqui et al. 2008; Lunello et al. 2009; Hakmaoui et al. 2012) and DNA viruses (Covey et al. 1997; Chellappan et al. 2004; Hagen et al. 2008; Rodriguez-Negrete et al. 2009) across the plant kingdom. Recovered tissues often contain less virus than symptomatic tissues, but there have also been reports of recovered tissues with higher or equal viral titers (Xin and Ding 2003; Jovel et al. 2007). Complete clearance of virus, as observed with recovery from viral disease in animals (Virgin et al. 2009), is normally not reported for recovered plants. Recovery in plants may be transient (Lu et al. 2012) and similar to a persistent viral infection in which host and virus are in a metastable equilibrium (Goic and Saleh 2012). The mechanisms leading to recovery long remained elusive and their further exploration had to wait until anti-viral RNA-silencing in plants was discovered (Ratcliff et al. 1997).

The RNA-silencing machinery of plants consists of several pathways of which only some have been shown to be directly involved in viral defense, while the main role of other pathways are regulation of endogenous mRNA. RNA-silencing may control RNA abundance either on the transcriptional, referred to as Transcriptional Gene Silencing (TGS) or the post-transcriptional level, referred to as Post Transcriptional Gene Silencing (PTGS) (Vazquez et al. 2010; Saze et al. 2012). Common to all RNA-silencing pathways is the role of 20-24 nt long small interfering RNA (siRNA) or microRNAs (miRNA) in down-regulating mRNA abundance or transcription rate in a sequence specific manner. miRNAs are produced from the structured single-stranded transcripts of specific miRNA-genes, while siRNAs are produced from long double-stranded RNA (dsRNA). Such dsRNA may originate from overlapping transcripts, inverted repeats, aberrant RNA processed by RNA-DEPENDENT-RNA-POLYMERASES (RDR) in complex with SUPPRESSOR OF GENE SILENCING 3 (SGS3) or from viral replication intermediates. Structured ssRNA or dsRNA are cleaved into miRNAs and siRNAs by DICER-like (DCL) proteins followed by methylation by HUA ENHANCER 1 (HEN1) (Fig. 1.1) (Yu et al. 2005). In the case of PTGS miRNAs or siRNAs are loaded into ARGONAUTE (AGO) within the RNA-induced silencing complex (RISC) to guide the cleavage or translational inhibition of perfect or near-perfect complementary mRNA (Palatnik et al. 2003; Brodersen et al. 2008). Cleaved RNA can then serve as template for dsRNA production viaRDRs (Willmann et al. 2011) that in turn may become substrates for new rounds of dicing mainly by DCL4 leading to the accumulation of secondary siRNAs (Fig.1.1). Secondary siRNAs are rarely observed for miRNA-regulated mRNA but they are abundant in the case of silenced transgenes. Aberrant RNA from transgenes may also serve as template for RDRs (Dalmay et al. 2000; Mourrain et al. 2000). In anti-viral defense double-stranded or structured single-stranded viral RNAs are processed by DCL proteins and viral siRNA accumulation is a

hallmark of active viral defense in plants. Even though DCL processing of viral RNA could harm the virus on its own, degradation of full-length viral RNA via AGO1 and amplification via RDR enhance the efficiency of silencing as an anti-viral defense mechanism. siRNAs are known to move cell-to-cell and in some cases long-distance (Brosnan and Voinnet 2011), and it has therefore been speculated that RNA-silencing could immunize naïve cells ahead of infection (Ding and Voinnet 2007).

To circumvent their degradation by the anti-viral RNA-silencing response of the host, plant viruses have evolved proteins with the ability to suppress silencing (Ding 2010). These silencing suppressors are crucial for viral infection and viruses with mutated silencing suppressor often have reduced fitness. Although the general function of silencing suppressors is conserved among viruses, the molecular mechanism of silencing suppression is not (Burgyan and Havelda 2011). However, most silencing suppressors do not block the production of primary siRNAs but target components downstream of DCLs (Alvarado and Scholthof 2009; Burgyan and Havelda 2011). The interference of viral silencing suppressors with endogenous silencing pathways involving miRNAs has been suggested to be the cause of virus-induced developmental symptoms (Dunoyer et al. 2004). However, the effect of silencing suppressor proteins on miRNA-regulation appears to differ from the effect on siRNA-mediated PTGS (Schott et al. 2012). The exact mechanism by which virus infection induces developmental symptoms may well include interference with other pathways than RNA-silencing.

Recovery in natural host-virus systems has been proposed to be mediated through silencing but only little direct evidence has been reported. Recovery from DNA-virus infection seems to be characterized by lower viral titer in recovered leaves (Covey et al. 1997; Chellappan et al. 2004; Hagen et al. 2008; Rodriguez-Negrete et al. 2009) and in one case it has been shown that the recovery phenotype of geminivirus *Cucurbit leaf crumple virus* in melon plants can be reverted by inoculation with non-related RNA virus *Cucumber mosaic virus* (Hagen et al. 2008), suggesting that recovery can be broken by *trans*-silencing suppression. In the case of recovery from RNA-virus infection there have been reports of both complete clearance of virus (Ratcliff et al. 1999; Lunello et al. 2009) as well as higher viral titers in recovered tissue (Xin and Ding 2003; Jovel et al. 2007), but in most cases a decrease in viral titer is observed (Ratcliff et al. 1997; Siddiqui et al. 2008; Lu et al. 2012). Recovery phenotypes are often observed for nepovirus infections, but contradicting observations have been made with regard to the involvement of silencing in nepovirus recovery. Neither overexpression of RDR1 nor knock-down of RDR6 affected recovery from *Tomato ringspot virus* in *Nicotiana benthamiana* (Jovel et al. 2007). However, recovery from *Tobacco ringspot virus* was blocked in *N. benthamiana* plants expressing the silencing suppressors HcPro and p25 (Siddiqui et al. 2008). *Nicotiana clevelandii* leaves recovered from *Tomato black ring nepovirus* W22 infection was resistant to re-infection with the same, closely related nepoviruses or non-related *Potato virus X* containing a W22 fragment while they remained susceptible to other non-related viruses (Ratcliff et al. 1997) suggesting that a sequence specific mechanism is involved in the maintenance of recovery. The observation, that plants infected with viruses with reduced or depleted silencing suppressor activity are

more prone to recover (Szittya et al. 2002; Raja et al. 2008; Wu et al. 2010), provides indirect evidence that establishment of recovery involves silencing. However, such mutant viruses often accumulate to lower titers than their corresponding WT virus and whether this sort of recovery is identical to that observed in natural virus-host interaction is not known. So far, no detailed genetic studies on recovery have been performed, which is mainly due to the lack of a natural virus-host recovery system in the model plant Arabidopsis.

Here we report that Arabidopsis plants infected with the tobamovirus *Oilseed mosaic virus* (ORMV) recover from symptoms at late stages of infection. Recovered leaves still contain infectious virus, but viral silencing suppression is no longer active. The recovery phenotype is siRNA-biogenesis dependent and RDR6, SGS3, HEN1, AGO1, DCL2 and DCL4 are essential for recovery. Accelerated recovery can be observed in mutants of the silencing antagonist EXONUCLEASE 4 (XRN4), confirming that silencing may also play a role in the onset of recovery. Surprisingly, RDR2 and DNA-dependent-RNA-polymerase IV (POLIV) are also needed for recovery, while other components of the TGS pathway are not essential.

2.3 Results

Arabidopsis infected with ORMV recovers from symptoms but viral titer remains high

Under our growth conditions Arabidopsis plants infected with the tobamovirus ORMV initially show severe symptoms such as growth retardation, necrosis and leaf deformation. However, at 23-25 days post inoculation (dpi) new leaves develop that are free of visible symptoms (Fig. 2.1 A). Leaves emerging after this time point remain symptom-free. Detailed analysis revealed the existence of transition leaves, where the distal tip was strongly symptomatic including margin serration while the proximal part of the same leaf was symptom-free (Fig. 2.1 A, arrow). To investigate if symptom-free tissue still contained virus, tissues from all types of infected leaves were used for virion purification. Virion isolations were analyzed by SDS-PAGE and coomassie staining. A clear band corresponding in size to the coat protein (CP, 17kDa) was observed in all tissues from ORMV-infected Arabidopsis but absent in mock-inoculated plants (Fig. 2.1 B). All virion isolations were infectious in *N.benthamiana* (not shown). We conclude that non-symptomatic leaves still contain infectious ORMV and a lack of symptoms is not due to absence of infectious virus.

Virion isolations are not quantitative and to investigate if viral titers were lower in recovered leaves, we isolated RNA from all types of symptomatic and recovered leaves from Arabidopsis at 28 dpi and measured viral RNA titer by qPCR. The total amount of viral RNA was similar in all tissue regardless of symptoms (Fig. 2.1 C). Virions of tobamoviruses are very stable and viral RNA detected by qPCR in non-symptomatic tissue could originate from dormant virions. To test for the presence of non-encapsidated viral RNA, we investigated if viral siRNAs could be detected in recovered leaves. Accumulation of viral siRNAs is a marker for the presence of uncoated viral RNA accessible to DCLs. Moreover, the silencing suppressor of ORMV sequesters 21 nt small RNAs and the presence of non-encapsidated viral RNA should correlate with active viral silencing suppression and, thus with the accumulation of 21 nt small RNAs. Northern blot using total RNA isolated from all symptom types was probed for viral siRNAs and strong signals corresponding to 21 nt viral siRNAs were obtained in symptom types 1 and 2 (Fig. 2.1 D). This supports the hypothesis that ORMV RNA is accessible to DCLs and stabilized by the silencing suppressor in symptomatic tissue. In contrast the viral siRNA signal was very weak in RNA from recovered tissue (Fig. 2.1 D). This suggests that viral siRNAs are no longer produced or no longer stabilized by the ORMV silencing suppressor. The viral RNA detected by qPCR could come from virus restricted to the vascular tissue in recovered tissue. To rule out that ORMV is restricted to vascular tissue of recovered leaves we performed *in situ* hybridization of all symptom types. Viral RNA could be detected in leaves of all symptom types (Fig. 2.1 E) showing that ORMV does not become phloem-limited during later stages of infection.

We wondered if the recovery phenotype is conserved for subgroup 3 tobamoviruses or ORMV-specific. Arabidopsis was therefore infected with two other tobamoviruses from subgroup 3, *Ribgrass mosaic virus* (RMV) and *Turnip vein clearing virus* (TVCV). Arabidopsis also recovered from RMV and TVCV infections (Fig. S2.1 A). However, symptoms in RMV-infected plants were much milder compared to ORMV- and TVCV-infected plants (Fig. S2.1

A). Plant age at infection also affected symptom formation. Older plants developed milder symptoms upon infection and did not recover from ORMV infection (Fig. S2.1 B).

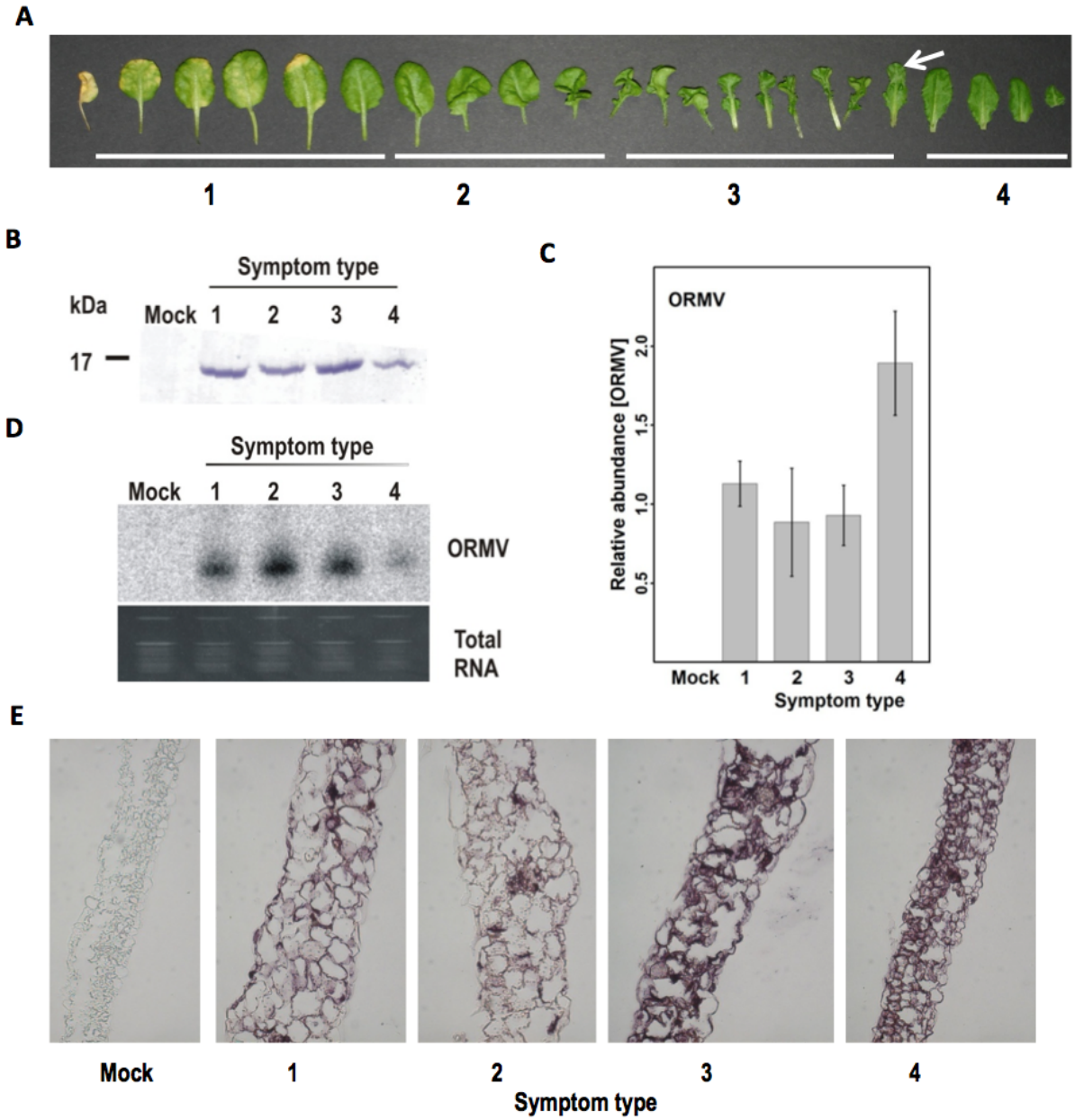

Figure 2.1 Arabidopsis recovers from ORMV infection but recovered tissue still contains virus. A) Leaves of an ORMV-infected plant at 28 dpi. The leaf series shows the leaves in order, starting with the bottom leaves (oldest leaves) on the left and ending with the top leaves (youngest leaves) on the right. The leaves show four distinct types of symptoms: necrotic leaves (1), curled leaves (2), curled leaves with serrated margins (3) and non-symptomatic leaves (4). The white arrow indicates a transition leaf showing both symptomatic and non-symptomatic areas. B) Viral CP accumulation visualized by coomassie staining of a SDS PAGE gel. The numbers above the lanes correspond to the symptom phenotypes shown in A. The gel was loaded with virions isolated from the infected leaves. C) Viral RNA levels in leaves showing the different symptom phenotypes were measured by qPCR, n=3 ±SE. D) Northern blot showing viral siRNAs detected with a virus-specific probe. Stained total RNA is shown as loading control. E) Localization of genomic viral RNA by *in situ* hybridization of leaves of all four symptom types shown in A. The colored signal indicates the presence of ORMV.

Viral silencing suppression is no longer active in recovered tissue

Viral silencing suppression is believed to play an important role in symptom formation during viral disease (Dunoyer et al. 2004) and we therefore speculated that viral silencing suppression is no longer active in recovered tissue. To test this, we infected the PTGS- green fluorescent protein (GFP)-reporter line L8Z2 (Glazov et al. 2003) with ORMV and followed silencing suppression during infection by monitoring GFP-signal under UV-light. At about 10-14 dpi green fluorescent tissue could be observed under UV-light in ORMV-infected, but not in mock-inoculated plants (not shown), indicating that the GFP-silencing was suppressed in infected tissue. At 28 dpi, symptomatic tissue appeared green under UV-light, while recovered tissue appeared red. This indicates that GFP silencing is re-established in recovered tissue (Fig. 2.2 A). In leaves undergoing transition to recovery, the symptomatic tip was green under UV-light while the non-symptomatic base was red. The clear boarder between the two regions made it possible to separate transition leaves in two parts, i.e. representing types 3a (symptomatic) and 3b (non-symptomatic) (Fig. 2.2 B). In order to verify that the green signal observed under UV-light corresponded to higher accumulation of GFP transcript and protein these were measured by qPCR and Western blot, respectively. In symptomatic tissue (1-3a), GFP transcript and protein levels were higher than in mock-inoculated tissue, while in non-symptomatic tissue GFP transcript and protein levels were similar to that of mock-inoculated plants (Fig. 2.2 B & C). As observed in Col-0, total viral RNA abundance was similar in all tissues and CP could be detected in all tissues apart from 3b (Fig. 2.2 B & C). A hallmark of active silencing suppression during ORMV infection is the accumulation of 21 nt siRNAs, miRNA* and some miRNAs (Blevins et al. 2006; Csorba et al. 2007; Hu et al. 2011). Northern blot analysis revealed that the amount of GFP siRNAs was only slightly increased in leaves displaying symptom type 1 while for symptom type 2 and 3a less GFP siRNAs were detected compared to mock-inoculated plants (Fig. 2.2 D). This observation may be explained by fact that the silencing suppression by subgroup 3 tobamoviruses blocks the production of RDR6-dependent secondary siRNAs (not shown) (Csorba et al. 2007) and the small RNAs accumulating during infection likely represent sequestered primary siRNAs. In the case of sense-PTGS, such as that of the L8Z2-line, the production of siRNA is RDR6-dependent (Mourrain et al. 2000; Luo and Chen 2007) and this may explain the low amount of GFP siRNAs in ORMV-infected L8Z2. Viral siRNAs and miRNA398* accumulated in leaves of symptom type 1 and 2, while only a very weak signal was observed for symptom types 3a, 3b and 4 (Fig. 2.2 D). This suggests that the silencing suppressor is not sequestering 21 nt siRNAs and miRNAs in recovered tissue.

To verify that the lack of silencing suppression in recovered tissue is a general phenomenon and also applies to silencing induced by RNAi hairpin constructs we infected Suc:Sul plants carrying an inverted repeat hairpin targeting a region of the *sulphur* gene for siRNA-guided silencing. The expression of the hairpin RNA is driven by a phloem companion cell-specific promoter from the gene *sucrose-proton symporter 2* and active RNAi leads to bleaching along the veins (Himber et al. 2003). In ORMV-infected leaves with strong symptoms, the bleaching along the veins was no longer observed, while vein bleaching was again visible in recovered leaves (Fig. S2.2). Taken together, these results suggest that ORMV no longer

suppresses silencing by sequestering siRNA and miRNAs or by blocking RDR6-activity in recovered leaves.

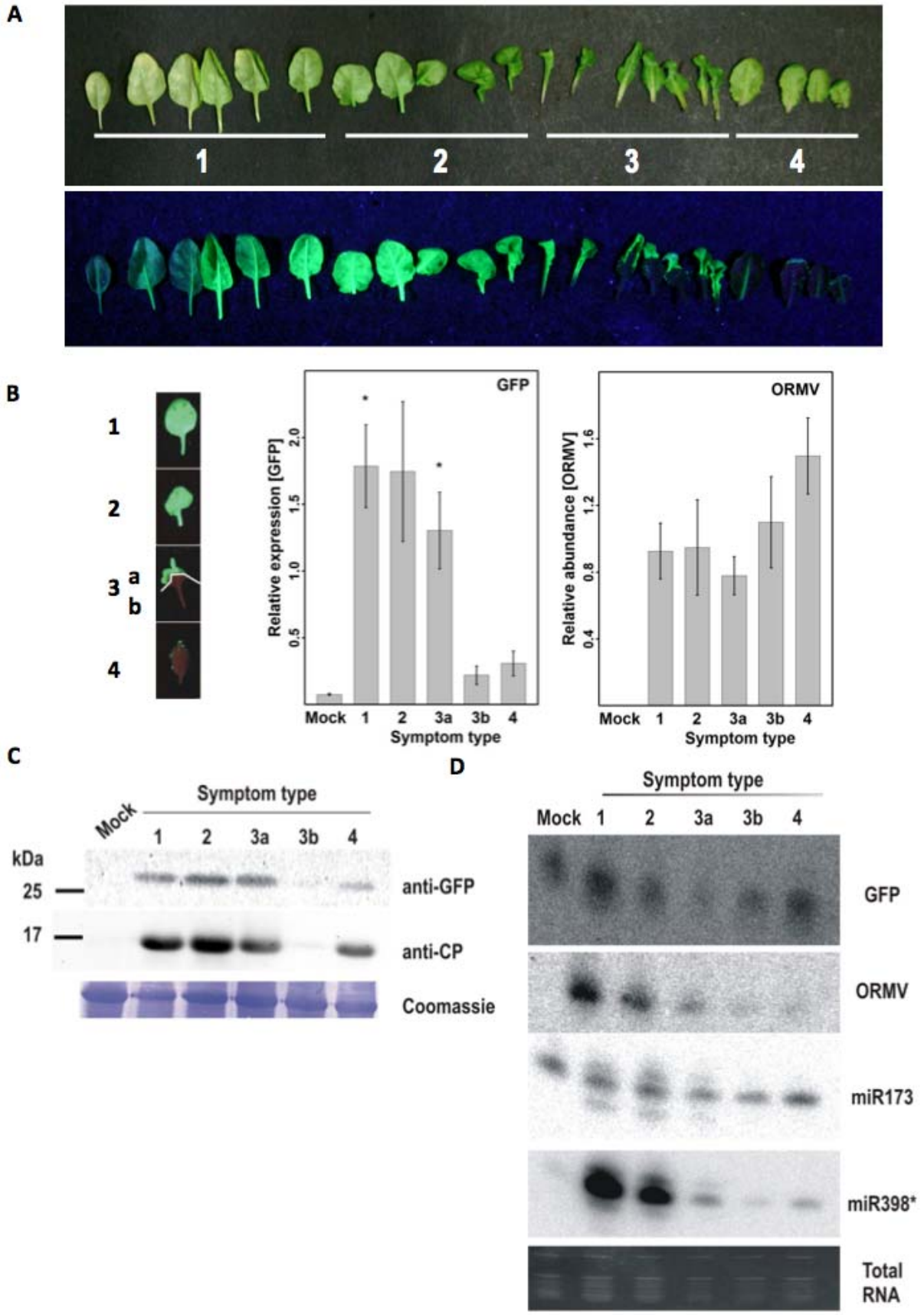

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Figure 2.2 ORMV silencing suppressor is not active in recovered tissue. Arabidopsis PTGS reporter line L8Z2 was inoculated with ORMV or water. A) Leaves of an ORMV-infected plant at 28 dpi. The leaf series shows the leaves in order, starting with the bottom leaves (oldest leaves) on the left and ending with the top leaves (youngest leaves) on the right. The leaves are shown in bright light (top) and under UV light (bottom). The GFP signal is indicative of silencing suppression by the virus. The ability of the virus to suppress silencing correlates with disease symptoms. B) Accumulation of GFP mRNA and viral RNA in leaves with different symptoms and silencing suppression phenotypes (indicated on the left) measured by qPCR; n= 3 ± SE, Student's t-test * $p<0.05$. C) Accumulation of GFP and viral CP in leaves with the specific symptom phenotypes detected by Western blot using GFP- and CP-specific antibodies. Coomassie staining of total protein is shown as control for equal loading. D) Northern blot showing the levels of GFP-derived siRNAs (GFP), viral siRNAs (ORMV), and of the miRNAs miR173 and miR398*. Stained total RNA is shown as loading control.

siRNA biogenesis is needed for recovery

The observation that ORMV-infected Arabidopsis plants recover made it possible to determine which parts of the RNA-silencing machinery are required for recovery. To do so, we infected a wide range of Arabidopsis mutants known to be impaired in PTGS, miRNA biogenesis or RNA decay with ORMV and monitored symptom formation during the following four weeks (table 2.1). Some mutants flowered before onset of recovery and were therefore excluded from the analysis. All mutants affected in miRNA biogenesis recovered as WT while *rdr6-15, sgs3-13, ago1-27, hen1-5* and *dcl2,4* mutants impaired in siRNA biogenesis did not recover and young emerging leaves remained symptomatic at 28 dpi (Fig. 2.3 A). These observation links recovery to genes involved in the biogenesis of viral siRNAs (Mourrain et al. 2000; Blevins et al. 2006; Donaire et al. 2008; Qi et al. 2009; Wang et al. 2010) and indicates that viral siRNAs are needed for the establishment of recovery or for maintenance of the recovered state.

Early recovery in xrn4/ein5 is partially dependent on RDR6

Of the PTGS mutants tested, only *xrn4-3* and *ein5-1*, both mutated in XRN4, recovered earlier than Col-0 (table 2.1). XRN4 is known to act as an endogenous silencing suppressor likely due to template competition with RDRs (Gazzani et al. 2004). However, XRN4 is also important for ethylene signaling, and *xrn4*-mutants are ethylene insensitive (Olmedo et al. 2006; Potuschak et al. 2006). Since ethylene together with other hormones plays an important role in general pathogen defense we infected other ethylene, salicylic acid (SA), jasmonic acid (JA) and auxin mutants with ORMV and followed symptom formation over time. However, all hormone mutants recovered in a manner similar to Col-0 (table S2.1), indicating that the accelerated recovery in *xrn4-3* and *ein5*-1 is due to enhanced silencing suppression and not caused by disturbance of ethylene signaling.

Table 2.1 Recovery phenotypes in PTGS mutants

Timing	ATG number	Mutant	Gene function
Early	AT1G54490	*ein5-1*	Ethylene signaling/ RNA decay
		xrn4-3	
Normal	AT1G05460	*sde3-4*	VIGS
	AT2G28380	*drb2*/N849 395	dsRNA binding
	AT3G26932	*drb3*/N022 644	
	AT3G62800	*drb4-1*	
	AT5G41070	*drb5*/N126 609	
	AT1G69440	*ago7-1*	tasiRNA production
	AT3G05040	*hasty-15*	miRNA transporter
	AT1G09700	*hyl1-2*	miRNA biogenesis
	AT1G01040	*dcl1-9*	
	AT2G27100	*se-1*	
	AT3G03300	*dcl2-5*	siRNA biogenesis
	AT1G14790	*rdr1-1*	
	AT2G13540	*abh1-8/cbp80*	cap binding
	AT5G44200	*cbp20*	
	-	*egs1-1*	enhanced silencing
	-	*egs2-2*	
	AT5G42540	*xrn2-3*	supressor of PTGS/RNA decay
	AT1G75660	*xrn3-3*	
	AT5G63980	*fry1-6*	
	AT1G31280	*ago2-1*	Viral defense (?)
	AT1G31290	*ago3-1*	unknown
	AT2G27880	*ago5-2*	unknown
	AT5G21030	*ago8-1*	unknown, pseudogene
	AT5G21150	*ago9-1*	ovule development
	AT5G43810	*ago10-1*	meristem regulation
Delayed	AT5G20320	*dcl4-2*	siRNA biogenesis
	AT3G15390	*sde5-2*	VIGS
	AT2G15790	*sqn-5*	vegetative phase change
None	AT1G48410	*ago1-27*	PTGS/miRNA
	AT4G20910	*hen1-5*	siRNA biogenesis
		dcl2,4	
		dcl 2,3,4	
	AT3G49500	*rdr6-15*	
		rdr1,6	
		rdr2,6	
		rdr1,2,6	
	AT5G23570	*sgs3-13*	

To investigate the role of XRN4/EIN5 in recovery further, *xrn4-3*, *ein5-1*, *ein5Xrdr6* and *rdr6-15* mutants were infected with ORMV. Visual observation showed that *ein5Xrdr6* recovered as WT around 23-25 dpi, while *ein5-1* and *xrn4-3* recovered before 21 dpi and *rdr6-15* did not recover. At 28 dpi, symptomatic and recovered leaves were counted and the percentage of recovered leaves was calculated for each genotype (Fig. 2.3 B). *rdr6-15* plants contained no recovered leaves and were therefore not included in this analysis. A significantly higher percentage of recovered leaves in *ein5-1* and *xrn4-3* compared to WT confirmed previous visual observations, while no differences were observed between WT and the double mutant *ein5xrdr6*. This supports our hypothesis that XRN4-mutants recover early due to enhance silencing and not because of disrupted ethylene signaling.

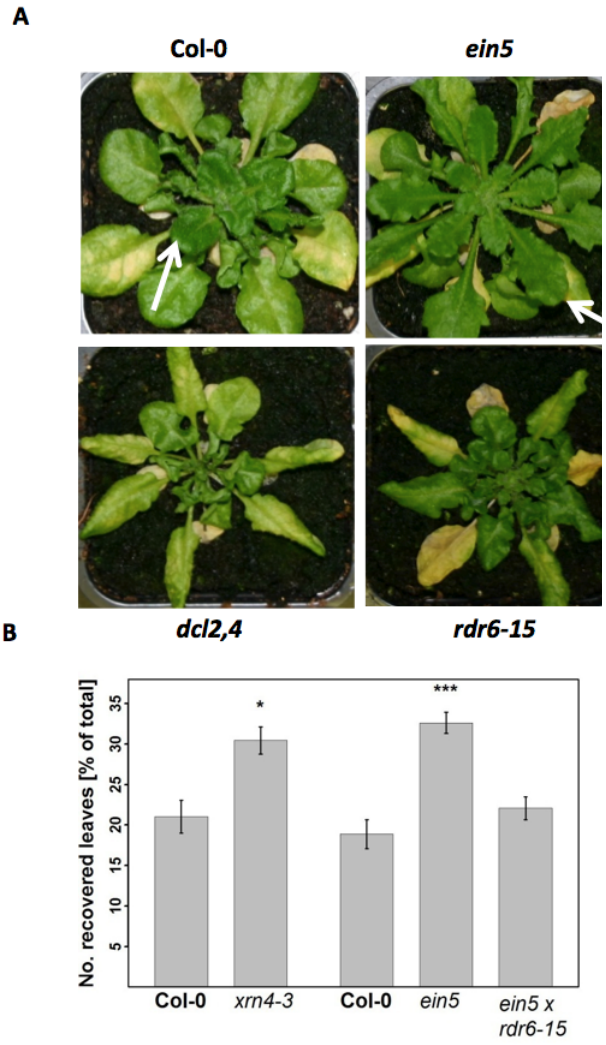

Figure 2.3 Early recovery in EIN5/XRN4-mutants are partially dependent on RDR6. A) ORMV-infected plants carrying mutations in genes involved in siRNA biogenesis or RNA decay were infected with ORMV. Pictures represents symptoms observed at 28 dpi. The white arrow indicates the first recovered leaf of WT and *ein5* plants. *dcl2xdcl4* and *rdr6-15* mutants did not display any recovered leaves at 28 dpi. B) The number of recovered leaves in relation to the total number of leaves in percent at 28 dpi in WT plants (Col-0), and in *xrn4-3*, *ein5*, and *ein5xrdr6* mutants. n=10 ±SE, binomial GLM, * p<0.05, *** p<0.001, compared to respective WT Col-0 plants.

Faster onset of recovery in dcl3-1 is DCL4-dependent, and RDR2 and POLIV are also essential for recovery

While PTGS have been shown to be involved in defense against both RNA and DNA viruses in plants (Diaz-Pendon and Ding 2008), TGS have so far only been linked to defense against DNA viruses (Raja et al. 2008). With the exception of HEN1 the two pathways are not believed to share components. In our initial screen of PTGS mutants we included the TGS mutant *dcl3-1* impaired in the production of 24 nt siRNAs guided DNA-methylation. Surprisingly, *dcl3-1* recovered much earlier than WT (Fig. 2.4 A). Further experiments scoring recovery visually in various plant genotypes including *dcl2x3* and *dcl3x4* double mutants revealed that the early recovery phenotype was lost in the *dcl3x4* double mutant only (table 2.2). To quantify this effect, we counted the number of recovered leaves at 28 dpi, and found that *dcl3-1* had significantly more recovered leaves, while *dcl4-2* and *dcl3x4* did not differ from Col-0 (Fig. 2.4 B). To rule out that early recovery in *dcl3-1* is dependent on siRNA-directed DNA methylation, we infected a range of TGS mutants known to act up- or downstream of DCL3 in the TGS pathway with ORMV. With the exception of a only slightly faster recovery in *drd1-6*'s, none of the TGS mutants downstream of DCL3 recovered earlier than WT (table 2.2) supporting that the early recovery in *dcl3-1* is DCL4-dependent and not due to reduced TGS activity. Strikingly, *rdr2-2* and several POLIV-subunit mutants did not

recover (table 2.2, Fig. 2.4 A). This suggests that, RDR2 and POLIV are involved in recovery in a manner independent of siRNA-directed DNA methylation.

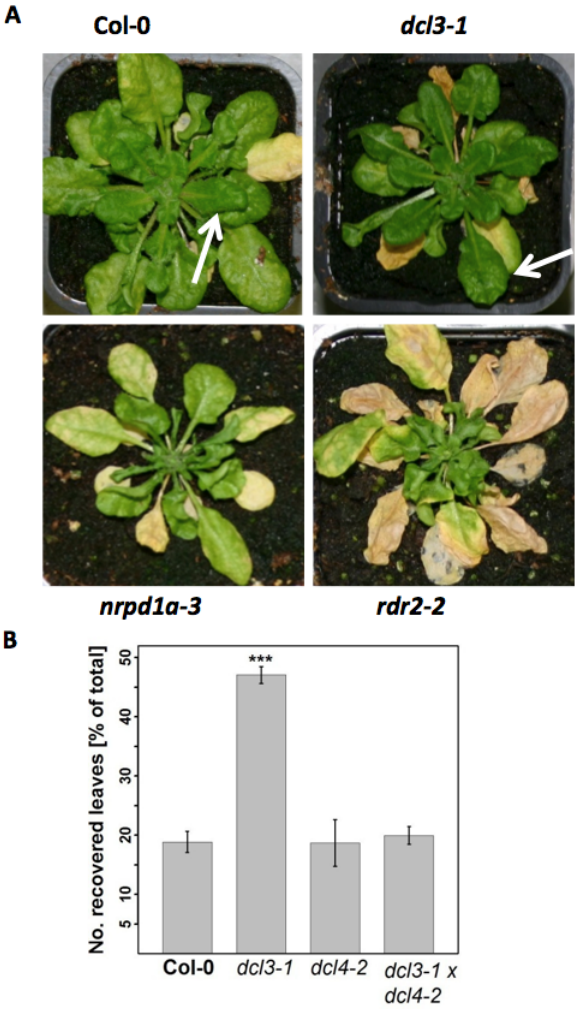

Figure 2.4 Components of the TGS pathway play different roles in recovery. A) ORMV-infected plants carrying mutations in genes involved in TGS at 28dpi. The white arrow indicates first recovered leaf in respective plant. *nrpd1a-3* and *rdr2-2* mutants failed to recover. B) The number of recovered leaves in relation to the total number of leaves in percent at 28 dpi in WT plants (Col-0) and *dcl3-1*, *dcl4-2* and *dcl3xdcl4* mutants, n=6-10 ±SE, binomial GLM, *** p<0.001.

Table 2.2 Recovery phenotypes in TGS mutants

Timing	ATG number	Mutant	Gene function
Early	AT3G43920	*dcl 3-1*	TGS, siRNA biogenesis
		dcl2,3	
	AT2G16390	*drd1-6*	chromatin remodelling/siRNA-directed DNA-methylation
Normal	AT2G40030	*drd3-7 (nrpd1b)*	siRNA-directed DNA-methylation; PolV
	AT3G42670	*classy 1-4*	spread of RNA-silencing
	AT2G36490	*ros1-4*	DNA de-methylation
	AT5G14620	*drm2-2*	methyltransferase,de novo DNA-methylation
	AT3G22680	*rdm1-4*	siRNA-directed DNA methylation
	AT3G49250	*dms3-4 (idn1)*	
	AT2G27040	*ago4-2*	
	AT2G32940	*ago6-3*	siRNA-directed DNA methylation, meristem
	AT5G63110	*rts1-1 (hda6)*	histone deacetylase
None	AT4G11130	*rdr2-2*	siRNA biogenesis
		rdr1,2	
	AT1G63020	*nrpd1a-3*	long distance PTGS/TGS (POLIV)
	AT3G23780	*nrpd2a-1*	
		drd2-4	

Gene expression of the PTGS and TGS pathway components are induced in recovered tissue

The low levels of viral siRNA in recovered tissue could be explained by the lack of stabilization by the silencing suppressor, decrease in PTGS activity or viral RNA being inaccessible for processing. Experiments with L8Z2 and Suc:Sul show that PTGS is active in recovered tissue but due to the on/off read-out of these systems it is not possible to determine increases in PTGS activity. Although components of the PTGS pathway are believed to be important for anti-viral defense only few have been shown to be transcriptionally induced during viral infection (Csorba et al. 2007). We investigated the transcriptional accumulation of several PTGS genes during ORMV infection (7, 14 and 21 dpi) in a previously published microarray study by our group (Hu et al. 2011) and found that a few genes such as AGO1 and SGS3 were induced by ORMV infection in Arabidopsis, while the expression of other genes related to PTGS including some found to be essential for recovery, did not change (Fig. S2.3 A,B,E). Since the microarray data did not include the transcriptional profile of recovered tissue, we decided to investigate the transcript accumulation of AGO1, SGS3, DCL4 and RDR6 in the different symptom types found at 28 dpi in ORMV-infected Arabidopsis by qPCR. In order to clearly distinguish recovered from non-recovered tissue we used the same cDNA as used for the analysis shown in figure 2.2, which was derived from infected L8Z2 plants and represented the symptomatic tissue types 1, 2 and 3a in which viral silencing suppression was active and the recovered tissue types 3b and 4, in which silencing suppression was inactive. As expected from the microarray data, AGO1 and SGS3 transcripts were induced in symptomatic tissue, while DCL4 and RDR6 were only induced in tissue type 3a, but not in the tissue types 1 and 2. However, all four genes were induced in recovered tissues (Fig. 2.5 A). Since some components of the TGS pathway are essential for recovery we investigated whether TGS-pathway components were induced in recovered tissue by determining transcript abundance of RDR2, POLIV-subunit NUCLEAR RNA POLYMERASE D 1a (NRDP1a), DNA-dependent-RNA-polymerase V complex (POLV)-subunit DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 3 (DRD3) and DCL3 by qPCR. None of the genes were induced in symptom types 1 or 2 and DRD3 and DCL3 transcript levels were only slightly higher in 3a (Fig. 2.5 B). In contrast, all four tested TGS genes were significantly up-regulated in recovered tissue (Fig. 2.5 B). Similar observations were made using cDNA from infected WT Col-0 plants (not shown). These results indicate that both TGS and PTGS pathways are induced in recovered tissue.

To investigate if the transcriptional induction of the silencing pathway observed in recovered leaves correlates with induced anti-viral defense, recovered leaves or leaves of similar size on mock-inoculated plants were inoculated with *Tobacco mosaic virus* (TMV). The inoculated leaves were harvested at four dpi and TMV RNA titers determined by qPCR. Although TMV could be detected in recovered leaves inoculated with TMV, viral titers were more than 200-fold higher in TMV-inoculated leaves of the mock plants (Fig. 2.5 C) and TMV titers in leaves harvested immediately after inoculation with virions were 15 fold higher (not shown). This indicates that one or more anti-viral pathways are induced in recovered leaves. Although the nucleotide sequences similarity between TMV and ORMV is low, stretches of

up to 17 nt with perfect match can be found, and the lower TMV levels in recovered leaves could be caused silencing mediate by ORMV-derived viral siRNAs.

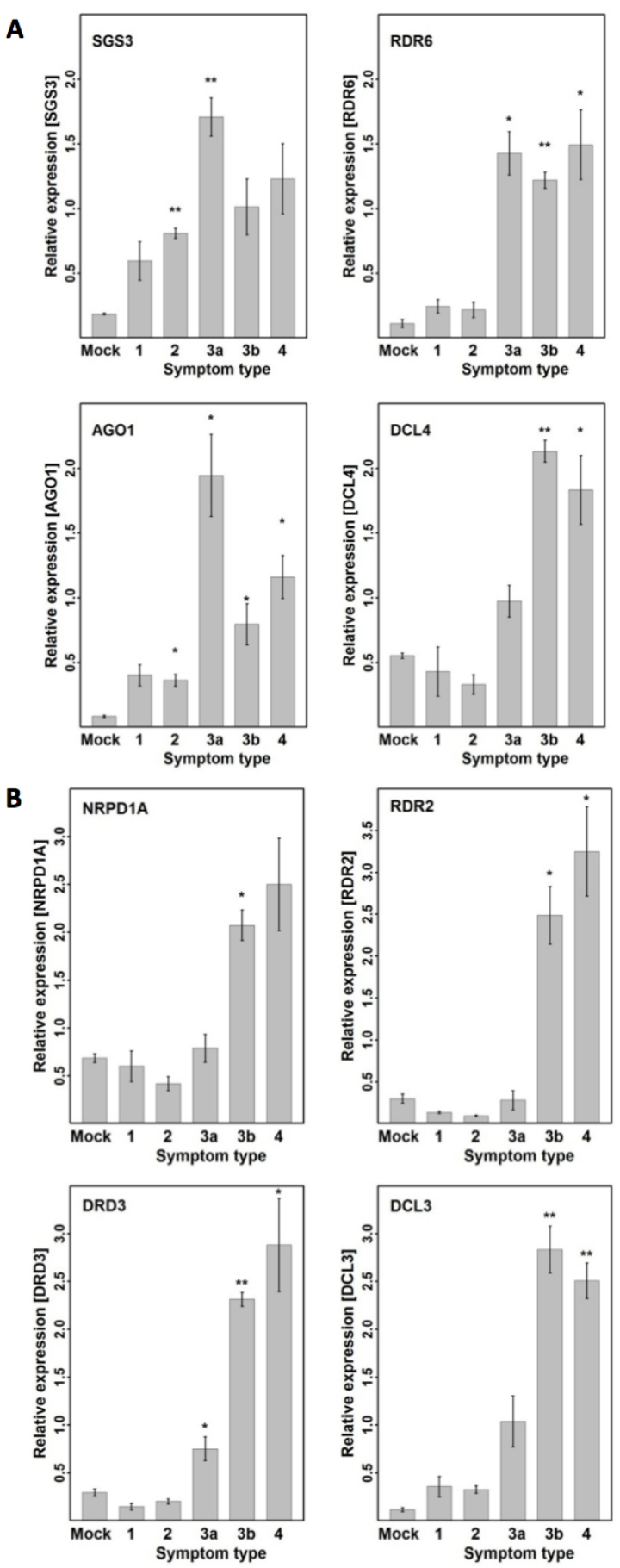

Figure 2.5 PTGS and TGS pathway genes are induced in recovered tissue. A & B) qPCR analysis of symptom-specific leaves (symptom categories as shown Fig. 2.2 B) of mock-treated or ORMV-infected L8Z2 plants at 28dpi, n=3, ±SE, Student's t-test, * p<0.05, **p<0.01. (A) Transcript levels of PTGS genes SGS3, RDR6, DCL4 and AGO1. (B) Transcript levels of TGS genes RDR2, NRPD1a, DRD3 and DCL3. C) qPCR analysis of TMV accumulation in leaves recovered from ORMV-infection and leaves of mock-inoculated plants, n=4, ±SE, Student's t-test, * p<0.05. Leaves were superinfected with TMV or mock-treated and harvested at four dpi.

2.4 Discussion

During compatible infections the defense mechanism of the host is successfully averted by the pathogen, as in the case of silencing and silencing suppression in plant-virus interactions. However, this balance of power may over time be tipped in the favor of the host leading to recovery. Such recovery events may be induced by environmental factors, plant development or presumably even reflect a viral strategy. In this study we present a host-virus interaction that displays the characteristics of recovery. Arabidopsis plants infected with the tobamovirus ORMV initially develop strong disease symptoms but at 23-25 dpi symptom-free leaves emerge. Recovered leaves still contain infectious virus but the lack of virus-derived siRNAs as well as viral silencing suppression activity leaves support the hypothesis that ORMV replication is reduced or absent in recovered leaves. Since plants were kept at the same light and temperature condition during the experiments, environmental factors can be ruled out as triggers for recovery onset in our system. The synchronal onset of recovery and the observation that older plants did not recover suggests a role of developmental age in the onset of recovery from ORMV disease. However, older plants also exhibited milder symptoms indicating lower viral titer, weak silencing suppressor activity or both, which could influence the onset of recovery.

The lack of recovery in siRNA biogenesis mutants shows that viral siRNAs are required for recovery. It seems unlikely that the absence of recovery in these mutants can be explained by higher viral levels suppressing the onset of recovery, as it has been reported that ORMV or closely related sub-group 3 tobamoviruses accumulate to WT levels in *rdr6-15*, *sde1*, *sgs2-1* and *sgs3-1* plants (Dalmay et al. 2000; Mourrain et al. 2000; Blevins et al. 2006). *dcl2,3,4* triple mutants, however, are more susceptible to ORMV (Blevins et al. 2006). Viral siRNA biogenesis is more likely needed for either triggering recovery by immunizing naïve tissue, to maintain the virus at lower levels in recovered tissue or both (Fig. 2.6). The observation that *rdr2-2* and several POLIV-subunit mutants do not recover supports the hypothesis that mobile viral siRNAs are needed for recovery. RDR2 and POLIV have been shown to be dispensable for the establishment of PTGS but important for maintenance and cell-to-cell movement of silencing independently of POLV (Dunoyer et al. 2005; Smith et al. 2007; Eamens et al. 2008). Grafting experiments in Arabidopsis investigating the perception of long-distance silencing signals found RDR2 and POLIV to be essential for establishing *de novo* transgene silencing in recipient scions (Brosnan et al. 2007). The visual phenotype of this system resembles that observed during recovery from ORMV, where the distal part may be symptomatic/not silenced while the proximal part is recovered/silenced. However, perception of the silencing signal was DCL3- and AGO4-dependent, which we did not find to be the case for the recovery phenotype. Short distance cell-to-cell spread of silencing is dependent on POLIV and RDR2, but seems to be independent of POLV and DCL3 (Dunoyer et al. 2007; Smith et al. 2007) as observed for recovery. In the above-mentioned transgene silencing systems, the produced siRNAs matched not only RNA but also DNA and it is possible that the perception of mobile siRNAs depends on amplification via homologous DNA and RDR2-POLIV. ORMV is a cytosolic RNA virus and would therefore be an unorthodox template for the nuclear POLIV/RDR2 complex. However, a direct role of POLIV and RDR2 in

viral siRNA biogenesis cannot be completely excluded, as it was recently shown that POLIV-RDR2 complexes can use RNA as template for dsRNA synthesis *in vitro* (Fig. 2.6) (Haag et al. 2012). Alternatively does ORMV contain short stretches (19-23 nt) with perfect or near-perfect matches to the Arabidopsis genome and viral siRNA with high similarity to host genes could trigger systemic amplification via RDR2-POLIV. A recent paper on the establishment of persistent infection with RNA viruses in Drosophila cell cultures showed that activation of retrotransposons leads to reverse transcription of viral RNA followed by genomic integration (Goic et al. 2013). Although we were not able to detect DNA with homology to ORMV by PCR in neither symptomatic nor recovered leaves (not shown), such a mechanism cannot be ruled out. A bioinformatic study has found evidence of widespread integration of fragments of RNA viruses into plant genomes (Chiba et al. 2011). Regardless of the origin of the template for the POLIV/RDR2-dependent dsRNA, the absence of recovery in *rdr2-2*, *nrpd1a-3*, *nrpd2a-1* and *drd2-4* shows that RDR2-derived dsRNA plays a unique role in recovery that cannot be replaced by RDR6-derived dsRNA (Fig. 2.6).

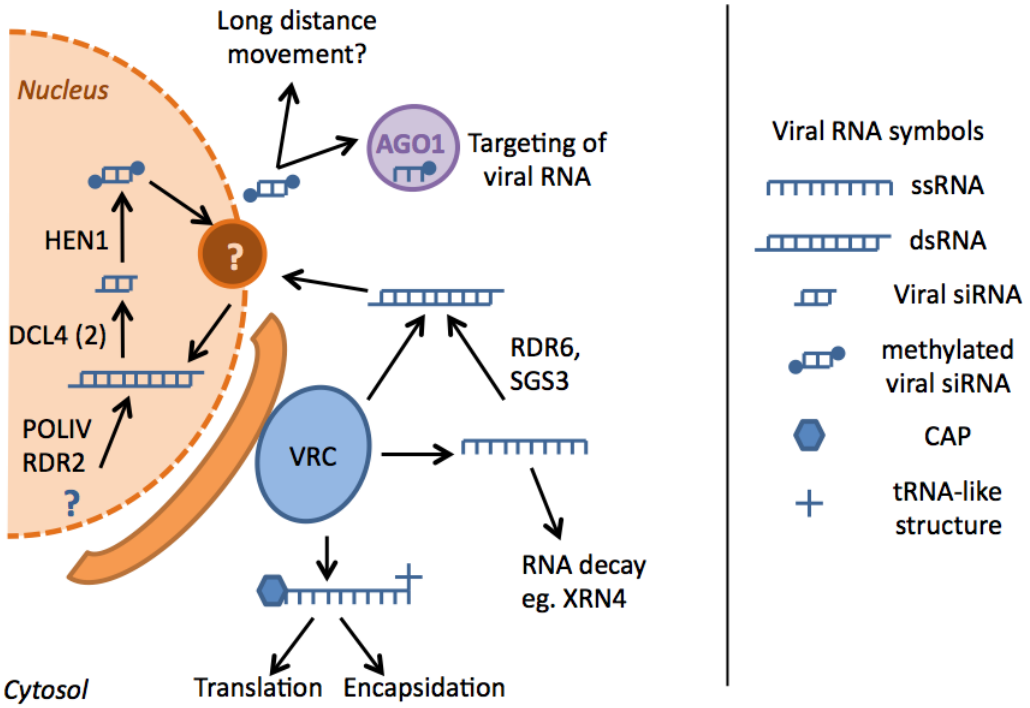

Figure 2.6 RNA-silencing components involved in recovery. Model of RNA-silencing components involved in recovery based on results from experiments with Arabidopsis mutants. Capped viral RNA from viral replication complexes (VRC) may be translated or encapsidated while abberant viral RNA may be degraded, most likely involving XRN4, or serve as template for dsRNA production by RDR6. Viral dsRNA is diced to viral siRNA by DCL4 in the nucleus, followed by methylation by HEN1. Methylated viral siRNAs are loaded into AGO1-containing RISC complexes or may potentially move between cells or long distance to program RISC complexes in naïve cells ahead of the spreading infection. Reduced degradation of aberrant viral RNA in XRN4 mutants enhances secondary viral siRNA production and recovery. POLIV and RDR2 are playing a distinctive role in recovery but the exact mechanism is unknown.

Once established, silencing can be stable, especially in the case where transgene hairpin constructs trigger silencing, but the strength of silencing can oscillate, as observed for some VIGS vectors (Hiriart et al. 2003), when the siRNA template itself is targeted by

silencing. A model for recovery, where induced silencing can act as the trigger, is supported by the mutants *ein5/xrn4* and *dcl3* showing early recovery. It has been speculated that enhanced silencing in *ein5* mutants is due to the availability of more template for RDR6 and thereby higher secondary siRNA production (Gazzani et al. 2004; Moreno et al. 2013); the loss of the early recovery phenotype in double mutants of RDR6 and EIN5 would support this hypothesis. The loss of the early recovery phenotype in double mutants of DCL3 and DCL4 and the normal recovery phenotypes of mutants downstream of DCL3 in the TGS pathway suggests that the involvement of DCL3 in recovery may be indirect. Early recovery in DCL3-mutant background could be explained by template competition between DCL3 and DCL4 (Deleris et al. 2006). However, very few viral DCL3-derived 24 nt viral siRNA are detected upon ORMV infection (Hu et al. 2011), thus questioning a direct competition for viral dsRNA during infection. Enhanced anti-viral silencing in DCL3 mutants could also be explained by a decrease in the level of DCL3-derived 24 nt siRNAs, which in turn could increase HEN1-capacity towards DCL4-derived siRNAs, including viral siRNAs (Yu et al. 2010).

Although silencing is believed to be important for anti-viral defense, only few Arabidopsis mutants impaired in silencing have been shown to be more susceptible to tobamoviruses (Dalmay et al. 2000; Mourrain et al. 2000; Blevins et al. 2006). This reflects the potency of the silencing suppressor to protect viral RNA from silencing during infection. However a shift in the balance between silencing and silencing suppression could lead to recovery. Such a shift could be caused by an imbalance between viral RNA levels and silencing suppression activity, allowing viral siRNAs to escape and immunize naïve tissue. However, it is also possible that a decrease in silencing suppression could be caused by an increase in viral encapsidation and leading to a decrease in translation of viral proteins including the silencing suppressor.

The establishment of a recovery system in the model plant Arabidopsis allowed the screening of recovery phenotypes in knock-out mutants from various defense pathways. This is a powerful tool to identify individual components or pathways needed for recovery, but in the case where mutants have lost their ability to recover, it cannot be distinguished if such mutants have lost the inducers or agents of recovery. More unbiased studies such as forward genetic screens or system biology experiments such as transcript- or proteomics could identify additional components involved in the onset of recovery.

2.5 Materials and Methods

Plant growth conditions and inoculation

Plants were grown in a Sanyo chamber (Panasonic, Japan) at 21 °C with 12 h/12 h light dark cycles. The fifth and sixth leaves of four week old plants were each rub-inoculated with 150 ng of virion or water, unless otherwise stated in the text. TVCV (pv-0361) and RMV (pv-0527) inocula was purchased from DSMZ (Braunschweig, Germany) and used for infection and virion isolation before use in described experiment. Emergence of recovered leaves was observed from 18 dpi and onwards. Pictures were taken at 28 dpi.

Western blotting

Total protein was isolated by extracting tissue ground in liquid N_2 in buffer with 100 mM TRIS, 150 mM NaCL, 10 percent glycerol, 0.04 percent NP-40 and Complete protease inhibitor (Roche, Switzerland). After centrifugation at 10.000 g for 10 minutes at 5 °C the supernatant was used for Bradford analysis (Biorad, USA). Extracts were diluted to 2 µg protein/µl in 2XLämmli sample buffer and denatured for 10 minutes at 95 °C. 30 µg of proteins were separated on 12 percent SDS-PAGE gels, followed by wet blotting to PVDF-membranes. ORMV CP was detected with polyclonal antibodies from DSMZ (Braunschweig, Germany) and GFP was detected with monoclonal antibodies from Roche (Switzerland) followed by HRP-conjugated secondary antibodies (Pierce, USA) and luminescence detection. After development membranes were stained with Coomassie as control for equal loading.

Quantitative reverse-transcriptase PCR

Mock-inoculated or virus-infected tissues were ground in liquid N_2 and total RNA was isolated with Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA quantities were determined spectrophotometrically with a Nanodrop (Thermo scientific, USA). 1-2 µg of RNA was reserve transcribed with random primers using High-capacity reverse transcriptase from Applied Biosystems (USA). Transcript abundance was measured by quantitative PCR using SYBR-green master mix (Roche, Switzerland) and gene specific primers (table S2) in a Lightcycler 480 (Roche, Switzerland). An internal standard was used for the calculation of relative expression. To take both developmental and virus infection effects into account three housekeeping genes (18S, AT1G13440 and AT4G26410) were used for normalization.

siRNA blotting

Total RNA was isolated as described above. 5 µg of RNA was separated on 12 percent Acrylamide UREA gels and transferred to Hybond N+ membranes (GE Healthcare, UK). GFP siRNAs were detected with a GFP-specific PCR fragment (tabe S2) labelled with $\alpha[^{32}P]$-dCTP. All other sRNAs were detected using short probes (table S2) end-labelled with $\gamma[^{32}P]$-dCTP. The membrane was hybridized over night at 45 °C (GFP)/37 °C in PerfectHyb Plus Hybridization Buffer (Sigma, USA) and washed five times in 2XSSC (300 mM NaCl, 30 mM $Na_3C_6H_5O_7$, pH 7) 0.5 percent SDS at 45 °C/ 37 °C. The membrane was exposed over night on a phosphorimaging screen (GE Healthcare, UK) and scanned with a Typhoon FLA 7000 phosphoimager (GE Healthcare, UK).

in situ hybridization

Leaves were fixed overnight at 4 °C in a FAA solution (formaldehyde 3.2 percent, acetic acid 5 percent, ethanol 50 percent, v/v), dehydrated gradually in 50, 70, 96 and 100 percent ethanol and infiltrated with paraplast/histoclear (v/v) solution (VWR International Labonord, France). Tissues were embedded in paraplast and sections (15 µm) obtained with a Leica microtome. Sections were mounted onto a silane (3-aminopropylsilane) coated microscopic slides and paraplast removed by immersion of the slides into histoclear solution. Sections were rehydrated trough 100, 96, 70, 50 and 30 percent ethanol solutions and distilled water. Prior to hybridization, sections were treated with protease K solution (1 µg/ml)(Sigma, USA) in TE buffer (20 mM Tris–HCl, 5 mM EDTA, pH 7.5) for 15 minutes at 37 °C, post-fixed with PFA 4 percent, dehydrated through an ethanol series and then air dried for 2 h. Probes were synthesized from PCR products obtained with the ORMV_insitu primers (table s2) amplifying 250 nt corresponding to the CP region using T7 RNA Pol and ROCHE Dig-RNA labeling mix (Roche, Switzerland). Sections were hybridized overnight at 42 °C with a denatured digoxigenin-labeled probe (0.5 µg/ml) diluted in the hybridization buffer (50 percent formamide desioniseed (v/v), 1xDenhardt's solution, 10 percent dextran sulfate, SSCx4, dextran sulfate 0.1 g/ml, tRNA 100 µg/ml, polyA µg/ml). Tissue sections were washed at 42 °C in 2×SSC, 2xSSC + 0.1 percent SDS, and 0.2xSSC. Tissues were saturated with blocking reagent 0.5 percent (Roche Diagnostics, Switzerland) diluted in buffer A (100 mM TrisHCl, 150 mM NaCl, pH 7.5) and incubated overnight at 4 °C with anti-DIG-antibodies-AP (dilution 1:1000) (Roche Diagnostics, Switzerland) in buffer A with BSA 1 percent (w/v), 0.1 percent TritonX-100 (v/v). After washing with buffer A, DIG were detected by incubating sections with NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt) (Roche Diagnostics, Switzerland) in 100 mM Tris–HCl (pH 9.5), NaCl 100 mM, $MgCl_2$ 50 mM. Progression of the color reaction was followed with a microscope and the reaction stopped with cold TE buffer (5 minutes) and washed with water. Slides were mounted with a mounting-media (glycerol 30 percent, TEx1) for microscopy analysis.

Virion isolation

Virions were isolated as described previously (Niehl et al. 2012). Virion isolation was used for SDS-PAGE analysis as described for Western blotting, followed by coomassie staining.

Statistical analysis

Changes in transcript abundance were analyzed by Student's t-test in Excell (Microsoft, USA). All other statistical analyzes were done in R (www-r-project.org).

2.6 Acknowledgements

We would like to thank Etienne Bucher, Patrice Dunoyer and Olivier Voinnet for helpful discussions and suggestions. Furthermore we would like to thank Martin di Donato, Annette Niehl, Khalid Amari and David Windels for technical assistance, discussions and critical comments. Funding was gratefully received from the Swiss National Science Foundation (SNF, grant 124940 and 144084 to MH, grant 126329 to FV).

2.7 Supplementary material

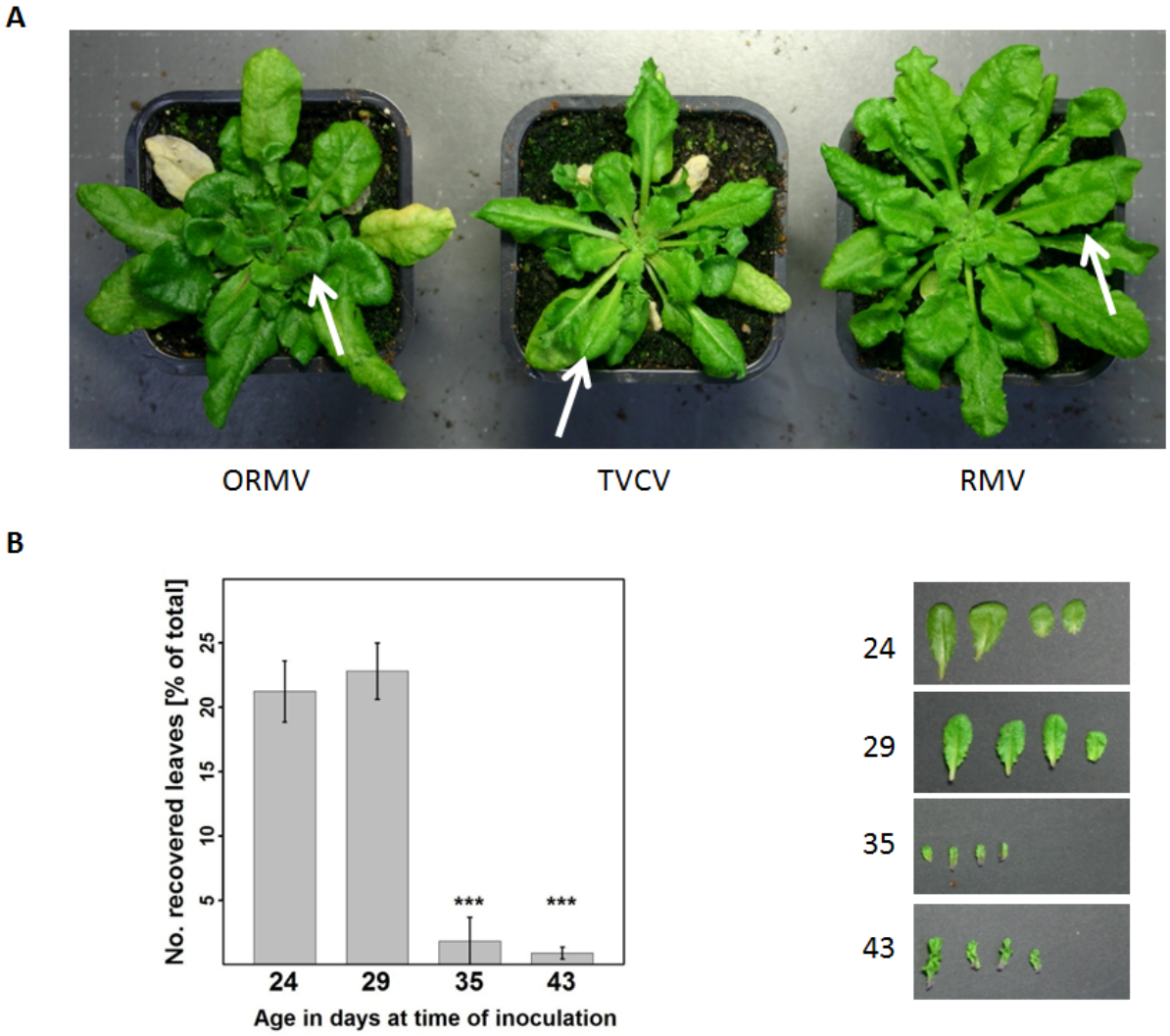

Figure S2.1 Recovery phenotype is conserved among subgroup 3 tobamoviruses but influenced by host age. A) Recovery of plants infected with *Oliseed rape mosaic virus* (ORMV), *Turnip vein clearing virus* (TVCV) or *Ribgrass mosaic virus* (RMV). Recovered leaves indicated with white arrows. Recovery from infection in Arabidopsis plants is observed for all three subgroup 3 tobamoviruses. B) 24, 29, 35 or 45 days old Arabidopsis plants were inoculated with ORMV virions. The number of recovered leaves at 28 dpi is presented as percent of total number of leaves; n= 7-10 ±SE, binomial GLM, *** $p<0.001$ compared to plants infected at the age of 29 days. The figure on the right shows the four youngest leaves at 28 dpi. The numbers indicate plant age in days at the time of inoculation.

Figure S2.2 ORMV does not suppress hairpin RNAi in recovered leaves. Leaves of ORMV-infected Suc:Sul plants at 28 dpi. Disease symptoms, silencing suppression and recovery phenotype of these plants mimics the phenotypes seen with the PTGS reporter line L8Z2 (Figure 2.2). The lower leaves are infected but do not show symptoms and no silencing suppression, i.e. vein bleaching (1), other leaves (stages 2 & 3) are symptomatic and show strong silencing suppression and loss of vein bleaching,while upper leaves (stage 4) show recovery and reappearance of vein bleaching indicating loss of silencing suppression in recovered leaves.

Table S2.1 Recovery phenotypes in hormone signaling mutants

Timing	ATG number	Mutant	Gene function
Early	AT1G54490	*ein5-1*	Ethylene signaling/ RNA decay
		xrn4-3	
Normal	AT5G03280	*ein2-1*	Ethylene signaling
	AT3G20770	*ein3-1*	
	AT3G51770	*eto1-1*	
	AT3G52430	*pad4-1*	SA-defense
	AT1G64280	*npr1-1*	
	-	*NahG*	
	AT4G33430	*bak1-4*	Innate immunity/ brassinosteroid
	AT1G30330	*arf6-1*	Auxin signaling
	AT5G37020	*arf8-2*	
	AT1G05180	*axr1-3*	
	AT3G62980	*tir1-10*	
	AT5G42650	*aos*	JA biosynthesis

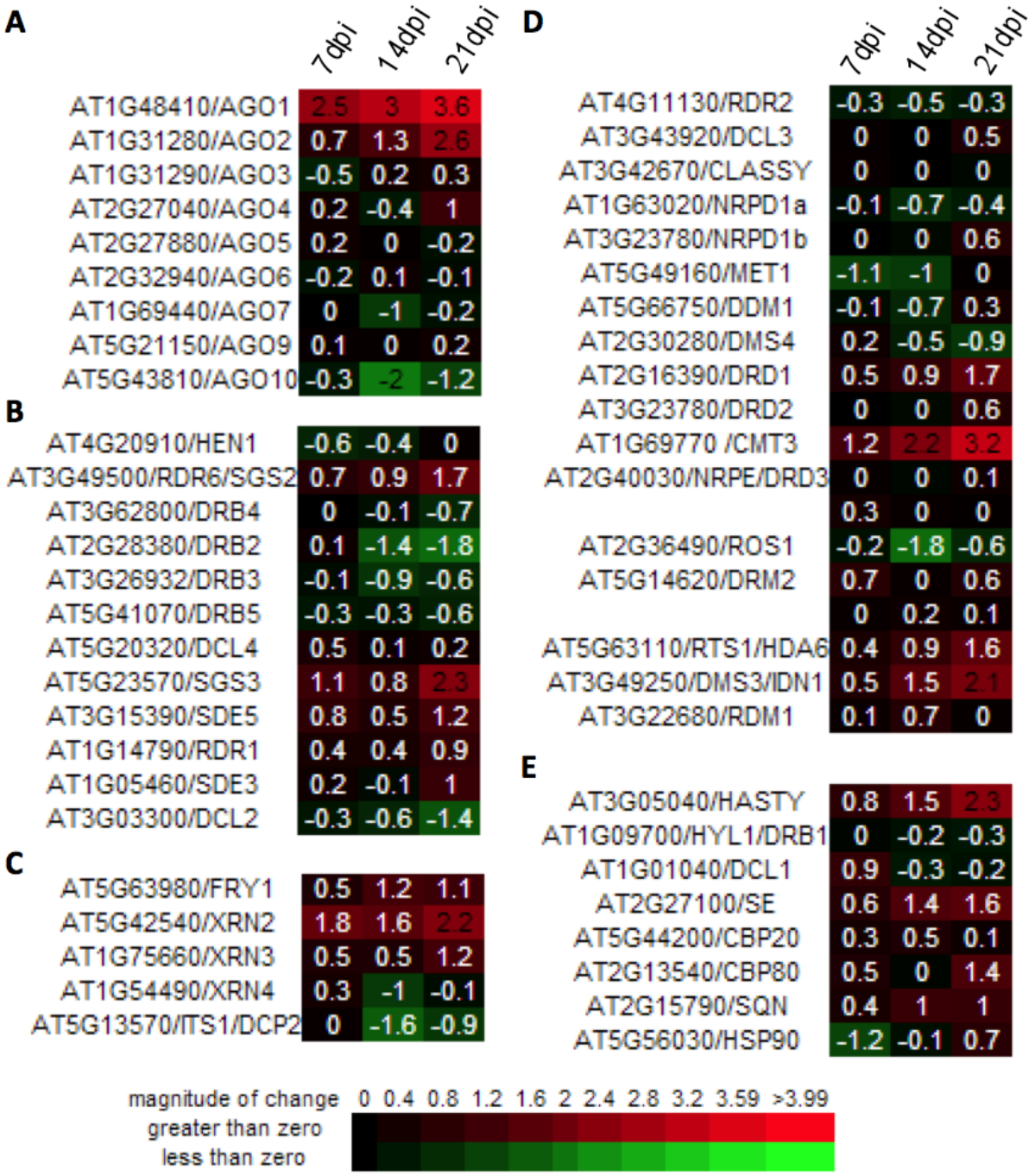

Figure S2.3 Changes in gene expression of genes involved in silencing during ORMV infection in Arabidopsis. A) AGO genes, B) siRNA biogenesis genes, C) genes encoding silencing antagonists, D) genes involved in TGS, E) miRNA biogenesis genes. Microarray data from Hu et al. (2011).

Table S2.2 Primers used in this study

	Name	ATG	Fwd	Rvs
qpcr	GAPDH	At1g13440	TTGGTGACAACAGGTCAAGCA	AAACTTGTCGCTCAATGCAATC
	EXP	At4g26410	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
	18S	At3g41768	CGTCCCTGCCCTTTGTACAC	CGAACACTTCACCGGATCATT
	SGS3	At5g23570	TTGGCAGGCAAGAGGAGGTTC	GCAAGGTCGTCATCAGAATCATCC
	RDR6	At3g49500	TCAGAAACTCATCCCTCCCAACAG	AATTGTGCCCAACTGCTCATTCG
	DCL4	At5g20320	TTTGACCATGCCGTACAGTGAAATC	AGAGGAAGAAGAAGTGAGGTGAAGG
	AGO1	At1g48410	AAGGAGGTCGAGGAGGGTATG	CAAATTGCTGAGCCAGAACAGTAG
	DLC3	At3g43920	TTGAAGTATGTTGCGAGCTGTCATC	CGATTGTCTCTGCCGTGATAGTTG
	RDR2	At4g31130	CTGATGGTGCTGATTACTGCTTCTC	TCGGAAGGAACTGCGGTCAAC
	NRPE/DRD3	At2g40030	ACCAAGATGCCACACCACCTG	GACTGAGCCTGAGCCTGAGATG
	NRPD1a	At1g63020	TGGATGGAGACACTGTGCTGATG	AAATCACCACGGAACGGCAAAC
	ORMV CP	-	CAAGCGGCAAGAGATACTGTTAGAC	GCTGGTTCGGTGTCACAATCG
	TMV	-	ATCTCAGTTCGTGTTCTTG	TCTAATACCGCATTGTACC
	GFP 5	-	GCCCGACAACCACTACCTGAG	GGCGGCGGTCACGAACTC
Probe	ORMV_siRNA	-	AATTGGTGCTCGTTGATGGTGTTC	TCTACCGTCCTCACATTGTCCTTG
	GFP_siRNA	-	CACATGAAGCAGCACGACTT	TGCTCAGGTAGTGGTTGTCG
	ORMV_insitu	-	CATCACGAGCTCGAATCAGT	TAATACGACTCACTATAGGG GGACTTCATGAGAGACTCGT
	U6	-	GGCCATGCTAATCTTCTCTGTATCGTT	CCAATTTTATCGGATGTCCCCGAAGGGAC
	miRNA398*	-	TGTTCACATGCCACTCCTTTG	
	miRNA173	-	GTGATTTCTCTCTGCAAGCGAA	

3 The Immunity Regulator BAK1 Contributes to Resistance against Diverse RNA Viruses

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3.1 Abstract

The plant innate immune system detects potential biotic threats through recognition of microbe-associated molecular patterns (MAMPs), or damage-associated molecular patterns (DAMPs), by pattern recognition receptors (PRRs). A central regulator of pattern-triggered immunity (PTI) is the BRI1 associated kinase 1 (BAK1) which undergoes complex formation with PRRs upon ligand binding. Although viral patterns inducing PTI are well known from animal systems, nothing similar has been reported for plants. Anti-viral defense in plants is rather thought to be mediated by posttranscriptional gene silencing of viral RNA, or through effector-triggered immunity, i.e. recognition of virus-specific "effectors" by resistance proteins. Nevertheless, infection by compatible viruses can also lead to the induction of defense gene expression, indicating that plants may also recognize viruses through PTI. Here we show that PTI, or at least the presence of the regulator BAK1, is important for anti-viral defense of Arabidopsis plants. Arabidopsis *bak1* mutants show increased susceptibility to three different RNA viruses during compatible interactions. Furthermore, crude viral extracts, but not purified virions, induce several PTI marker responses in a BAK1-dependent manner. Overall, we conclude that BAK1-dependent PTI contributes to anti-viral resistance in plants.

3.2 Introduction

Both plants and animals perceive microbe-associated molecular patterns (MAMPs) as a first line of defense against potential microbial pathogens (Janeway and Medzhitov 2002; Zipfel and Felix 2005; Boller and Felix 2009). Such MAMPs are broadly conserved molecular microbial structures essential for the microbial life style, and absent from the host. Upon MAMP perception by pattern recognition receptors (PRRs), a signaling cascade is initiated, which ultimately leads to PTI, i.e. "pattern-triggered immunity" (Janeway and Medzhitov 2002; Boller and Felix 2009).

PTI is well studied in animals and active against a wide range of different pathogens, including viruses. The best characterized PRRs in animals are Toll-like receptors (TLRs), which recognize a wide range of MAMPs, including viral RNA and DNA (Song and Lee 2012). In order to permit efficient detection of extracellular and intracellular microbes, animal TLRs are either localized to the plasma membrane or associated with the endomembrane system (Jensen and Thomsen 2012). As viruses are obligate intracellular pathogens, they are predominately recognized by intracellular receptors, such as TLR3, TLR7 and TLR8 for viral RNA and TLR9 for viral DNA (Rathinam and Fitzgerald 2011; Jensen and Thomsen 2012).
In contrast to anti-viral innate immunity in animals, no similar mechanism has been described in plants. In contrast, anti-viral defense strategies in plants are mediated by recognition and subsequent degradation of viral genomic RNA or replication intermediates by the host post-transcriptional gene silencing machinery, leading to the generation of viral small interference RNAs (vsRNA) (Ruiz-Ferrer and Voinnet 2009). The production of vsRNAs leads to sequence specific degradation or translational inhibition of viral target RNAs (Ding 2010). However, whether or not viral RNA is directly recognized by innate immune receptors in plants such as the case in animal systems is still unknown.

Although a role for PTI in the plants' defense against viruses has not been studied, PTI is well examined in respect to microbes and known to significantly contribute to microbial resistance. The molecular mechanisms underlying PTI in plants share a number of features known from MAMP perception systems in animals (Boller and Felix 2009). Today, several MAMPs from fungi, oomycetes and bacteria, as well as a number of plant PRRs involved in their perception are known. All known PRRs of plants appear to be plasma membrane proteins with an extracellular receptor domain and either an intracellular kinase domain (receptor-like kinases, RLK) or without a structured intracellular domain (receptor-like proteins, RLP). Interestingly, a number of these RLKs and RLPs have been shown or are believed to interact with the LRR-RLK BAK1 (for BRI1-associated kinase 1) upon ligand binding (Chinchilla et al. 2007; Heese et al. 2007; Schulze et al. 2010). This interaction likely leads to cross phosphorylation between the two kinases and subsequent activation of downstream signaling (Schulze et al. 2010; Schwessinger et al. 2011). As BAK1 interacts with several PRRs, it is regarded as a general regulator of plant immunity (Chinchilla et al. 2007; Heese et al. 2007; Chinchilla et al. 2009).

Plants also have surveillance systems to monitor cell integrity in a mechanistically similar manner to PTI. Wounding of Arabidopsis plants, for example, is believed to induce the production of *At*PEPs, small peptides that are relatively poorly conserved amongst

higher plants and derived from longer peptides called *ProPEPs* (Wasternack et al. 2006; Huffaker and Ryan 2007; Krol et al. 2010; Yamaguchi and Huffaker 2011). Similarly to many MAMPs, *At*Peps are perceived by PRRs, namely Pep-Receptor 1 (PEPR1) and PEPR2 (Krol et al. 2010). Interestingly, PEPR1 and PEPR2 also interact with BAK1 upon *At*Pep recognition, which leads to the induction of a stereotypical defense response reminiscent of PTI (Boller and Felix 2009; Krol et al. 2010). Thus, BAK1 is a central player in different aspects of immunity, including classical PTI as well as Damage-associated molecular patterns (DAMP) signaling and thus constitutes an ideal molecular tool to fish for novel components of immunity.

Given the similarities between plant and animal innate immune systems, we hypothesized that plant viruses also induce PTI by yet unknown mechanisms. This hypothesis is supported by the induction of gene expression associated with PTI and salicylic acid signaling during compatible viral infections (Whitham et al. 2003; Love et al. 2005; Carr et al. 2010; Hanssen et al. 2011). To test this hypothesis we infected a range of PTI signaling mutants with three different RNA viruses and found that plants mutated for the common regulator of PTI, BAK1, exhibited increased susceptibility to virus infection. This suggested that viral elicitors, or DAMPs produced in response to the virus, were recognized in a BAK1-dependent manner and that specific recognition of these molecular patterns contributed to resistance against these viruses. Consistently, we found that extracts of infected plants induced PTI responses in Arabidopsis, such as MAPK activation, increased production of the plant hormone ethylene or root growth inhibition in a BAK1-dependent manner when applied to healthy plants. In conclusion, we show that BAK1 is involved in anti-viral defenses in plants and provide evidence that virus encoded MAMPs or virus-induced DAMPs elicit classical PTI responses.

3.3 Results

Components of DAMP-signaling are transcriptional up-regulated during virus infections

Analysis of publicly available microarray data (https://www.genevestigator.com/gv/; (Whitham et al. 2003; Love et al. 2005; Carr et al. 2010; Hanssen et al. 2011)) and previously published microarrays on cDNA derived from tissue of Arabidopsis plants infected with tobamorvirus *Oilseed rape mosaic virus* (ORMV) (Hu et al. 2011) revealed that genes related to innate immunity exhibited modulated expression during compatible virus infections, and that these gene expression changes increased over time (Fig. 3.1, Fig. S3.1).

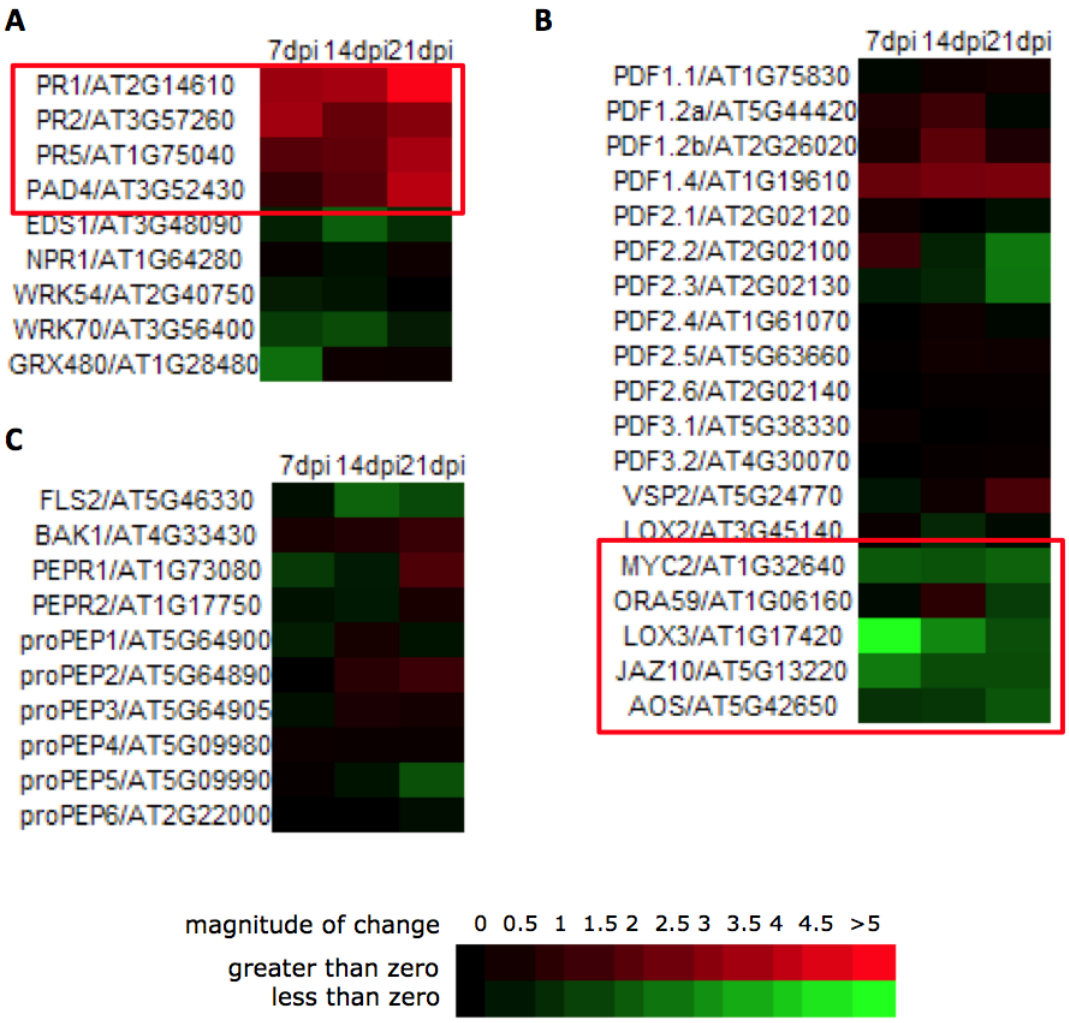

Figure 3.1 Expression of SA-. JA- and PTI-pathway genes upon ORMV infection. Heat maps show $\log_2$ expression levels of genes involved in defense responses via SA (A), JA (B) or PTI (C) at 7, 14, 21 dpi. Microarray data from Hu et al. (2011).

Interestingly, also *PEPR1* and *ProPEP2* were induced. Hence, we speculated that compatible virus infection could also interfere with DAMP signaling. To test whether DAMP-signaling was indeed induced upon virus infection, we infected Arabidopsis wildtype (WT) Col-0 plants with purified virions of three different RNA viruses, namely the tobamoviruses *Tobacco mosaic virus* (TMV) strain U1 and ORMV, and the tombusvirus *Turnip crinkle virus* (TCV). TCV and ORMV both cause strong symptoms in Arabidopsis plants, while TMV causes only very

mild symptoms in Arabidopsis (Dardick et al. 2000). The transcripts of the precursor proteins *AtproPEP2* and *AtproPEP3*, which are known to be induced by wounding, biotic stress and MAMP signaling (Huffaker and Ryan 2007), were strongly up-regulated by all three RNA viruses in systemic infected Arabidopsis leaf tissue compared to mock treated tissue (Fig. 3.2 A). To define the potential involvement of PEP signaling in anti-viral defenses more closely, we also analyzed the expression of both *PEPR1* and *PEPR2*. Interestingly, infection with TMV which causes only mild symptoms on Arabidopsis, significantly induced *PEPR1* and *PEPR2* at 21 days post inoculation (dpi), while the slight enhancement observed for TCV and ORMV infections was statistically not significant (Fig. 3.2 B and 3.2 C). Overall, these results indicate that components of the danger signaling pathway can be induced by RNA virus infections.

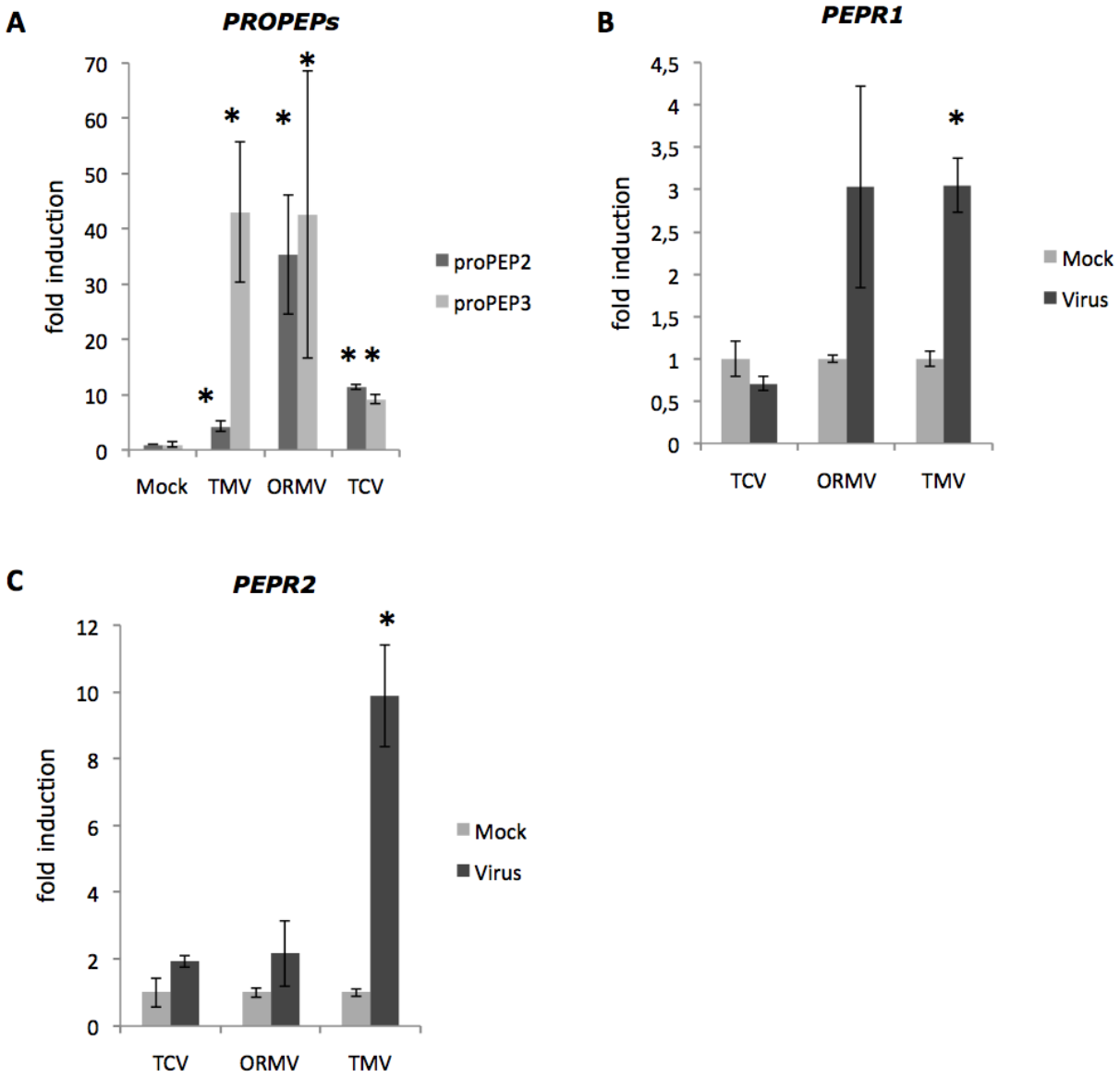

Figure 3.2 Components of PRR-triggered immunity are induced during viral infection. qPCR analysis of components of PRR-triggered immunity. cDNA was generated from tissue of Arabidopsis Col-0 plants infected with the respective virus harvested at 21 dpi, n=3, ±SEM, Student's t-test, * $p<0.05$. Fold changes upon virus infection of A) *PROPEP2* and *PROPEP3*, B) *PEPR1* and C) *PEPR2*.

bak1-mutants are more susceptible to RNA viruses

To investigate a potential role of *At*PEP signaling in anti-viral defenses we infected WT, the *pepr1/2* double mutant as well as mutants of BAK1, a common regulator of PTI responses and interactor of PEPR1/2 upon ligand binding, with TCV sap inoculations (Fig. S3.4 A). We chose two different BAK1 mutants, namely the knockout mutant *bak1-4* (Chinchilla et al., 2007; Heese et al., 2007) and the single amino acid mutant *bak1-5* (Chinchilla et al. 2007; Heese et al. 2007; Schwessinger et al. 2011), which is impaired in PTI signaling but functions normally in brassinosteroid (BR)-signaling. We also included the flg22-specific MAMP receptor mutant *fls2* as a negative control, since an involvement of FLAGELLIN-SENSITIVE 2 (FLS2) in anti-viral defenses seems unlikely (Gomez-Gomez and Boller 2000). At 21 dpi, TCV infection causes growth retardation and altered leaf morphology with rounder and slightly crinkled leaves in Col-0 WT, while the petiole length remains largely unaltered (Fig. 3.3 A). TCV infection of *pepr1/2* and *fls2* mutants produced similar symptoms as observed in the Col-0 WT suggesting that neither *pepr1/2* nor *fls2* mutant plants exhibit increased susceptibility to TCV (Fig. S3.2 B). Interestingly, disease symptoms such as growth retardation and altered leaf morphology were strongly enhanced in the *bak1-4* and *bak1-5* mutants upon TCV infection, suggesting that BAK1-dependent DAMP- and/or MAMP- signaling may be involved in anti-viral defense. Consistent with the assumption that disease symptoms would represent a measure for susceptibility, we observed a correlation between disease symptoms and virus accumulation. There was a tendency towards increased accumulation of viral coat protein (CP) in *bak1* mutants (Fig. 3.3 B), and a clearly increased accumulation of viral RNA in *bak1* mutants, compared to Col-0 WT, *pepr1/2* and *fls2* (Fig. 3.3 C). Together, these results demonstrate that *bak1* mutants are hypersusceptible to TCV, while *pepr1/2* or *fls2* mutants exhibit similar susceptibility to TCV as WT plants, suggesting that AtPEP signaling is not sufficient to dampen infections by this virus.

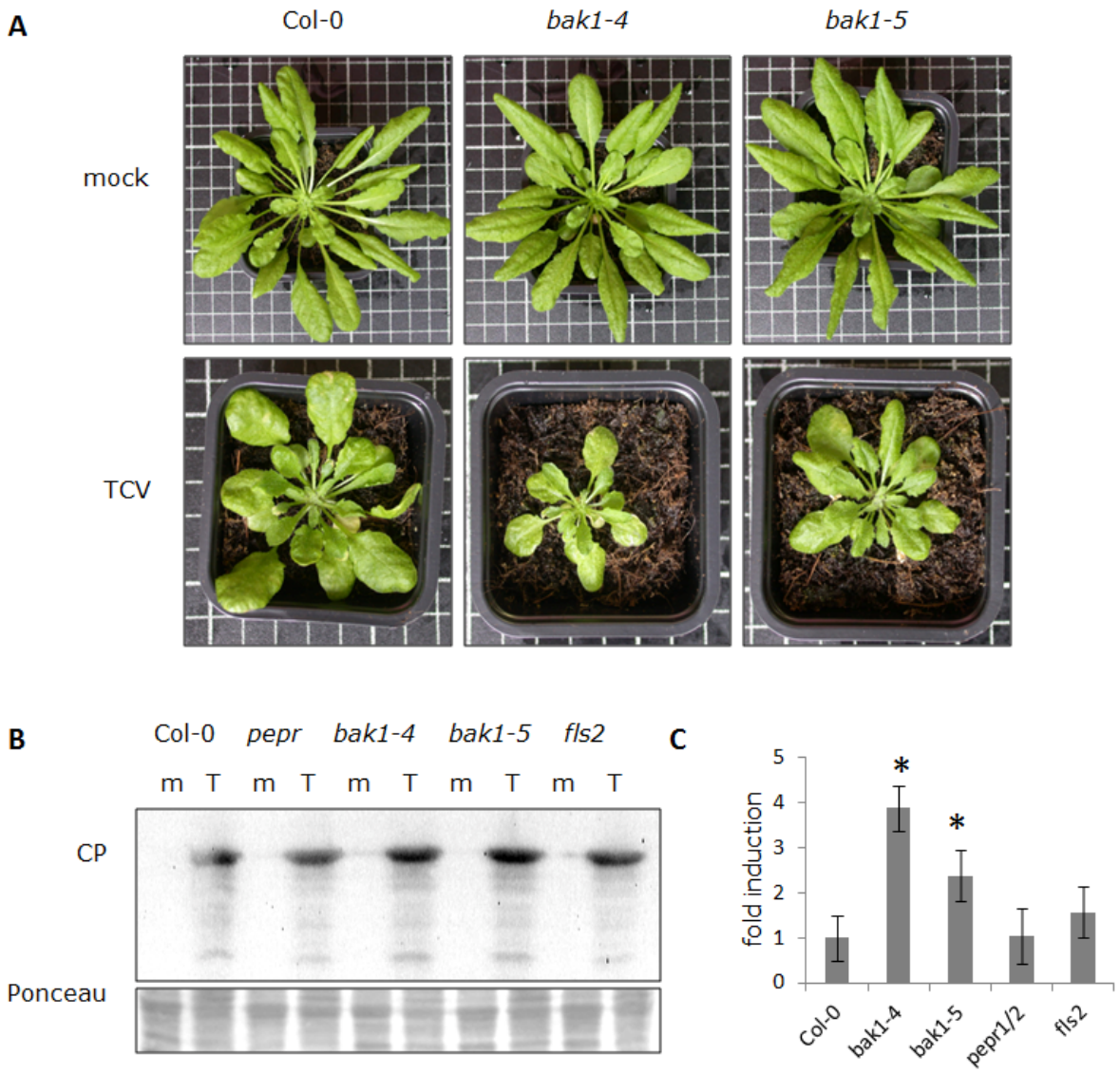

Figure 3.3 Characterization of TCV infections at 21 dpi in Col-0 and *bak1* mutant Arabidopsis lines on symptom and viral titer level. A) Col-0 WT, the *bak1-4* knockout and the *bak1-5* point mutation mutants were infected with TCV crude extract and symptoms were scored 21 dpi. The pictures present the average phenotype observed in the different lines. B) Accumulation of the TCV CP detected by anti-CP antibodies (DSMZ) on Western blots loaded with leaf tissue extracts derived from the mock (m) and TCV (T) infected lines shown above and in and supplementary figure (S3.2). Ponceau S staining of total protein is shown as control for equal loading. C) qPCR analysis of TCV CP accumulation in the infected lines shown in A and S3.2, n=3, ±SEM, Student's t-test, * $p<0.05$.

To investigate whether the increased susceptibility of *bak1* mutants was specific for TCV or whether *bak1* mutants were generally more susceptible to virus infection, we analyzed virus-induced symptoms and virus accumulation of the tobamovirus ORMV, which is well adapted to Arabidopsis as indicated by strong viral replication and symptom development. Infection of WT plants with ORMV led to growth retardation, severely curled and serrated leaves with shortened petioles and compact rosette formation. These symptoms developed similarly in WT as well as in *fls2* and *pepr1/2* mutants (Fig. S3.3), but were again enhanced in *bak1-4* and *bak1-5* mutants (Fig. 3.4A). Enhanced symptom

development was paralleled by increased virus accumulation in *bak1* mutants compared to the widltype; we found a tendency towards higher levels of ORMV CP (Fig. 3.4 B) and a two-fold increase in ORMV RNA in *bak1* mutant plants compared to WT, *fls2* and *pepr1/2* mutants (Fig. 3.4 C). Taken together, the *bak1* mutants were not only more susceptible to TCV but also to ORMV infection.

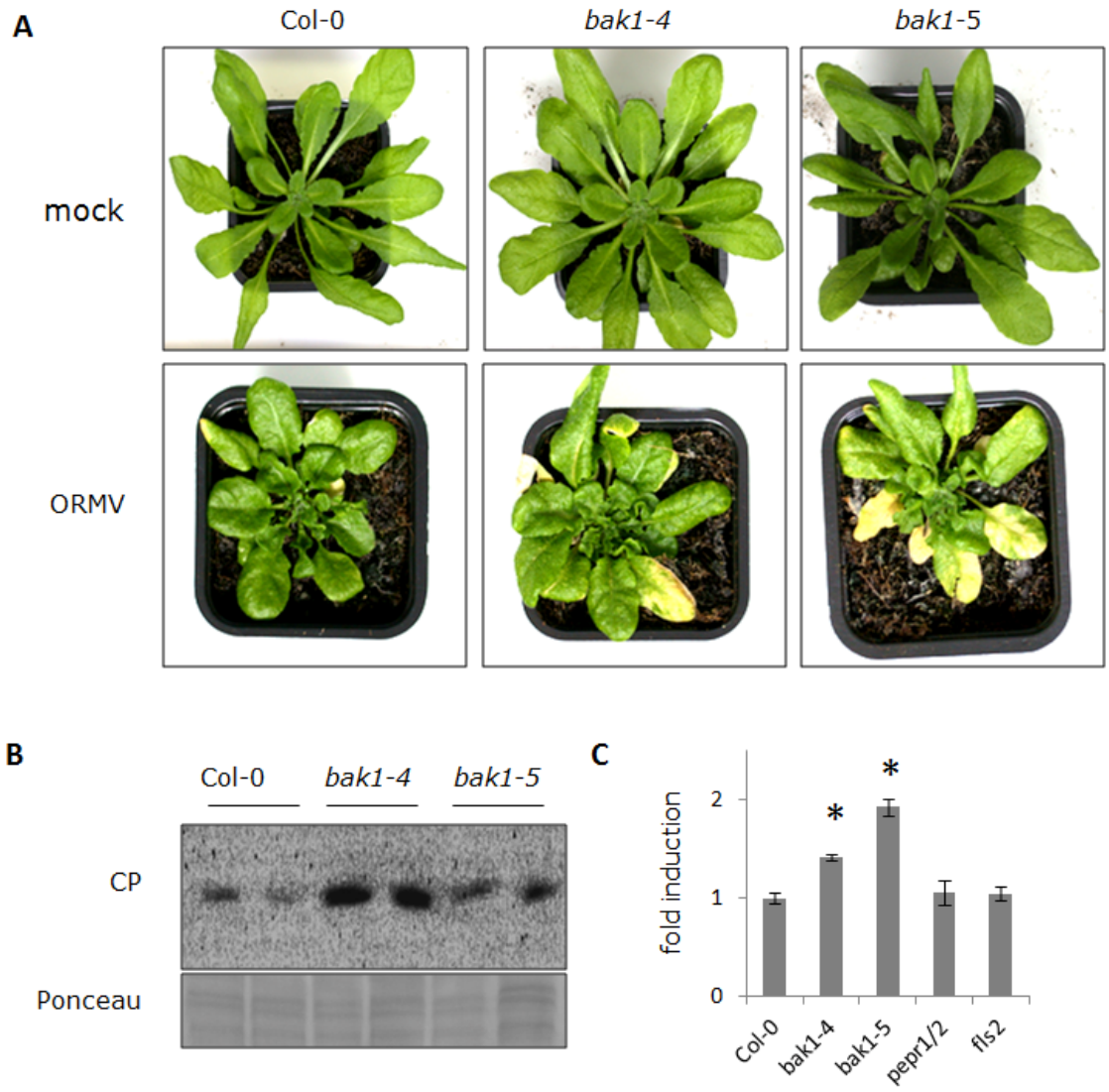

Figure 3.4 Characterization of ORMV infections at 21 dpi in Col-0 and *bak1* mutant Arabidopsis lines on symptom and viral titer level. A) Col-0 WT, *bak1-4* knockout and *bak1-5* point mutation mutants were infected with ORMV virions and symptoms were scored 21 dpi. The pictures present the average phenotype observed in the different lines. B) Accumulation of the ORMV CP detected by anti-CP antibodies (DSMZ) on Western blots of leaf tissue extracts from the infected lines shown in A. The samples were loaded as duplicates. Ponceau S staining of total protein is shown as control for equal loading. C) qPCR analysis of ORMV RNA accumulation in the infected lines shown in A and S3.3, n=3, ±SEM, Student's t-test, * $p<0.05$.

Arabidopsis Col-0 plants infected with TCV or ORMV display apparent viral symptoms (Dempsey et al. 1997; Cai et al. 2009). In contrast, TMV infection of Arabidopsis Col-0 plants causes only mild symptoms, including shortened petioles and an overall more compact rosette (Fig. 3.5 A) (Dardick et al. 2000; Pereda et al. 2000; Serrano et al. 2008). Given the

strong symptom formation in WT plants upon ORMV and TCV infections, we included the symptomatically weaker TMV in our assays. Similar to the results obtained with ORMV and TCV, TMV-associated symptoms were also enhanced in *bak1* mutants characterized by an even more compact rosette than in virus infected Col-0 plants and altered leaf morphology upon infection (Fig. 3.5 A), while *fls2* and *pepr1/2* mutants displayed similar infection symptoms as the WT control (Fig. S3.3). Consistent with the observation obtained by TCV and ORMV infection that stronger disease symptoms correlate with higher virus titers, the TMV CP appeared slightly more abundant while the genomic RNA accumulated to significantly higher levels in the *bak1* mutants compared to WT or *fls2* and *pepr1/2* mutants (Fig. 3.5 B and C).

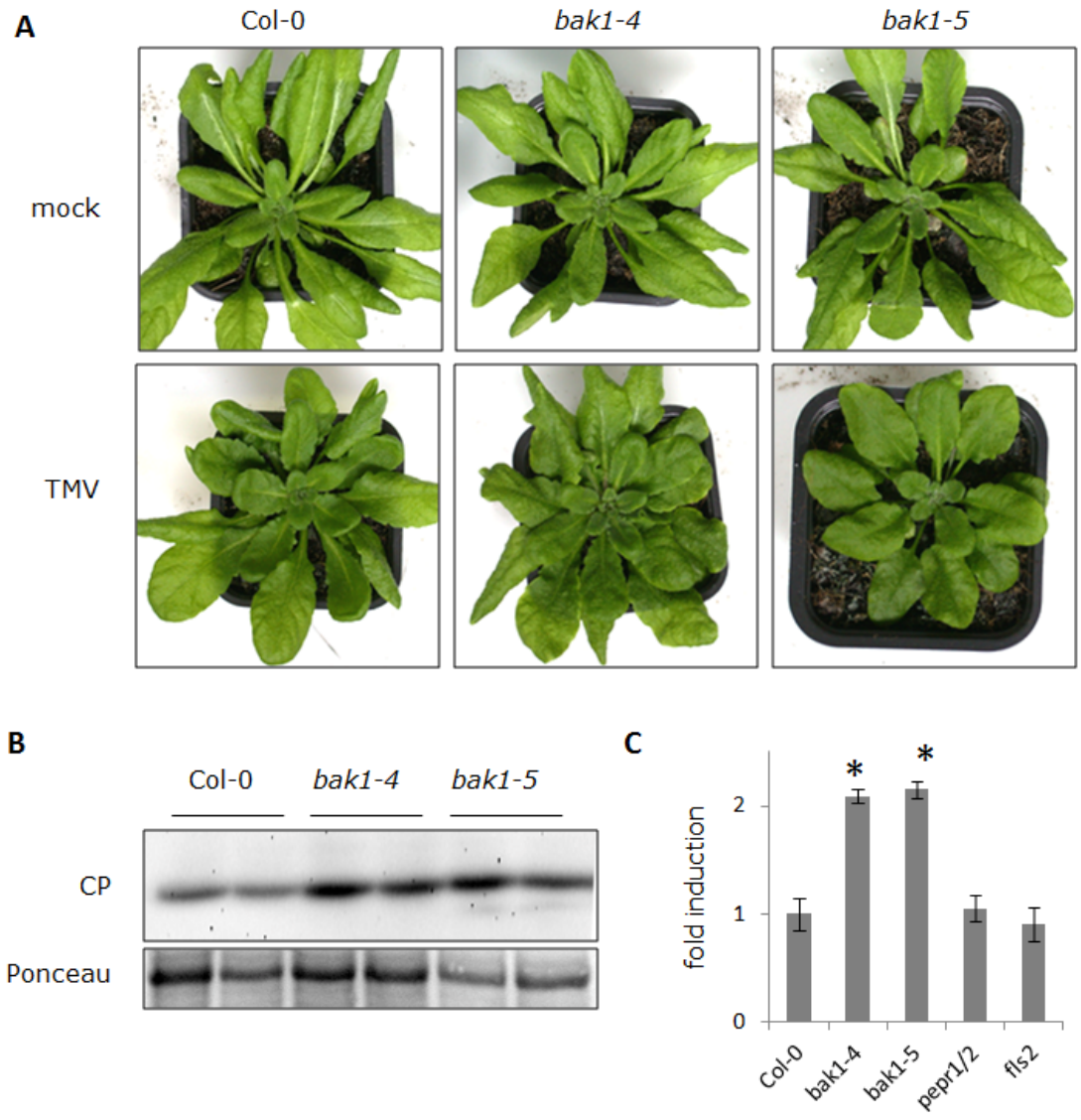

Figure 3.5 Characterization of TMV infections at 21 dpi in Col-0 and *bak1* mutant Arabidopsis lines on symptom and viral titer level. A) Col-0 WT, *bak1-4* knockout and the *bak1-5* point mutation mutants were infected with TMV virions and symptoms were scored 21 dpi. The pictures present the average phenotype observed in the different lines. B) Accumulation of the TMV CP detected by anti-CP antibodies (DSMZ) on Western blots of leaf tissue extracts derived from the infected lines shown in A. The samples were loaded as duplicates. Ponceau S staining of total protein is shown as control for equal loading. C) qPCR analysis of TMV RNA accumulation in the infected lines shown in A, n=3, ±SEM, Student's t-test, * $p<0.05$.

Taken together, our results suggest that a BAK1-dependent pathway is important in the plants' defenses against viruses. By contrast, although induction of *AtproPEPs* and PEPR suggested that PEP signaling might be involved in anti-viral defense, unaffected virus titers and symptoms in *pepr1x2* mutants demonstrate that PEP signaling alone is not sufficient to restrict virus infection in Arabidopsis.

Crude extracts from virus infected plants induce PTI-responses in Arabidopsis

Considering the increased susceptibility of *bak1* mutants to RNA viruses, we wondered whether extracts of virus infected leaf tissues contain MAMPs/DAMPs that induce PTI responses in plants. To address this question we prepared crude extracts of TCV-infected or mock-treated *Nicotiana benthamiana* leaves (Fig. S3.4 A) and tested whether these extracts were able to induce typical PTI responses such as MAPK activation, ethylene production and seedling growth inhibition in Arabidopsis. In plant innate immunity PRR activation often leads to the downstream phosphorylation of mitogen-activated protein kinases (MAPKs) which occurs within minutes of MAMP/DAMP treatment (Nuhse et al. 2000). To monitor MAPK activation upon crude extract application, we infiltrated highly diluted extracts of mock-infected and TCV-infected *N. benthamiana* plants into Arabidopsis leaves for 15 minutes. Proteins were extracted and subjected to Western blot analysis with a commercially available antibody detecting the dually phosphorylated MAPKs. Very little activated MAPK was detected when the leaf tissue was treated with 5 percent v/v mock extract. In contrast, high amounts of activated MAPKs were detected when the leaf tissue was treated with 5 percent v/v TCV extract (Fig. 3.6 A).

Besides early responses such as MAPK activation, MAMPs and DAMPs also induce the production of phytohormones. During PTI the production of ethylene has been particularly well studied and offers a reliable and sensitive method to detect MAMP/DAMP activity (Zipfel et al. 2004; Chinchilla et al. 2006; Chinchilla et al. 2007). Therefore, we also analyzed whether highly diluted TCV extracts were able to induce ethylene production in Arabidopsis. While the mock extract was unable to induce ethylene production in Arabidopsis samples, the TCV extract consistently led to increased levels of ethylene production, although the effect was not as strong as in the positive control, the flg22 treatment (Fig. 3.6 B). This difference in activity is not surprising considering that flg22 is a highly purified synthetic peptide applied at very high concentrations (1 μM), while the TCV extract represents a complex mixture in which the active compound might only be present in diminutive amounts. Unfortunately, we could not increase the amount of TCV extract to provide stronger ethylene peaks, because the mock extract showed some activity when applied at higher concentrations, possibly due to the detergent used for the extractions. The eliciting molecule may be of proteinaceous nature as no ethylene response was observed when the crude extracts where pre-treated with proteinase K (Fig. S3.4 B).

To evaluate the BAK1-dependency of the ethylene response, we also investigated the potential of crude viral extracts to induce ethylene in *bak1-4*, *bak1-5*, *fls2*, *pepr1/2* double mutant plants. The diluted TCV extract induced strong ethylene production in the WT plants *fls2* and *pepr1/2* mutants, but only a small ethylene production in the *bak1-4* mutant and

none at all in the *bak1-5* mutant (Fig. 3.6 B). These results indicate that one or more components of these extracts induce(s) ethylene production in a BAK1-dependent, but PEPR1/2-independent manner.

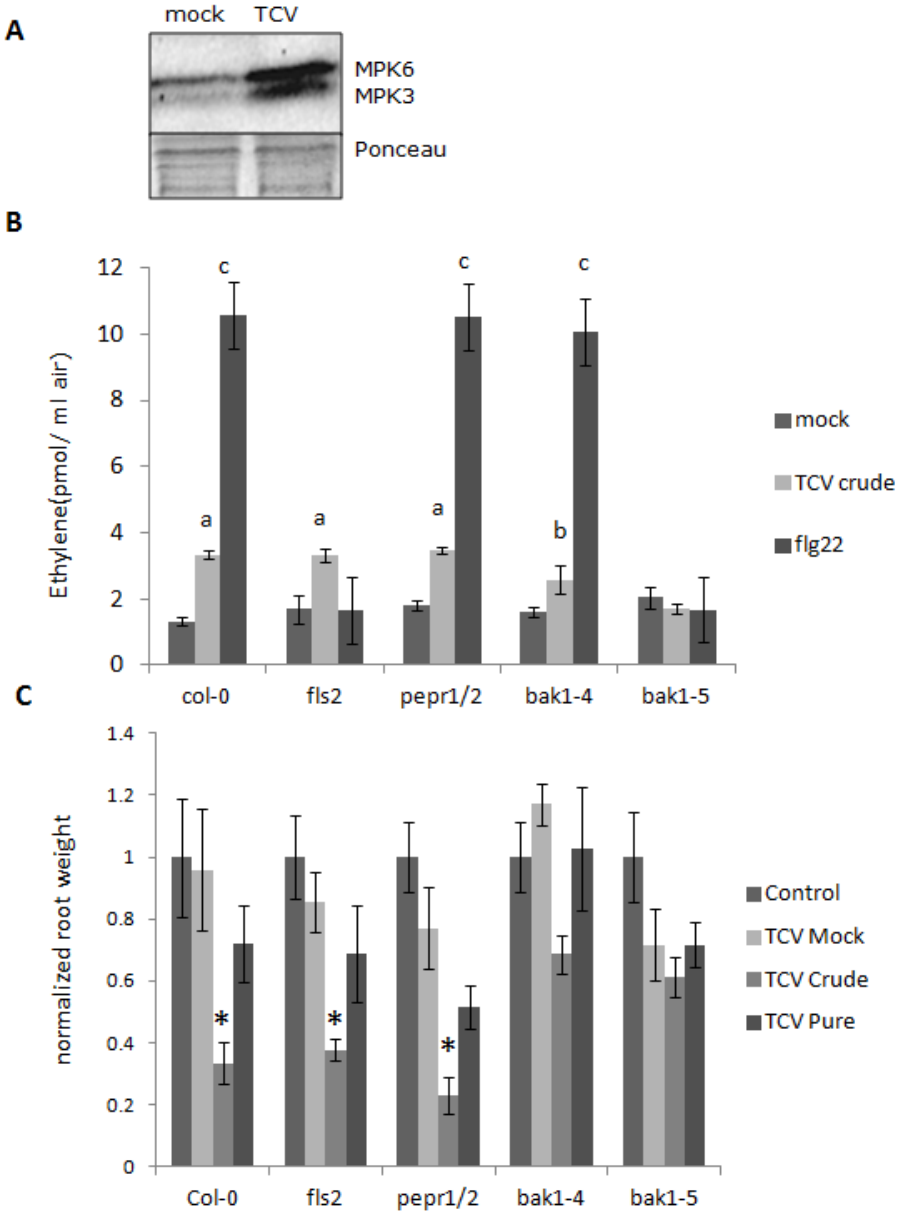

Figure 3.6 Compound(s) within crude extracts of TCV infected *N. benthamiana* leaves induce immune responses in Arabidopsis in a BAK1-dependent manner. A) Four weeks old Arabidopsis leaves (n =3) were infiltrated with crude extracts from mock-infected or TCV-infected *N. benthamiana* leaves. After 15 minutes, the tissue was harvested, extracted and subjected to Western blot analysis using anti-pERK1-2 monoclonal antibodies. B) Leaf strips of four week old Arabidopsis plants of Col-0 WT, *pepr1/pepr2* double mutant, *fls2*, *bak1-4* knockout and the *bak1-5* point mutation mutants were treated with "TCV extract", "mock extract" or a positive flg22 control and ethylene accumulation was measured four hours after treatment, n=4, ±SEM, one-way ANOVA, significant differences are marked by different letters. C) One week old Arabidopsis seedlings of Col-0, *pepr1/pepr2* double mutant, *fls2*, *bak1-4* knockout and *bak1-5* point mutation mutant were treated with crude extracts of TCV-infected leaves or with purified virions in sterile multiplates containing liquid MS medium and root weight was measured 10 days post treatment, n=8, ±SEM, Student's t-test, * $p<0.05$.

Based on these experiments it is not clear whether the eliciting activity comes from the plant and therefore constitutes a DAMP or is a component of the virus. To address this question we prepared crude as well as highly purified extracts of TCV or mock-infected *N. benthamiana* leaves (Fig. S3.4 A) and compared their activities in a third bioassay to look at the activity of elicitors of the PTI response, namely the seedling growth inhibition assay (Gomez-Gomez et al. 1999). We applied crude as well as highly purified extracts of TCV (5 percent v/v) or mock-infected (5 percent v/v) *N. benthamiana* leaves to one week old seedlings of WT, *pepr1/2*, *fls2*, *bak1-4* and *bak1-5* and continued to grow these seedlings under continuous light for ten days. While the overall seedling growth was only slightly affected by crude viral extracts, the roots of WT, *fls2* and *pepr1/2* showed severe growth inhibition (Fig. 3.6 C). Whether or not the seedlings became infected by TCV due to the long incubations with TCV sap extract has not been investigated. Conversely, the *bak1-4* and *bak1-5* mutants did not display any significant root growth inhibition suggesting that a *BAK1*-dependent signaling pathway is activated by the crude extract. Interestingly, highly purified extracts of TCV did not induce significant root growth inhibition (Fig. 3.6 C). This suggests that the elicitor of root growth inhibition and potentially PTI is not derived from intact TCV virions but is co-extracted from plant tissue or an intermediate product of TCV. Taken together these results show that a compound(s) in the crude extract from TCV infected *N. benthamiana* elicit(s) several PTI-responses; this activity is BAK1-dependent, and the elicitor perceived is not the virion itself.

3.4 Discussion

Innate immunity constitutes the first line of defense against biotic threats and protects eukaryotes from a wide range of potential pathogens. The perception of MAMPs by PRRs activates a range of fast and efficient defense responses which collectively lead to PTI in both, animals and plants (Boller and Felix 2009). Plants also sense danger by monitoring cell integrity. Is cell integrity disturbed, plant endogenous DAMPs are either actively or passively released and recognized by PRRs at the plasma membrane, leading to PTI-like defense responses (Huffaker and Ryan 2007; Boller and Felix 2009; Krol et al. 2010). Interestingly, compatible virus-host interactions are associated with upregulation of PTI marker genes, indicating that the presence of the viral pathogen is sensed by the host by a yet unknown mechanism (Whitham et al. 2003; Love et al. 2005).

Here we present first evidence that viruses are either directly or indirectly recognized by the PTI surveillance system in a BAK1-dependent manner. As a first indication, we observed that mutants in the central PTI regulator BAK1 are more susceptible to three different RNA viruses, namely ORMV, TMV and TCV. However, BAK1 is not only important for regulation of innate immunity, but also involved in cell death control and BR-signaling, a phytohormone important for plant growth (Wang et al. 2008). To exclude the possibility, that the increased susceptibility of *bak1-4* mutants (knockout mutant) to RNA viruses results from an impairment in BR signaling, we also included the *bak1-5* mutant in our analysis, which is strongly impaired in PTI but not affected in BR responses (Schwessinger et al. 2011). This is in contrast to the *bak1-4* mutant, which is impaired in both signaling pathways (Chinchilla et al. 2007; Heese et al. 2007). In all our pathogen assays, the *bak1-4* and *bak1-5* mutants were similarly affected, suggesting that BR signaling does not contribute to viral replication and symptom development. Interestingly, a viral nuclear shuttle protein (NSP) of the DNA virus *Tomato yellow spot begomovirus* interacted with the kinase domain of tomato BAK1 in yeast-two hybrid assays, suggesting that it might be an important virulence target for plant viruses (Sakamoto et al. 2012). In addition, this result suggests that BAK1 not only plays an important role in anti-viral defense to RNA viruses but also to DNA viruses. Thus, BAK1 seems to contribute to anti-viral defenses due to its role as a central regulator of immunity and independent of its ability to enhance BR signaling. However, since BAK1 appears to be involved in many RLK-mediated pathways, the increased symptom development in *bak1* mutants could still be indirect and caused by effects on convergent but unrelated pathways.

Our results did not reveal differences in susceptibility of *pepr1/2* mutants to virus infection, although the receptors as well as *AtPep2* and *AtPep3* are up-regulated during viral infections. This could be consequence of functional redundancy among different DAMP perception systems or indicating that the virus-induced PTI response is *At*PEP independent. Still, the involvement of BAK1 in anti-viral immunity suggests that either a viral MAMP or a virus-induced DAMP may be recognized by an unknown BAK1-interacting receptor. To test this, we obtained crude and highly purified TCV extracts from infected *N. benthamiana* plants and used these for standard PTI assays in Arabidopsis. Crude but not highly purified TCV extracts induced a number of PTI marker responses including ethylene production, MAPK activation and seedling growth inhibition. Based on these observations, we conclude

that TCV-infected tissue contains an elicitor active compound sufficient for extracellular PTI induction and that intact TCV virions alone are not perceived as a MAMP in Arabidopsis Col-0. Interestingly, a previous study had shown that TMV coat protein is perceived extracellular and induces ROS production in epidermal peels of tobacco plants shortly after application (Allan et al. 2001). Consistent with the infection assays, ethylene production and seedling growth inhibition upon crude extract application were BAK1-dependent. The requirement of BAK1 for signaling further suggests that the elicitor is perceived at the plasma membrane. Overall, these results support the hypothesis that PTI-responses are induced by PRR-mediated recognition of MAMPs/DAMPs present in the crude extracts.

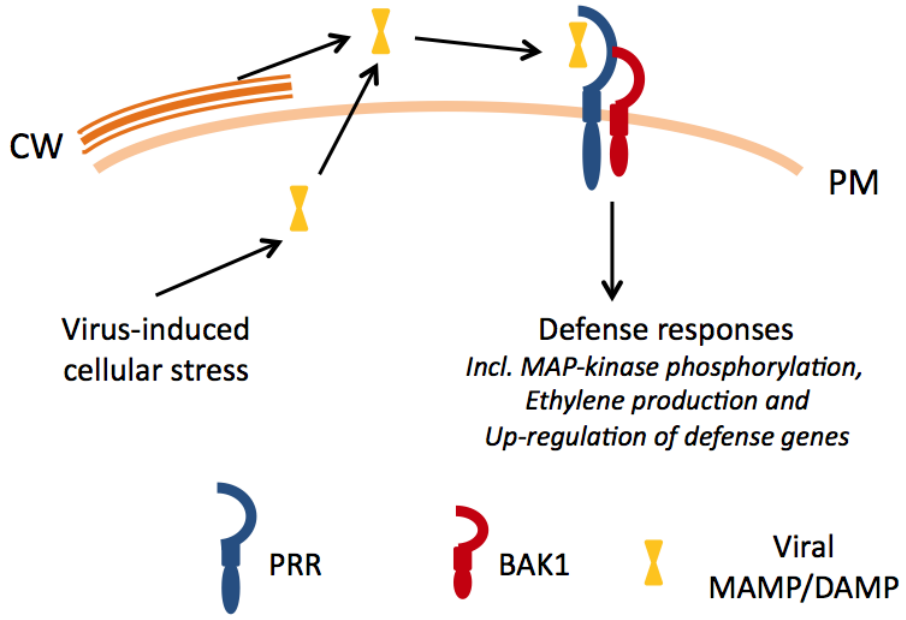

Figure 3.7 Proposed model for BAK1-dependent PTI response during viral infection. During viral infections compounds with MAMP/DAMP properties appear. The nature and origin of these MAMP/DAMP are not known but they could be products of virus-induced cellular stress or cell-wall (CW) fragment originating from virus-induced plasmodesmata changes. The viral MAMP/DAMP is recognized in a BAK1-dependent manner, likely through a Pattern Recognition Receptor (PRR) in the plasma membrane (PM).

Since viruses are strictly intracellular one explanation for the observed PTI induction is that TCV infections lead to the production of a plant derived DAMP. In support of this hypothesis, only crude extracts resulted in PTI responses while highly purified virions did not. However, the crude TCV extract also contains virus encoded proteins, viral RNA or sRNA in their free form. In purified virions these compounds are not accessible, because there are tightly packed and assembled. Thus, it is equally well possible that viral proteins or the viral RNA induce PTI responses before assembly. However, a conserved recognition mechanism between Arabidopsis and *N. benthamiana* is likely since the active compound is recognized in Arabidopsis despite of being obtained from infected tissue of *N. benthamiana*. Overall we suggest a model in which virus infections continuously release a DAMP and or MAMP into the apoplast where the eliciting activity is then recognized by a yet unknown BAK1-dependent receptor (Fig. 3.7). Further research will be needed to identify the anti-viral

MAMP/DAMP receptor. Our finding that BAK1 is involved in anti-viral innate immunity against several RNA viruses makes BAK1 a powerful tool in the search for the virus MAMP/DAMP and its receptor.

3.5 Material & Methods

Plants, growth conditions, and virus inoculation

Arabidopsis thaliana plants were grown in a growth chambers (Sanyo, Japan) at 21°C with 8 h/16 h light/dark cycle. To initiate virus infection, two leaves of four weeks old plants were rub-inoculated either with sap from healthy or TCV-infected plants or with 150ng purified TMV or ORMV virion particles per leaf.

TCV crude extracts

N. benthamiana plants were infected with TCV by infiltration of agrobacterium GV3101 carrying pBIN19-TCV or an empty vector construct. Two to three days post infiltration non infiltrated systemic tissue was harvested and frozen in liquid nitrogen. The tissue was ground and extracted in 1/10 (w/v) PBS-Tween (0.5 percent) overnight on a rotation wheel at 4 °C. The extracts were centrifuged three times at 4000 g for 15 minutes to remove cellular debris and analysed by SDS-page and coomassie staining.

TCV pure extracts

Highly purified TCV extracts were prepared according to the protocol of Leberman (Leberman 1966).

TMV and ORMV virion preparation

TMV- or ORMV-infected leaves were frozen and ground in liquid nitrogen. For each gram tissue powder one ml buffer A (0.5M NaP pH 7; 0.1 percent 2-ME) and one volume of butanol/chloroform 50/50 was added and mixed by shaking. The mixture was centrifuged for 10 minutes at 14000 rpm. The upper aqueous phase was removed and centrifuged at 15000 rpm for 15 minutes. Following addition of 1/10 volume of 40 percent PEG8000 to the upper aqueous phase, incubation for 10 minutes on ice, and centrifugation for10 minutes at 14000 rpm, supernatant was discarded and the pellet resuspended in 0.2 volume 10 mM Na_2HPO_4 pH 7. The sample was again centrifuged for 10 minutes at 5000 g and the supernatant transferred into a fresh tube. Sodium chloride was added to a total of 1 percent and PEG to a total of 4 percent followed by centrifugation at 5000 g for 10 minutes and the removal of the supernatant. The final pellet was resuspended in 10 mM Na_2HPO_4 pH 7 and the concentration measured photometrically, whereby an OD_{260} = 3 equals 1 mg/ml of virion particles.

Quantitative RT-PCR

Arabidopsis total RNA was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel, Germany) and treated with DNase according to the manufacturer's specifications. cDNA was synthesized from 500 ng of RNA with random primers using the AMV reverse transcriptase system according to the manufacturer's instructions (Promega, USA). Quantitative RT-PCR was performed in a 96-well format using a Light Cycler 480 machine (Roche Applied Science, Switzerland). On the basis of the obtained C_T values, normalized expression to the reference gene *UBQ10* (AT4G05320) was calculated using the qGene protocol. The gene-specific primers used were as follows: *UBQ10* (AT4G05320) with UBQ_fw (5'-GGCCTTGTATAATCCCTGATGAATAAG) and UBQ_rv (5'-AAAGAGATAACAGGAACGGAAACATAG), *PEPR1* (AT1G73080) with PEPR1_fw (5'-CAACAACAATGTGGAGGATA) and PEPR1_rv (5'-AACGAGATTACCGAACTGAA), *PEPR2*

(AT1G17750) with PEPR2_fw (5'-AAGAAGATGGCTTAATGCTG) and PEPR2_rv (5'-GAGTTGTGCCAGTAACAGTG), *ProPEP2* (AT5G64890) with ProPEP2_fw (5'-TCACCAAACTATTGGATTTCAA) and ProPEP2_rv (5'-GACTCAATTGACTTCTTAATC), *ProPEP3* (AT5G64905) with ProPEP3_fw (5'-CAACGATGGAGAATCTCAGA) and ProPEP3_rv (5'-CTAATTGTGTTTGCCTCCTTT), *BAK1* (AT4G33430) with BAK1_fw (5'-GACCTTGGGAATGCAAATCTATC) and BAK1_rv (5'-AAAACTGATTGGAGTGAAAAGTGAAA). For the quantification of viral RNA, the following primer combinations were used: TMV with TMV_fw (5'-GACCTGACAAAAATGGAGAAGATCT) and TMV_rev (5'-GAAAGCGGACAGAAACCCGCTG), ORMV with ORMV_fw (5-AGGTGGGGTAACAGTGAGCGTGA) and ORMV_rev (5'-GCTTTCGCTTGGCATCCGCG), TCV with TCV_fw (5'-GTCGATTTCGGCAAACTCAT) and TCV_rev (5'-GCTGGTTGAGCCAGTTCTGT).

MAP kinase activation assays

Arabidopsis leaves of four to six week old plants were infiltrated with crude extract from mock-treated or TCV-infected *N.benthamiana* plants (5 percent v/v in water) for 15 minutes. Leaf discs (50 mg) were then frozen in liquid nitrogen and proteins were extracted in 100 µl extraction buffer (50 mM Tris-HCl pH 7.5, 150 nM NaCl, protease inhibitor cocktail (Sigma-Aldrich, USA) for 30 minutes at 4°C. Subsequently 100 µl Lämmli loading buffer (2x) were added to each sample. Samples were subjected to immunoblot analysis using the anti-p42/44-phospho-ERK antibody (Cell Signaling, USA). Blots were developed using CDP-star technology (NEB, USA).

Ethylene production

Ethylene production was measured as described previously (Felix et al. 1999). For the induction of ethylene by crude viral extracts, the extracts were added to the water in which the leaf strips were floating at 5 percent (v/v).

Seedling growth inhibition assay

Five days after germination, sterile seedlings were exposed to liquid MS medium supplied with viral extracts of 5 percent (v/v) final concentration or to a control treatment with buffer containing 1 µM flg22 (one seedling per 500 µl of medium in 24-well plates). The effect of the treatment on seedling growth was analyzed after ten days by weighing the fresh weight of the roots.

3.6 Acknowledgements

We would like to thank David Baulcombe and Anne Simon for providing TCV clones and Cyril Zipfel for the *bak1-5* mutant. Funding was gratefully received from the Swiss National Science Foundation (SNF, grant 31003A_140694 and SNF, grant 144084).

3.7 Supplementary material

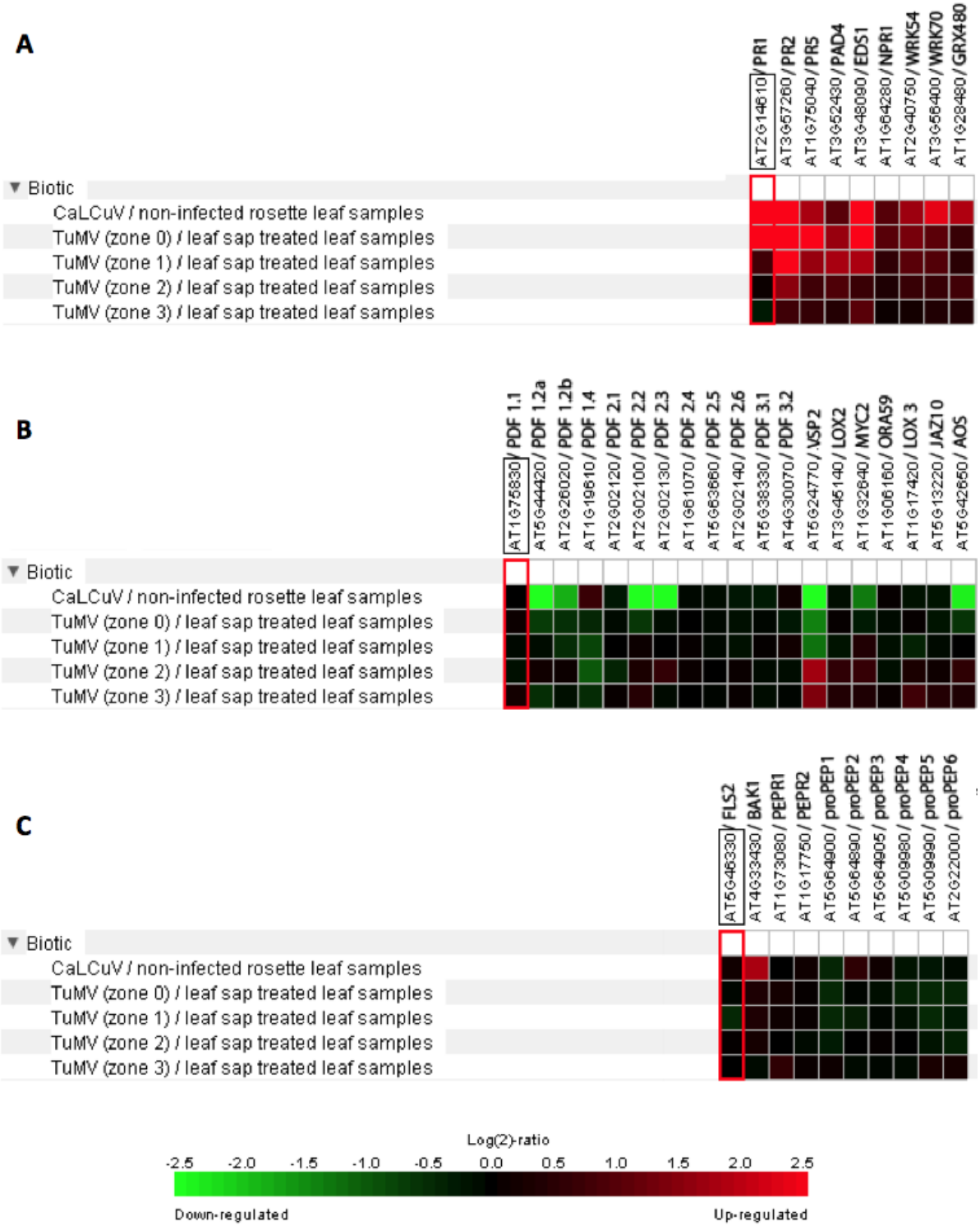

Figure S3.1 Expression of SA-, JA- and PTI-pathway genes upon viral infection. Using array data from plants infected with viral pathogens (*Cabbage leaf curl virus* (CalCuV) and *Turnip mosaic virus* (TuMV)) deposited in the Genevestigator database the expression levels of genes involved in defense response via SA (A), JA (B) or PTI (C) were retrieved.

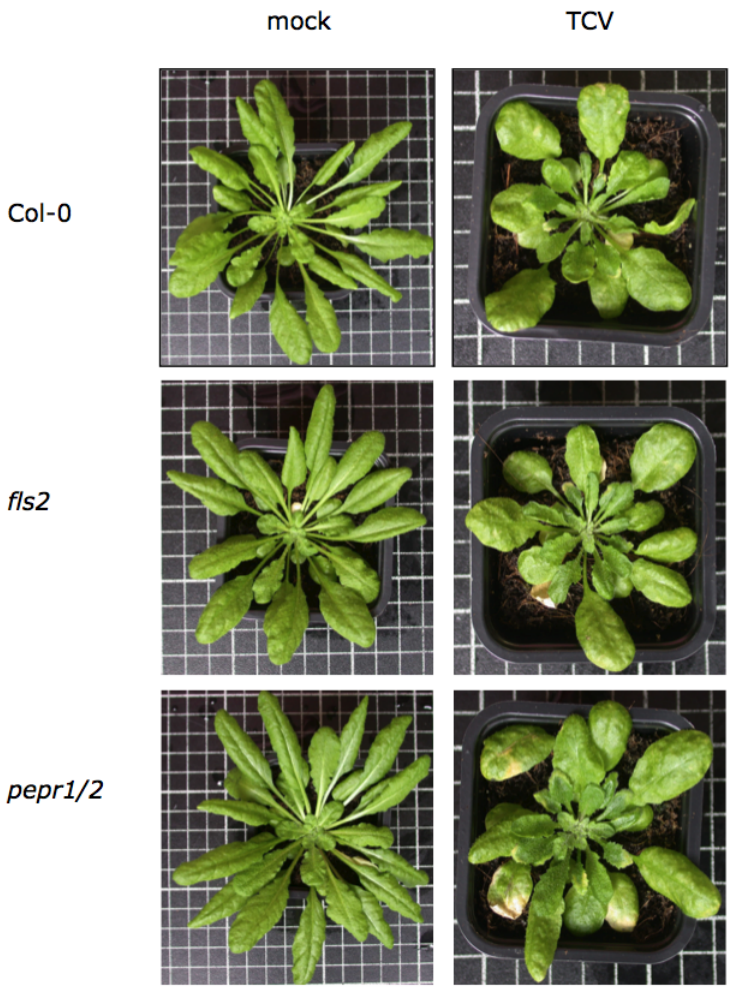

Figure S3.2 Symptomatic characterization TCV infections at 21 dpi in *fls2* and *pepr1/pepr2* mutant lines. Col-0, the *fls2* and *pepr1/2* knockout lines were infected with TCV crude extract and symptoms were scored 21 dpi. The pictures present the average phenotype observed in the different lines. The pictures shown here are derived from the same infection as in Figure 3.2 A.

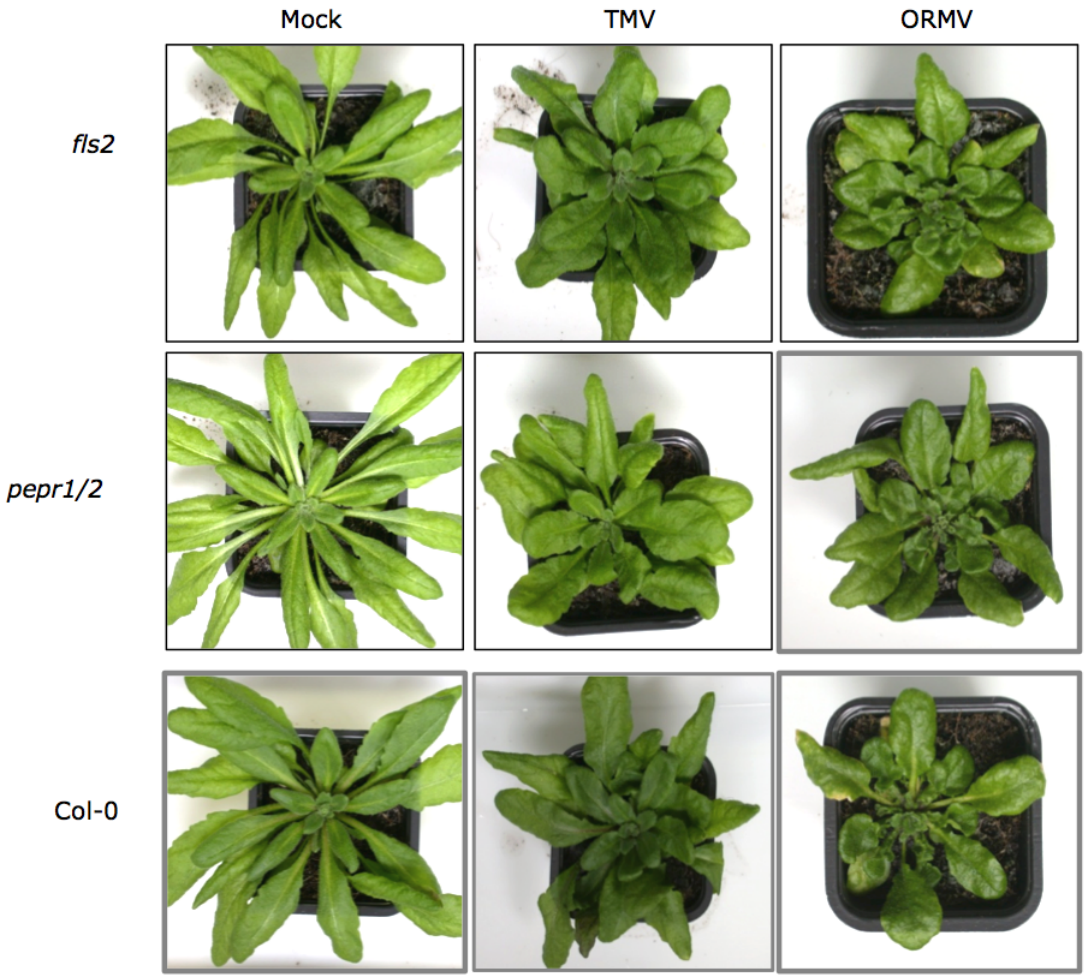

Figure S3.3 Symptomatic characterization of ORMV and TMV infections at 21 dpi in *fls2* and *pepr1/pepr2* mutant lines. The *fls2* and *pepr1/2* knockout lines were infected with TMV or ORMV virions and symptoms were scored 21 dpi. The pictures present the average phenotype observed in the different lines. The pictures shown here are derived from the same infection as in Figure 3.4 and 3.5.

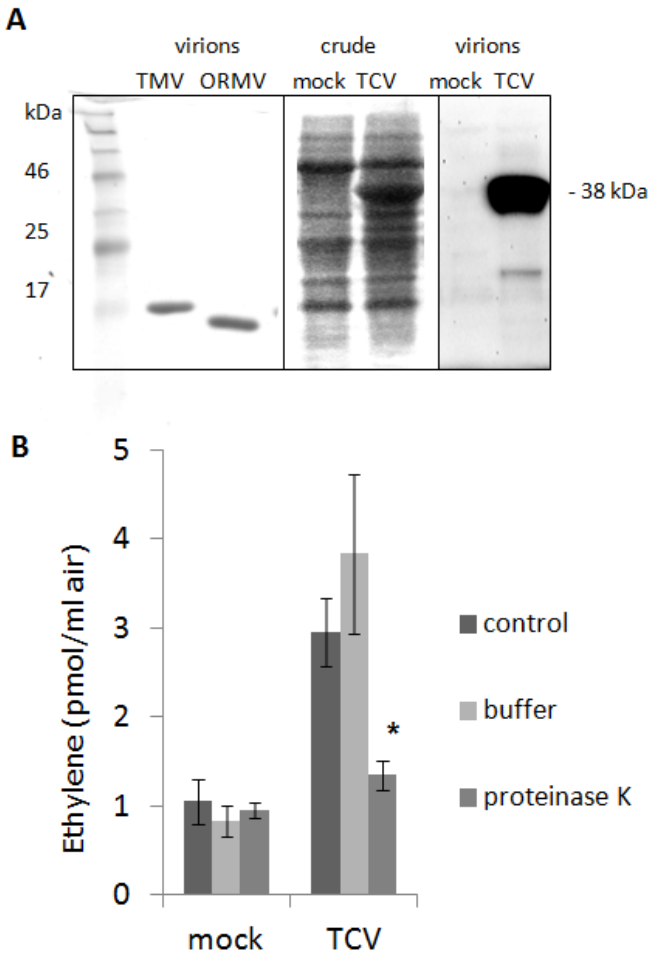

Figure S3.4 Purity of viron preparations and TCV crude extracts. A) Approximately 1 µg of TMV or ORMV virions were separated on 15 percent SDS-PAGE gels followed by Coomassie staining. Only the ~17 kDa coat proteins were detectable in the virion preparations. Likewise, 10 µl of highly purified TCV virions resulted in bands corresponding to the size of viral coat protein (38 kDa). In contrast the crude extract shows a highly complex mixture containing various plant proteins. B) Leaf strips of four week old Arabidopsis plants were treated with mock or TCV crude extract diluted in water, the proteinase K buffer or proteinase K buffer plus proteinase K and ethylene content was measured four hours after treatment. Significant differences ($p < 0.05$, student t-Test) are marked by an asterisk.

4 Characterization of Tolerance to *Tobaccos mosaic virus* in *Arabidopsis thaliana*

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4.1 Abstract

In tolerant hosts specific systemic viral infections do not cause symptoms. Tolerance is not always linked to low viral titer and the mechanism by which the host achieves tolerance is not understood. *Tobacco mosaic virus* (TMV) infections in *Arabidopsis thaliana* are non-symptomatic in most ecotypes. To understand the mechanisms behind tolerance to TMV in Arabidopsis, a tolerant ecotype (Col-0) and a non-tolerant (Sha) differing with respect to the development of disease symptoms in response to TMV infection as well as RNA-silencing and hormone signaling mutants were characterized upon TMV infection. Tolerance to TMV in Arabidopsis correlates with the absence of induction of defense-related genes and slower movement out of the inoculated leaf leading to later systemic infection. However, functional RNA-silencing and Salicylic acid (SA) signaling are dispensable for tolerance to TMV in Arabidopsis. These results suggest that mechanisms other than RNA-silencing and SA-signaling are involved in tolerance to TMV in Arabidopsis.

4.2 Introduction

Systemic virus infection in plants may cause severe disease symptoms such a growth retardation, necrosis, chlorosis and developmental abnormalities but in many cases the infection occurs without symptoms. Next-generation-sequencing of plant material have revealed that healthy plants in nature are often infected with virus (Pantaleo et al. 2010; Thapa et al. 2012). Host in which viruses establish infection and move systemically without causing disease symptoms are referred to as tolerant (Palukaitis and Carr 2008). The mechanism behind tolerance is not understood but may in some cases be due to lower viral accumulation. The genetic variation found between natural populations, such as *Arabidopsis thaliana* ecotypes, can be explored to understand the mechanisms behind resistance as well as tolerance to virus. Such approaches have identified several gene encoding resistance proteins against specific viruses, incl. HYPERSENSITIVE RESPONSE TO TCV (HRT) against *Turnip crinkle virus*, RESISTANT TO CMV (Y) (RCY1) allelic to HRT against *Cucumber mosaic virus Y*, RESTRICTED TEV MOVEMENT 1 (RTM1) against *Tobacco etch virus* and REQUIRED FOR POTEXVIRUS RESTITANCE 1 (JAX1) against viruses of the genus potexvirus (Dempsey et al. 1997; Mahajan et al. 1998; Chisholm et al. 2000; Takahashi et al. 2002; Yamaji et al. 2012). Ecotype studies may also be used to identify more susceptible ecotypes for viruses not causing symptoms in model ecotypes (Lee et al. 1996; Dardick et al. 2000). Arabidopsis is a tolerant host for the tobamovirus *Tobacco mosaic virus* (TMV). Most Arabidopsis ecotypes are infected by TMV but the infection is symptomless with the exception of the ecotype Shahdara (Sha), which develops strong symptoms when infected with TMV (Dardick et al. 2000; Pereda et al. 2000). Virus chimeras between TMV and symptom-inducing tobamovirus *Oilseed rape mosaic virus* (ORMV) mapped symptom development in Arabidopsis ecotypes RLD and Can-0 to the replicase protein (Mansilla et al. 2009), but no host loci linked to a loss-of-tolerance have been mapped.

In order to establish an infection viruses have to suppress the defense mechanisms of the host. One example of this is viral suppression of anti-viral Post Transcriptional Gene Silencing (PTGS) to avoid degradation of the viral RNA (Burgyan and Havelda 2011). PTGS is initiated by the presence of double-stranded RNA (dsRNA), which is processed by DICER-like endonucleases (DCL) to 21-24 nt long small RNAs (sRNA). After methylation by Hua Enhancer 1 (HEN1), sRNAs are loaded into ARGONAUTE (AGO) proteins within the RNA-Induced Silencing Complex (RISC) to target complementary RNA for cleavage or translation inhibition. Cleaved RNA can serve as template for the production of dsRNA molecules by RNA-dependent-RNA-polymerases (RDR). The RDR-derived dsRNA is processed by DCL enzymes into secondary sRNAs (Diaz-Pendon and Ding 2008; Burgyan and Havelda 2011). The processing of viral dsRNA is identical to that of some endogenous sRNAs, and the same proteins are involved in both processes (Ding 2010; Vazquez et al. 2010). Processing of viral sRNA (vsRNA) in Arabidopsis involves DCL2, 3 and 4, HEN1 and RDR1, 2 and 6, while AGO1 and 2 are the main anti-viral AGOs (Ding 2010). Viral suppression of the anti-viral silencing defenses of the host is therefore crucial for successful establishment of local infection as well as for viral invasion of systemic tissue. The importance of silencing as an anti-viral defense is supported by the finding that plant viruses encode silencing suppressor proteins

(Diaz-Pendon and Ding 2008). Furthermore, several Arabidopsis mutants with disrupted silencing pathways are hypersusceptible to some viruses (Blevins et al. 2006; Deleris et al. 2006; Diaz-Pendon et al. 2007; Donaire et al. 2008; Jaubert et al. 2011). Attenuated viruses, which do not cause strong disease symptoms and therefore can be used for cross-protection against more virulent strains, are often found to encode less efficient silencing suppressors (Nishiguchi and Kobayashi 2011), which suggests that one factor playing a role in determinating the tolerance of the plants to specific viruses involves the interplay between the host silencing response and viral silencing suppression. In the case of tobamoviruses the involvement of the 126k replicase subunit in silencing suppression was first described for *Tomato mosaic virus* (ToMV) (Kubota et al. 2003), a virus closely related to TMV. Since then this observation has been confirmed for several other tobamoviruses, including TMV (Ding et al. 2004; Csorba et al. 2007; Vogler et al. 2007). A hallmark of active tobamoviral silencing suppression is stabilization and accumulation of 21 nt long sRNAs from both virus and plant (Csorba et al. 2007; Kurihara et al. 2007; Vogler et al. 2007; Hu et al. 2011). Many of these accumulating small RNAs are unmethylated (Blevins et al. 2006; Csorba et al. 2007; Vogler et al. 2007) and based on these observations it has been suggested that the small subunit of the replicase suppresses silencing by sequestering 21 nt long small RNAs either before methylation or after methylation, in which case it may actively de-methylate bound sRNAs. The small replicase subunit of TMV-cg (122k) has been shown to bind small RNA duplexes *in vitro* (Csorba et al. 2007; Kurihara et al. 2007) but it is not known if direct binding is the primary mode-of-action *in vivo*. In ToMV a C349Y mutation in the replicase originally found in an attenuated non-symptomatic strain of ToMV was shown to interfere with the ability of the virus to suppress its own silencing as well as PTGS silencing of a green fluorescent protein (GFP)-reporter upon infection in *Nicotiana tabacum* and *Nicotiana benthamiana* (Kubota et al. 2003). In a similar study comparing symptomatic and non-symptomatic strains of TMV in *N. tabacum,* a S360T mutation in the replicase protein was found crucial for TMV symptom formation (Ding et al. 2004). Introducing the C349Y mutation found in ToMV into the 126k protein of TMV disrupted local silencing suppression within infection sites in *N.benthamiana* leaves and also upon ectopic expression in *N.benthamiana* agro-infiltration patch assays (Vogler et al. 2007).

Although RNA-silencing is a potent anti-viral defense, other defense pathways are also activated upon virus infection. During symptomatic virus infections the induction of pathogenesis-related genes such as PATHOGENESIS RELATED PROTEIN (PR)1, PR2 and PR5 are often observed (Whitham et al. 2003; Love et al. 2005; Ascencio-Ibanez et al. 2008; Hanssen et al. 2011; Hu et al. 2011; Lu et al. 2012; Mandadi and Scholthof 2012). PR-genes are known markers for the activation of defense hormone Salicylic Acid (SA) and pre-treatment of plants with SA can inhibit compatible viruses (Singh et al. 2004; Lewsey and Carr 2009). Viral infection of Arabidopsis mutants impaired in SA-signaling revealed that PR1, PR2 and PR5 induction in inoculated leaves depends on SA-signaling (Huang et al. 2005). However, no increase in viral titer was observed in these mutants, questioning if the hypothesis that pathogenesis-related genes are part of an active anti-viral defense response (Huang et al. 2005). Although PR1, PR2 and PR5 are known to be induced by SA (Thomma et

al. 2001) abiotic stresses may also induce their expression in a partially SA-independent manner (Liu et al. 2013). A SA-dependent up-regulation of defense-related genes during viral infection is supported by reports showing that *Solanum lycopersicum* shoots infected with *Tomato spotted wilt virus* and infection of *Potato virus Y* or *Potato virus X* (PVX) in susceptible *Solanum tuberosum* lead to increases in SA levels in systemic tissue (Krecic-Stres et al. 2005; Niehl et al. 2006; Miozzi et al. 2011). A decrease in the expression of jasmonic acid (JA)-responsive genes is often observed during viral infections (Ascencio-Ibanez et al. 2008; Hu et al. 2011) and has been regarded as a consequence of the activation of the antagonistic SA-pathway (Pieterse et al. 2012). However, a recent report showing that the C2 protein of geminiviruses actively represses the JA-pathway and that JA-treatment decreases geminivirus *Beet curly top virus* symptoms and titers (Lozano-Duran et al. 2011) suggests that the JA-pathway plays a more direct role in viral defense. The activation of SA-responsive genes by virus infections is observed across the plant kingdom, but it is not known if this activation represents a direct response to the viral pathogen or is a by-product of cellular remodeling and damage induced by the virus (Laliberte and Sanfacon 2010). Many plant pathogens are sensed by the plant through pathogen-associated molecular patterns (PAMP), which are molecules perceived as “non-self” (Boller and Felix 2009). The recognition of PAMPs triggers defense responses including induction of defense-related genes (Tsuda et al. 2008). No virus-derived PAMP has been described so far in plants. Plant-derived damage-associated molecular patterns (DAMP) released upon cell damage act similarly to PAMPs and wounding-induced DAMPs trigger responses in gene expression similar to those observed during pathogen attack (Cheong et al. 2002; Sun et al. 2011). The observation, that extracts from virus-infected plants induce PAMP/DAMP-like responses in Arabidopsis (chapter 3) suggests that viral infection activates DAMP-signaling. In turn this may explain the induction of defense genes seen during compatible virus infections. Mutants of a core-regulator of PAMP/DAMP-signaling BRI1-Associated Receptor Kinase1 (BAK1) are more susceptible to TMV (chapter 3), but it is not known if the anti-viral BAK1 mode-of-action is SA-dependent.

Despite these findings indicating that virus infection triggers defense-related mechanisms in the host, the mechanism by which a tolerant host restrains the infecting virus and prevents disease is not known. We used TMV mutants with reduced silencing suppression as well as tolerant and susceptible Arabidopsis ecotypes and Arabidopsis mutants impaired in RNA-silencing and SA-signaling to characterize tolerance to TMV in Arabidopsis. We found that a mutation in the 126k replicase of TMV previously found to reduce the silencing suppression activity of the virus did not affect virus accumulation in neither tolerant nor susceptible Arabidopsis. Moreover mutants impaired in vsRNA biogenesis did not show an increased susceptibility to TMV. Symptomatic TMV infections induce SA-responsive defense-related genes but SA-production or –signaling mutants were not more susceptible. The susceptible ecotype Sha and the tolerant ecotype Col-0 both perceived the phytohormones JA and ethylene as well as an unidentified PAMP/DAMP found in extracts of virus-infected plants. Together these results indicate that tolerance to TMV is not mediated through RNA-silencing or SA-signaling.

4.3 Results

Tolerance to TMV in Arabidopsis is linked to delayed systemic movement

A study comparing susceptibility to TMV in 14 different Arabidopsis ecotypes identified the ecotype Sha as a symptomatic host (Dardick et al. 2000). When infected with TMV Sha displays distinct symptoms compared to other ecotypes including Col-0 (Fig. 4.1 A) (Dardick et al. 2000; Padmanabhan et al. 2005). Northern blot analysis with RNA isolated from Col-0 and Sha revealed slightly higher amounts of sub-genomic viral RNA in Sha at 21 days post inoculation (dpi) (Fig. 4.1 B). In a previous report the TMV coat protein (CP) was detected in systemic tissue of Sha plants seven days earlier than in any other ecotype tested, and this phenotype was found to be genetically linked to symptom development (Dardick et al. 2000). However, the set-up used in this report could not distinguish if movement out of the inoculated leaf or accumulation of TMV CP within systemic tissue was enhanced in Sha plants. To investigate if TMV spreads more efficiently in Sha compared to Col-0, a leaf-detachment assay for systemic movement was established. Two leaves of four weeks old plants were rub-inoculated with TMV virions and both inoculated leaves were removed after one, three or five days. At 28 dpi systemic tissue was harvested and used for ELISA to identify infected plants. In both Col-0 and Sha TMV had moved out of the inoculated leaves in about 20 percent of the plants within the first 24 hours. However, while this number remained unchanged at three dpi for Col-0, Sha plants had supported the systemic spread of TMV from the inoculated leaves in 70 percent of the plants. At five dpi, TMV had moved out of the inoculated leaves in all Sha plants whereas only 70 percent of the Col-0 plants had supported systemic movement at this time point (Fig. 4.1 C). This experiment was repeated four times with similar results. To validate the observations CP-levels were determined by Western blot in inoculated leaves at three dpi and in systemic tissue at seven dpi. In inoculated leaves slightly higher levels of CP were detected in Sha leaves as compared to Col-0 (Fig. 4.1 D upper panel). As expected based on the leaf-detachment assays TMV CP could be detected in systemic tissue of Sha at seven dpi whereas systemic leaves of Col-0 plants were still free of virus at this time point (Fig. 4.1 D lower panel). These results show that TMV moves out the inoculated leaf faster in Sha compared to Col-0, as suggested previously by Dardick et al. (2000). Faster systemic movement could be caused by accelerated cell-to-cell movement or more efficient phloem entry in inoculated leaves of the ecotype Sha. Furthermore both SA-signaling and RNA-silencing have been linked to suppression of viral systemic movement (Alamillo et al. 2006; Deleris et al. 2006).

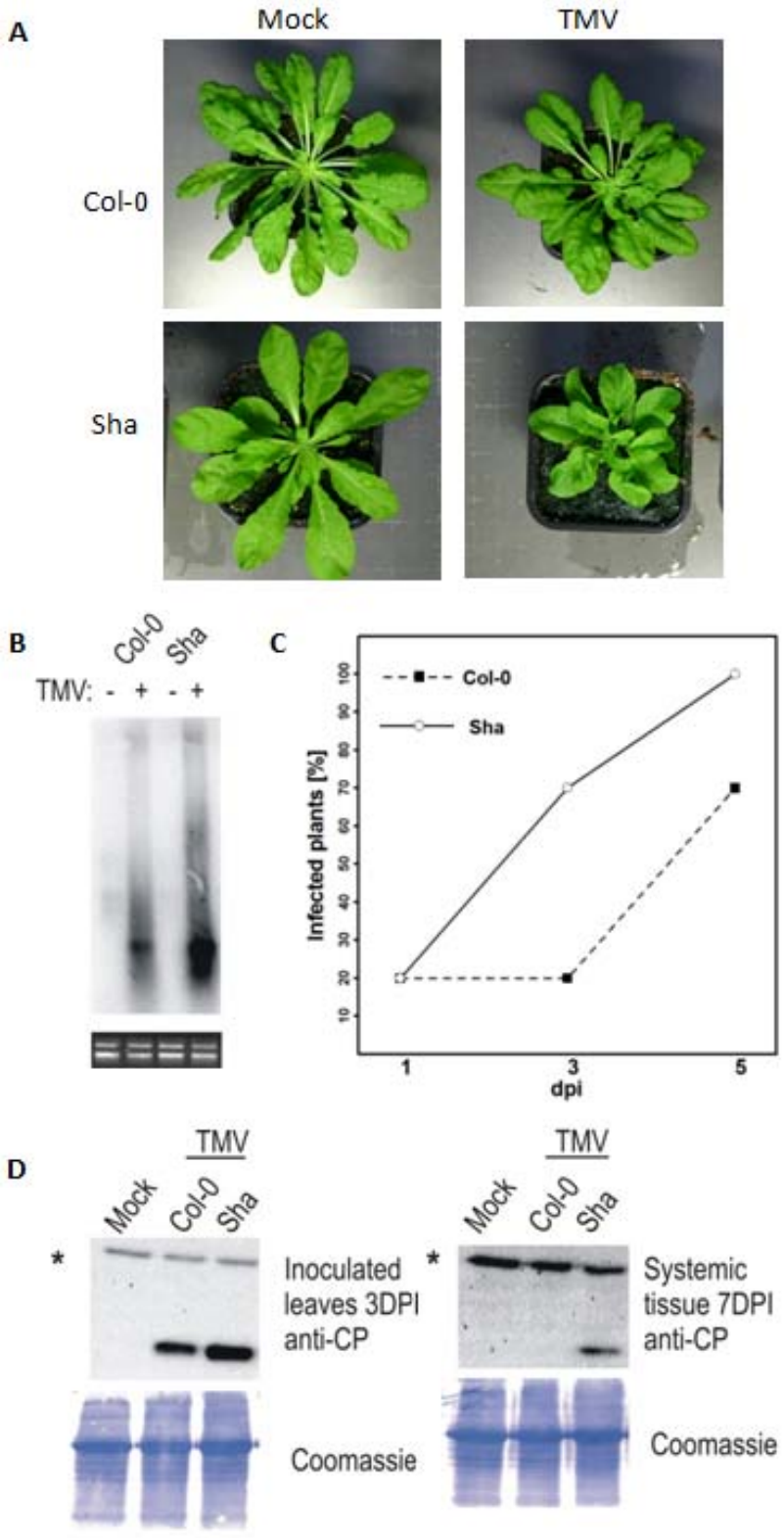

Figure 4.1 Tolerance to TMV is linked to delayed systemic movement. A) Four weeks old Arabidopsis plants of the ecotype Col-0 and Sha were infected with TMV. The pictures present the symptom at 21 dpi. B) Viral accumulation detected by Northern blot of RNA isolated from plants shown in A. C)To determine the rate of systemic movement in the two ecotypes four weeks old Col-0 and Sha plants were infected with TMV and inoculated leaves were removed at one, three or five dpi. Infections were verified by ELISA at 28 dpi, n=10, black squares - Col-0; open circles -Sha. D) Western blot of inoculated leaves (three dpi, left panel) and systemic leaves (seven dpi, right panel) with anti-CP, asterisk indicates non-specific cross-reacting band. Coomassie staining of total protein is shown as control for equal loading.

The C349Y mutation in the 126k protein affects viral fitness in N.tabacum (n/n) but not in Arabidopsis

Viruses with a reduced capacity to suppress silencing often fail to accumulate and to cause symptoms. One example of this is the mild "Holmes" strain of TMV, where mild symptom formation correlates with weak silencing suppression (Ding et al. 2004). Viruses carrying mutation disrupting their silencing suppression activity can therefore be used as tools to investigate the role of host RNA-silencing in anti-viral defense (Wang et al. 2010). TMV silencing suppression activity is provided by the small replicase subunit (126k) and a C349Y (G1411A) mutation that reduces the silencing suppression activity of this protein was previously described and introduced into TMV U1 (Vogler et al. 2007). *In vitro* transcribed RNA of this mutant virus (TMV_{Repmt}) was infectious in *N. benthamiana* and virions were purified and used for further experiments. To verify previous reports that the C349Y mutation affects viral accumulation and symptom development in *N. tabacum* (n/n) (Vogler et al. 2007), six weeks old *N. tabacum* (n/n) plants were inoculated with equal amounts of TMV or TMV_{Repmt} virions. At 14 dpi, plants inoculated with TMV exhibited systemic disease symptoms showing mosaic and strongly deformed leaves, whereas plants inoculated with TMV_{Repmt} displayed no symptoms except for occasional patches of chlorotic tissue (Fig. 4.2 A). Viral CP- and RNA-levels were lower in systemic tissue of TMV_{Repmt} infected plants compared to systemic tissue infected with WT TMV (Fig. 4.2 B & C).

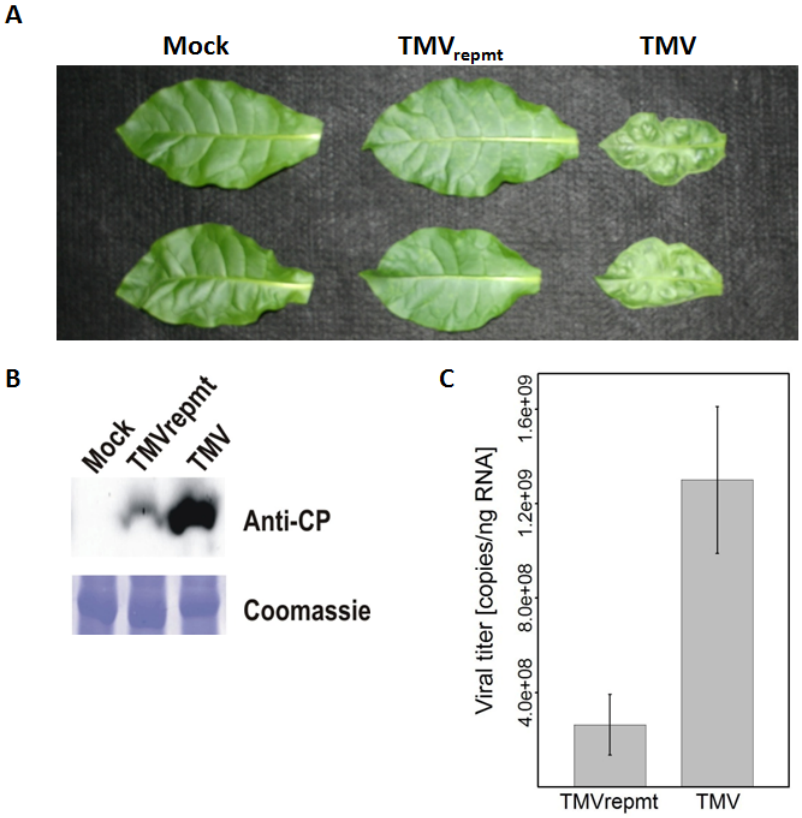

Figure 4.2 TMV_{repmt} causes less symptoms in tobacco than WT TMV and this correlates with lower viral titer. A) Pictures present symptoms in systemic leaves of tobacco plants infected with TMV_{repmt} or TMV virions at 14 dpi. B) Accumulation of the TMV CP detected by anti-CP antibodies (DSMZ) on Western blots of protein extracts from the infected tissue shown in A. Coomassie staining of total protein is shown as control for equal loading. C) TaqMan qPCR analysis of viral titers in RNA isolated from tissues shown in A, n= 3 ± SE.

The lack of symptom development in TMV_{Repmt}-infected *N.tabacum* (n/n) may therefore be explained by reduced virus accumulation and it can be concluded that the C349Y mutation reduces viral fitness in *N.tabacum* (n/n). We speculate that in hosts where RNA-silencing limits TMV and wild type (WT) TMV can suppress host RNA-silencing, a difference in viral accumulation between TMV and TMV_{Repmt} would be observed. However, we cannot rule out that the effect of the C349Y mutation observed in *N.tabacum* (n/n) is caused by less efficient replication. To test if WT and mutant virus show the same differences in accumulation and symptom development in Arabidopsis, we first infected the tolerant Arabidopsis ecotype Col-0 with TMV or TMV_{Repmt} virions. Symptoms of Arabidopsis plants infected with TMV_{Repmt} did not differ from those infected with TMV (Fig. 4.3 A) and similar CP- and RNA-levels were obtained by Western blot and qPCR (Fig. 4.3 B & C).

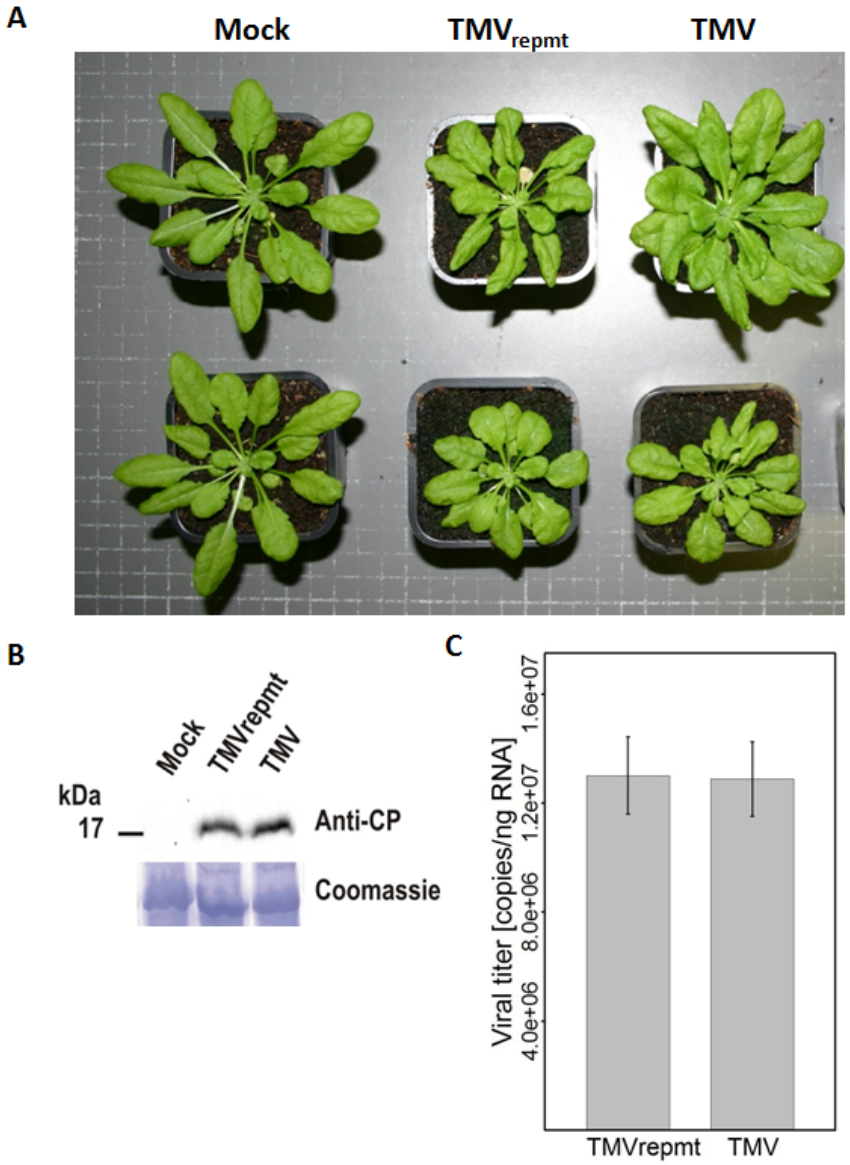

Figure 4.3 TMV and TMV_{repmt} infections in Arabidopsis Col-0 lead to similar symptoms and viral titers. A) Four week old Arabidopsis Col-0 plants were infected with TMV_{repmt} or TMV virions. Pictures represent symptoms observed at 21 dpi. B) Accumulation of the TMV CP detected by anti-CP antibodies (DSMZ) on Western blots of protein extracts from the infected tissue shown in A. Coomassie staining of total protein is shown as control for equal loading. C) TaqMan qPCR analysis of viral titers in RNA isolated from tissue of plants shown in A, n= 3 ± SE.

Next, we performed the same experiment with the Sha ecotype. However, symptoms caused by TMV_{Repmt} were again identical to those caused by WT TMV (Fig. 4.4 A) and similar CP- and RNA-levels were observed by Western blot and qPCR, respectively (Fig. 4.4 B & C). Viruses are known to have high mutation rates during infection. To verify that the C349Y mutation was stable in Arabidopsis, RNA from infected Sha plants was isolated and used for cDNA synthesis. The cDNA was used to amplify a fragment of the replicase by PCR followed by sequencing. The C349Y (G1411A) mutation was found still present in systemic tissue of all biological samples infected with TMV_{Repmt}. The equal symptoms and titers observed upon infection with WT TMV and TMV_{Repmt} can therefore not be explained by a loss of the C349Y (G1411A) mutation. Overall the results show that unlike in *N. tabacum* the C349Y mutation does not affect TMV fitness in Arabidopsis. Apparently, the two hosts operate different mechanisms to control TMV accumulation.

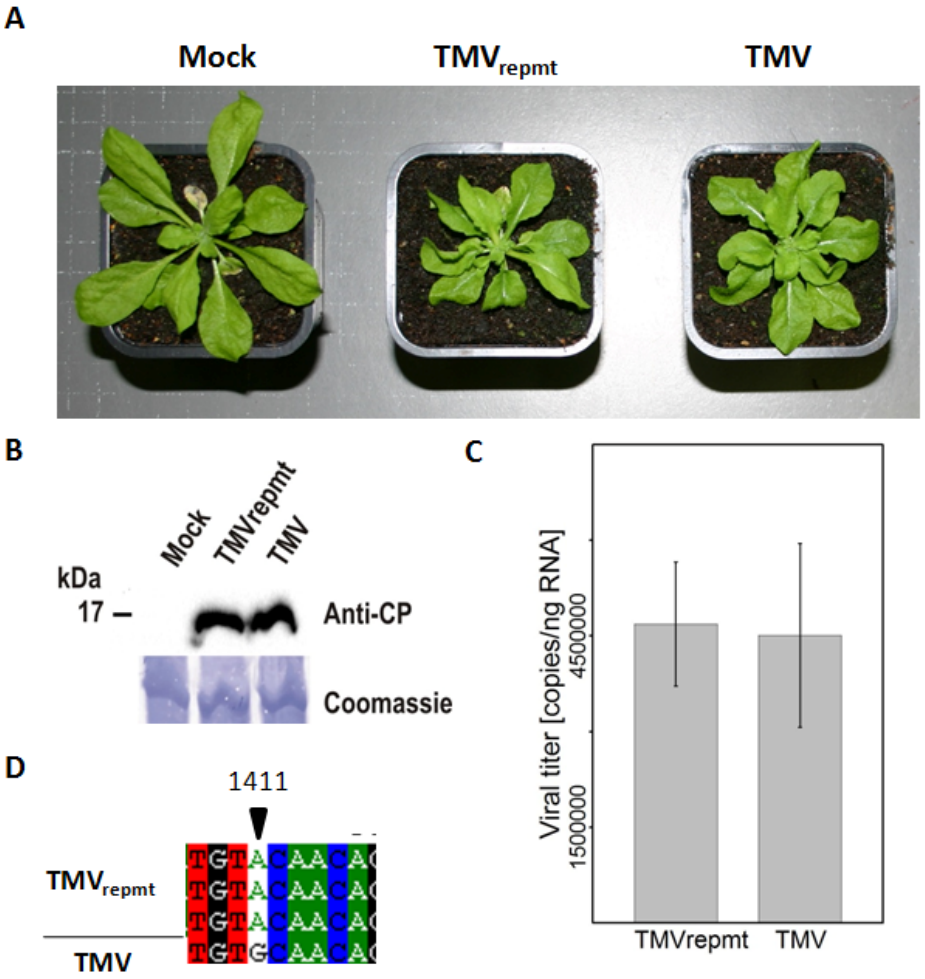

Figure 4.4 TMV and TMV_{repmt} infection in Arabidopsis Sha lead to similar symptoms and viral titers. A) Four week old Arabidopsis Sha plants were infected with TMV_{repmt} or TMV virions. Pictures represent symptoms observed at 21 dpi. B) Accumulation of the TMV CP detected by anti-CP antibodies (DSMZ) on Western blots of protein extracts from the infected tissue shown in A. Coomassie staining of total protein is shown as control for equal loading. C) TaqMan qPCR analysis of viral titers in RNA isolated from tissue of plants shown in A, n= 3 ± SE. D) The stability of A to G mutation in TMV_{repmt} was verified by sequencing of PCR amplification products of cDNA made from three different RNA extracts of TMV_{repmt}-infected plants.

RNA-silencing does not affect TMV accumulation in Arabidopsis Col-0

The experiments with TMV and TMV_{Repmt} suggest that the silencing suppressor of TMV is not active in Arabidopsis during infection or that RNA-silencing is not the limiting factor for TMV infection in this host. To further investigate a potential role of RNA-silencing in providing tolerance to TMV in Arabidopsis, we infected several RNA-silencing mutants with TMV and TMV_{Repmt}. In *dcl2,3,4* triple mutants no vsRNAs are produced and the mutant is known to be hypersusceptible to several viruses (Deleris et al. 2006; Garcia-Ruiz et al. 2010; Wang et al. 2010), including the tobamovirus ORMV, which causes symptoms in Arabidopsis (Blevins et al. 2006). The *rdr1,2,6* triple mutant does not produce secondary siRNA and is hypersusceptible to viruses with reduced silencing suppression (Garcia-Ruiz et al. 2010; Wang et al. 2010). We also included a *hen1* mutant in our experiment since non-methylated siRNA and miRNAs accumulate during tobamovirus infection (Csorba et al. 2007; Vogler et al. 2007) and interference with HEN1 activity has been suggested as a mode of action for 126k. Both sRNA and microRNA (miRNA) biogenesis are blocked in *hen1-5,* whereas the effects of the *dcl2,3,4* and *rdr1,2,6* triple mutants are restricted to siRNA biogenesis. Col-0, *dcl2,3,4* and *rdr1,2,6* showed typical weak symptoms of TMV infection at 21 dpi and no differences between plants inoculated with TMV_{Repmt} or TMV were observed (Fig. 4.5 A). In *hen1-5* symptoms were not observed but this may be due to the strong developmental phenotype of the mutant (Fig. 4.5 A). Regardless of the host genotype, TMV_{Repmt} and TMV accumulated to approximately equal levels (Fig. 4.5 B), as seen in previous experiments with Col-0 (Fig. 4.3). The results confirm the conclusion that TMV infection in Arabidopsis is not limited by RNA-silencing. Interestingly, however in *hen1-5* virus levels were significantly higher compared to all other genotypes tested (ANOVA $p=0.0001$, Fig. 4.5 B). This may indicate that HEN1 activity provides resistance to TMV in Arabidopsis; nevertheless, given that *hen1-5* mutants have a strong developmental phenotype which includes smaller cell size (Chen et al. 2002) it is difficult to make conclusions regarding HEN1 activity on viral fitness in this host.

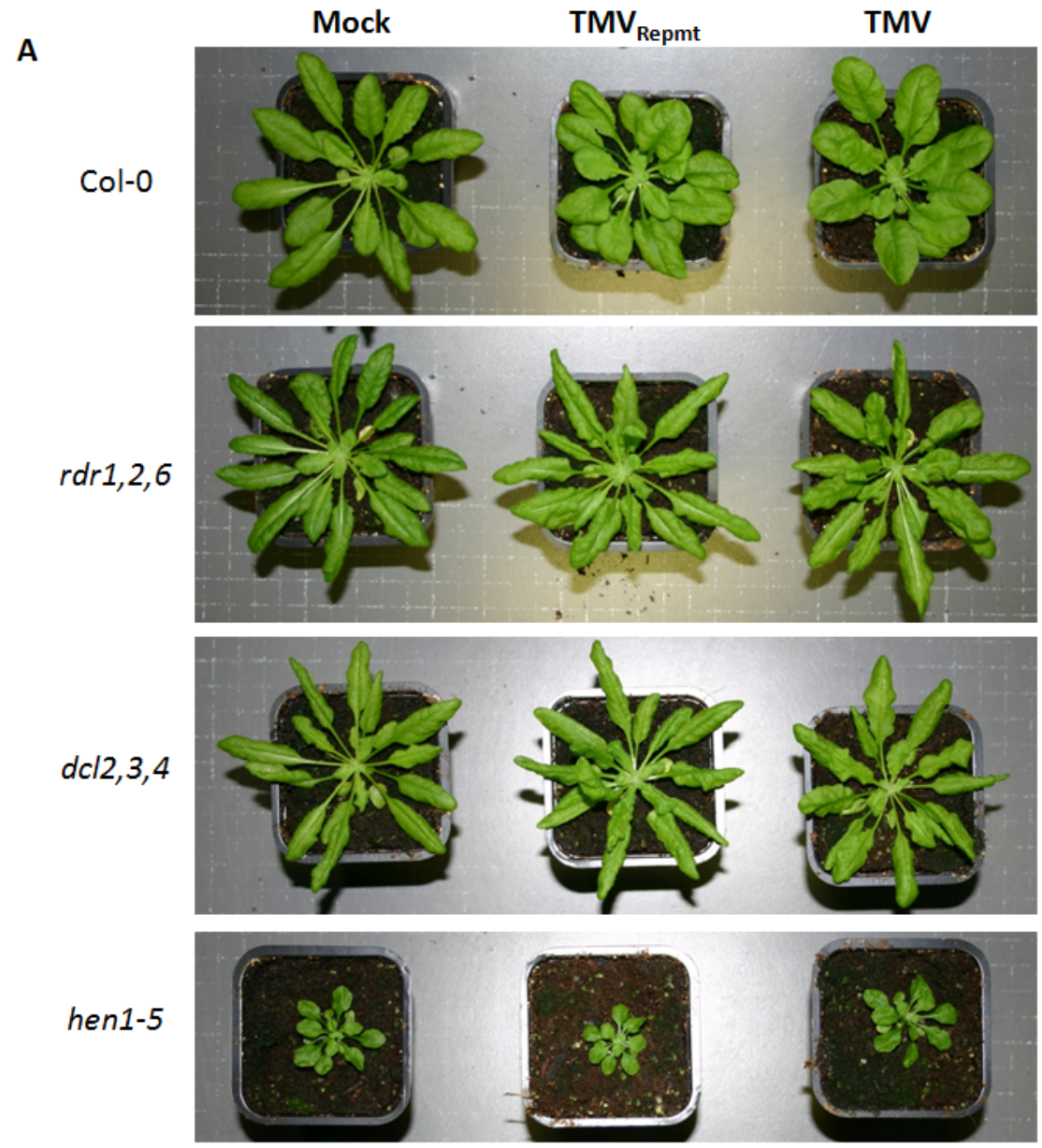

Figure 4.5 TMV and TMV$_{repmt}$ accumulate to similar levels in Arabidopsis silencing mutants. Arabidopsis Col-0, *dcl2xdcl3xdcl4*, *rdr1xrdr2xrdr6* and *hen1-5* plants were inoculated with virions of TMV$_{repmt}$ or WT TMV. A) The pictures present the average phenotype observed in the different lines at 21 dpi infected with either TMV$_{repmt}$ or TMV or mock-treated. B) Accumulation of viral RNA was measured by TaqMan qPCR analysis of RNA isolated from tissues of plants shown in A, n=3 ± SE, ANOVA, *** p<0.001, grey bars TMV$_{repmt}$, black bars TMV.

Symptomatic, but not tolerant, TMV infection induces known defense marker genes

Symptomatic virus infections are known to induce a range of defense-related genes linked to SA-signaling (Whitham et al. 2003; Love et al. 2005; Ascencio-Ibanez et al. 2008; Hanssen et al. 2011; Mandadi and Scholthof 2012). To investigate if TMV infection activates similar defense mechanisms, the expression of several genes known to be induced upon symptomatic ORMV infection (Hu et al. 2011; Niehl et al. 2012) was analyzed upon TMV infection in Col-0 and Sha plants at 21 dpi. In addition to the two pathogenesis-related markers PR1 and PR2 also the Cell Division Cycle 48b (CDC48b) and AGO1 transcript abundance was addressed. Induction of CDC48b is linked to ER-stress and viral protein stability (Niehl et al. 2012), while the induction of AGO1 is linked to anti-viral RNA-silencing (Csorba et al. 2007). Upon TMV infection none of the four genes were induced in Col-0 at 21dpi (Fig. 4.6 A, upper panel). However, TMV infection of the Sha ecotype led to a strong four-five fold induction of PR1 and PR2 and also CDC48b and AGO1 were slightly induced (Fig. 4.6 A, lower panel). Interestingly, these observations correlate the induction of PR protein genes with increased virus accumulation and stronger disease symptoms in Sha rather than with a potential defense mechanism in Col-0. Thus it appears that the induction of pathogenesis-related proteins does not represent an active mechanisms restricting TMV in Arabidopsis Col-0, but that the activation of these genes is linked to symptom severity.

SA-signaling does not restrict TMV in Col-0

Hormone defense signaling in plants is predominately linked to the hormones SA, JA and ET but other hormones may modulate the responses as well (Pieterse et al. 2012). Both synergistic and antagonistic relationships between SA, JA and ET have been reported for various pathogens. The PR1 and PR2 genes are known markers of induced SA-signaling, suggesting that symptomatic TMV infection elicits a SA-dependent response. To investigate the role of SA in defense against TMV in Col-0 a mutant impaired in SA-signaling (*npr1*) and an Arabidopsis line over-expressing the bacterial protein SALICYLATE HYDROXYLASE (NahG), which diminishes SA-levels, were inoculated with TMV. At 21 dpi, no visible symptoms could be observed in these mutants (Fig. 4.6 B) but systemic infection could be confirmed by Western blot (not shown). These results support the hypothesis that the mechanism involved in tolerance to TMV in Col-0 is SA-independent.

Hormone perception in the Arabidopsis ecotype Sha

It is well established that abscisic acid (ABA)-perception in Sha is reduced (Clerkx et al. 2004) and that this ABA-insensitivity affects Sha's germination rate as well as drought and salt tolerance (Clerkx et al. 2004; Bouchabke et al. 2008; Ren et al. 2010). Sha is also more susceptible to necrotrophic fungi *Sclerotinia sclerotiorum*, a trait linked to impaired ABA- and JA-signaling (Perchepied et al. 2010). Taken together these observations indicate that hormone signaling in Sha differs from that of other ecotypes such as Col-0 or Ler, and this could influence the tolerance to TMV. To explore this further, we compared JA perception and signaling between Sha and Col-0. The hormone JA is known to be involved in defense against necrotrophic pathogens, wounding responses and acts as a repressor of SA-signaling. A classic test for JA perception is seedling growth inhibition in the presence of JA in growth medium (Feys et al. 1994).

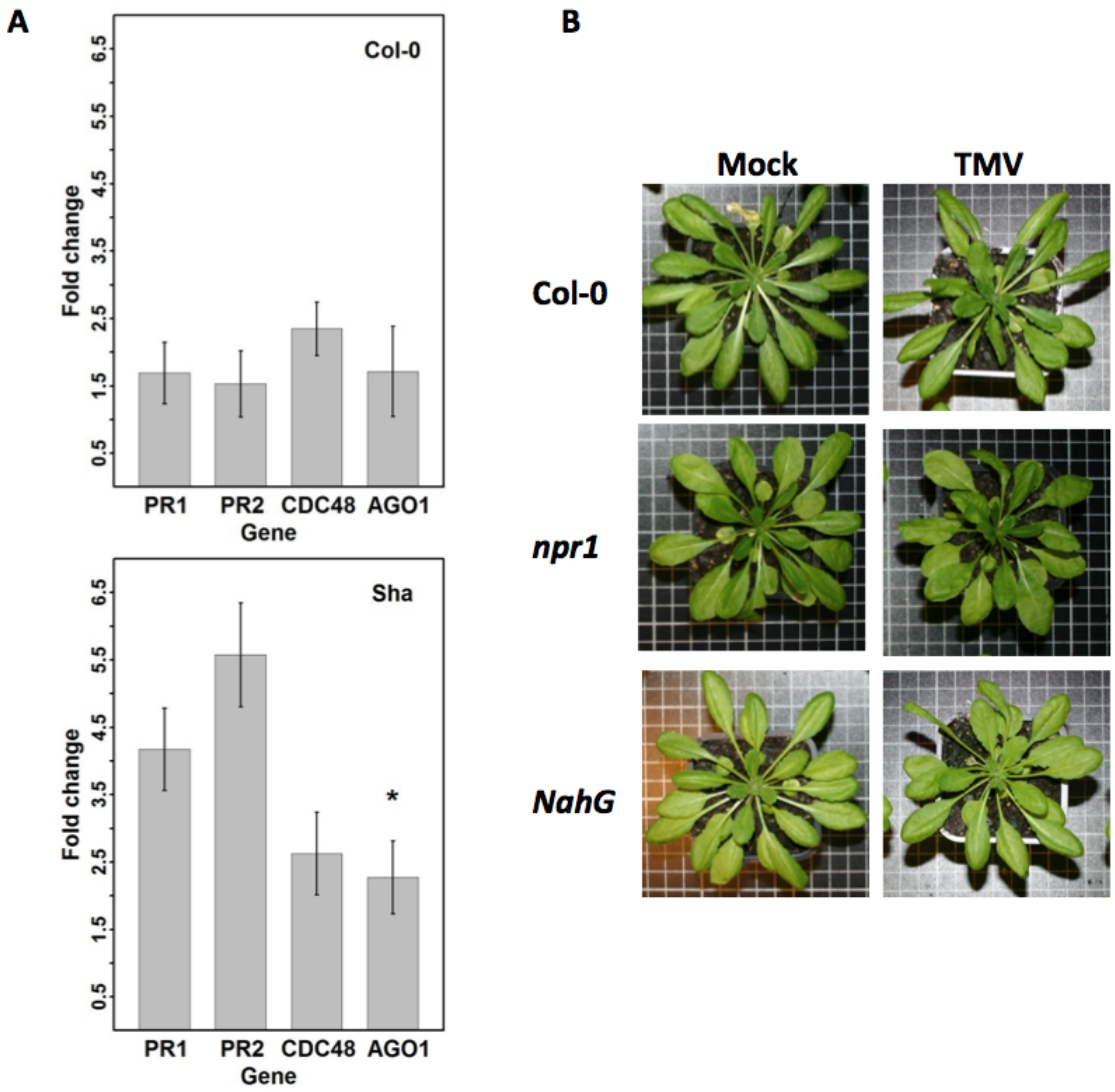

Figure 4.6 Symptomatic TMV infection in Arabidopsis ecotype Sha induces the expression of SA-associated defense marker genes, but SA-production and signaling mutants are not more susceptible to TMV. A) Gene expression of marker genes was measured by qPCR using cDNA made with RNA isolated from Col-0 and Sha plants infected with TMV at 21 dpi. The fold change in comparison to mock-inoculated plants is shown, n=3, ±SE, Student's t-test, * $p<0.05$. B) Four weeks old Col-0, NahG-overexpressing plants and *npr1* mutant plants were infected with TMV virions. Pictures show plants infected with TMV or mock-treated at 21 dpi.

Seedlings of Col-0 and Sha grown with MeJA exhibited different phenotypes. A 40 percent reduction in growth was observed for Col-0 at 10 µM of MeJA while only 12 percent growth reduction was observed in Sha seedlings after ten days of MeJA treatment (Fig. 4.7 A). At higher concentrations of MeJA both ecotypes responded similarly to MeJA with regard to growth inhibition but Sha seedlings accumulated less anthocyanins (not shown). Another physiological response to application of MeJA is leaf senescence (He et al. 2002). Col-0 leaf disks incubated with MeJA appeared yellow-brownish after three-four days indicating accelerated break-down of chloroplasts, while Sha leaf disks remained green (Fig. 4.7 B). Reduced seedling growth inhibition and delayed senescence in Sha indicates that this ecotype is less sensitive to applied JA as compared to Col-0, but this may not reflect the response to changes in endogenous JA levels. Systemic wound responses depend on the

induction and perception of endogenous JA (Glauser et al. 2009). To test if endogenous JA-signaling was impaired in Sha, leaves of four weeks old Sha and Col-0 plants were either not treated or briefly wounded by rub-inoculation and systemic tissue was harvested two hours later. Systemic wound responses in Arabidopsis include the transcriptional induction of the JA-biosynthesis enzymes LIPOXYGENASE (LOX) 2 and LOX3 as well as JASMONATE ZIP DOMAIN 10 (JAZ10) and VEGETATIVE STORAGE PROTEIN 2 (VSP2) (Berger et al. 2002; Glauser et al. 2009). The basal expression levels of LOX2, LOX3 and JAZ10 were similar for untreated Col-0 and Sha but VSP2 expression was 27-fold higher in Col-0 compared to Sha. Wounding induced all four marker genes in systemic tissue of both Col-0 and Sha (Fig. 4.7 C). The higher fold change for VSP2 in Sha can be explained by the low basal levels. These results show that the systemic wound response is not impaired in Sha.

During senescence JA interacts with the plant hormone ethylene (ET) (Fischer 2012) and to rule out that the effect observed for Sha in the senescence assay was not caused by ET-insensitivity, the classic triple response ET-signaling assay was performed with Sha and Col-0 seedlings. When germinated in the dark seedlings become etiolated. In the presence of ET-precursor 1-Amino-Cyclopropane-Carboxylate (ACC) this response is inhibited and seedlings have shorter, thicker hypocotyls and the hypocotyl forms a hook (Fig. 4.7 D). ET-insensitive mutants remain elongated even under high ACC concentrations (Guzman and Ecker 1990). When germinated on ½ MS plates with 20 µM ACC both Col-0 and Sha seedlings displayed the classical triple response (Fig. 4.7 D). Taken together these results suggest that some JA-responses are dampened in Sha but JA-dependent systemic wound signaling and ET perception are functional.

Arabidopsis ecotype Sha senses viral-induced DAMP

Viral pathogens in animals are recognized by innate immunity receptors (Jensen and Thomsen 2012) whereas no similar mechanism has been described in plants. However, Arabidopsis mutants of the central innate immunity regulator BAK1 are more susceptible to several RNA viruses, including TMV, and crude extracts from virus-infected plant elicits classical innate immunity responses such as ET burst, growth inhibition and mitogen-activated protein kinase (MAPK) activation in a BAK1-dependent manner (chapter 3). To investigate if the enhanced susceptibility of Sha to TMV could be linked to weakened innate immunity responses, the response of Sha to crude extracts from *Turnip crinkle virus*(TCV) infected *N.benthamiana* was tested. The ET-bursts in respond to the classical elicitor flg22 and to crude extracts from virus-infected plants were similar in Col-0 and Sha (Fig. 4.7 E) showing that the perception of virus-induced DAMP is not impaired in Sha.

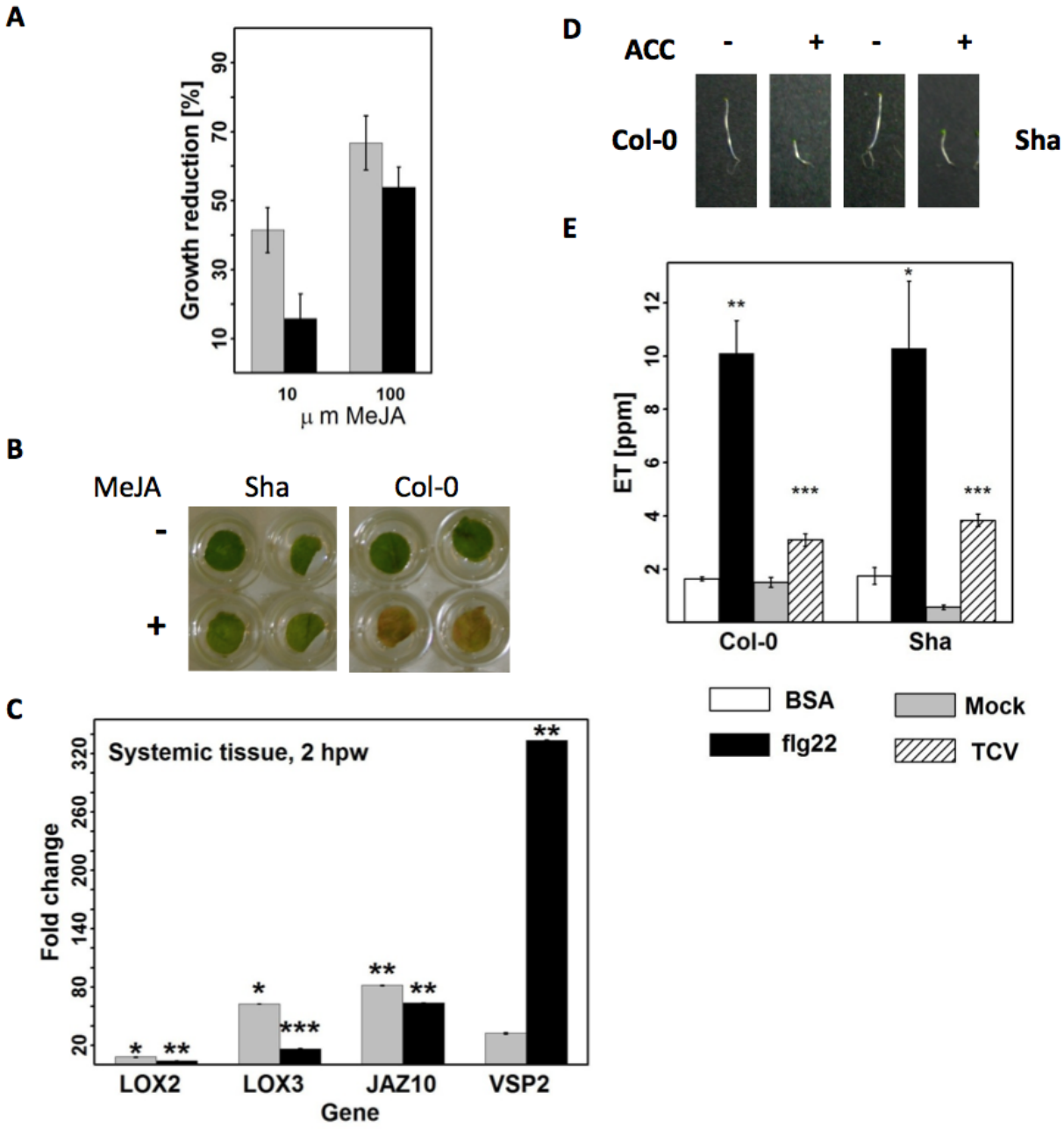

Figure 4.7 Characterization of JA, ET and PAMP perception in Sha plants. A) Five days old seedlings were grown in ½ MS medium containing 10 or 100 µm MeJA. Growth reduction in percent compared to seedlings grown without MeJA. Mean of ratios from four independent experiments ±SE, grey bars Col-0, black bars Sha. B) Leaf disks from six-eight weeks old plants were incubated with or without 100 µm MeJA to induce senescence. Picture shows senescence in leaf disks after three - four days of treatment. C) Two leaves of four weeks old plants were either not treated or mock inoculated. Systemic tissue was harvested after two hours and expression of wounding response marker genes measured by qPCR, n=2-3 ± SE, Student's t-test comparing each treatment to their corresponding untreated control, * p<0.05, ** p<0.01, *** p< 0.001. The data are shown as fold change compared to untreated plants, hpw – hour post wounding, grey bars Col-0, black bars Sha. D) To investigate ET perception Col-0 and Sha seeds were germinated at room temperate without light on plates with or without 20 µM ACC. Pictures taken five days after germination. E) Crude extracts from TCV infected *N.benthamiana* plants and positive control flg22 was used to induce ET production in leaf stripe of Col-0 and Sha plants, n= 5 ±SE, Student's t-test comparing each treatment to their corresponding mock treatment, * p<0.05, ** p<0.01, *** p< 0.001.

4.4 Discussion

In compatible virus-host interactions the virus infection often causes disease symptoms. However, plants may also remain without symptoms despite the presence of the virus, a phenomenon referred to as tolerance (Palukaitis and Carr 2008). It has been suggested that tolerance is due to the failure of the virus to suppress the defense mechanism of the tolerant host and thus accumulate to lower levels (Agudelo-Romero et al. 2008). RNA-silencing is a conserved defense mechanism against viruses but the role of silencing in plant tolerance to viruses has not been addressed until now. In this study we show that a mutation disrupting silencing suppression in TMV affects viral fitness in the symptomatic host, *N. tabacum* (n/n), whereas silencing had no effect on symptoms and virus accumulation in the tolerant host Arabidopsis. It cannot be ruled out that the activity or mode-of-action by which the TMV 126k protein suppresses RNA-silencing depends on host factors not conserved between *N. tabacum* (n/n) and Arabidopsis. Until now detailed molecular studies of RNA-silencing components have mainly been carried out in Arabidopsis and it remains to be shown whether the knowledge obtained from Arabidopsis with regard to RNA-silencing applies across the plant kingdom. However, both the 126k of TMV and 122k, the silencing suppressor of the ORMV-related crTMV, can suppress silencing when transiently expressed in *N. benthamiana* (Csorba et al. 2007; Vogler et al. 2007). Furthermore, overexpression of viral silencing suppressors in Arabidopsis can lead to strong developmental phenotypes also in cases where the virus does not infect Arabidopsis (Dunoyer et al. 2004). In a study using TMV/ORMV virus chimera, it was shown that infection of the Arabidopsis ecotypes Can-0 and RLD with TMV carrying the replicase of ORMV caused ORMV-like symptoms (Mansilla et al. 2009). It could therefore be speculated that TMV fails to accumulate in Arabidopsis because of insufficient suppression of the host silencing machinery. However, if this was the case we should expect Arabidopsis silencing mutants to be more susceptible to TMV. *dcl2,3,4* triple mutants are known to be hypersusceptible to viruses causing symptoms (Blevins et al. 2006) as well as to viruses such as *Tobacco rattle virus* (TRV), PVX and *Cucumber mosaic virus* strain-Q (CMV-Q) which are not symptomatic in Arabidopsis (Deleris et al. 2006; Diaz-Pendon et al. 2007; Donaire et al. 2008; Jaubert et al. 2011) and viruses with reduced silencing suppression (Diaz-Pendon et al. 2007; Garcia-Ruiz et al. 2010; Wang et al. 2010). *rdr1* and *rdr6* single and double mutants have been shown to be more susceptible to the non-adapted virus TRV (Donaire et al. 2008). Also a mutant of CMV Fny-strain with reduced silencing suppression (CMVΔ2b) and *Turnip mosaic virus* accumulated to higher levels in *rdr1x6* mutants (Garcia-Ruiz et al. 2010; Wang et al. 2010). However, in the case of TMV an effect of silencing pathway mutations on viral infection could not be discerned. Both the *dcl2,3,4* triple mutant incapable of producing primary viral sRNAs and host sRNAs and the *rdr1,2,6* triple mutant incapable to produce secondary vsRNAs, did not show changes in susceptibility to either TMV_{Repmt} or TMV in comparison to WT. Due to the higher viral titers of both TMV_{Repmt} and TMV in the *hen1-5* mutant it cannot be excluded that silencing does inhibit TMV infection in Arabidopsis, but *hen1*-mutants are compromised in several pathways not directly linked to the anti-viral defense such as the miRNA pathway. The *hen1-5* mutant also exhibits much stronger developmental phenotypes

compared to *dcl2,3,4* and *rdr1,2,6* triple mutants and the observed accumulation of TMV in *hen1-5* may therefore be caused by secondary effects. Viral susceptibility is not commonly tested in *hen1*- mutants and so far it has only been reported that *hen1-4* is more susceptible to CMV (Boutet et al. 2003). Considering the complete blockage of anti-viral silencing in the *dcl2,3,4* triple mutant the results presented here indicate that anti-viral silencing is not directly limiting the accumulation of TMV in Arabidopsis. Given the hypersusceptibility of *hen1-5* to TMV, a role of the miRNA pathway in restricting TMV in Arabidopsis cannot be excluded.

The hormone SA induces another defense pathway believed to act against viral pathogens. In Arabidopsis the role of SA-regulated defense genes in viral defense has remained elusive since Arabidopsis SA-production or –signaling mutants do not show increased susceptibility to compatible viruses (Revers et al. 2003; Huang et al. 2005; Love et al. 2007; Rajakaruna et al. 2007). We have shown here that TMV induces SA-responsive PR-genes; however this induction is positively correlated with symptom severity. Furthermore SA-mutants are not more susceptible to TMV, thus indicating that SA-mediated defense is not involved in tolerance of Col-0 to TMV. Our observations are in agreement with a recent report showing that overexpression of PR2 does not interfere with the progression of tobamovirus *Turnip vein clearing virus* infection (Zavaliev et al. 2013). Taken together, the observations question a functional role for PR-proteins in restricting compatible RNA-viruses. Seemingly, this may be in contradiction with the observation that treatment with SA prior to inoculation inhibits TMV accumulation during early stages of infection in Arabidopsis (Lewsey and Carr 2009). The mechanism by which SA inhibits TMV is independent of RNA-silencing but it is not known if PR proteins are functionally involved (Lewsey and Carr 2009). Although PR-proteins are themselves regarded as anti-microbial agents, they unlikely act anti-viral, and defense mechanisms downstream of *pr* genes have not been described (Carr et al. 2010).

SA and JA-signaling pathways are known to act antagonistic and activation of the SA-pathway could represent a viral strategy to suppress JA-signaling. Perception of JA was impaired in Sha as shown by reduced growth inhibition and delayed senescence upon JA-treatment but JA-dependent long-distance wound signaling was not affected in Sha. Impaired JA-signaling is unlikely to be the cause of Sha's increased susceptibility towards TMV, but further experiments with JA-signaling mutants in Col-0 would be needed to further confirm this conclusion. The hormone ABA is involved in abiotic stress responses but ABA also acts as a negative regulation of SA-induced defense mechanism (Yasuda et al. 2008). Intriguingly, ABA treatment has been shown to induce resistance against *Tobacco necrosis virus* in *Phaseolus vulgaris* (Iriti and Faoro 2008), as well as delay systemic movement of TMV-cg in Arabidopsis (Chen et al. 2013). Given the ABA-insensitivity of Sha it would be interesting to see if ABA-signaling mutants in Col-0 background are more susceptible to TMV.

It is also possible that tolerance to TMV in Arabidopsis is independent of hormone signaling. RTM1, JAX1 and Tm-1 are believed to confer resistance to specific viruses independently of hormone signaling (Chisholm et al. 2000; Ishibashi et al. 2007; Yamaji et al.

2012). Furthermore, *myo*-inositol hexaphosphate can inhibit viral accumulation independently of SA (Murphy et al. 2008).

It has been reported that systemic movement of TMV is slow in most Arabidopsis ecotypes. In the symptomatic ecotype Sha, faster systemic movement is genetically linked to symptom development, but in the ecotype Uk-4, TMV moves efficiently without causing symptoms (Dardick et al. 2000; Serrano et al. 2008). Previous studies have used viral detection in systemic tissue as marker for systemic movement. This approach does not provide resolution with respect to the efficiencies by which the given virus moves out of the inoculated leaf and enters the phloem, movement into the systemic leaves, or accumulation above detection limit in systemic leaves. By our approach, in which inoculated leaves are detached at certain days after infection, we show that the efficiency by which TMV moves out of the inoculated leaf and enters the phloem is faster in Sha compared to Col-0. Movement out of the inoculated leaf could be influenced by structural features such as vein density or phloem architecture, but could also reflect the ability of the virus to move cell-to-cell. Examples of viruses able to move cell-to-cell within the inoculated leaf but unable to spread systemically suggest that viruses must actively suppress or hijack host mechanisms to achieve systemic movement (Hipper et al. 2013). Mapping of the loci responsible for more efficient movement in Sha would greatly enhance our understanding of viral systemic movement. The identification of “loss-of-tolerance” loci could be done by using one of the already established recombinant inbred lines with Sha as one of the founding parents (Clerkx et al. 2004; Simon et al. 2008; Huang et al. 2011). Two other loci restricting TMV movement in Arabidopsis, Virus Systemic Movement 1 (*vsm1*) (Lartey et al. 1998) and Delayed Systemic Tobamovirus Movement 1 (*DSTM1*) (Pereda et al. 2000; Serrano et al. 2008) also await mapping.

4.5 Materials and Methods

Plant growth conditions and virus inoculation

Arabidopsis plants were grown in a Sanyo phytochamber (Panasonic, Japan) at 21 °C with 12 h/12 h light dark cycles. The fifth and sixth leaves of four week old plants were each rub-inoculated with carborundum and 150 ng purified virions dissolved in water or water. For the leaf detachment assay both inoculated leaves were removed one, three or five dpi. Systemic wound responses were elicited by rub-inoculation of two leaves as described above with water. Two systemic leaves were harvested two hours later. *N.tabacum* Xanthi sx (n/n) plants were grown in greenhouse at 25 °C with 16 h/8 h light/dark. Third and fourth leaves of six week old plants were rub-inoculated with 150 ng of virion or water.

Western blotting

Total protein was isolated by extracting tissue ground in liquid N_2 in buffer with 100 mM TRIS, 150 mM NaCL, 10 percent glycerol, 0.04 percent NP-40 and Complete protease inhibitor (Roche, Switzerland). After centrifugation at 10.000g for 10 minutes at 5 °C the supernatant was used for Bradford analysis (Biorad, USA). Extracts were diluted to 2 µg/µl in 2XLämmli samples buffer and denatured for 10 minutes at 95 °C. Equal amounts of proteins were separated on 12 percent SDS-PAGE gels, followed by wet blotting to PVDF-membranes. TMV CP was detected with polyclonal antibodies from DSMZ (Germany) followed by HRP-conjugated secondary antibodies (Pierce, USA) and luminescence detection. After development membranes were stained with Coomassie as control for equal loading.

RNA isolation and cDNA synthesis

Total RNA was isolated with Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA quantities were determined spectrophotometrically with a Nanodrop (Thermo scientific, USA). 1-2 µg of RNA was reserve transcripted with random primers using High-capacity reverse transcriptase from Applied Biosystems (USA).

Quantification of endogenous RNA transcript levels

Transcript abundance was measured by quantitative PCR using SYBR-green master mix (Roche, Switzerland) and gene specific primers, PR1 (fwd: TTCTTCCCTCGAAAGCTCAA rvs: AAGGCCCACCAGAGTGTATG) , PR2 (fwd: ATAGCCACTGACACCACCACTG, rvs: TAAGGGTAGAGATTCACGAGCAAGG), AGO1 (fwd: AAGGAGGTCGAGGAGGGTATG, rvs: CAAATTGCTGAGCCAGAACAGTAG), CDC48 (fwd: TATTCGACAAGGCACGACAG, rvs: CTCTATCTGCTGCACCACCA), LOX2 (fwd: CTATGGAATCTTCGTAAGACTCATG rvs: CGGCTGAACTTAGCTCTAATGCATA), LOX3 (fwd: CGGATAGAGAAAGAGATTGAGAAAAGGAAC, rvs: AGGTACACCTCTACACGTAACACCAGG), JAZ10 (fwd: TCGCAAGGAGAAAGTCACTGCAAC, rvs: CGATTTAGCAACGACGAAGAAGGC), VSP2 (fwd: CAAAATATGGATACGGGACA, rvs: ATTGCCAACGATGTTGTATC) in a Lightcycler 480 (Roche, Switzerland). The housekeeping gene AT1G13440 was used for normalization (fwd: TTGGTGACAACAGGTCAAGCA, rvs: AAACTTGTCGCTCAATGCAATC). Fold changes in gene expression levels were calculated using a relative standard curve (Livak and Schmittgen 2001).

Viral titer measured by TaqMan -qPCR

TMV titers were determined by TaqMan RT-qPCR in a one-step procedure. 100 ng of RNA was reverse transcribed with SuperScript III (Invitrogen,USA) followed by qPCR with TMV

specific primers (fwd: CCCTACACCAGTCTCCATCATTG, rvs: CGAACAGGTGTGCCTTGACA) and detected with TMV-specific probe ([6-FAM] AGACAGCCCACATGTTTTGGTCGCA [TAM]) from Sigma, USA). An internal standard curve with *in vitro* transcribed TMV RNA was used to calculate the concentration of viral RNA.

Northern blotting

4 µg of total RNA was run on 1 percent agarose formaldehyde gels and transferred to nylon membranes N+ (Amersham, USA). TMV-specific probe was made by PCR using following primers: fwd: ATCTCAGTTCGTGTTCTTG, rvs: TCTAATACCGCATTGTACC. The PCR fragment was cleaned up with Wizard SV PCR clean-up system (Promega, USA) and used for radioactive labelling with α[^{32}P]-dCTP. The membrane was hybridized over night at 45°C in PerfectHyb Plus Hybridization Buffer (Sigma, USA) and washed five times in 2XSSC (300 mM NaCl, 30 mM $Na_3C_6H_5O_7$, pH 7) 0.5 percent SDS at 45 °C. The membrane was exposed over night on a phosphorimaging screen (GE Healthcare, UK) and scanned with a Typhoon FLA 7000 phosphoimager (GE Healthcare, UK).

Verification of the G1411A mutation in TMV_{repmt}

A TMV replicase PCR fragment was amplified with the primers AATACCTGGTTTTGTAAGTTTTC (fwd) and AACTTGCTAATCAGTAAGTCATC (rvs) from cDNA made with RNA from infected plants. PCR fragments were cleaned up with Wizard SV PCR clean-up system (Promega, USA) and sequenced using an ABI 3500 capillary sequencer (Applied Biosystems, USA).

ELISA-detection of TMV virions

Infection of TMV was verified by double-sandwich ELISA with AP-labeled antibodies (DSMZ, Germany) following the manufacturer's instructions. Colorimetric alkaline phosphotase assay was performed with Sigma FAST (Sigma, USA).

JA-induced seedling growth inhibition

Five days after germination on ½ MS plates, sterile seedlings were transferred to liquid MS medium supplied with MeJA to a final concentration of 10 or 100 µM. The effect of the treatment on seedling growth was analyzed after ten days by weighing the seedlings.

JA-induced senescence

Leaf disks of six to eight week old Col-0 and Sha plants were floated water with 0 or 100 µM MeJA in growth chamber conditions described above for three to four days.

Ethylene perception

Sterile seeds were placed on ½ MS plates with 0, 10 or 20 µM of ACC and place at 5 °C for two days. Plates were transferred to room temperature but kept dark and five days later seedling elongation was recorded.

Ethylene production

Ethylene production was measured as described previously (Felix et al. 1999). For the induction of ethylene by crude viral extracts, the extracts were added to the water in which the leaf strips were floating at 5 percent (v/v).

Statistical analysis

Changes in transcript abundance were analyzed by Student's t-test in Excell (Microsoft, USA). All other statistical analyzes were done in R (www-r-project.org)

5 Discussion

5.1 General discussion

Research on molecular plant-virus interactions in plants has largely been focused on the initial establishment of infection and cell-to-cell movement of the virus within the inoculated leaf. Less attention has been paid to the tug-of-war between host and virus during systemic infections (Mandadi and Scholthof 2013). In plant tissue systemically infected with viruses several anti-viral defense pathways are activated (Carr et al. 2010) and viral pathogens must efficiently circumvent these mechanisms to establish and maintain infection. Although viruses have evolved to suppress or circumvent host defense responses (Burgyan and Havelda 2011), the host may during later stages of infection regain control over the invading virus leading to recovery from viral disease, phenotypically observed by the emergence of non-symptomatic tissue (Fig. 5.1). Host tolerance is another example where the plant controls the viral pathogen. During infection in a tolerant host the virus can replicate and move but no disease symptoms are observed (Little et al. 2010). The identification of the molecular mechanisms underlying plant immune responses leading to recovery and tolerance are of general biological interest and can guide the development of new strategies for crop protection against viral pathogens.

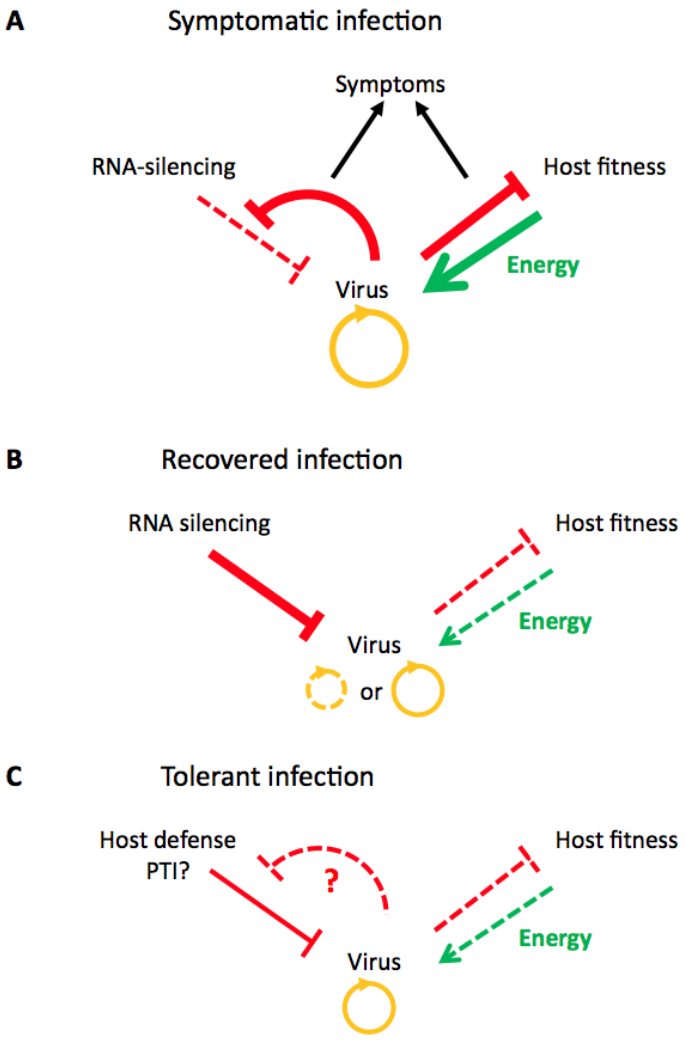

Figure 5.1 Model describing the origin of disease symptoms caused by viruses. During symptomatic infection high viral replication (represented by yellow circle) is maintained through the suppression of the host anti-viral RNA-silencing pathway. High viral replication negatively influences host fitness leading growth inhibition. This, together with suppression of RNA-silencing involved in plant development, leads to the development of disease symptoms (A). In the recovered stage the silencing suppressor is not active and often little or no viral replication takes place. The presence of the virus therefore no longer causes disease symptoms (B). In a tolerant host the virus replicates but causes no symptoms (C). Defense mechanisms involved in tolerance are unknown and most likely host-virus specific and is in some cases linked to lower viral titer.

Compatible virus infections trigger defense pathways that are either based on RNA-silencing or mediated by hormones such as SA (Carr et al. 2010). RNA-silencing is an efficient anti-viral defense response leading to the degradation of viral RNA. The RNA-silencing pathway plays a general role in sRNA-mediated gene regulation and expression of genes belonging to the RNA-silencing pathway is generally not induced by viral infection (chapter 2). This is in contrast to the expression of defense-related genes which gradually increases during virus infection (Whitham et al. 2003; Love et al. 2007; Ascencio-Ibanez et al. 2008; Mandadi and Scholthof 2012). Most defense-related genes induced by virus infection are down-stream of the accumulation of the hormone SA, but it has proven difficult to deduce the role of SA and the defense-related gene products during compatible virus infections (Carr et al. 2010). The importance of RNA-silencing for plant viral defense is better understood and the suppression of the host silencing machinery is considered essential for a plant virus to establish an infection (Burgyan and Havelda 2011). The degree to which RNA-silencing is involved in conferring host-specific tolerance is not known and host-specific resistance mediated by RNA-silencing has been described only very recently (Jaubert et al. 2011). Recovery on the other hand is believed to be at least partly dependent on RNA-silencing (Ratcliff et al. 1997; Ratcliff et al. 1999). The observation that silencing is a pre-requisite for recovery described in chapter 2 of this thesis supports this hypothesis. As described in chapter 2 recovery also correlates with a loss of viral silencing suppression activity, but it remains unknown how the host overcomes the established viral silencing suppression during late stages of the infection and further work is needed to answer this question. Several mechanisms could potentially trigger recovery such as oscillations in RNA-silencing capacity of the host, host recognition of the viral pathogen or cellular changes induced by the virus. Also non-viral plant pathogens encode proteins which suppress or circumvent host defense. Such pathogen encode proteins, referred to as effectors, and they can directly target defense pathway components to promote infection (Bozkurt et al. 2012; Deslandes and Rivas 2012; Donofrio and Raman 2012). Plant R-proteins convey immunity either by direct recognition of effector proteins or by monitoring the integrity of protein(s) targeted by effectors, the latter is referred to as the Guard Model (Dangl and Jones 2001). A mechanism, which monitors either the integrity of core components or the activity of a defense pathway represents a potent mechanism to detect invading pathogens capable of suppressing host defense pathways, and several observations suggest that viral silencing suppression activity is sensed by such host mechanisms. Host proteins recognizing VSRs due to their sRNA-binding activity have been described (Nakahara et al. 2012; Sansregret et al. 2013), implying that sRNA binding is perceived by the host, even if the biological role of this mechanism during infection is still unclear. In tomato several R-proteins are targets of miRNAs and viral silencing suppression interfering with miRNA activity therefore leads to an increase in R-protein mRNA. It was suggested that the release of R-proteins from miRNA regulation represents a second layer of anti-viral defense, where viral silencing suppression activity leads to a general induction of R-proteins (Shivaprasad et al. 2012). As recovery is initiated during systemic infection, characterized by active silencing suppression, a host mechanism monitoring RNA-silencing activity could serve as the inducer of recovery.

Another hypothesis based on the observation that SA can induce gene expression of RDR1 in plants (Xie et al. 2001; Yu et al. 2003; Yang et al. 2004) and an increase in SA-levels during virus infection (Krecic-Stres et al. 2005; Niehl et al. 2006; Miozzi et al. 2011), is that SA-signaling could induce RNA-silencing and hereby stimulate recovery. However, we did not observe altered recovery patterns for the SA-signaling mutant *npr1* and NahG-overexpressing plants, which argues against a role of SA in recovery.

Host tolerance to viral infection has not received much attention despite the fact that in natural ecosystems many plants have been found to be infected by viruses without displaying any disease symptoms (Roossinck 2013). Experiments testing the susceptibility of a range of Arabidopsis ecotypes to specific viruses have identified both resistant and tolerant ecotypes for several viruses (Martin et al. 1997; Dardick et al. 2000; Park et al. 2002), but only loci conveying resistance have been mapped so far (Dempsey et al. 1997; Chisholm et al. 2000; Takahashi et al. 2002; Yamaji et al. 2012). Most of the tested Arabidopsis ecotypes were found to be tolerant to TMV and this tolerance correlates with delayed systemic movement (chapter 4) (Dardick et al. 2000). Arabidopsis tolerance to TMV does likely not involve RNA-silencing, as mutants impaired in RNA-silencing did not display symptoms or higher viral accumulation upon infection (chapter 4). Intriguingly, in the tolerant ecotype Col-0 TMV infection did not induce the expression of SA-regulated defense-related genes and mutants impaired in SA-signaling did not develop symptoms upon infection (chapter 4). This suggests that TMV movement and symptom induction in Arabidopsis is independent of RNA-silencing and SA-signaling. One possibility could be incompatibility with host proteins needed for completion of the viral life cycle, as incompatibility with the host translation system is known to cause recessive resistance (Maule et al. 2007; Palukaitis and Carr 2008) and non-host resistance (Nieto et al. 2011). However, TMV both replicates and moves systemically in tolerant Arabidopsis ecotypes suggesting some degree of compatibility between most host and viral proteins exists. The two-fold increase in TMV accumulation observed in mutants of BAK1 (chapter 3) shows that PTI restricts TMV in Arabidopsis and could explain Arabidopsis tolerance to TMV. However, also viruses causing symptoms in Arabidopsis accumulated to higher levels in BAK1 mutants, questioning if PTI is specifically involved in tolerance towards TMV in Arabidopsis or if PTI represents a more general anti-viral defense.

The mechanism by which virus-induced PTI-signaling restricts virus infection is not known, but PTI could act up-stream of the induction of defense-related genes during systemic virus infection. However, the biological function of the induction of defense-related genes in anti-viral defense remains elusive (Huang et al. 2005; Love et al. 2005). The elicitor of PTI during viral infection still needs to be identified. The loss of elicitor activity in proteinase K treated extracts suggests that the elicitor is a peptide or protein, but unlikely the DAMP *At*PEP, as PEP-receptor mutants responded to TCV-extracts in all PTI-assays tested. However, Arabidopsis likely contain more proteinaceous DAMPs that those identified at present and the DAMP induced by viral infection may be among these. Another group of DAMPs described in Arabidopsis are the cell-wall derived oligogalacturonide (OG) fragments (Boller and Felix 2009). OGs are perceived by Wall-Associated-Kinase1 (WAK1) (Brutus et al.

2010) and their perception elicit classical PTI-responses (Denoux et al. 2008). WAK1 itself is up-regulated by SA-treatment (He et al. 1998). Interestingly, gene expression of WAK1 is also 1.5-3 log_2fold higher in ORMV-infected Arabidopsis (Kørner & Heinlein, unpublished data). Virus infection can lead to changes in cell-wall composition (Niehl, unpublished data) and virus-induced PD-modification could theoretically also lead to the release of cell-wall components such as OGs (Fig. 3.7). Identification of the virus-induced DAMP or its receptor would greatly improve the understanding of plant host defenses acting during compatible interactions.

5.2 Concluding remarks

In this thesis the role of different defense mechanisms restricting compatible viruses during infection was examined.

A system to study recovery from tobamovirus ORMV disease in the model plant Arabidopsis was indentified and characterized. We could show that recovered tissue contains infectious virus but the virus does no longer suppress RNA-silencing in recovered tissue and the virus detected in recovered tissue likely represents dormant virions. Furthermore, we could show through infections of mutants impaired in silencing show that functional RNA-silencing is a pre-requisite for recovery from ORMV disease. In contrast, a similar approach using several mutants impaired in various hormone signaling suggests that hormone signaling is not involved in recovery. Intriguingly, plant age at time of infection affected the ability of the host to recover. Further work to identify the exact molecular mechanisms behind the onset and maintenance of recovery would be of great interest. Such knowledge would both enhance our understanding of viral disease dynamics and potentially help to improve crop protection. The identification of such a mechanism would allow the testing of the generality of the results obtained with ORMV in Arabidopsis by including analysis of recovery from viral disease in other plants. Several virus diseases have been reported to lead to recovery under experimental conditions in a wide range of plants (Ratcliff et al. 1999; Xin and Ding 2003; Chellappan et al. 2004; Jovel et al. 2007; Hagen et al. 2008; Siddiqui et al. 2008; Rodriguez-Negrete et al. 2009; Lu et al. 2012), but the lack of mutants in these plants have impede the identification of the molecular mechanisms behind recovery so far.

PTI is known to play an important role in the defense against invading pathogens in both plants and animals. It is well-established that PTI restricts virus infections in animals (Osorio and Sousa 2011; Jensen and Thomsen 2012) but until now PTI has not been linked to anti-viral defense in plants. Here we show that Arabidopsis BAK1 mutants impaired in PTI-responses are hypersucceptible to several RNA-viruses. Furthermore, crude extracts from virus infected plants contain compound(s) eliciting classical PTI-responses. This suggests that virus infection induces the production of one or more elicitors which activate BAK1-dependent anti-viral defense mechanisms. Further work is needed to identify the elicitor and its corresponding receptor. One approach would be to isolate and identify BAK1-interacting proteins after treatment with crude extracts from virus-infected plants. The mechanism by

which PTI restricts virus infection also awaits identification. Analysis of infection progression in plants carrying mutations in genes acting down-stream of BAK1 or transcriptome analysis comparing WT and BAK1-mutants infected with virus could aid the identification of such mechanisms.

Host tolerance to virus infection is a neglected research topic. In this thesis Arabidopsis was explored as a tolerant host for TMV infection. Previous reports on the loss-of-tolerance phenotype of ecotype Sha to TMV linked this to accelerated movement (Dardick et al. 2000). Using a different experimental set-up this was confirmed for our growth conditions. Both RNA-silencing and SA-signaling have been shown to play a role in restricting viruses in plants (Alamillo et al. 2006) but mutants of RNA-silencing and SA-signaling in the tolerant ecotype Col-0 background were not more susceptible to TMV. This suggests that other mechanisms control tolerance to TMV in Arabidopsis. The mapping of the loss-of-tolerance loci in Sha is therefore likely to provide new knowledge on how plants restrict viral infections.

Taken together, the results presented in this thesis highlight the diversity of plant defense pathways modulating the outcome of compatible virus infection. A more detailed understanding of these pathways and their cross-talk would greatly aid the development of new approaches to improve crop protection against viral pathogens.

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Lundmark M,Kørner CJ,Nielsen TH (2010) ”Global analysis of microRNA in Arabidopsis in response to phosphate starvation as studied by locked nucleic acid-based microarrays”, Physiologia Plantarum:140(1):57-68.

Presentations

”Role of plant silencing pathways in recovery from viral disease symptoms” at BSPP Presidential Meeting 2012: Fitness Costs and Trade-offs in Plant-Parasite Interactions, 16th to 18th December 2012, Norwich UK, invited speaker

”Role of plant silencing pathways in recovery from viral disease symptoms” Department seminar, Institute de Biologie Moléculaire des Plantes, Strasbourg, 7th February 2013.

Other publications

"Significance of Copper for the Uptake and Detoxification of Atrazine by Poplar Tree Species", Kørner,C., Knillmann, S., Ehlting, B., Wennrich, R., Brüggemann, N., Wesenberg, D., Krauss, G-J., & Rennenberg, H. Abstract at the 7th Workshop on Sulphur Metabolism in Plants, Warsaw, Poland, May 13-17, 2008

Teaching experience

Coordinator of student journal club
Technical assistance during practical exercises
Supervision of master student

Language skills

Danish (mother tongue)
English (excellent)
German (good)

Other skills

Basic knowledge of R

8. Literature

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Appendix

Predicting Targets of Tobamoviral vsRNAs in *Arabidopsis thaliana* and *Nicotiana tabacum*

Introduction

During infection viruses are targeted by the RNA-silencing machinery of the host. Viral RNA is processed into viral small RNAs (vsRNAs) of 21-24 nt by host endonucleases. vsRNAs are loaded into ARGONAUTE (AGO) proteins within the RNA Induced Silencing Complex (RISC) and can hereafter target complementary RNA for degradation or translational inhibition (Ding 2010). To evade this defense mechanism plant viruses and some animal viruses viral encode silencing suppressors (VSR) that interfere with RNA-silencing at various steps (Soldan et al. 2005; Burgyan and Havelda 2011; Fabozzi et al. 2011; Jing et al. 2011). However, several mammalian DNA viruses do not block RNA-silencing but utilizes the RNA-silencing system to silence specific host genes through virus-encoded microRNAs (miRNA) (Cullen 2013). In contrast to virus-encoded miRNA targeting host mRNA, vsRNAs are believed to target the viral RNA through RISC, but in the presence of VSR vsRNA are predominately considered to be non-functional. However a more differential model of vsRNAs has recently been proposed. This model predicts that some vsRNAs may modulate the host transcriptome at specific stages of infection (Amari et al. 2012). Indeed, although virus infections in plants lead to massive production of vsRNAs their role in modulating the host transcriptome is still not understood (Llave 2010) and a general role of vsRNA in host transcrptome modulation has not been established in plants. However, there are several examples of single natural occurring vsRNA targeting host transcripts. The leader of *Cauliflower mosaic virus* (CaMV) was shown to produce large amounts of vsRNAs that potentially could target several host genes. Many of the predicted targets were down-regulated during CaMV infection (Moissiard and Voinnet 2006). Two distinct vsRNAs of a crucifer infecting strain *Tobacco mosaic virus*-cg (TMV-Cg) likely target separate Arabidopsis transcripts for RISC-cleavage (Qi et al. 2009). Next-generation-sequencing of small RNAs and non-capped RNA in grapevine revealed a range of host transcripts potentially targeted by vsRNA from two different grapevine infecting viruses (Miozzi et al. 2013). However, unlike for miRNAs encoded by mammalian viruses, in none of the above cases has vsRNA targeting been linked to a biological function. Not only viral vsRNAs but also small RNAs from a non-coding satellite virus and from non-coding viroids may target host transcripts. Studies on symptom severities induced by non-coding *Cucumber mosaic virus* (CMV) *Y satellite RNA* (Y-sat) identified a Y-sat vsRNA that targets the mRNA of MAGNESIUM PROTOPORPHYRIN CHELATASE SUBUNIT I (CHL1) involved chlorophyll biosynthesis (Shimura et al. 2011; Smith et al. 2011). Also sRNAs of non-coding *Peach latent mosaic viroid* (PLMVd) were shown to target a host mRNA and hereby induce strong albino symptoms (Navarro et al. 2012).

Results and Discussion

To investigate if vsRNAs modulate the host transcriptome during *Oilseed mosaic virus* (ORMV) infection in Arabidopsis, a list of all potential 21 nt vsRNA from ORMV plus and minus strands was created *in silico* and used as input for target prediction with the psRNATarget software (Dai and Zhao 2011). The Arabidopsis TAIR9 library and a cut-off score of 3 were used. The score statistically evaluates the individual predictions based on criteria deducted from known miRNA and target site with 0 being the best, often perfect matching, predictions (Dai and Zhao 2011).This approach resulted in 13.488 unique gene candidates, including some targets which were predicted to be targeted by several vsRNAs. To minimize false positives a more stringent cut-off score of 1.5 or below was used for further analysis (494 targets) (Fig. A1).

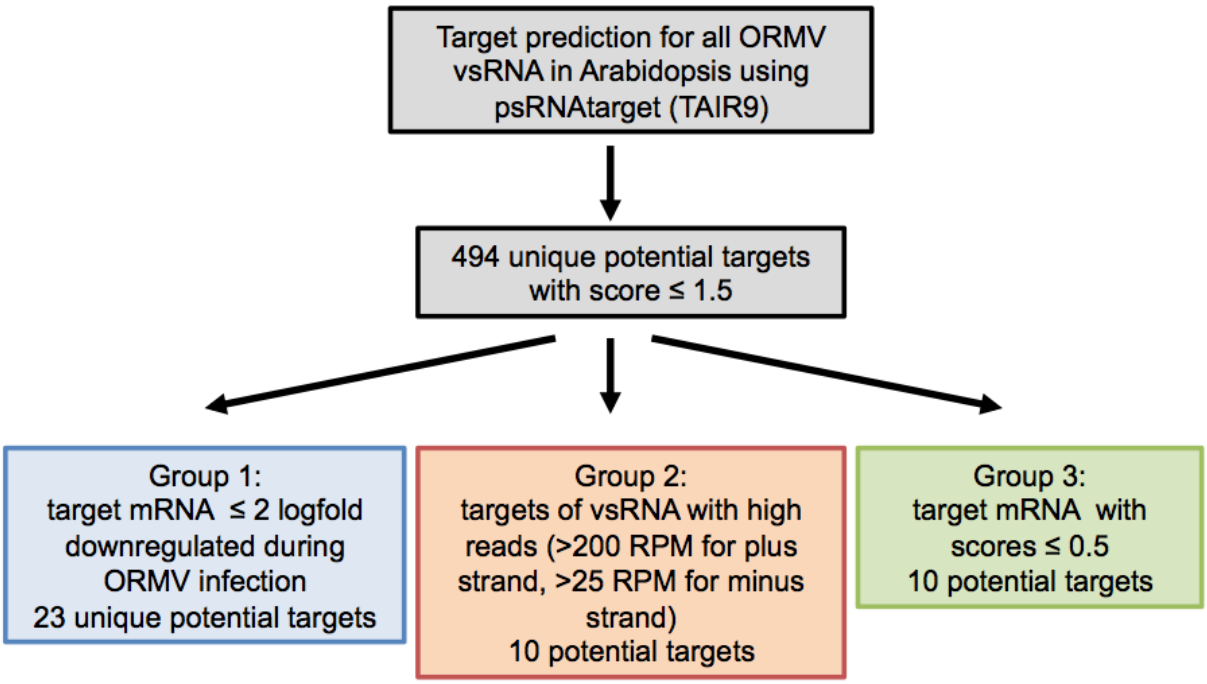

Figure A1. Work flow chart for the prediction of targets of ORMV vsRNAs in Arabidopsis. All theoretically possible vsRNA from both plus and minus strand of ORMV were constructed *in silico* and used for target prediction with the psRNAtarget. Arabidopsis TAIR9 was used as target library.

To even more narrow the list of potential candidates three approaches was used. We speculated that the successful targeting of a host transcript would lead to a decrease in transcript abundance during viral infection. We therefore filtered the list of potential targets based on their expression pattern during ORMV infection based on previous microarray experiments (Hu et al. 2011). In the first group of potential targets (group 1) 23 unique potential targets were found to be down-regulated by a $\log_2$ factor of two or more in ORMV-infected tissue compared to mock-treated tissue at 7, 14 or 21 days post inoculation (dpi) (table A1). As a second approach (group 2) potential targets were filtered based on the abundance of the corresponding vsRNA as measured by deep-sequencing at 7 dpi (Hu et al. 2011). Using a cut-off

of 200 RPM for the plus strand and 25 RPM for the minus strand, ten potential targets was identified, one of them (AT1G01080) shared with group 1 (table A2). A third group was created with the most robust prediction scores of 0.5 or 0, identifying ten potential targets (table A3).

Table A1 Predicted targets of ORMV vsRNA in Arabidopsis belonging to group 1. Targets in bold were chosen for RLM-RACE analysis.

vsRNA_ID	Target	Score	Annotation
36	AT4G11960	1	PGRL1B PGR5-like B
1118	**AT1G59910**	**1**	**Actin-binding FH2 (formin homology 2) family protein**
6022	AT5G18170	1	GDH1 glutamate dehydrogenase 1
10143	AT1G29670	1	GDSL-like Lipase/Acylhydrolase superfamily protein
10436	**AT4G16990**	**1**	**RLM3 disease resistance protein (TIR-NBS class), putative**
11400	AT2G32030	1	Acyl-CoA N-acyltransferases (NAT) superfamily protein
132	**AT5G45750**	**1,5**	**AtRABA1c, RAB GTPase homolog A1C**
1181	**AT3G07180**	**1,5**	**GPI transamidase component PIG-S-related**
1601	AT4G34560	1,5	unknown protein
1842	AT1G24100	1,5	UGT74B1, UDP-glucosyl transferase 74B1
2143	**AT4G36630**	**1,5**	**EMB2754, Vacuolar sorting protein 39**
5147	AT5G53530	1,5	VPS26A, vacuolar protein sorting 26A
5499	**AT1G01080**	**1,5**	**RNA-binding (RRM/RBD/RNP motifs) family protein**
5537	AT3G60640	1,5	ATG8G, Ubiquitin-like superfamily protein
6604	AT5G14970	1,5	unknown protein
7079	AT1G24120	1,5	ARL1, ARG1-like 1
7376	AT4G24800	1,5	MA3 domain-containing protein
8322	AT3G18080	1,5	BGLU44, B-S glucosidase 44
10669	AT4G33740	1,5	unknown protein
10977	AT4G18480	1,5	Nucleoside triphosphate hydrolases superfamily protein
11332	AT5G63030	1,5	Thioredoxin superfamily protein
11851	AT4G03510	1,5	ATRMA1, RING membrane-anchor 1
1831	AT5G16050	1- 1,5	GF14 UPSILON, general regulatory factor 5

Table A2 Predicted targets of ORMV vsRNA in Arabidopsis belonging to group 2. Targets in bold were chosen for RLM-RACE analysis.

vsRNA_ID	Target	Score	Annotation
5293	AT1G36450	1	transposable element gene
2076	**AT5G37530**	**1,5**	**NAD(P)-binding Rossmann-fold superfamily protein**
2650	**AT2G17290**	**1,5**	**Encodes calcium dependent protein kinase 6 (CPK6)**
2900	**AT5G06530**	**1,5**	**ABC-2 type transporter family protein**
3439	**AT5G24460**	**1,5**	**unknown protein**
3795	**AT1G02810**	**1,5**	**Plant invertase/pectin methylesterase inhibitor superfamily**
5499	AT1G01080	1,5	RNA-binding (RRM/RBD/RNP motifs) family protein
5499	**AT5G38900**	**1,5**	**Thioredoxin superfamily protein**
10605	AT4G26280	1,5	P-loop containing nucleoside triphosphate hydrolases superfamily protein

Table A3 Predicted targets of ORMV vsRNA in Arabidopsis belonging to group 3. Targets in bold were chosen for RLM-RACE analysis.

vsRNA_ID	Target	Score	Annotation
5490	**AT3G01750**	**0**	**Ankyrin repeat family protein**
2323	**AT2G01390**	**0,5**	**Tetratricopeptide repeat (TPR)-like superfamily protein**
6756	AT2G25170	0,5	PKL_Encodes a SWI/SWF nuclear-localized chromatin remodeling factor
47	AT2G35945	0,5	Potential natural antisense gene, locus overlaps with AT2G35940
4270	AT3G05340	0,5	Tetratricopeptide repeat (TPR)-like superfamily protein
11138	AT3G22104	0,5	Phototropic-responsive NPH3 family protein
10031	**AT3G47780**	**0,5**	**ATH6_member of ATH subfamily**
3658	**AT3G56660**	**0,5**	**basic region/leucine zipper motif protein 49 (BZIP49)**
9673	**AT5G42440**	**0,5**	**Protein kinase superfamily protein**
1121	**AT5G45410**	**0,5**	**unknown protein**

Small RNA-guided cleavage leads to the production of RNA fragments. Fragments originating from the 3’ part of the full-length mRNA contain a non-capped 5’end. Therefore, a standard method for verifying sRNA-guided cleavage is the identification of such non-capped RNA fragment by 5' RNA-Ligase Mediated-Rapid Amplification of cDNA Ends (RLM-RACE), which involves the ligation of a RNA adaptor to non-capped phosphorylated 5' ends of RNA followed by cDNA synthesis. PCRs with adaptor and gene specific primer followed by cloning and sequencing can afterwards determine RNA cleavage sites. In our experiments, RLM-RACE cDNA was created using RNA from mock-treated or ORMV-infected Arabidopsis tissue harvested at 21 dpi and six targets from each of the candidate targets gene groups mentioned above were selected for RACE analysis (marked in bold in respective tables). Of the six targets belonging to group 1, four targets (AT1G16990, AT5G45750, AT3G07180, AT1G01080) were found to give infection-specific PCR fragments of approximately the expected size based on the predicted cleavage site mediated by the specific vsRNA. The PCR-fragments were cloned and the 5'ends cleavage site was determined by sequencing. The cloned sequences matched the target genes, verifying the specificity of the primers and the quality of the RLM-RACE library, but none of the cloned 5'ends matched the predicted cleavage sites of the vsRNAs (Fig. A2). Some RNA fragments from cleaved mRNA accumulate in mutants of exonuclease XRN4 (German et al. 2008) and to enrich the pool of cleaved mRNA RLM-RACE cDNA was prepared with RNA from *xrn4-5* plants infected with ORMV and used this for PCR with gene specific primers. This approach identified two additional cleavage sites in AT4G16990 but still not matching the predicted vsRNA site (Fig. A2).

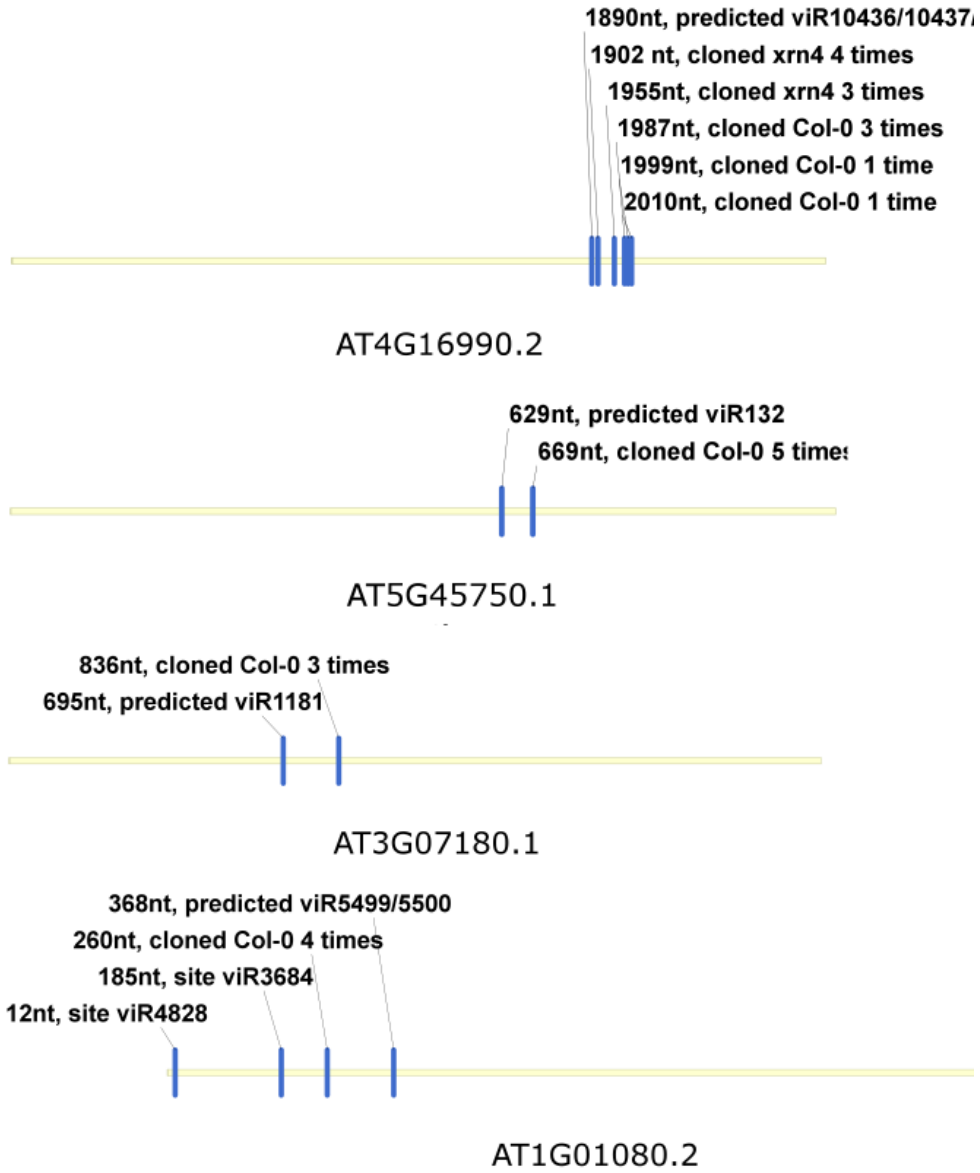

Figure A2 Cleavage sites obtained by RLM-RACE for Group 1 targets. RNA from mock-treated and ORMV-infected tissue (21 dpi) was used for RACE analysis with adaptor and gene specific primers. PCR-products of expected size in ORMV samples were cloned and sequenced. PCR products were obtained for four out of six tested genes. The ends of the cloned cDNAs are shown as well as predicted vsRNA target sites.

To verify the down-regulation observed in the microarray, transcript abundance of AT1G16990, AT5G45750, AT3G07180, AT1G01080 was measured by qPCR using cDNA made from RNA originating from an independent experiment. At 21 dpi, AT1G16990, AT3G07180 and AT1G01080 were found to be down-regulated in ORMV-infected tissue compared to mock-treated tissue, while the down-regulation of AT5G45750 could not be verified by qPCR (Fig. A3).

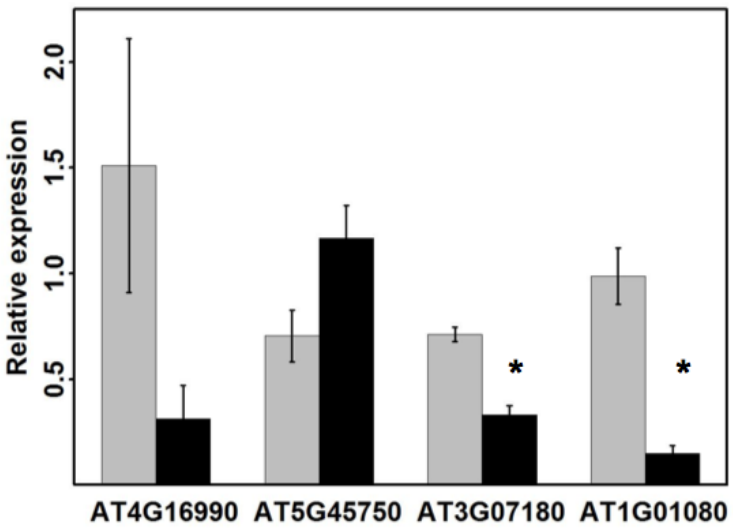

Figure A3 Confirmation of down-regulation of Group 1 targets during ORMV infection. Transcript abundance of the four genes from which RACE products could be amplified was measured by qPCR with cDNA made from RNA isolated from mock-treated or ORMV-infected Arabidopsis tissue at 21 dpi, n= 3 ± SE, * p<0.05 Student's t-test, grey bars mock, black bars ORMV.

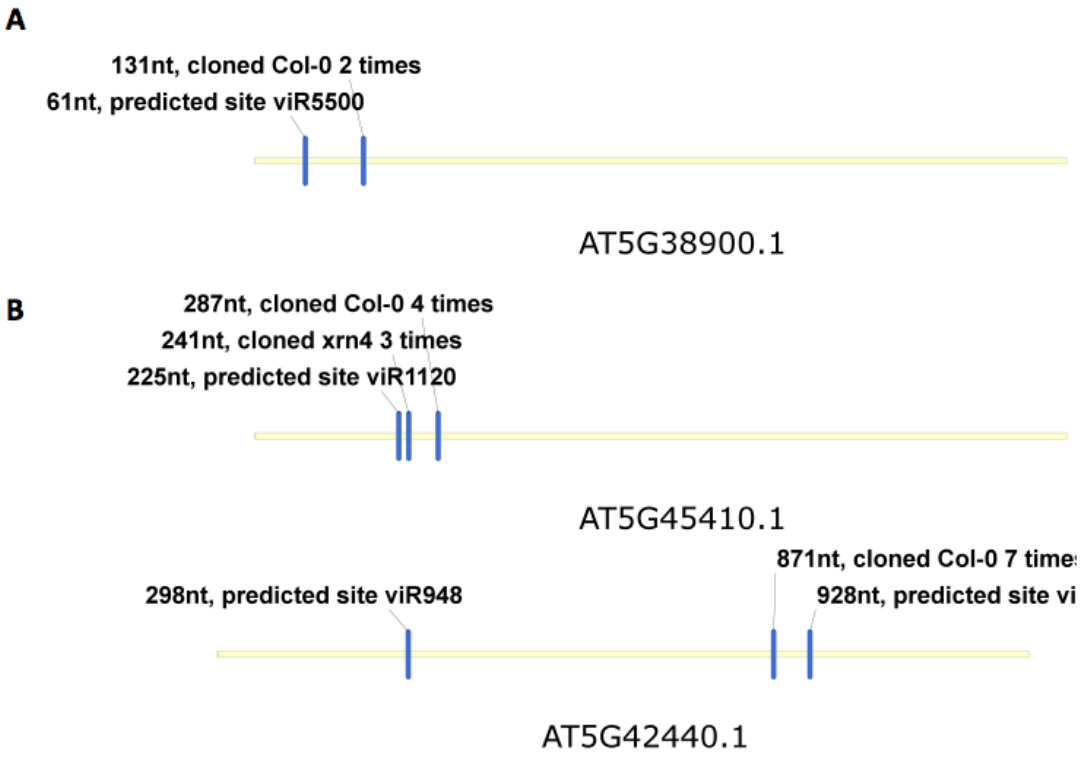

Figure A4 Cleavage sites obtained by RLM-RACE for Group 2 and 3 targets. RNA from mock and ORMV-infected tissue (21 dpi) was used for RACE analysis with adaptor and gene specific primers. PCR-products of correct size in ORMV samples was cloned and sequenced. A) One out of the six tested predicted targets from group 2 gave ORMV-specific bands. B) Two out of six tested predicted targets from group 3 gave ORMV-specific bands. The ends of the cloned cDNAs are shown as well as predicted vsRNA target sites.

Of the six predicted targets belonging to group 2 only one (AT5G38900) gave rise to PCR products of the expected size. Cloning and sequencing determined a 5'end at 131 nt, thus 71 nt away from the predicted vsRNA site (Fig. A4 A). In group 3, the 5'ends of two candidates were cloned but again neither matched the predicted vsRNA sites (Fig. A4 B). In conclusion none of the predicted targets tested here could be verified as true targets of vsRNAs.

A similar target analysis approach was adapted for *Tobacco mosaic virus* (TMV) in *N. tabacum.* The UNIGENE SGN genome library and a cut-off limit of 3 were used. This returned 10.153 unique predicted targets. The list of predicted TMV vsRNAs targets with a score of 1 or below is shown in table A4. This list includes U368942, a hydroxyproline-rich glycoprotein and U383342, a protein containing leucine-rich-repeats. Both proteins belong to protein families involved in defense (Pearce and Ryan 2003; Deepak et al. 2007; Elmore et al. 2011). Five potential targets were chosen for verification by measuring the respective transcript levels by qPCR. Six week old *N.tabacum* plants were infected with TMV_{Repmt} or TMV. TMV_{Repmt} carries a point mutation that reduces the silencing suppression activity of the 126k replicase subunit (Vogler et al. 2007). Thus, in tissues infected with the mutant virus, the vsRNAs are expected to be more active as compared to tissue infected with wild type virus. However, as compared to wild type TMV, TMV_{Repmt} accumulates to lower titers, less vsRNAs accumulate during infection as compared to wild type TMV (Vogler et al. 2007), and at 14 dpi systemic leaves of plants infected with TMV display strong symptoms, while those infected with TMV_{Repmt} do not (see Fig. 4.2). Nevertheless, RNA was isolated from systemic leaves of plants infected with TMV_{Repmt},TMV or mock-treated at 14 dpi and used for cDNA synthesis and further qPCR analysis. U368942 and U367497 were slightly but significantly down-regulated only in tissue infected with wild type TMV, while U383342 showed a tendency of down-regulation in TMV-infected tissue compared to mock. With the exception of U367497, that shown a tendency of down-regulation, relative expression was not changed in TMV_{Repmt}-infected tissue compared to mock-treated tissue. U369299 transcript abundance in TMV_{Repmt}- and TMV infected tissue was similar to that of mock-treated tissue, while U383886 relative expression was slightly higher in TMV-infected but not in TMV_{Repmt}-infected tissue. The decrease in abundance of U368942, U367497 and U383342 mRNA in TMV-infected tissue could indicate that these targets are targeted by TMV vsRNAS. To investigate this further important to determine the activity of these vsRNAs by RLM-RACE or degradome analysis.

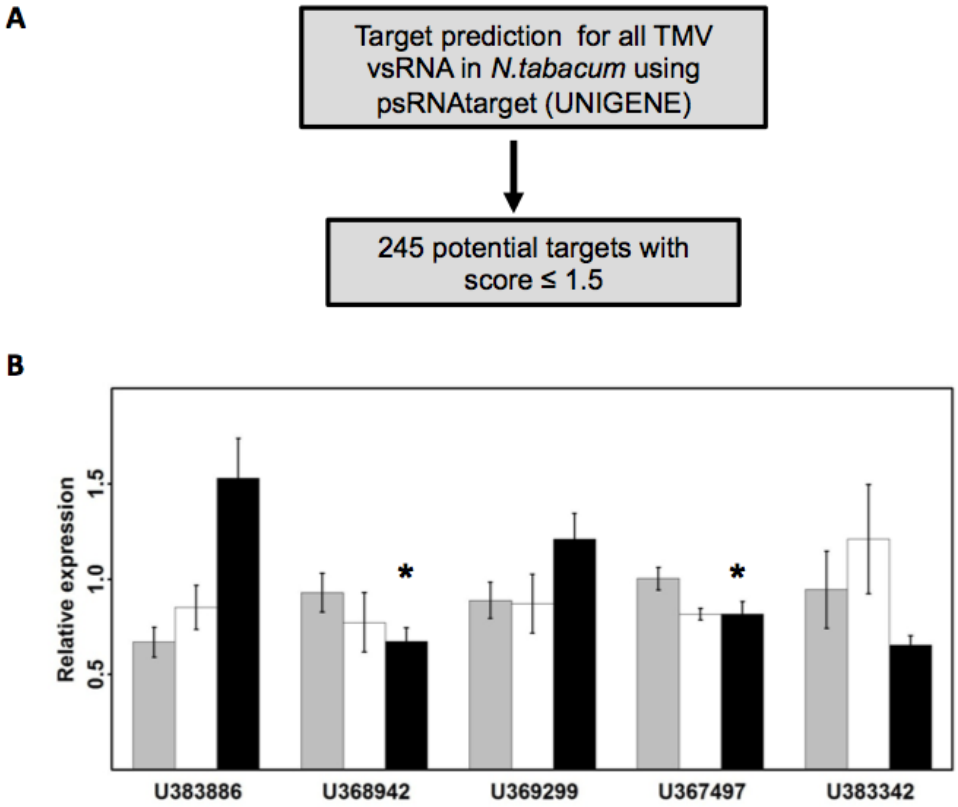

Figure A5. Predictions of targets of TMV vsRNAs in *N. tabacum.* A) All theoretically possible vsRNA from both plus and minus strand of TMV were constructed *in silico* and used for target prediction with psRNAtarget. The *N.tabacum* UNIGENE database was used as target library. 245 unique potential targets with a score ≤1.5 were retrieved. B) Gene expression of five predicted gene targets was measured by qPCR with cDNA made from RNA isolated from mock-treated or TMV_{Repmt} - or TMV-infected *N.tabacum* tissue harvested at 14 dpi, n=3 ±SE, * p<0.05 Student's t-test, grey bars mock, white bars TMV_{repmt}, black bars TMV.

Table A4 Predicted targets of TMV vsRNA in *N.tabacum*. Targets in bold were chosen for qPCR analysis.

vsRNA_ID	Target	Score	Annotation
13008	U374904	0	viral protein
19107	**U383886**	**0**	**encodes a member of the DREB subfamily A-6 of ERF/AP2 family**
15270	**U368942**	**0,5**	**hydroxyproline-rich glycoprotein family protein**
17346	**U369299**	**0,5**	**phosphoglucomutase**
4864	U363036	1	similar to cullin family protein
17420	U363155	1	myb family transcription factor
15295	U363578	1	protease inhibitor, putative
1051	U363984	1	farnesyl pyrophosphate synthetase
14476	U364465	1	unknown
16233	U364652	1	unknown
1050	U364938	1	farnesyl pyrophosphate synthetase
17411	U366455	1	cinnamoyl-CoA reductase-related
1798	U366774	1	photosystem II 22kDa protein
17441	U367084	1	23.5 kDa mitochondrial small heat shock protein (HSP23.5-M)
14702	U367450	1	4'-phosphopantetheinyl transferase family protein
1786	**U367497**	**1**	**extra-large guanine nucleotide binding protein**
16193	U369790	1	proline-rich family protein
3829	U371672	1	Encodes a protein disulfide isomerase-like (PDIL) protein
5234	U371729	1	zinc finger (AN1-like) family protein
1471	U371964	1	expressed protein
3983	U372731	1	cytochrome P450 83B1 (CYP83B1)
3446	U374816	1	Encodes a gibberellin 2-oxidase
18284	U375770	1	senescence-associated family protein
5565	U375885	1	basic helix-loop-helix (bHLH) family protein
1131	U377528	1	unknown
16745	U380150	1	UVB-resistance protein-related / regulator of chromosome condensation
4911	U380776	1	unknown
21	U380881	1	unknown
14174	U381134	1	Encodes a protein with similarity to carotenoid cleaving deoxygenases
18786	U381626	1	lactoylglutathione lyase family protein / glyoxalase I family protein
13707	U381674	1	CCAAT displacement protein-related / CDP-related
3445	**U383342**	**1**	**disease resistance family protein, contains LRR domains**
13707	U383461	1	CCAAT displacement protein-related / CDP-related
3825	U384158	1	unknown
13139	U387914	1	ubiquitin-like protein (SMT3)

Together these data suggest that vsRNAs can potentially target a large range of host transcripts. That none of the 18 Arabidopsis transcript predicted to be targeted by ORMV vsRNAs examined by RLM-RACE could be verified as true targets could indicate that vsRNAs are not functional due to VSR activity in the systemically infected tissue used for this analysis. This is in contrast to a previous report, where two Arabidopsis mRNA targets of TMV-cg vsRNAs, a virus very similar to ORMV, were verified by RLM-RACE (Qi et al. 2009). The two verified targets were

also found in our bioinformatic analysis. In the analysis of Qi et al. (2009) the predicted target site for mRNA of AT1G30460 was situated from 190-221 nt and targeted by a plus strand vsRNA. Our analysis predicted that a vsRNA of the minus strand would target AT1G30460 mRNA at a different position (2313-2333 nt) with a prediction score of 2. The second verified target AT2G16595 identified by Qi et al. (2009) was in our analysis predicted to be targeted at the same position (520-540 nt) but given a two nt divergence between the vsRNA of TMV-cg and ORMV, the score in our prediction was 2, and AT2G16595 was therefore not considered a likely *in vivo* target. In both cases the respective vsRNAs from TMV-cg and ORMV differed in only two nucleotides. This highlights that small changes in the viral genome can affect the pool of predicted vsRNA targets dramatically. It should be kept in mind that RNA with phosphorylated 5'ends cloned by RLM-RACE can originate from RISC-mediated cleavage events but may also be caused by mechanical shearing of the RNA. Cloned RLM-RACE 5'ends matching vsRNA target site should therefore be validated with RLM-RACE cDNA from several independent experiments and by other analysis such as measuring target mRNA or protein abundance by qPCR or western blot, respectively.

Although no function could be assigned to the vsRNAs investigated here, vsRNAs may successfully target host genes. Recombinant plant viruses carrying 200 – 1300 nt long fragments of a specific host gene are known to cause down-regulation of host gene through homologous vsRNAs during infection, referred to as Virus Induced Gene Silencing (VIGS) (Becker and Lange 2010). VIGS is an artificial system producing a large population of perfect-match vsRNA targeting a specific gene, unlike endogenous miRNAs that target only 21 nt of their target, in turn this may explain the efficiency of VIGS compared to single vsRNAs with homology with host transcripts. It has been shown that a VIGS-vector carrying a 21 nt homologous fragment could induce VIGS of the corresponding host genes, if the homologous fragment was inserted in a plant miRNA precursor backbone (Tang et al. 2010). In Arabidopsis miRNA precursors are processed by endonuclease DICER-like 1 (DCL), while viral dsRNA are processed by DCL 2, 3 and 4, depending on the virus (Blevins et al. 2006; Deleris et al. 2006). That a 21 nt VIGS-miRNA, presumably produced by DCL1, has the same efficiency as a VIGS vector carrying a fragment of 100-300nt, where vsRNA are produced by other members of the DICER-like family, suggests that not abundance but biogenesis pathway determine the potency of vsRNAs. vsRNAs produced through the miRNA biogenesis pathway could therefore represent active vsRNAs targeting endogenous transcripts. However, at least in Arabidopsis DCL1-dependent vsRNAs are hardly detected by Northern blot (Blevins et al. 2006; Deleris et al. 2006) and potentially active DCL1-dependent vsRNAs would represent a small fraction of the total vsRNA population.

In summary, the present analysis demonstrates that a high number of potential vsRNA targets can be predicted *in silico*, but further studies are needed to investigate whether these indeed represent true targets *in vivo*. Target validation by RLM-RACE is a gene-specific and rather labor- and time-consuming procedure. Moreover, in case of negative results (i.e. a predicted 5’end is not found) it is difficult to determine if this indicates the absence of

vsRNA-guided cleavage or sub-optimal PCR conditions. High throughput methods such as degradome analysis combined with transcriptome analysis (German et al. 2008; Miozzi et al. 2013) are promising new methods for the identification of true targets of vsRNAs at a whole-genome scale.

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