Interactions of *Potato virus A* with Host Plants: Recombination, Gene Silencing and Non-Hypersensitive Resistance

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Abstract

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In this thesis molecular interactions between *Potato virus A* (PVA) and its hosts *Nicotiana benthamiana* and potato (*Solanum tuberosum*) have been studied using PVA recombinants, gene silencing and non-hypersensitive resistance (nnr).

The full-length cDNA clone pPVA-B11 infects *N. benthamiana* systemically but is restricted to inoculated leaves in potato. Therefore, a new infectious clone based on the isolate PVA-U, which infects potato systemically, was constructed by replacing parts of pPVA-B11 with parts of PVA-U. Chimeric viruses produced during this process were used to study how recombination of two closely related viral strains might alter their virus-host interactions. Our results suggest that recombination between homologous viral genomes can result in new potyviral strains with novel phenotypic traits. The full-length infectious clone based on PVA-U, pUFL, was able to infect potato systemically and could be further used for studying nnr resistance in potato.

To be able to study PVA movement in nnr plants *gfp* was inserted into the P1 region of pUFL. PVA-GFP could not infect any potato genotypes, but was still highly infectious in *N. benthamiana*. Infection of *gfp* transgenic *N. benthamiana* (line 16c) resulted in transgene silencing despite the strong silencing suppressor HC-Pro. In this model system systemic progression of gene silencing and antiviral defense was analyzed in a novel manner. Use of GFP as a visual marker revealed a mosaic-like recovery phenotype in the top leaves. Leaf areas appearing red or purple under UV-light (no GFP expression) corresponded to dark green islands under visible light and they contained little PVA and *gfp* mRNA. The surrounding green fluorescent tissue contained replicating viral deletion mutants that suppressed *gfp* silencing.

In plants with nnr resistance PVA moves cell-to-cell and accumulates to high titres in inoculated leaves, but cannot move systemically and no necrotic lesions develop on inoculated leaves. Suppression subtractive hybridization was used to extract transcripts of genes that had higher expression levels in the inoculated leaves of nnr genotypes compared to susceptible (S) genotypes 24 hpi. Hybridization of the extracted transcripts to a cDNA array containing 10 000 potato cDNAs and sequencing of randomly picked clones identified 645 genes expressed at a higher level in nnr than S. The sequenced cDNAs were spotted to a microarray and used together with quantitative PCR to study differentially expressed genes in repeated experiments. According to microarrays and quantitative PCR a family of *proteinase inhibitor 2* (*pin2*) were the only genes expressed to significantly higher levels in nnr than S plants. Since SSH is a more sensitive method than microarray it cannot be excluded that the genes detected by SSH can be involved in a basal defense activated by putative virus-induced molecular patterns resulting in a low resistance response that is high enough to inhibit spread of PVA in the nnr genotypes. *PR-1* and SAR were not induced in systemic leaves in nnr plants.

Keywords: basal defense, chimeric viruses, infectious cDNA clone, microarray, non-hypersensitive resistance, *Potato virus A*, *proteinase inhibitor 2*, silencing suppressor, suppression subtractive hybridization, systemic silencing, virus-induced gene silencing

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Papers I-III

The thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Paalme, V., Gammelgård, E., Järvekülg, L. & Valkonen, J. P. T. 2004. *In vitro* recombinants of two nearly identical potyviral isolates express novel virulence and symptom phenotypes in plants. *Journal of General Virology 85*, 739-74

II. Gammelgård, E., Mohan, M. & Valkonen, J. P. T. 2007. Potyvirus-induced gene silencing: the dynamic process of systemic silencing and silencing suppression. *Journal of General Virology 88*, 2337-2346

III. Gammelgård, E., Vuorinen, A., Mohan, M. L., Auvinen, P., Somervuo, P. & Valkonen, J. P. T. Inhibition of potyvirus translocation from inoculated potato leaves: analysis of induction of defense responses. (Submitted)

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Abbreviations

6K1 and 6K2	proteins of 6 kDa
AgMV	*Agropyron mosaic virus*
BSMV	*Barley stripe mosaic virus*
BS	bundle sheet cells
CC	companion cell
CI	cylindrical inclusion
CP	coat protein
CyRSV	*Cymbidium ringspot virus*
DCLs	dicer-like genes
DGI	dark green island
DRGs	defence related genes
dsRNA	double stranded RNA
eIF	eukaryotic translation initiation factor
EP	epidermal cells
ER	extreme resistance
GFP	green fluorescent protein
HR	hypersensitive response
HC-Pro	helper-component proteinase
HoMV	*Hordeum mosaic virus*
kDa	kilodalton
LMV	*Lettuce mosaic virus*
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
miRNA	microRNA
MP	movement protein
MSV	*Maize streak virus*
NBS	nucleotide binding site
NIa	nuclear inclusion protein a
NIa-Pro	C-terminal proteinase domain of NIa
NIb	nuclear inclusion protein b
nnr	non-necrotic resistance
ONMV	*Oat necrotic mottle virus*
P1	P1 protein
P3	P3 protein
PAMPs	pathogen activated molecular patterns
PD	plasmodesm
Pin	proteinase inhibitor
PP	phloem parenchyma
PPU	pore-plasmodesm unit
PPV	*Plum pox virus*
PR-1	pathogenesis related protein 1
PSbMV	*Pea seedborne mosaic virus*
PSRP	phloem-specific small RNA binding protein
PTGS	post transcriptional gene silencing
PVA	*Potato virus A*
PVX	*Potato virus X*

PVY	*Potato virus Y*
RdRp	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex
RLKs	receptor like kinases
RSS	RNA silencing suppressor
S	susceptible
SA	salicylic acid
SAR	systemic acquired resistance
SE	sieve element
SEL	size exclusion limit
siRNA	short interference RNA
SMV	*Soybean mosaic virus*
SPI	serine proteinase inhibitor
SSH	suppression subtractive hybridization
STNV	*Satellite tobacco necrosis virus*
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*
TRV	*Tobacco rattle virus*
TuMV	*Turnip mosaic virus*
VIGS	virus-induced gene silencing
VPg	viral genome linked protein
WSWM	*Wheat streak mosaic virus*
WTV	*Wound tumour virus*
ZYMV	*Zucchini yellow mosaic potyvirus*

Introduction

Already when people were nomads plant diseases were probably a problem for them. This became even worse when people settled down as farmers and began growing a few food crops in small plots, making the crop more prone to infections by plant pathogens. Diseases caused by viruses, bacteria, fungi, nematodes and protozoa reduce crop yields by 10-20% at the global level, despite the use of pesticides and preventive measures. These losses affect individual farmers as well as national food production and economies (Agrios, 1997).

Plant viruses are particularly difficult to control since they are intracellular parasites and cannot be controlled with chemicals. The best ways to control virus diseases are virus-free planting material, virus-resistant cultivars and appropriate agricultural practices in the field (Jones, 2006). When a virus infects a plant it must enter a plant cell and utilize the host machinery for nucleic acid and protein synthesis to be able to replicate and accumulate in the host (Agrios, 1997). It also uses the endogenous pathways of the host to move within cells, between cells and between different parts of the plant (Carrington *et al.,* 1996). A successful interaction might interfere with the normal cellular processes resulting in diseased plants. To avoid disease, plants have evolved different defense mechanisms against viruses (Hull, 2002). For developing sustainable virus control strategies it is important to understand the interactions between plants and viruses resulting in disease or no disease. Today biotechnology and functional genomics have made it possible to study these interactions in detail at the molecular level.

This thesis illuminates new examples of the complex molecular interactions between viruses and hosts and evolution of these interactions.

Potato virus A (PVA)

Potato virus A (PVA) belongs to the genus *Potyvirus* (family *Potyviridae*), one of the largest groups of plant viruses in the world. Potyviruses cause disease and economical damage in agricultural, horticultural, ornamental and pasture crops (Ward & Shukla, 1991). The host range of individual members of genus *Potyvirus* is usually limited. The main host species for PVA is potato (*Solanum tuberosum*). PVA can decrease the potato yields by up to 40% in synergistic infections with *Potato virus X* (PVX) or *Potato virus Y* (PVY) (Bartels, 1971). The symptoms vary from mild mosaic to rugosity of the leaves.

The virions of potyviruses are flexuos and rod-shaped, 680-900 nm long and 11-15 nm wide, made up of about 2000 units of a single structural protein (coat protein, CP) encapsidating a single molecule of positive single stranded (ss) RNA of approximately 10 kb (Dougherty & Carrington, 1988) (Fig. 1). The particles of PVA are approximately 730 nm long and 15 nm wide (Fribourg & De Zoeten, 1970). The genome is 9565 nucleotides long and contains a single open reading frame encoding a polyprotein of 3059 amino acids (aa) (Puurand *et al.,* 1994). The potyviral RNA strand has a poly-A tail at the 3´end (Hari *et al.,* 1979) and a viral

genome-linked protein VPg at the 5´end (Siaw *et al.*, 1985; Riechmann *et al.*, 1989; Murphy *et al.*, 1990). The VPg of PVA has been identified (Oruetxebarria *et al.*, 2001). It is exposed at one end of the PVA virion, being accessible to protein-protein interactions. VPg is phosphorylated by host kinases when it is bound to the virus particle and the phosphorylation might play a role in the VPg mediated functions during the infection cycle (Puustinen *et al.*, 2002).

The potyvirus genome contains one single open reading frame that is translated into a large polyprotein, which is subsequently cleaved into smaller polypeptides by three virus-encoded proteinases (Shukla *et al.*, 1994). The protein 1 (P1) proteinase and helper-component proteinase (HC-Pro) catalyze their own cleavage at the C-terminus (Carrington *et al.*, 1989a, 1989b; Verchot *et al.*, 1991) while nuclear inclusion protein a (NIa) is the main proteinase responsible for cleavage in the C-terminal two-thirds of the polyprotein (Dougherty & Carrington, 1988). In PVA it has been shown that NIa mediates both cis- and trans-cleavages, but the cis-cleavages may be preferred. The sites at P3/6K1, CI/6K2 and VPg/NIaPro junction were processed slowly, while the other sites 6K1/CI, 6K2/VPg, NIa-Pro/NIb and NIb/CP were quickly processed (Merits *et al.*, 2002). Potyvirus proteins are multifunctional and their functions are presented in Table 1.

Potyviruses are transmitted by aphids in a non-persistent manner, i.e. aphids acquire virus particles feeding on an infected plant and can transmit them only for a number of hours (Robert *et al.*, 2000). The virions are retained in the stylet and are transmitted via the saliva into the phloem when the aphid is feeding on a healthy plant. The virus cannot propagate in the vector and the transmission must occur fast. Some potyviruses can also be transmitted through the seeds of their hosts (Johansen *et al.*, 1996). Potyviruses can also be transmitted in infected plant material, such as cuttings and tubers, which is important in vegetatively propagated plants.

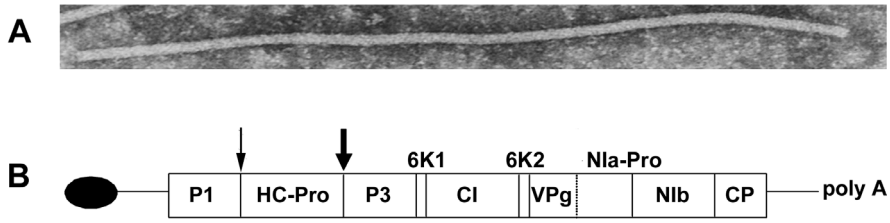

Fig. 1. Particle morphology (A) and genome organization (B) of potyviruses. The viral RNA is encapsidated into filamentous particles. The genome contains one ORF, which is processed into ten mature proteins. The processing sites are indicated by vertical lines. The P1 and HC-Pro cleavage sites are indicated by thin and thick arrow, respectively. All other cleavage sites are processed by NIa-Pro. VPg, presented by a black circle, is covalently attached to the 5´end of the viral RNA and the 3´end terminates with a poly (A) tail. The mature proteins are [protein 1 (P1), helper component proteinase (HC-Pro), third protein (P3), the 6kDa protein 1 (6K1), cylindrical inclusion protein (CI), the 6kDa protein 2 (6K2), nuclear inclusion protein a (NIa) which is processed into viral genome linked protein (VPg) and NIa proteinase (NIa-Pro), nuclear inclusion protein b (NIb), coat protein (CP)].

Table 1. *Known functions of the mature potyviral proteins*

Name	Function*	References
5´NTR	Enhancement of translation	10
P1	Proteinase Accessory factor for suppression of RNA silencing	36 1, 6, 22
HC-Pro	Proteinase Cell-to-cell and long-distance movement Suppressor of RNA silencing Aphid transmission Symptom expression Avirulence determinant in SMV (*Rsv*) Interaction with calmodulin-like protein Interaction with ring-finger protein and HIP2	7, 8 4, 31 1, 6, 23 28 4, 29 17 2 18
P3	Avirulence determinant	17, 21
6K1	Avirulence determinant	21
CI	Helicase Cell-to-cell movement Avirulence determinant	16, 27 9 20
6K2	Long-distance movement	39
VPg	Cell-to-cell and long-distance movement Avirulence determinant Interaction with the eukaryotic translation factor eIF4E and eIF(iso)4E Interaction with PVIP	26 5, 23, 26, 33 24, 32, 38 15
NIa-Pro	Proteinase Elicitor of Ry-mediated resistance	13, 14 25
NIb	RNA-dependent RNA polymerase Interaction with poly(A)binding protein (PABP)	19 37
CP	Encapsidation of viral RNA Cell-to-cell and long-distance movement Aphid transmission	34 11, 12, 31, 35 3
3´NTR	Symptom induction	30

*All proteins and non-translated regions (NTR´s) are necessary for virus propagation (Shukla *et al.,* 1994; Kekarainen *et al.,* 2002). All main proteins except P3 are known to bind RNA (Merits *et al.,* 1998). Most proteins and the NTR´s are involved in symptom expression (Riechmann *et al.,* 1995; Johansen *et al.,* 1996; Chu *et al.,* 1997; Simón-Buela *et al.,* 1997; Sáenz *et al.,* 2000). 1) Anandalakshmi *et al.,* 1998; 2) Anandalakshmi *et al.,* 2000; 3) Atreya *et al.,* 1990; 4) Atreya *et al.,* 1992; 5) Borgström & Johansen, 2001; 6) Brigneti *et al.,* 1998; 7) Carrington *et al.,* 1989a; 8) Carrington *et al.,* 1989b; 9) Carrington *et al.,* 1998; 10) Carrington & Freed, 1990; 11) Dolja *et al.,* 1994; 12) Dolja *et al.,* 1995; 13) Dougherty & Carrington, 1988; 14) Dougherty *et al.,* 1989; 15) Dunoyer *et al.,* 2004; 16) Eagles *et al.,* 1994; 17) Eggenberger & Hill, 1997;18) Guo *et al.,* 2003; 19) Hong & Hunt, 1996; 20) Jenner *et al.,* 2000; 21) Johansen *et al.,* 2001; 22) Kasschau & Carrington, 1998; 23) Keller *et al.,* 1998; 24) Leonard *et al.,* 2000; 25) Mestré *et al.,* 2000; 26) Nicolas *et al.,* 1997; 27) Laín *et al.,* 1990; 28) Pirone & Thornbury, 1984; 29) Redondo *et al.,* 2001; 30) Rodriguez-Cerezo *et al.,* 1991; 31) Rojas *et al.,* 1997; 32) Schaad *et al.,* 1997; 33) Schaad *et al.,* 2000; 34) Shukla & Ward 1989; 35) Varrelmann & Maiss, 2000; 36) Verchot *et al.,* 1991; 37) Wang *et al.,* 2000; 38) Wittmann *et al.,* 1997; 39) Rajamäki & Valkonen, 1999

Potyviruses can also be transmitted mechanically, e.g., through wounds in contact between plants (Shukla *et al.,* 1994). Aphid-transmissibility of PVA is controlled by aa 5-7 at the CP N-terminus. An Asp-Ala-Ser (DAS) motif at these positions increases the accumulation of PVA in inoculated leaves, whereas an Asp-Ala-Gly

(DAG) motif is required for aphid transmissibility. If DAS is mutated to DAG the virus accumulation will be reduced, but the virus transmissibility will be restored (Andrejeva *et al.*, 1999).

PVA isolates PVA-U, PVA-M and PVA-B11 represent different strain groups of PVA, collected in Michigan, Maine and Hungary, respectively (Valkonen *et al.*, 1995). The strain groups can be distinguished from each other by coat protein sequence, different abilities to trigger hypersensitive response in potato cv. King Edward, and ability to infect potato systemically (Valkonen *et al.*, 1995; Rajamäki *et al.*, 1998). PVA-B11 is in contrast to PVA-U and PVA-M not able to spread systemically in susceptible potato plants. For example, PVA-M can move systemically in *Solanum commersonii* but not PVA-B11. However, a single aa substitution in VPg, His118Tyr, allows PVA-B11 to move systemically in *S. commersonii* (Rajamäki & Valkonen, 2002). Similarly, another aa substitution in VPg, Val116Met, makes it possible for PVA-M to move systemically in *Nicandra physaloides* where PVA-M is usually restricted to the inoculated leaves (Rajamäki & Valkonen, 1999). It has been shown that kinases from *S. commersonii* phosphorylates VPg in different patterns depending on if there is a leucin or serine residue at position 185, while kinases from *Nicotiana tabacum* phosphorylates both forms of VPg in the same pattern (Puustinen *et al.*, 2002). These findings might explain the different abilities of VPg to support vascular movement and accumulation of PVA in different hosts.

Viral infection

Replication, movement within cells and cell-to-cell transport

Potyviruses enter cells through wounds, usually those made by feeding aphid vectors. Inside the cell the virus needs to replicate to establish infection. The viral particle is uncoated, the RNA is released to the cytoplasm and then translated on ribosomes to proteins required for viral replication (Shukla *et al.*, 1994). The viral replicase, RNA-dependent RNA polymerase (RdRp), synthesizes a negative strand using the positive strand as a template. The negative strand is then used to generate positive-stranded RNA molecules (Hull, 2002). The replication is followed by intracellular movement of viral RNA from the ribosomes to the plasmodesmata (PD) (Lucas & Wolfe, 1993), and this is probably facilitated by the endoplasmatic reticulum and cytosceletal elements. The plasmodesmata have an opening of about 2.5 nm, which limits molecular transport between plant cells to small molecules with a molecular mass of about 1 kDa. The plasmodesmal size exclusion limit (SEL) can be increased by viral movement proteins (MP) to allow intercellular movement of large endogenous proteins or invading viruses (Wolf *et al.*, 1989). The virus moves from cell-to-cell through the plasmodesmata of several cell types: epidermal (EP), mesophyll (MS), bundle sheet (BS), phloem parenchyma (PP) and companion cells (CC). Finally, it is loaded into the sieve elements (SE) for long-distance movement (Fig. 2) (Gilbertson & Lucas, 1996;.Carrington *et al.*, 1996).

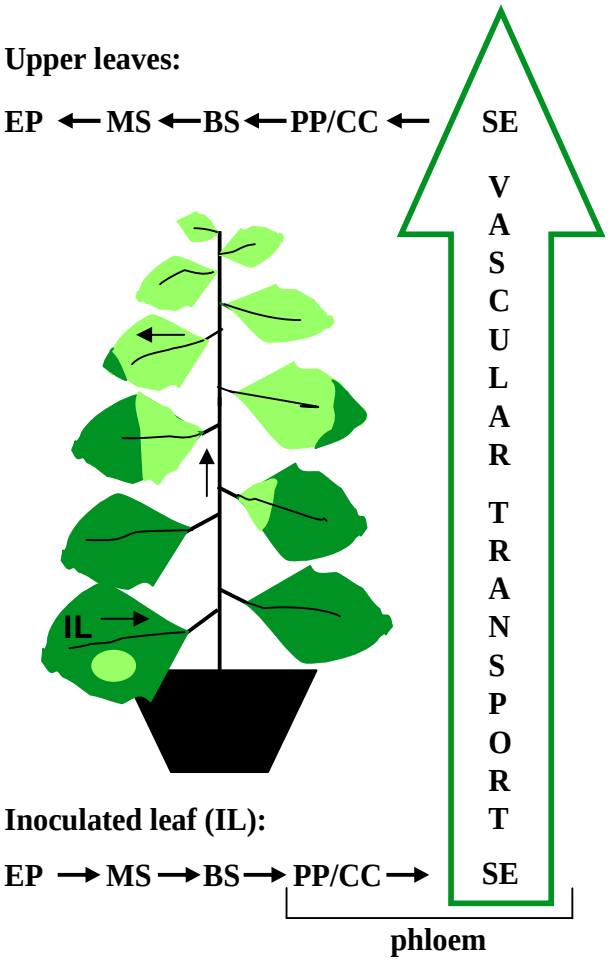

Fig. 2. From the inoculated leaf (IL) a virus spreads from the initial infection site (light green circle) to distal parts of the plant via phloem. Virus moves cell-to-cell through different types of cells [epidermal cells (EP), mesophyll cells (MS), bundle sheet cells (BS), phloem parenchyma cells (PP), companion cells (CC)] until it is loaded into sieve elements (SE). In SEs, virus follows the phloem translocation stream. Sink tissue of the plant where virus is transported and unloaded is light green and source tissue is dark green (modified from Rajamäki, 2002).

For successful cell-to-cell and long-distance movement PVA CP must be phosphorylated by protein kinase CK2 (Ivanov *et al.*, 2003). Using *in vitro* assays it was demonstrated that CK2 phosphorylation inhibited the binding of PVA CP to RNA. VPg is an important domain required for systemic invasion of plants with potyviruses. A single aa substitution His118Tyr overcome strain-specific resistance to PVA in *S. commersonii* and also controls virus accumulation in infected plant leaves and phloem loading (Rajamäki & Valkonen, 2002). VPg is translocated from inoculated source leaves to sink leaves, where it accumulates in CC at an early stage of virus infection, no virus particles or other viral proteins were found in CC at this infection stage. This suggests that VPg might be a phloem protein,

which specifically acts in CC in the sink leaves to facilitate virus unloading (Rajamäki & Valkonen, 2003). Infection foci at an initial stage of infection were associated with both major and minor veins suggesting that both may unload PVA in the sink leaves (Rajamäki & Valkonen, 2003).

Phloem transport

The phloem is constructed of long enucleate SEs, closely connected to CC and associated PP (Oparka & Santa Cruz, 2000). SEs are joined by perforated end walls, which form sieve tubes (Fig. 3b). SEs have lost many organelles and contain endoplasmatic reticulum, modified mitochondria and plastids pressed against the cell wall (Sjölund 1997; Oparka & Turgeon, 1999; Oparka & Santa Cruz, 2000). They do not contain any ribosomes and therefore the virus cannot replicate in the SE. SEs are connected to CC through special plasmodesmata called pore-plasmodesm units (PPUs) (van Bel, 1996). PPUs have an unusually high SEL, allowing passage of proteins in the range of 10-40 kDa (Kempers *et al.*, 1993; Kempers & van Bel, 1997). The SE-CC complex is viewed as a single functional unit due to the close structural and functional connections between the SE and CC (Fig. 3b) (Oparka & Turgeon, 1999). The SE-CC complex is surrounded by xylem vessels (X), BS and PP (Fig. 3a).

Following entry to the phloem the virus moves cell to cell in SEs. Little is known about the vascular transport of viruses compared to cell-to-cell movement. The host and viral factors required for cell-to-cell and vascular movement are probably different since some viruses spread cell-to-cell but can not establish systemic infection (Gilbertson & Lucas, 1996). The transportable form of potyviruses is unknown but moving as a ribonucleoprotein seems more probable because it would protect the RNA from silencing (Carrington *et al.*, 1996; 1998; Rajamäki & Valkonen, 2004). It is hypothesized that movement of PVA proceeds in repeated cycles including loading to the phloem for a short transport followed by unloading to phloem cells for replication and possibly silencing (Germundsson & Valkonen, 2006). Finally, the virus exits the phloem and initiates infection in sink tissues (Cronin *et al.*, 1995). The exit of the virus to the sink tissues seems to occur from major veins (Roberts *et al.*, 1997; Imlau *et al.*, 1999; Oparka *et al.*, 1999).

As leaves mature they undergo a developmentally programmed transition from net carbon importers (sink) to net carbon exporters (source). The transition proceeds in a basipetal manner from the leaf tip to the base (Turgeon, 1989), as the minor veins of the leaves mature. The phloem transport follows the sink-source transition (Fig. 2). At the transition boundary, import ceases and export is initiated (Turgeon, 1989). The viruses follow the sink-source relationship and use the same transport pathway as photoassmilates, proteins, hormones and RNAs (Crawford & Zambryski, 1999; Thompson & Schulz, 1999; Santa Cruz, 1999; Oparka & Santa Cruz, 2000; Ruiz-Medrano *et al.*, 2001).

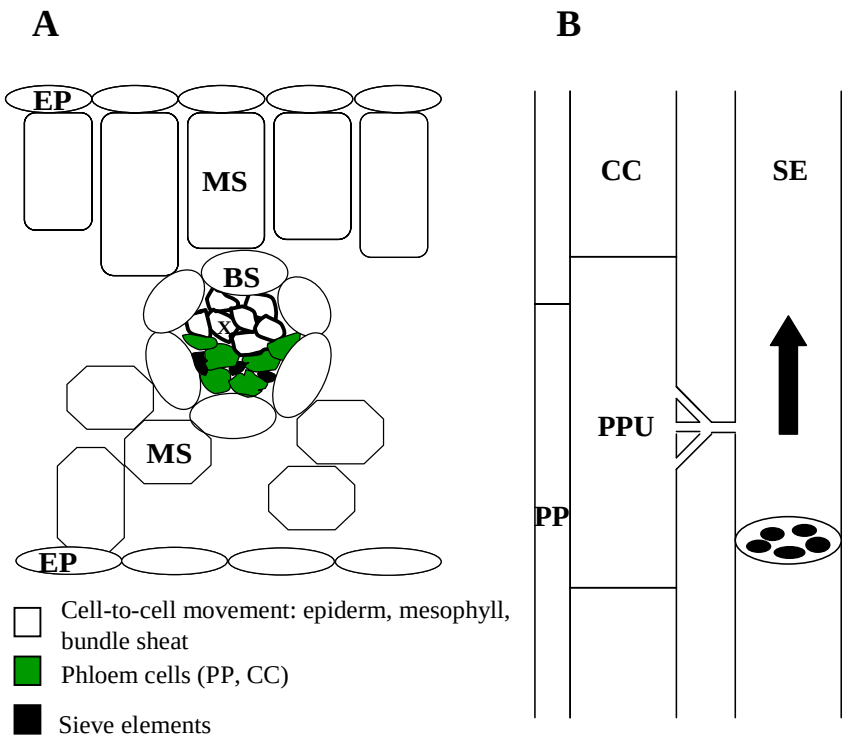

Fig. 3. A) Schematic picture of a transverse section of a minor vein. BS, bundle sheath cell; EP, epidermal cell; MS, mesophyll cell; X, xylem vessel B) Schematic picture of a longitudinal section of the phloem. A pore-plasmodesm unit (PPU) connects companion cell (CC) and sieve element (SE). PP, phloem parenchyma cell (modified from Rajamäki, 2002).

Transport regulations

The entry and exit of the plant vasculature is strongly regulated to control photoassimilate loading, entry of foreign molecules and exit of endogenous phloem proteins. Therefore, the SE-CC complex has been suggested to form the control point of phloem traffic (Oparka & Turgeon 1999; Santa Cruz, 1999). Transport through plasmodesmata in source leaves is tightly regulated (Wolf *et al.,* 1989; Deom *et al.,* 1990), whereas in sink leaves plasmodesmata are more permissive (Oparka *et al.,* 1999; Crawford & Zambryski, 2000; 2001). The movement in the phloem is greatly affected by plant species, physiological state of the plant and leaf age, but also the size, abundance and subcellular localization of proteins (Crawford & Zambryski, 2000; 2001). In some cases viruses have problems to move from one cell type to another, which defines critical cell boundaries. One of them is between the BS and PP. For example, the 30 kDa movement protein (MP) of *Tobacco mosaic virus* (TMV) (genus *Tobamovirus*) increases plasmodesmal SEL between MS and BS cells, but cannot move in plasmodesmata connecting BS and PP cells (Ding *et al,*. 1992). The boundary between BS and PP also restricts movement of

Cowpea chlorotic mottle virus (CCMV) (genus *Bromovirus*) in resistant soybean (Goodrick *et al.,* 1991).

Plants that do not support a specific step in viral movement can help in elucidating viral determinants for movement. *Tobacco etch virus* (TEV) (genus *Potyvirus*) with mutations in the central region of HC-Pro can enter phloem cells but is unable to establish systemic infection in *Nicotiana tabacum* (Cronin *et al.,* 1995; Andersen & Johansen, 1998; Guerini & Murphy, 1999). *Pea seed-borne mosaic virus* (PSbMV) isolate NY (genus *Potyvirus*) moves cell-to-cell throughout inoculated leaf including the petiole of *Chenopodium quinoa* but is unable to establish systemic infection due to mutation in CP (Andersen & Johansen, 1998).

Potyviral movement proteins

Movement of the viral nucleic acid is dependent on MP in both source and sink leaves (Oparka *et al.,* 1999). Several types of viral movement proteins have been found to support cell-to-cell spread of viruses through PD (Lucas, 1995; Carrington *et al.,* 1996). Some viruses encode specific MPs, whereas several potyviral proteins are involved in the viral movement, including the cylindrical inclusion protein (CI), HC-Pro, CP, 6 kDa protein (6K2) and VPg. Formation of specific structures by potyviral CI is required for intercellular passage of viruses (Rodriguez-Cerezo *et al.,* 1997). CI is suggested to interact with plasmodesmata and capsid protein-containing ribonucleoprotein complexes to facilitate potyvirus cell-to-cell movement (Carrington *et al.,* 1998). Mutations in the central region of HC-Pro inhibit systemic movement of the virus but only slow down cell-to-cell movement (Cronin *et al.,* 1995). These mutants are also defective in RNA silencing suggesting that the importance of HC-Pro in systemic movement is associated with its RNA silencing suppressor activity (Kasschau & Carrington, 2001). Mutations in the N-terminal part of the CP affect cell-to-cell movement and long-distance movement (Dolja *et al.,* 1994). Single aa mutations in 6K2 make it possible for the PVA-M to overcome resistance to vascular movement (Rajamäki & Valkonen, 1999). Deletion of various portions or insertion of six histidine residues into various positions of the 6K2 protein inhibited systemic infection in *Nicotiana benthamiana* and *N. tabacum* (Spetz & Valkonen, 2004). Spontaneous mutants revealed that the PVA 6K2 protein affects viral long-distance movement and symptom induction independently and in a host-specific manner. Several studies have identified VPg as the avirulence determinant in potyvirus-host interactions where systemic infection is restricted (for example Nicolas *et al.,* 1997; Schaad *et al.,* 1997; Keller *et al.,* 1998; Matsuta *et al.,* 1999; Borgström & Johansen, 2001).

Virus evolution

Populations of RNA viruses are genetically heterogenous and consist of a complex mixture of mutant and recombinant genomes. This type of a population structure is known as a quasispecies (reviewed in Domingo *et al.,* 1998). Mutations and recombination in viruses are generated by errors during the replication of genomes (reviewed in Roossinck *et al.,* 1997). Mutation rates are relatively high during RNA virus replication due to the lack of proofreading activity in the viral RNA-

dependent RNA polymerase, a characteristic that is also seen in potyviruses (García-Arenal *et al.,* 2001).

Recombination is the process by which segments of genetic information are switched between nucleotide strands of different genetic variants during the replication. Recombination has been proposed to be a relatively common process in some plant RNA viruses, especially the potyviruses (Chare & Holmes, 2006). The high mutation rates and high frequency of recombination in potyviruses might explain their successful adaptation to many different hosts and environments.

The distribution of genetic variants generated by mutation or recombination in a viral population will depend on two major evolutionary processes: genetic drift and selection. Genetic drift is the name of the random process leading to omittance of genetic variants from the next generation (reviewed in García-Arenal *et al.,* 2001). The numbers of individuals that actually pass on their genes to the next generation are called the effective population. In a population of viruses, the effective population size may be much smaller than the actual population size, since a large fraction of the population will consist of mutants that can not multiply. Infection of a new host will be started by only a few virus particles, which will reduce the effective population size even more. Population bottlenecks causing genetic drift will also occur in different moments of the history of the virus, e.g., at each time when a new host plant is infected. Other examples of bottleneck situations are aphid transmission and geographic subdivision. It was recently shown that also systemic infection of the plant is an important genetic bottleneck. The population diversity of *Cucumber mosaic virus* (CMV) (genus *Cucumovirus*) was stochastically and significantly reduced during systemic infection of *N. tabacum* (Li & Rossnick, 2004).

Virus evolution is often explained by selection, but this is not always based on evidence. As in genetic drift, selection can decrease or increase population diversity and therefore it is often difficult to separate selection from genetic drift (García-Arenal *et al.,* 2001). Selection can be associated with several factors in the infection cycle of the virus. Factors that can cause selection are, e.g., host plants, over-coming of resistance genes and maintenance of structural features of the virus.

There is evidence suggesting that quasispecies evolution may lead to the selection of virulent viruses and to emergence of new viral pathogens (reviewed in Domingo *et al.,* 1998). However, the high potential of genetic variation in plant viruses due to recombination or mutations does not necessarily result in high diversity of virus populations. Several studies show that plant RNA virus populations are genetically stable rather than diverse and that virus-encoded proteins are not more variable than those of their host and vectors (Fraile, *et al.,* 1997; Hillman *et al.,* 1991; Keese *et al.,* 1989; Ambrós *et al.,* 1998). Furthermore, populations of plant RNA viruses are not more varied than populations of plant DNA viruses despite the higher mutation rates (García-Arenal *et al.,* 2001).

Plant defense against viruses

Resistance to viruses is based on both RNA silencing and innate immunity. Innate immunity is activated either when pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors or upon resistance (R) protein mediated recognition of pathogen race-specific effector molecules (Nürnberger & Kemmerling, 2006) (Fig. 5). RNA silencing involves several pathways important for endogenous gene regulation, transposon taming, viral defense and heterochromatin formation (reviewed in Brodersen & Voinnet, 2006). It is possible that there are several layers of defense involving both silencing and innate immunity (Whitham *et al.,* 2006). For successful infection viruses must suppress all layers of defense. To complicate the picture further there are also uncharacterized defense mechanisms, many of them are suggested to result from defective virus-host interactions (e.g. Rajamäki & Valkonen, 2002; Ruffel *et al.,* 2004). There are also several indications that silencing and innate immunity might be partly connected (e.g. Ji & Ding, 2001; Pruss *et al.,* 2004).

RNA silencing

Posttranscriptional gene silencing (PTGS) or RNA silencing was first discovered in plants (Napoli *et al.,* 1990; van der Krol *et al.,* 1990) but was later found to be important in fungi and animals (reviewed in Cogoni & Macino, 2000). RNA silencing pathways are triggered by double-stranded (ds)RNA (Fire & Mello, 1998). The dsRNA is processed into short interfering (si)RNA of 20-26 nucleotides (nt) (Hamilton & Baulcombe, 1999). The siRNA is incorporated into an RNA-induced silencing complex (RISC), which cleaves the complementary RNA into siRNA (Hammond *et al.,* 2000; Liu *et al.,* 2004). There is also transcriptional gene silencing (TGS) through methylation of DNA and chromatin (Jones *et al.,* 1999; Jones *et al.,* 2001).

RNA silencing is a natural defense mechanism against viruses, induced by double-stranded structures of the viral RNA (Molnar *et al.,* 2005). Virus-induced gene silencing (VIGS) can be separated into a primary and secondary phase (Fig. 4). In primary VIGS the virus is recognized and spliced into siRNA by RISC. In secondary VIGS the primary signal is amplified resulting is systemic silencing.

Primary VIGS

Replication of RNA viruses produces double-stranded hybrids of plus-strand and negative-strand genomic RNA, so called replicative forms. It was first believed that processing of replicative forms of the virus was the base of VIGS in plants, but the situation can be more complex. For example, in plants infected by TMV and *Cymbidium ringspot virus* (CymRSV) (genus *Tombusvirus*) the siRNA produced are not distributed homogenously along the viral genome. Instead it maps preferentially to short, imperfect hairpins that result from interactions in the genomic ssRNA (Lacomme *et al.,* 2003; Molnár *et al.,* 2005).

The dsRNAs is cleaved into siRNAs by the Dicer-like proteins (DCLs). In *Arabidopsis thaliana* there are four DCLs involved in different silencing pathways (reviewed in Brodersen & Voinnet, 2006). At least two size classes of primary siRNA are produced during PTGS in plants: siRNAs 21-22 nt and siRNAs 24-26 nt (Hamilton *et al.,* 2002; Tang *et al.,* 2003). In silencing induced by exogenously derived dsRNA DCL4 seems to be the preferred enzyme for producing 21-22 nt siRNA (Dunoyer *et al.,* 2005). However, it has been suggested that the high levels of dsRNA produced in this kind of PTGS would promote activites of different Dicers and RISCs, which would normally act in different pathways (Brodersen & Voinnet, 2006). Recent analyses of combinatorial Dicer knock-outs support this idea (Gasciolli *et al.,* 2005; Xie *et al.,* 2005). These 21-22 nt siRNAs are believed to be involved in the degradation of mRNA (Hamilton *et al.,* 2002) and cell-to-cell movement of the silencing signal (Himber *et al.,* 2003). The 24 nt siRNAs are produced by DCL3 and believed to mediate exclusively chromatin modifications (Hamilton *et al.,* 2002; Zilberman, *et al.,* 2003; Moissiard *et al.,* 2007). The siRNAs are unwinded by an ATP-dependent RNA helicase and then one of the strands is incorporated into RISC (Hammond *et al.,* 2000; Bernstein *et al.,* 2001; Tabara *et al.,* 2002). RISC contains at least two components, Argonaute proteins and siRNA. The siRNA guides sequence-specific binding of mRNA (Baulcombe, 2004). The bound mRNA is then spliced into siRNA by Argonaute proteins (Liu *et al.,* 2004). Once cleaved some of the siRNA is further degraded by exonuclease activity in the cytoplasm, thus depleting the cell of the target RNA, sometimes to undetectable levels (Glazov *et al.,* 2004).

Secondary VIGS and systemic silencing

The newly produced siRNA can also be used to further amplify the RNA silencing reaction by the action of RdRp:s, which have been identified in plants, *Caenorhabditis elegans* and fungi, but not insects or vertebrates (Dalmay *et al.,* 2000; Sijen *et al.,* 2001; Makeyev & Bamford, 2002; Schwach *et al.,* 2005). This means that viral replication induces primary VIGS. Viral siRNAs produced in the primary VIGS trigger host-directed secondary silencing reactions, which leads to accumulation of 21 nt and 22 nt siRNA corresponding to sequences outside the silencing target sequence. The target RNA is processed by Dicer 2 and 4 and RISC in the same process that occurs in primary VIGS leading to degradation of the corresponding viral RNA (Vaistij *et al.,* 2002; Himber *et al.,* 2003). This phenomenon called transitivity requires an RdRp also known as SGS2 or SDE1, SDE3, a protein with RNA-helicase signatures, and SGS3, a coiled-coil protein of unknown function (Mourrain *et al.,* 2000). Several viral suppressors inhibit the RDR6-dependent amplification of VIGS (Moissiard *et al.,* 2007).

In plants, transitivity is bidirectional but in *C.elegans* it proceeds in the 3´-5´direction (Voinnet *et al.,* 1998; Vaistij *et al.,* 2002). In transgenic plants transitivity is followed by methylation of the transgene and extensive movement of silencing throughout the plant (Vaistij *et al.,* 2002; Himber *et al.,* 2003). Transitivity is usually observed along transgenes but most studies have not been able to provide evidence for transitivity along endogenous sequences (Ruiz *et al.,* 1998; Jones *et al.,* 1999; Vaistij *et al.,* 2002; Himber *et al.,* 2003; Koscianska *et al.,* 2005; Miki *et al.,* 2005; Petersen & Albrechtsen, 2005). Recently it was

reported that a plant gene expressed as a transgene was silenced by transitivity but not when it was expressed as an endogene, suggesting that transitivity is affected by the context in which genes are expressed (Bleys *et al.*, 2006).

The siRNAs produced in secondary VIGS are believed to move from cell-to-cell through plasmodesmata and initiate RNA silencing or further signal amplification in the new cells. This is followed by long-distance movement in the vasculature, unloading of the signal into new leaves and movement via plasmodesmata from cell-to-cell (see section above and Fig. 2) (Palauquai *et al.*, 1997; Voinnet & Baulcombe, 1997). Evidence suggests that the long distance signal might be small RNAs, a range of siRNAs (18-25 nt) has been found to enter and move in cucurbit phloem (Hamilton & Baulcombe 1999; Hamilton *et al.*, 2002; Himber *et al.*, 2003; Yoo *et al.*, 2004; Dunoyer *et al.*, 2006; Shaharuddin *et al.*, 2006). One study also reported a phloem-specific small RNA binding protein (PSRP), which preferentially binds short, single-strand RNA and likely plays a role in trafficking them in the phloem (Yoo *et al.*, 2004).

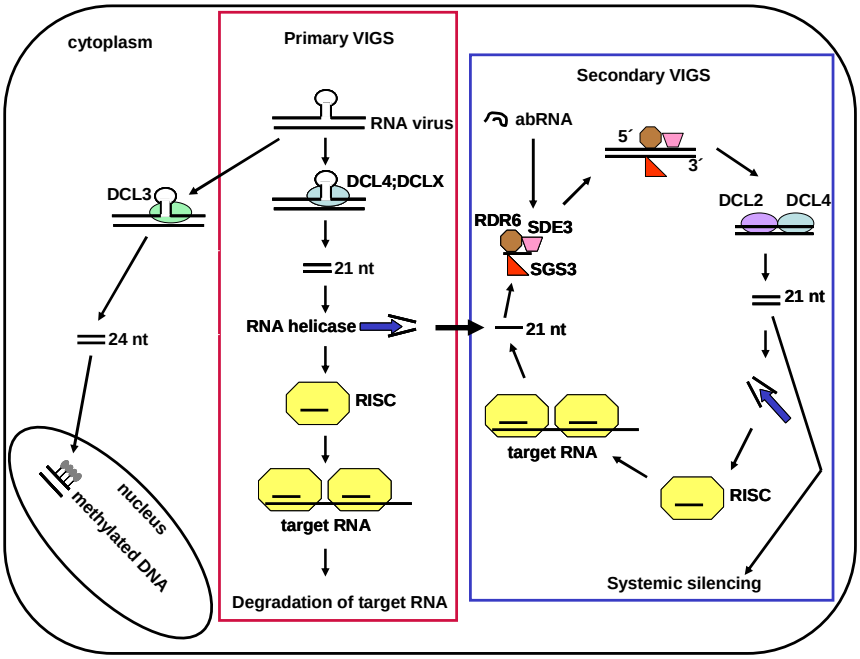

Fig. 4. A simplified model of virus-induced gene silencing in plants. RNA silencing is triggered by dsRNA originating from viruses. In primary VIGS dsRNA is cleaved by Dicer-like enzymes into small interfering (si)RNA that are incorporated into the RNA-induced silencing complex (RISC). The siRNA guides sequence-specific binding of mRNA, which is spliced into more siRNA. The siRNA can be further degraded or used to amplify the silencing signal in secondary VIGS. This process called transitivity requires RDR6, SDE3, and SGS3 and leads to silencing of sequences outside the target sequence and to systemic silencing.

Plants with decreased RDR6 activity are hypersusceptible to several viruses (Mourrain *et al.*, 2000; Muangsan, *et al.*, 2004) suggesting that transitivity is an important mechanism for dealing with the high replication rates of the virus. The systemic response might immunize cells that are about to become infected and thereby stop the virus infection. For example the meristems of *N. benthamiana* with reduced RDR6 activity are invaded by several viruses, whereas these tissues normally are immune to infection.

Finally, one should note that there might be many entry points into VIGS pathways. Some RNA viruses might be much affected by primary VIGS while others escape the primary VIGS but remain susceptible to secondary VIGS which can be induced by aberrant RNA (abRNA) from the virus. The number of RdRps and DCLs probably also varies between plant species providing flexibility in the initiation and implementation of VIGS (Voinnet, 2005).

Recovery and dark green islands

Plants containing virus-derived transgenes can sometimes be triggered to recover from infection with the transgene-homologous virus. In recovered plants new healthy and virus-free leaves appear in the earlier virus-infected plant, 3-5 weeks after virus inoculation (Lindbo & Dougherty, 1992a; 1992b; Dougherty *et al.*, 1994; Swaney *et al.* 1995; Guo & Garcia, 1997). The recovered tissue is resistant to subsequent infection by the same virus. No viral protein is detected and a drastic decrease of the transgene-derived mRNA is observed. However, it has been shown that the transcription rate is not affected (Lindbo *et al.*, 1993). Dark green islands (DGIs) occur when a virus systemically infects a plant and induces silencing in some of the infected tissues. DGIs give rise to mosaic symptoms that include chlorotic, virus-infected leaf tissue surrounding discrete regions of dark-green, virus-free tissue. Cytological and morphological studies reveal that DGIs have a phenotype similar to healthy tissue and that DGI formation occurs at random sites across a leaf (Hull, 2002). Recovery and DGIs are both caused by PTGS. Moore *et al.* (2001) concluded that DGIs and recovery are related phenomena differing in the ability to transport the silencing signal.

Viral suppression of RNA silencing

Many viruses have evolved mechanisms to overcome PTGS. The silencing suppression phenomenon and the first silencing suppressor were discovered when potyviral HC-Pro was found to enhance the accumulation of many unrelated viruses (Pruss *et al.*, 1997; Kasschau *et al.*, 1997). The ability of HC-Pro to suppress silencing was later confirmed in tobacco (Anandalakshmi *et al.*, 1998; Kasschau & Carrington, 1998; Brigneti *et al.*, 1998). Later it was discovered that suppression of RNA silencing is a general mechanism used by both RNA and DNA viruses in plants (Voinnet *et al.*, 1999). This was the beginning for the identification of additional silencing suppressors, many of which had earlier been characterized as pathogenicity determinants. However, besides being pathogenicity determinants there are few similarities between the silencing suppressors. Many different viral proteins with distinct sequences and structures can be silencing

suppressors. Their functions are also diverse and they target many different steps in the silencing pathways. The ability to suppress RNA silencing has probably evolved as features of proteins that already had many different functions (Moissard & Voinnet 2004; Roth *et al.,* 2004).

The potyviral HC-Pro is a dsRNA-binding protein that interacts physically with siRNA duplexes and thereby prevents RISC formation (Lakatos *et al.,* 2006). However, HC-Pro cannot inhibit the activity of already assembled RISC (Lakatos *et al.,* 2006). HC-Pro is a strong silencing suppressor capable of suppressing silencing (Anandalakshmi *et al.,* 1998) and reverse established transgene-induced silencing (Brigneti *et al.,* 1998).

miRNA and silencing suppressors

Almost all eukaryotes express 20-22 nt imperfectly paired dsRNA, so called miRNA (reviewed in Bartel, 2004). These miRNAs target many regulators of plant development, such as transcription factors and protein degradation regulators, in a sequence-specific manner (reviewed in Baulcombe, 2004). They derive from 70 nt precursor primary miRNAs that are transcribed from non-protein coding genes and cleaved by Dicer-like proteins in the nucleus (Kurihara & Watanabe, 2004). miRNA are incorporated into RISC and target complementary RNA. In plants the target RNA is usually degraded (reviewed in Baulcombe, 2004), but in animals it most often results in repressed translation (Chen *et al.,* 2004). Several unrelated suppressors from multiple viruses have been shown to inhibit miRNA activities in plants when expressed from transgenes and, at the same time they trigger developmental defects (Kasschau *et al.,* 2003; Zilberman *et al.,* 2003; Chapman *et al.,* 2004; Chen *et al.,* 2004; Dunoyer *et al.,* 2004). Some of these defects resemble virus symptoms and suggest that perturbation of endogenous silencing pathways is at least one of the mechanisms by which viruses can induce symptoms on infected plants. However, some defects fall outside the commonly observed virus symptoms (Dunoyer *et al.,* 2004).

Vectors for virus induced gene silencing (VIGS)

Virus-derived vectors can be used to silence gene expression without transformation and selection. However, the viruses alter gene expression in their host, and therefore the process of VIGS must be understood. Kumagai *et al.* (1995) were first to use VIGS to knock down expression of endogenous plant genes. When designing a VIGS vector the region of the gene to be targeted for silencing must be carefully considered. If the sequence is from a region unique to the target gene the silencing phenotype will be gene specific, but if the insert includes regions that are similar in related genes the specificity may be decreased (Lu *et al.,* 2003). Theoretically, VIGS requires a very short target sequence, but the lower limit of insert size has been shown to be 23 nucleotides (Thomas *et al.,* 2001). The upper limit size probably depends on size constraints of the virus to move from cell-to-cell and may vary between viruses and plant species, but the practical limit seems to be around 1.5 kb (Burch-Smith *et al.,* 2004). The insert must also be cloned into the virus without compromising viral replication and movement. An efficient VIGS vector should replicate and accumulate to sufficient levels in the host plant to

generate dsRNA and should also be devoid of strong silencing-suppressors. *Potato virus X* (PVX) (genus *Potexvirus*), TMV and TMV satellite virus do not have strong silencing suppressors, whereas for example the CMV, TEV and CymRSV have RNA silencing suppressors that can reverse transgene silencing. They are therefore not considered suitable as VIGS vectors (Vance & Vaucheret, 2001). It is an advantage if the virus does not cause severe symptoms, since they would make the silencing phenotype difficult to interpret. Detailed protocols for constructing and using virus-derived silencing vectors have been published (Lu *et al.*, 2003; Rui *et al.*, 2003).

RNA virus derived vectors can be gene-replacement vectors or insertion vectors. Since viral proteins usually are multifunctional and each RNA virus-encoded protein is required for efficient movement and replication, gene replacement vectors have not been extensively used for silencing, even though there are functional gene-replacement vectors described (Pogue *et al.*, 2002; Turnage *et al.*, 2002). Most vectors derived from RNA viruses are insertion vectors (Pogue *et al.*, 2002) and contain a duplicated subgenomic promoter preceding the insertion site. TMV was the first VIGS vector (Kumagai *et al.*, 1995). TMV induces severe viral symptoms and the silencing efficiency is low, even if the silencing efficiency has been stabilized by incorporation of direct inverted repeats of 40-60 bases to form dsRNA hairpins (Lacomme *et al.*, 2003). PVX cloned into a binary plasmid can be inoculated by agroinfection. PVX VIGS causes moderate disease symptoms and is a more stable vector than TMV, but has a narrower host range. Both PVX and TMV are excluded from the meristems of their hosts, and can therefore not silence genes in those tissues. The limitations of meristem exclusions, host range, and symptom induction were overcome when a VIGS vector based on *Tobacco rattle virus* (TRV) were developed (Ratcliff *et al.*, 2001; Liu *et al.*, 2002).

VIGS has been a powerful tool for identification of signaling components involved in disease resistance (Shirasu *et al.*, 1999; Romeis *et al.*, 2001; Liu *et al.*, 2002; Peart *et al.*, 2002,; Jin *et al.*, 2003; Sharma *et al.*, 2003; He *et al.*, 2004). The power of VIGS is its rapid initiation of silencing in intact wildtype (wt) or transgenic plants. An advantage of using a virus vector for reverse genetics is that most crop plants that are difficult to transform are susceptible to viruses (Voinnet, 2005). Another advantage of viral vectors is that there is no need for screening large populations to identify a specific phenotype and only one plant generation is needed to find a plant with the desired phenotype. Loss-of-function mutants of some genes that have been silenced by VIGS would be embryo lethal. It is possible to silence a whole gene family by targeting the most conserved sequence of these genes. VIGS can also be rapidly tested in many genotypes of the same species, whereas gene silencing induced with stably transformed transgene constructs are usually generated in a single genotype. Despite its advantages, VIGS also has some limitations. VIGS seldom results in complete suppression of the target gene, which makes it impossible to detect a phenotype if a low level of gene expression is sufficient for maintaining the function. VIGS does often not result in uniform silencing of a gene throughout an infected plant, and the levels of silencing can vary between different plants and experiments. It should also be noted that VIGS is dependent on a virus-host interaction and the virus alone could alter development

and mask phenotypes. Finally, VIGS might also silence non-target genes, even if this possibility decreases with increased knowledge about the genome sequence and silencing mechanisms of different organisms (Burch-Smith *et al.*, 2004).

Innate immunity

Non-host resistance

Generally, most plant species are resistant to most microbial species. This phenomenon is called non-host resistance. It is durable against a broad range of pathogens, perhaps due to the large number of mechanisms involved (reviewed in Nürnberger *et al.,* 2004; Thordal-Cristensen, 2003). Non-host resistance is conferred by preformed barriers and induced resistance.

The preformed barriers, such as wax layers and rigid cell walls, are constitutively present on the plant surface, and protects the plant from becoming infected (Heath, 2000; Dixon, 2001; Kamoun, 2001; Nürnberger *et al.*, 2004). However, pathogens have evolved mechanisms to overcome the preformed barriers. Viruses can do this through wounds introduced by vectors (Agrios, 1997). If the pathogen manages to overcome the constitutive defense and penetrate the cell walls it might successfully infect the plant. Alternatively, the pathogen activated molecular patterns (PAMPs) can be recognized by receptors at the plant cell surface and induce a resistance reaction, PAMP-triggered immunity (PTI) (Jones & Dangl, 2006) (Fig. 5). PAMP-based non-self recognition is found in almost all eukaryotes (Aderem & Ulevitch, 2000; Cook *et al.,* 2004). A large number of PAMPs have been shown to probably trigger receptor-mediated defense-responses in natural plant-microbe interactions (Gomez-Gomez & Boller, 2000; Nürnberger & Brunner, 2002; Parker, 2003; Espinosa & Alfano, 2004; Jones & Takemoto, 2004). PAMPs are functionally important for the microbe. They are structurally conserved across a wide range of microbes, and are not normally present in the host (Aderem & Ulevitch, 2000; Medzhitov & Janeway, 2002; Underhill & Ozinsky, 2002).

Viral PAMPs have not been as extensively characterized as PAMPs in bacteria and fungi, but there are some examples. *Cauliflower mosaic virus* (CaMV) in *A. thaliana* (Love *et al.,* 2005) and TMV in tobacco (Allan *et al.,* 2001) induce PAMP-like activities. The PAMPs is predicted to be peptides of the viral coat protein.

The plant cell receptors recognizing PAMPs have in some cases been shown to be receptor-like kinases (RLKs) (Gomez-Gomez & Boller, 2000; Zipfel & Felix, 2005) and it has been suggested that other so far uncharacterized PAMP receptors also are RLKs (Nűrnberger & Kemmerling, 2006). The molecular mechanisms involved in non-host resistance are not completely understood but its induction is connected to calcium influx, production of reactive oxygen species (ROI), GTP binding proteins, production of nitric oxide (NO), MAP kinase signaling, transcriptional induction of pathogen-responsive genes, and deposition of callose to reinforce cell walls at the site of infection, all of which contribute to prevention of microbial growth (Jonak *et al.,* 2002; Abramovitch & Martin, 2004; Jones & Takemoto, 2004; Nürnberger *et al.,* 2004).

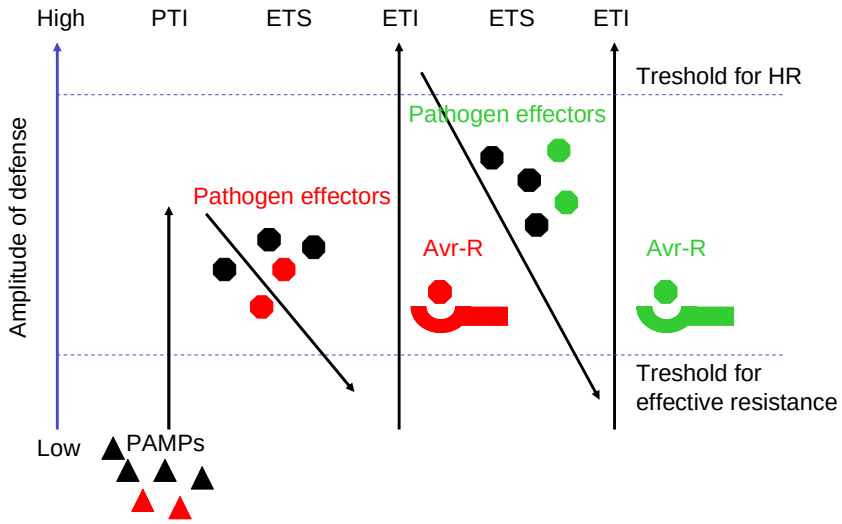

Fig. 5. Zigzag model illustrating the quantitative output of the plant immune system. The ultimate amplitude of disease resistance or susceptibility is proportional to [PTI-ETS+ETI]. Plant receptors detect pathogen-associated molecular patterns (PAMPs) inducing PAMP-triggered immunity (PTI). Successful pathogens deliver effectors interfering with PTI resulting in effector-triggered susceptibility (ETS). One effector can be recognized by an R protein activating effector-triggered immunity (ETI). If pathogens acquire new effectors, ETI can be suppressed, but selection favors new R-genes resulting again in ETI (modified from Jones & Dangl, 2006).

Gene-for-gene resistance

The non-host resistance can be overcome by a pathogen species or individual races or strains of a given pathogen species through acquisition of virulence factors, so called effectors (Fig. 5). The effectors enable the pathogens to either evade or suppress the PAMP-triggered immunity (PTI). The effector will make the plant susceptible again; therefore, this is called effector-triggered susceptibility (ETS). The PTI can still be detected in susceptible plants, but it cannot stop infection; nonetheless it is referred to as basal defense. The importance of basal defense against viruses has not been established. *A. thaliana* mutants defective in SA-mediated defense responses have not been found to have enhanced virus susceptibility (Laird *et al.*, 2004; Huang *et al.*, 2005). In contrast, a tomato (enhanced disease susceptibility) *eds1* mutant was reported to be more susceptible to TMV, suggesting there can be a role for SA in basal defenses (Hu *et al.*, 2005).

The selective pressure on host plants caused by the effectors of virulent pathogens has resulted in evolution of plant resistance (R) genes, which specifically recognize pathogen strain -or race-specific factors. This is called effector triggered immunity (ETI) (Jones & Dangl, 2006) (Fig. 5). Most effector proteins are virulence factors required for the colonization of host plants. An effector that is recognized by an R protein is termed avirulence (Avr) protein. To avoid resistance, pathogens may evolve new races leading to the establishment of

race/plant cultivar-specific resistance (Abramovitch & Martin, 2004; Espinosa & Alfano 2004; Jones & Takemoto, 2004). This type of resistance is genetically determined by complementary pairs of pathogen-encoded avirulence genes and plant R genes (Gabriel & Rolfe, 1990) and conforms to the gene-for-gene resistance (Flor, 1946). Matching R-Avr genes results in resistance (incompatible interaction), while lack of matching R-Avr gene pairs result in disease (compatible interaction). (Abramovitch & Martin, 2004; Alfano & Collmer, 2004; Jones & Takemoto, 2004).

Recently, a zigzag model illustrating the quantitative output of the plant innate immune system was described (Jones & Dangl, 2006) (Fig. 5). In this model the disease level is proportional to [PTI-ETS+ETI]. Thus, resistance and susceptibility are not described as a fixed permanent condition, but as something that can take many different levels depending on PAMPs, effectors and resistance genes. If the sum of the equation reaches a certain threshold the plant will be resistant, but below the threshold the plant remains susceptible.

For gene-for-gene resistance there is a large body of information available. Over the past 10-15 years many novel genes, proteins and molecules have been discovered through the use of gene expression studies and reverse genetics (reviewed in Hammond-Kosack & Parker, 2003). Several R genes have been cloned. They are largely conserved both in monocotyledons and dicotyledonous plants and are grouped into different classes based on the conserved domains they contain. Effectors have been cloned from bacteria, fungi and viruses. They may directly encode the elicitor (Van den Ackervecken *et al.*, 1992) or they encode an enzyme that catalyses the production of the elicitor molecule (Keen *et al.*, 1993). Viral CPs, replicases and MPs have been identified as viral effectors (reviewed by Culver, 1997), but also the viral virulence determinants that suppress silencing can be considered effectors.

The simplest biochemical interpretation of the gene-for-gene hypothesis involves direct interaction of a receptor and a ligand, which was demonstrated in a few cases (Cohn *et al.*, 2001; Jia *et al.*, 2000). In several studies it has been found that R proteins constitute components of larger signaling complexes, but these proteins may not necessarily bind directly to the matching Avr protein (Axtell & Staskawicz, 2002; Mackey *et al.*, 2002; Van der Hoorn *et al.*, 2002). These studies have led to the guard hypothesis. According to the guard hypothesis the effectors function as elicitors of cultivar-specific resistance only when the complementary R protein is recruited into a functional signal perception complex (Van der Biezen & Jones, 1998; Cohn *et al.*, 2001; Dangl & Jones 2001; Bonas & Lahaye, 2002; Holt *et al.*, 2003; Van der Hoorn *et al.*, 2002). Thus, the role of the R protein is to monitor/guard the Avr-mediated perturbance of cellular functions.

Different R gene and avirulence gene interactions activate different resistance pathways. They are dependent on diverse genes and signaling molecules to establish successful resistance. However, the different signaling networks seem to be highly interactive, which makes the situation complex (reviewed in Hammond-Kosack & Parker, 2003).

Inducible responses associated with gene-for-gene resistance

The most well-known response in gene-for-gene resistance is the frequently observed, highly localized hypersensitive response (HR). HR occurs against all known groups of pathogens (Keen, 1990). HR to viruses results in development of necrotic lesions at the initial infection sites of inoculated leaves (Jones, 1990). Cell death might not be essential for the mechanism that prevents virus infection. HR against CaMV in *Nicotiana edwardsonii* and *Nicotiana bigelovii* can be uncoupled from cell death (Cawly *et al.*, 2005).

There are also other indications that the cell death is not essential. *A. thaliana* mutants *dnd1*, *dnd2*, *hlm1* (Yu *et al.*, 1998; Clough *et al.*, 2000; Balague *et al.*, 2003; Jurowski *et al.*, 2004) and *RSS1-R* in *A. thaliana* (Deslandes *et al.*, 2002) lack the capacity to respond to *Pseudomonas syringae* with HR–related cell death but do exhibit typical gene-for-gene resistance responses. In HR, a range of plant defense-related pathways are activated that lead to cell death and to the restriction of the pathogen at the initial site of infection (Hammond-Kosack & Jones, 1996; Heath, 2000). The cell death response has been considered to be a form of programmed cell death, which may have similarities with animal apoptosis (Gilchrist, 1998; Greenberg & Yao, 2004). A rapid induction of active oxygen species (oxidative burst) is typical of HR in plants. Ion fluxes across the plasma membrane (Ca^{2+} and H^{+} influx; $K^{+}$and Cl^{-} efflux) activate an NADPH oxidase, which produces reactive oxygen intermediates (ROI). The active oxygen species can also be produced by peroxidases and extracellular enzymes, such as oxalate and amine oxidases. The active oxygen species may be directly toxic to the pathogen or may lead to crosslinking of the cell walls. They also act as secondary messengers in the activation of transcription of defense genes. It is believed that NO starts the oxidative burst. NO also induce defense genes like PR-1 and PAL (reviewed in Hammond-Kosack & Jones, 1996). Other important early signals are the G-proteins, which activate the Ca^{2+} signaling through phospholipase C (PLC) (reviewed in Jones, 2002). When the Ca^{2+} level increases in the cytosol many calcium binding proteins are activated. Calmodulin is a calcium sensor transmitting changes in cytosolic calcium levels to cellular metabolic processes. Calmodulin activates several targets including transcription factors, protein kinases and Ca^{2+} ATPases (reviewed in Yang & Poovaiah, 2003). One calmodulin-related protein also interacts with HC-Pro and has been suggested to function as an endogenous silencing suppressor through an uncharacterized calcium-dependent pathway (Anandalakshmi *et al.*, 2000). The calcium signaling activates different protein kinases, like mitogen-activated protein kinases (MAPK) and calcium-dependent protein kinases (CDPK). The signaling of kinases is complex. Specific isoforms become activated by environmental stimuli and race -and non-race specific resistance. (Desikan *et al.*, 1999; Suzuki *et al.*, 1999). The early signaling described above activates transcription factors and regulate defense related genes (DRGs), such as those involved in ubiquitination, production of PR-proteins and proteinase inhibitors.

Extreme resistance (ER) to viruses is another resistance response inducing signal transduction pathways and is based on interactions between dominant resistance genes and avirulence genes. In contrast to HR which is virus-strain specific, ER typically acts against all strains of a virus. ER efficiently reduces virus replication in infected cells and no cell death is observed in inoculated leaves (Cockerham, 1970; Ross, 1986; Köhm *et al.*, 1993).

Other resistance responses

There are also resistances that do not function on a gene-for-gene basis, but still confer resistance to virus replication, cell-to-cell movement or vascular movement. The exact mechanisms are not very well studied, but many of these resistance genes have been shown to be recessive. Already 1905 Biffen discovered that resistance against yellow rust in a specific wheat variety was due to a single recessive gene. The barley *mlo* gene is one of the most studied recessive resistance genes and functions as a negative regulator of cell death (Büschges *et al.*, 1997). Resistance genes that do not induce HR, SAR or cell death have usually been suggested to be passive and the resistance to result from defective host-virus interactions. This has been supported by data indicating that the resistance is overcome by mutations in viral proteins. Usually VPg is the important virulence determinant, in which mutations of host-specific aa can overcome the resistance (for example Gibb *et al.*, 1989; Nicolas *et al.*, 1997; Schaad *et al.*, 1997; Keller *et al.*, 1998; Rajamäki & Valkonen, 1999; Rajamäki & Valkonen, 2002). It has been shown that the natural recessive resistance genes *pvr2* and *pot1* (Ruffel *et al.*, 2002; Moury *et al*, 2004; Ruffel *et al.*, 2005) correspond to the eukaryotic initiation factor 4E (eIF4E). The interaction between VPg and eIF4E has been shown in several virus-host combinations (Wittman *et al.*, 1997; Léonard *et al.*, 2000; Schaad *et al.*, 2000; Beauchemin *et al.*, 2007; Roudert-Tavert *et al.*, 2007). Ruffel *et al.* (2004) suggested that the resistance could be due to incompatibility between the VPg and eIF4E.

Systemic acquired resistance

The localized HR induces systemic acquired resistance (SAR), which is a broad-spectrum resistance in uninfected parts of the plant (reviewed in Hammond-Kosack & Parker, 2003; Durrant & Dong, 2004). SAR is long-lasting, sometimes for the life-time of the plant, and effective against a broad spectrum of pathogens including viruses, bacteria, fungi and oomycetes (Ryals *et al.*, 1996; Sticher *et al.*, 1997). SAR is characterized by the increased expression of pathogenesis-related genes (Van Loon *et al.*, 1998; Van Loon & Van Strien, 1999). Upregulation of PR-1 indicates SAR induction (Van Loon & Van Strien, 1999; Durrant & Dong, 2004). SAR requires the signal molecule SA (Gaffney *et al.*, 1993; Delaney *et al.*, 1994), but SA is not the systemic signal (Dempsey *et al.*, 1997). The signal might be a lipid (Falk *et al.*, 1999; Jirage *et al.*, 1999; Maldonado *et al.*, 2002) and recently it was also suggested that jasmonate might be involved in systemic signaling inducing SAR (Truman *et al.*, 2007). The systemic signals induce SA accumulation throughout the plant. To further induce SAR the *NPR1* (non-expressor of PR-1) is required (Cao *et al.*, 1994; Delaney *et al.*, 1995; Glazebrook

et al., 1996; Shah *et al.*, 1997). NPR1 relocates from the cytoplasm to the nucleus (Kinkema *et al.,* 2000), binds with various TGA class transcription factors and induces PR gene expression (Subramaniam *et al.,* 2001; Fan & Dong, 2002).

Pathogenesis-related protein 1 (PR-1)

PR-1 is one of the 17 PR-protein families that have been defined so far (reviewed in Van Loon & Strien, 1999). PR-proteins are induced by a pathogen in tissues that normally do not express the PR-proteins. The expression must have been shown to occur in at least two different plant-pathogen combinations. For a single plant-pathogen combination the expression must have been independently detected in two different laboratories. PR-1 has been shown to be encoded by small multigene families in tomato, tobacco and potato. There are both basic and acidic PR-1 proteins. PR-1 has a high stability due to a unique compact structure. Most PR proteins function as β-1,3-glucanase, chitinases, endoproteinases, defensins, thionin, peroxidases, thaumatin-like proteins, ribonuclease-like proteins, lipid transfer proteins and oxalate oxidase proteins. The function of PR-1 is not known. There are indications that PR-1 might participate in pathogen resistance. Transgenic tobacco plants expressing PR-1 to high levels exhibited increased tolerance to some oomycete pathogens (Alexander *et al.,* 1993, Niderman *et al.,* 1995). PR-1 shows homology to some proteins of non-plant origin, such as; allergen, mammalian sperm coating glycoproteins and human glioma pathogenesis-related protein (GliPR) (Broekaert *et al.,* 2000).

Connections between RNA silencing and innate immunity

There are several indications that silencing and innate immunity might be partly connected. It has for example been shown that viral RNA-silencing suppressors can induce HR (Scholthof *et al.,* 1995; Li *et al.,* 1999). CMV silencing suppressor 2b suppresses both RNA silencing and SA-induced defense pathways (Ji & Ding, 2001). Tobacco plants expressing HC-Pro show enhanced resistance to a broad range of pathogens (Pruss *et al.,* 2004). It is proposed that HC-Pro suppresses the effect of miRNAs that are targeted against negative regulators of R-protein mediated defense. Another explanation could be that HC-Pro releases a silencing-based mechanism that directly restricts constitutive R-gene expression (Stokes *et al.,* 2002).

Aims of the study

The aim of this study was to examine molecular interactions between potyviruses and their hosts using PVA and its hosts *N. benthamiana* and potato as the model system. The more specific aims were to:

- Obtain a new infectious clone of PVA based on the isolate PVA-U that infects potato plants systemically.

- Use the novel viral chimeras produced during the process of construction of the PVA-U clone for studying how recombination of two closely related viral strains might alter their virus-host interactions.

- Study whether PVA can be used as a VIGS vector and analyze the systemic progression of viral infection and RNA silencing.

- Use functional genomics to study the genetic bases of non necrotic resistance, nnr, which inhibits transport of PVA from the inoculated leaves in potato.

Results and discussion

Studies of PVA *in vitro* recombinants (I)

The full-length cDNA clone pPVA-B11 (Puurand *et al.*, 1996) infects *N. benthamiana* but not potatoes systemically. Previous studies have shown that pPVA-B11 is able to infect inoculated leaves of the diploid potato plants in population v2, but that it is unable to systemically spread throughout any plant, including those genotypes that are susceptible to PVA-U (Hämäläinen *et al.*, 2000). To obtain a new infectious clone based on PVA-U, parts of the full-length clone of pPVA-B11 were replaced stepwise with parts of PVA-U until the new clone was ready. During this process six novel chimeric viruses, pBUI, pBUII, pBUIII, pBUI+pBUII, pBUI+pBUIII and pBUII+pBUIII were produced. These six chimeras were used for studying how recombination of two closely related viral strains might alter their virus-host interactions. The UI, UII and UIII segments correspond to nucleotides 1-3012, 2905-6823 and 6055-9565 of PVA-U, respectively.

Infectivity of the full-length PVA-U clone (pUFL), pPVA-B11 and the virus chimeras were tested in *N. benthamiana* and potato lines v2-134 and v2-51. pUFL infected the susceptible potato line v2-134 and *N. benthamiana* systemically. However, it was not able to systemically infect the resistant (nnr) potato line v2-51 although quite high virus titers were detected in inoculated leaves, as also found in a previous study (Hämäläinen *et al.*, 2000).

The *N. benthamiana* plants inoculated with pUFL and pPVA-B11 showed similar symptoms of severe mosaic and leaf malformation. All chimeras infected *N. benthamiana*, but their infectivity varied. Some chimeras caused similar phenotypes as the parental viruses, but there were also chimeras causing completely new phenotypes that were not like any of those found with the parental viruses. pBUI+III caused severe mosaic and leaf malformation as the parental viruses, while very severe mosaic and leaf malformation were observed in leaves infected with pBUII and pBUIII, even though the titer of pBUII was only half of that of pUFL. Infection with pBUI+II resulted in novel symptoms of yellow vein chlorosis (net chlorosis) without leaf malformation, although viral titers were equivalent to those of the parental viruses. pBUI produced titers that were 2.5-fold less than the titers of pBUI+II and pUFL and its infection caused mild mosaic without leaf malformation. pBUII+UIII showed a systemic infection delayed by two weeks and the viral titers were low.

pBUII+III was not inoculated to potato because of its low infectivity in *N. benthamiana*. All other chimeras, except pBUI+II, infected the susceptible line v2-134 systemically, but none of them infected the resistant line v2-51 systemically. The titers of pBUI+II in inoculated leaves were significantly lower than the titers of the other chimeras. pBUI accumulated to similar levels as the other chimeras in both inoculated and upper non-inoculated leaves of potato and the titers were not significantly lower compared to the other chimeras as they were in *N. benthamiana*

(see section above). In general, the viral titers in potato were lower than in *N. benthamiana* for all chimeras but they were quite high as compared to the PVA titers previously observed in potato plants (Andrejeva *et al.*, 1999). No symptoms were induced in v2-51 and v2-134 by the chimeras or the parental viruses.

In our study *N. benthamiana* had higher PVA titers than potato and also tolerated more changes in the viral genome. The fact that *N. benthamiana* is extremely susceptible to viruses is well-known (van Dijk *et al.*, 1987; Dawson & Hilf, 1992). Yang *et al.*, (2004) found that one of two RdRps in *N. benthamiana* is modified by an extra 72 nt insert and not induced by SA and viruses. RdRp:s have been shown to be important both for SA-inducible defenses and RNA silencing (Moissard *et al.*, 2007; Xie *et al.*, 2001). Therefore, the mutated RdRp might explain the extreme susceptibility of *N. benthamiana* to viruses.

PVA-B11 could not infect v2-134 systemically, but the new chimeras made of PVA-U and PVA-B11 in this study could overcome resistance to vascular movement in v2-134. This was a novel finding because in a previous study a chimera of PVA-B11 carrying the CP encoding region of isolate U (B11-Ucp) was not able to infect v2-134 systemically (Andrejeva *et al.*, 1999). When a few aa residues of HC-Pro and VPg were mutated to make B11-Ucp more similar to PVA-U, the new mutated chimera moved systemically but at a slow rate (Hämäläinen *et al.*, 2000). This indicates that complex coordinated functions between different parts of the PVA polyprotein might be required for a successful infection cycle.

The sequence of pUFL has a few aa changes compared to PVA-U. Fourteen changes are predicted to result in aa changes or a short frameshift region. All changed aa residues were identical to PVA-B11, making pUFL more similar to PVA-B11 than PVA-U, probably because of host adaptation. pPVA-B11 has been maintained in *N. benthamiana* and pUFL was constructed via intermediary chimeras pBUI, pBUII and pBUIII, which were preselected for infectivity in *N. benthamiana*. For this reason the aa which have changed in pUFL compared to PVA-U may have greater compatibility for interactions with *N. benthamiana* and with the rest of the virus genome derived from pPVA-B11. Host adaptation studied by Yarwood (1979) showed that serial passaging of the virus in different hosts could change viral properties. The experiments often involved a host shift, in which the virus adapted to one particular host was inoculated to and passaged in a different host. The resulting changes of virus traits were taken as evidence for host adaptation. It has also been shown at the molecular level, using the DNA oligomer-directed RNase H cleavage method and sequencing that *Satellite tobacco necrosis virus* (STNV, single-stranded RNA satellite viruses; family 81) from tobacco adapted to its new host mung bean, *Vigna radiate*, by gradual selection of sequence variants from the tobacco-adapted population (Donis-Keller *et al.*, 1981).

It has been shown in many studies that small changes in the potyviral genome can make it possible for the virus to overcome host resistance, possibly because these changes restore virus interactions with the host. For example, a single aa substitution His118Tyr in the VPg of PVA-B11 allows the virus to move systemically in *S. commersonii*, a host where it is usually restricted to inoculated

leaves (Rajamäki & Valkonen, 2002). Similarly, another aa substitution in VPg, Val116Met, makes it possible for PVA-M to move systemically in *N. physaloides* where PVA-M is usually restricted to the inoculated leaves (Rajamäki & Valkonen, 1999). The Val116Met substitution has also been found to occur *in vivo* during replication of PVA-M in infected *N. physaloides* plants (Rajamäki & Valkonen, 2004). *Pisum sativum*, accession PI 269818, is resistant to the potyvirus PSbMV pathotype P1, but susceptible to pathotype P4 isolates. Mutation analysis showed that the region influencing virulence was VPg, more specifically aa 105 to 117 (Borgström & Johansen, 2001). When pathotype P1 was allowed to pass through PI 269818, mutants overcoming resistance appeared. Mutations were only found at codons from aa 105 to 117. No changes in virus sequence were observed when pathotype P4 passed through PI 269818, suggesting that the changes in P1 were due to host adaptation. The recessive gene *pot-1* in *Lycopersicon hirsutum* can be overcome by a single mutation, Arg119His, in VPg (Moury *et al*., 2004). With changes in five aa between codons 105-123 in the VPg PVY could circumvent the resistance conferred by the gene *pvr2* in *Capsicum annuum* (Moury *et al*., 2004).

The virulence determinant can, however, also be located in other parts of the genome. Viral determinants for overcoming recessive resistance (*sbm-2*) in *P. sativum* against PSbMV reside in the region encoding the N-terminal part of the P3 protein (Hjulsager *et al.*, 2006). The 3´terminal region of the *Plum pox virus* (PPV) genome, from nucleotide 7677 to the end appears to be responsible for the ability of isolate PPV-R to overcome *rpv1*-mediated resistance in *A. thaliana* (Decroocq *et al*., 2006). This region corresponds to the end of the NIb, the whole CP and the 3´NTR region.

The titers of pUFL were among the highest of all viruses tested in this study. This might be because pUFL as well as PVA-B11 contain an Asp-Ala-Ser (DAS) motif at the CP N-terminus, The motif DAS increases the accumulation of PVA in inoculated leaves, as compared to DAG, but DAS is not compatible for aphid transmissibility which requires DAG (Rajamäki *et al.*, 1998; Andrejeva *et al.*, 1999; Valkonen *et al.*, 2002). Virus maintained artificially through mechanical passage or vegetative propagation may lose the ability to be vector-transmitted. This was first reported for *Wound tumour virus* (WTV) (Reddy & Black, 1977), and has also been shown in several other cases (reviewed in Pirone & Blanc, 1996). Loss of vector transmissibility is also more common in viruses whose transmission is dependent on viral helper components besides the CP, which is the case with potyviruses (Pirone & Blanc, 1996).

In this study, there was no direct correlation between the viral titers and symptom severity, suggesting that interactions between the chimeras and specific hosts are more important determinants for the phenotypic difference than the viral titer. This conclusion is supported by another study (Kagiwada *et al.*, 2005). They constructed chimeras between PVX-OS causing necrosis and mosaic and PVX-BS causing mild mosaic in *N. benthamiana* and *N. tabacum*. Western blot analysis of viral CP in the uppermost leaves of *N. tabacum* 7, 10 and 15 days post inoculation (dpi) showed no difference in accumulation of CP between PVX-BS and PVX-OS. There were also no differences in local lesions induced in *C. quinoa* after sap

inoculation from uppermost leaves 7, 10 or 15 dpi. The symptom determinant was a single aa residue in the RdRp. In PPV the symptom determinant of vein necrosis in *Nicotiana clevelandiii* is residing within a 173 aa long region from the C-terminal part of P3 and 6K1 (Sàenz *et al.,* 2000). In the same region a stretch of 74 aa contains the symptom determinant for PPV infection in *P. sativum* (Sàenz *et al.,* 2000). PVY^N causes veinal necrosis in tobacco, while mosaic symptoms are observed with PVY^O. By constructing chimeric viruses between PVY^N and PVY^O the determinant for necrosis induction was mapped to the two aa residues K_{400} and E_{419} in the C-terminal part of HC-Pro (Tribodet *et al.,* 2005). The isolate AF199 of LMV causes local lesions followed by wilting and plant death in *L. sativa* cultivars Ithaca and Vanguard 75. Analysis of viral chimeras revealed that nucleotides 112-386 in P1 and/or nucleotides 5496-5855 in CI are sufficient for causing wilting in Ithaca but not Vanguard 75, indicating that the determinants were different in these two cultivars (Krause-Sakrate *et al.,* 2005).

Our results suggest that virulence depends also on compatible coordinated functions between different parts of the PVA genome. In some of our chimeras interactions between different parts of the polyprotein or between mature proteins produced from different parts of the polyprotein might have been disrupted and resulted in reduced virulence. This model is supported by previous studies. Gomez de Cedron *et al*. (2006) introduced a series of point mutations to CI of PPV. CI could still self-interact in all mutants but with reduced binding strength, which correlated with reduced efficiency of movement, whereas virus replication was not affected. The authors suggest that the CI-CI interactions putatively required for RNA replication and virus movement could be rather different.

The same PVA chimera could cause completely different phenotypes in potato and *N. benthamiana* suggesting that host factors play an important role for virus-host interactions. This is also found in recent publications studying Potyviridae-host interactions. One study by Stenger & French (2004) involved another member of the Potyviridae family, strain Sidney 81 of *Wheat streak mosaic virus* (WSMV) (genus *Tritimovirus*) infecting wheat, oat and barley. When HC-Pro of WSMV-Sidney 81 was replaced with the corresponding region derived from four strains of WSMV or the related tritimovirus *Oat necrotic mottle virus* (ONMV) changes in symptom severity and aphid transmission, but not in host range, could be observed. It was first when HC-Pro from the potyviruses TEV and *Turnip mosaic virus* (TuMV) or rymoviruses *Hordeum mosaic virus* (HoMV) and *Agropyron mosaic virus* (AgMV) replaced the HC-Pro of WSMW-Sidney 81 that changes in host range of WSMV could be detected. However, all chimeric viruses were able to infect wheat. It was suggested that the host-virus interactions mediated by HC-Pro are less specific in wheat than in the two other hosts, barley and oat. As in the present study, they could not predict virulence of a certain chimeric WSMV from the virulence of the parental viruses and concluded that replacements with distantly related HC-Pro may perturb virus-host interactions and alter virulence in an unpredictable manner. Another example is a study where twelve *A. thaliana* accessions were challenged with PPV isolates representing four PPV strains. Each accession supported local and systemic infection by at least some of the PPV isolates, but high variability was observed in the interaction between PPV isolates

and the 12 *A. thaliana* accessions (Decroocq *et al.*, 2006). Resistance to local infection or long-distance movement was detected in 40 % of the accession-isolate combinations analyzed. In a few cases the resistance genes were already known. For example resistance to long-distance movement of PPV-El Amar in Col-0 requires the *RTM* genes and resistance to long-distance movement in Cvi-1 to PPV-PS is controlled by the single recessive gene *rpv1*.

Our results suggest that recombination between homologous viral genomes can result in new potyviral strains with novel phenotypic traits. This is supported by a study by Chare & Holmes (2006) who conducted a phylogenetic study of recombination frequency in plant RNA viruses. The results suggested that recombination is relatively common in some plant RNA viruses, most particularly the potyviruses. They also concluded that recombination occurs less often between phylogenetically divergent strains. Another recent study where recombination sites in 92 isolates of the potyvirus TuMV were studied shows that recombination occurred throughout the genomes (Ohshima *et al.*, 2007). Some recent results indicate that close homology might be a prerequisite for successful recombination. Martin *et al.* (2005) used engineered recombinants of a DNA virus, *Maize streak virus* (MSV), to demonstrate that the virus need to maintain intragenomic interaction networks and that this limits the evolutionary potential of recombination for MSV and probably for genomes in general. To function optimally fragments of genetic material should reside within genomes similar to those in which they evolved. The similarity necessary for optimal functionality correlates with the complexity of the intragenomic interaction networks in which the genome fragments must function.

PVA as a vector for virus-induced gene silencing (II)

Viruses encoding strong RNA-silencing suppressors (RSS), such as the potyviral HC-Pro (Anandalakshmi *et al.*, 1998; Kasschau & Carrington, 1998), have been anticipated to be poor VIGS vectors. However, there are also indications that potyviruses might be useful as VIGS vectors. Arazi *et al.* (2001) constructed a vector based on *Zucchini yellow mosaic virus* (ZYMV-AGII) (genus *Potyvirus*), which maintained systemic infectivity in cucumber when a human c-Myc peptide or hexahistidine peptide was inserted between the NIb and CP coding regions. This vector was used to lower or enhance expression of a MAPK in cucumber (Shoresh *et al.*, 2006). However, the mechanism behind the 50-60 % reduction in MAPK expression was not analyzed in detail, although it was likely caused by VIGS. There are also several reports on transgenic plants that recover from infection with a potyvirus homologous to the transgene sequence despite the HC-Pro encoded by the potyvirus (e.g Dougherty *et al.*, 1994;. Swaney *et al.*, 1995; van den Boogart *et al.*, 2004; Germundsson & Valkonen, 2006). Carrington *et al.* (2001) concluded that many viruses encoding functional suppressors trigger RNA silencing during infection. This might be explained by the fact that the suppressor proteins do not affect all steps in the silencing pathway and thereby allow RNA silencing to be induced and function to some extent.

In order to test whether potyviruses can be used as VIGS vectors, transgenic *N. benthamiana* (line 16c) showing strong constitutive expression of the green fluorescent protein (GFP) (Brigneti *et al.,* 1998) was infected with an infectious cDNA of PVA that contains the *gfp* gene inserted to the N-terminus of the P1 region. The chimera was called PVA-GFP. Our results show that PVA-GFP can induce systemic silencing of the transgene *gfp*, even if HC-Pro also is expressed by PVA-GFP.

Our data suggest that siRNAs were produced from *gfp* mRNA and viral *gfp* already in the inoculated leaves. The virus-derived siRNA was probably produced from the complete PVA genome, because it accumulated in equal amounts from the 5´and 3´proximal parts of the genome. However, we do not know if PVA-GFP was silenced by transitivity, i.e., secondary silencing triggered by primary silencing of the viral *gfp* gene (Vaistij *et al.,* 2002; Himber *et al.,* 2003) or natural silencing based resistance that is induced by the dsRNA of PVA (Hamilton & Baulcombe 1999; Molnar *et al.,* 2005) or both mechanisms. VIGS has been shown to spread over a distance of at least 1000 nucleotides from the 5´end towards the 3´end of the target mRNA, while 3´ to 5´spread can extend at least 332 nucleotides with a possible limit of 600 nucleotides (Vaistij *et al.,* 2002; Petersen & Albrechtsen, 2005). The PVA genome is nearly 10 000 nt long and therefore it is hard to believe that the complete viral genome was silenced only due to transitivity. This makes the natural resistance theory more probable, but there are no data supporting or rejecting the idea that both mechanisms could be active at the same time.

Viruses (Roberts *et al.,* 1997; Santa Cruz, 1999) and silencing signals (Yoo *et al.,* 2004; Tournier *et al.,* 2006) move in plants according to the source-sink relationship (Turgeon, 1989). This was also observed in the present study. PVA-GFP and the silencing followed the phloem transport. In the first and second leaf (leaves 1 and 2) above the inoculated leaf there was no silencing detected, which was expected since they were source tissues already at the time of plant inoculation. PVA-GFP was systemically transported to and replicating in the basal part of leaf 3 and 4, which caused a more intense GFP fluorescence in these tissues. Silencing was not induced in these leaves, which might be because transgene mediated PTGS is induced in tissues that have a high level of target mRNA expression (Palauqui & Vaucheret, 1998; Vaucheret *et al.,* 1998; Hiriart *et al.,* 2003) whereas leaves 3 and 4 were full-grown and hence had relatively low levels of target mRNA.

In leaves 5 and 6 the first signs of *gfp* silencing were seen in the sink tissue. In leaves 7-15 the transgene *gfp* was suppressed to such a high extent that the leaves were completely red under UV-light, but still low levels of *gfp* mRNA were detectable. PTGS does not usually result in complete abolishment of target RNAs, but there is some degree of expression remaining (e.g., Elmayan & Vaucheret 1996; Hamilton *et al.,* 1998). The silencing was maintained for at least 2 weeks and the silenced tissue was also free of viral symptoms. For a VIGS vector this is an advantage, because viral symptoms can mask a transgene phenotype. The systemic silencing was efficient enough for suggesting that PVA could be used as a VIGS vector in *N. benthamiana*. The alleviated symptoms of PVA-GFP were

caused by the insert in P1 (Rajamäki *et al.,* 2005). Since P1 is suggested to enhance the silencing suppressor function of HC-Pro (Pruss *et al.,* 1997; Anandalakshmi *et al.,* 1998; Brigneti *et al.,* 1998; Kasschau & Carrington 1998; Savenkov & Valkonen 2002; Rajamäki *et al.,* 2005) the insert in P1 might also have made silencing possible due to weakened HC-Pro (Rajamäki *et al.,* 2005). Our results suggest that other potyviruses with strong silencing suppression also could be used for VIGS if they were modified in an appropriate way. Foreign insertions have caused alleviated symptoms and reduced RNA silencing suppression efficiency in several potyviruses, for example TEV (Dolja *et al.,* 1993), PPV (Guo *et al.,* 1998) and LMV (German-Retana *et al.,* 2000).

In leaves at position 9 and above the PVA-GFP lost the insert and the accumulating deletion mutants released GFP expression, which was observed as green spots in the previously silenced, red leaves when observed under UV-light. Sequenced PCR-products from leaves 9-14 revealed deletion of most or all *gfp* and in one case even deletion of 45 nucleotides of the P1 region. Barajas *et al.* (2006) infected HC-Pro transgenic and non-transgenic *N. benthamiana* plants with a PVX vector carrying HC-Pro (PVX-HCT). As in the present study they detected intact PVX-HCT and deletion mutants in both types of plants, but the deletion mutants could be seen already at 9 d.p.i. as compared to 28 d.p.i. in our study. The insert was lost earlier in transgenic plants than non-transgenic plants, and the authors suggested that this could be because the transgene possibly promoted a higher selective pressure against the recombinant virus. However, in our study there were no clear differences between the appearance of deletion mutants in transgenic and non-transgenic plants. The previous authors also suggested that the HC-Pro deletion mutants accumulating in the plants probably were less efficiently targeted by silencing than PVX-HCT, and therefore capable of evading the plant silencing (Barajas *et al.,* 2006). In the present study, a dot-blot detecting the negative viral strand confirmed that after loss of the insert PVA quickly accumulated to higher levels. However, we could also see that PVA siRNA accumulated to higher levels indicating that silencing of PVA increased at the same time. This suggests that the deletion mutants were not less efficiently targeted by silencing than the intact virus, but that the silencing was not efficient enough to decrease virus accumulation. In the study by Barajas *et al.,* (2006) virus accumulation and silencing in the same leaf and between different leaves were not compared, making it difficult to compare their results to ours.

In the PVA deletion mutants lacking the *gfp* insert, HC-Pro was able to reverse silencing. In the new emerging top leaves a mosaic with dark green islands (DGIs) and surrounding light green tissue were detected. In transgenic plants the DGIs corresponded to red silenced tissue observed under UV light, while the surrounding tissue showed a strong GFP expression. Accumulation of viral RNA was very high in green tissues, but low in red tissues. *gfp* mRNA was rarely detectable in red tissue, but in the green tissue the amounts of GFP transgene RNA were modest. The red tissues had higher accumulation of *gfp* siRNA than the green tissues. Moore *et al.,* (2001) concluded that DGIs are the result of antiviral PTGS, however, our study is the first one in which silencing in DGIs could be detected by a visible marker, e.g. change in color from green to red under UV-light. In the

surrounding tissue the strong GFP expression from the transgene was possible due to RNA silencing suppression caused by replicating PVA. Silencing of *gfp* was maintained in DGI because accumulation of PVA was inhibited in them by a virus resistance mechanism. Since all viruses in leaves with DGIs had lost the *gfp* insert the *gfp* silencing must be due to a systemic signal. However, it is not known if the DGIs were induced locally as a response to PVA replication or by a systemic silencing signal. We think systemic signaling is the preferred explanation since HC-Pro enforces RNA silencing suppression by binding siRNA and hence cannot inhibit the activity of preassembled RISC (Lakatos *et al.*, 2006).
Transport of preassembled RISC from lower leaves or incorporation of transported siRNA into RISC could cause *gfp* transgene silencing in the top leaves despite HC-Pro. Upon arrival of PVA that has lost the *gfp* insert, the silencing is suppressed in those tissues where it replicates. However, in DGIs silencing is active and prevent virus replication. The localization of DGIs along the veins and observation of the red-green pattern as soon as the leaves open supports this theory.

Hiriart *et al.*, (2003) silenced the *ChlC* gene using TMV VIGS. Two weeks post inoculation they saw some mosaic and chlorosis. Three weeks post inoculation the apical stem and leaves turned white indicating lack of chlorophyll and *ChlC* silencing. By six weeks new leaves were composed of green and white mosaic. Probably as in our case this corresponded to DGIs even if it was not notified by the authors. However, instead of all the new leaves showing DGIs as in our study, leaves developing 8 weeks post infection were completely silenced. After that the new apical growth shifted between completely white and green or white-green mosaic in about 2-weeks intervals. The virus levels also fluctuated, in the green leaves the levels were very high, in the white-green leaves they were high and in the white leaves they were very low. Apparently replication of the TMV vector was able to repeatedly induce silencing in the study by Hiriart *et al.*, (2003) whereas in our study the silencing only was induced once. One reason for the difference could be that Hiriart *et al.* (2003) silenced an endogene whereas we silenced a transgene. It has been shown that elimination of a PVX VIGS vector did not occur when an endogenous gene was targeted (Ruiz *et al.*, 1998) but if the VIGS vector target a transgene, both the vector and the transgene will be silenced (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998). This explains the fact that the VIGS vector was able to repeatedly accumulate to high levels in the study by Hiriart *et al.* (2003), while in our study the virus levels were very low until the GFP insert was lost. However, silencing in our plants was maintained in larger part of the plants despite the low levels of PVA. Silencing of endogenes often persists just as long as the virus infection permits (Ruiz *et al.*, 1998; Jones *et al.*, 1999) and there is no transitivity or systemic silencing induced in the plants. In our study silencing was maintained despite low levels of PVA because silencing of transgenes persists longer than the virus infection due to transitivity and systemic silencing. However, endogenes can be systemically silenced when they are expressed as transgenes (Bleys *et al.*, 2006).

Resistance to potyvirus translocation in a diploid potato population (III)

In previous studies a cross between diploid parental lines expressing hypersensitive resistance (HR) and susceptibility (S) to PVA resulted in 78 progeny genotypes that were characterized for resistance to PVA (Valkonen, 1994; Watanabe *et al.*, 1994; Hämäläinen *et al.*, 1998; 2000). This population included 6 genotypes failing to support translocation of PVA from inoculated leaves, in which the virus accumulated to high levels similar to S plants. The infected leaves developed no detectable symptoms. This resistance was called non-necrotic resistance (nnr). The resistance was not PVA strain-specific (unpublished data).

In order to characterize nnr, plants were grown in the greenhouse and inoculated with PVA. Samples from inoculated leaves were collected at certain time intervals after inoculation and analyzed using different methods of functional genomics.

Genes differentially expressed in the nnr genotypes as compared to S plants upon PVA infection were detected by suppression subtractive hybridization (SSH) in samples collected 24 hours post-inoculation. Two cDNA libraries, SSH-nnr and SSH-S, were obtained containing genes differentially induced upon infection in nnr and S plants, respectively. The content of the SSH-nnr library was analyzed using the TIGR potato cDNA array containing 10 000 non-redundant potato cDNAs as a diagnostic tool (Potato Functional Genomics Project of The Institute for Genomic Research, TIGR; USA). Over 1000 cDNAs gave positive signal, and of them 542 genes were exclusively detected in the SSH-nnr library. Putative function could be assigned to 351 genes and many of those were putative defense-related genes. To investigate further the gene induction, 384 sequenced cDNAs from the SSH library, corresponding to 146 genes, were spotted on a microarray and used to analyze the results from three repeated plant experiments at 12 and 24 hours post inoculation. Approximately one third (43 cDNAs) of these were identical or had high sequence identity to the cDNAs that gave positive signal in the hybridization of the SSH-nnr library of the TIGR potato cDNA array. Taken together, a total of 645 genes (542+146-43) were expressed at a higher level in nnr than S genotypes according to the SSH analysis. The induction of genes in nnr was, however, only slightly higher compared to S plants. Only a family of *serine proteinase inhibitor 2* (*Pin2*) were expressed at a significantly higher level at both 12 and 24 hours according to microarray analysis and quantitative PCR.

Many of the genes that have a slight differential induction in nnr according to the SSH are typical for HR and SAR, which might indicate that there is activation of similar defense pathways as in R-gene mediated virus resistance, but without cell death. This is probably the case for the recessive resistance genes *dnd1* and *RSS1-R* in *A. thaliana* (Yu *et al.*, 1998; Deslandes *et al.*, 2002). To test this hypothesis, *PR-1* induction, which is considered to be a marker for SAR, was analyzed in upper non-inoculated leaves. There was no significant systemic *PR-1* induction. The upper non-inoculated leaves in PVA-inoculated nnr plants were not resistant to PVA infection, suggesting that nnr was not an HR-like response without cell death.

According to our SSH results, defense genes are induced to a slightly higher level in nnr genotypes compared to S genotypes. Many of these genes encode defense-related proteins, for example PR-proteins, proteins involved in signal transduction and ubiquitination. This means that even if it cannot be proved that the genes in the SSH-nnr library are induced to a significantly higher level, they may still be part of a basal disease resistance induced by pathogen-associated molecular patterns (PAMPs) (reviewed in Whitham *et al.*, 2003; Chisholm *et al.*, 2006; Jones & Dangl, 2006) (Fig. 5). The basal resistance, PAMP-triggered immunity (PTI) resulting in the slightly higher gene induction seen in the SSH-nnr compared to the SSH-S library might be enough to inhibit vascular movement of PVA. However, PTI is not always enough to stop virus infection and it can be induced even in susceptible plants without limiting viral infection (Laird *et al.*, 2004; Huang *et al.*, 2005). Recently a model illustrating the plant innate immune system as a zig-zag model was described (Jones & Dangl, 2006). In this model PTI can be overcome by effectors produced by the pathogen. This means that the plant will become susceptible again, which is called effector-triggered susceptibility, (ETS). The plants can then involve resistance genes recognizing the effectors, resulting in effector-triggered immunity (ETI). If the nnr resistance has an ETI response there will be an R gene recognizing the effector resulting in the gene induction and nnr resistance. This may be the case with the genes *RTM1* and *RTM2* that restrict the movement of TEV in the phloem and prevent systemic infection of *A. thaliana* plants without involving HR or SAR (Chisholm *et al.*, 2001). However, the nnr genotypes are not known to carry PVA-specific R-genes and *PR-1*, HR and SAR are not induced, suggesting that PTI is responsible for the nnr resistance phenotype. Another explanation is that nnr is due to a missing interaction or other unknown factors and the low induction of defense genes is not involved in nnr. This defense gene induction could then be explained as a basal resistance which in this case is not involved in inhibiting vascular movement of PVA.

The zig-zag model illustrates the quantitative output of the plant immune system (Jones & Dangl, 2006). In this model the disease level is proportional to [PTI-ETS+ETI] (Fig. 5). Thus, resistance and susceptibility are not described as a fixed permanent condition but as something that can take many different types of amplitude depending on PAMPs, effectors and resistance genes. If the sum of the equation reaches above a certain threshold the plant will be resistant, but below the threshold the plant remains susceptible. For example HR is considered to be a very efficient resistance with a high level of defense, even if this model is open for the possibility that different HR responses have different defense levels. The resistance level of nnr is apparently high enough to inhibit systemic virus infection. If nnr is a PTI response it might be activated also in S plants, but the amplitude in the S plants might not reach the level acquired to stop systemic infection.

The difference between S and nnr plants might be too low to be detected with microarrays and quantitative PCR, but possible to detect with the more sensitive SSH. These results are in line with Pan *et al.* (2006). They compared differential gene expression in the same material by hybridizing the SSH library directly to the pre-made microarrays or printing sequenced SSH clones on a chip. The direct

hybridization identified more than twice as many differentially expressed genes as the chips. Cao *et al.* (2004) also concluded the importance of complementing microarrays with SSH, especially in identifying novel genes and transcripts of low abundance.

Pin2 was the most abundant gene in the SSH library, and also the only gene which was found to be clearly induced based on microarray and quantitative PCR assays. *Pin2* has eight homologues in *S. tuberosum* with different expression patterns (Barta *et al.,* 2002). *Pin2* encodes a serine proteinase inhibitor (SPI) involved in defense against herbivores and it is also induced by wounding, various forms of stress and pathogen infection in barley, potato, tobacco, tomato and pepper (Graham *et al.,* 1985; Sanchez-Serrano *et al.,* 1986; Pena-Cortes *et al.,* 1992; Balandin *et al.,* 1995; Lee *et al.,* 1996; Kim *et al.,* 2001). *Pin2* has also been shown to be induced in response to virus infection. *CaPinII* was induced in pepper plants during hypersensitive response to TMV (Shin *et al.,* 2001). Potyviruses contain a cysteine proteinase domain, NIa, and Gutierrez-Campos *et al,* (1999) used cysteine proteinase inhibitors to engineer resistance against potyviruses in transgenic tobacco plants.

SaPin2 in *Solanum americanum* is expressed in the phloem suggesting that it could possibly regulate proteolysis in the sieve elements (Xu *et al.,* 2001; Sin & Chye, 2004). To investigate where in the inoculated leaves PVA movement was stopped we tried to monitor infection with PVA-GFP (I). The virus chimera was, however, not able to infect potato plants of the v2 mapping population systemically (unpublished data). Therefore only immunostaining for the PVA CP was conducted in the inoculated leaves at the edges of the bombarded areas. Signal for the virus was detected in all types of leaf cells, even the companion cells, and the results with nnr and S genotypes were similar. These results suggest that nnr might have a role in transport of PVA in the phloem. Multigenic recessive genes conferring strain-specific resistance to TEV inhibit vascular transport in *N. tabacum* (Schaad & Carrington, 1996). The viral determinant controlling vascular transport has been mapped to VPg (Schaad *et al.,* 1997). As in the present study TEV can enter PP and CC suggesting that resistance is functional at or beyond the entry into SE (Schaad & Carrington, 1996).

Resistance to potyvirus translocation, replication or cell-to-cell movement without HR, cell death, SAR and any known dominant R gene has usually been suggested to be passive, resulting from defective host-virus interactions. This hypothesis has been based also on the observation that resistance is overcome by mutations in viral proteins (for example in Gibb *et al.,* 1989; Nicolas *et al.,* 1997; Rajamäki & Valkonen, 2002). Usually VPg is the crucial virulence determinant, in which mutations of aa can overcome the resistance. It has been shown that the natural recessive resistance genes *pvr2* and *pot1* (Ruffel *et al.,* 2002; Ruffel *et al.,* 2005; Moury *et al,* 2004) correspond to the eukaryotic initiation factor 4E (eIF4E). The VPg-eIF4E interaction has been found in several potyvirus-host combinations (Wittman *et al.,* 1997; Léonard *et al.,* 2000; Schaad *et al.,* 2000, Beauchemin *et al.,* 2007; Roudert-Tavert *et al.,* 2007). Ruffel *et al.* (2004) suggested that the resistance could be due to incompatibility between the VPg and eIF4E. No study,

however, has to our knowledge suggested that this incompatibility would lead to gene induction or report a gene expression study in this kind of resistance. We have performed the first gene induction study on a so-called passive resistance and cannot rule out an active mechanism, which suggests that these potentially passive resistances could in some cases be a new kind of incompatible interaction resulting in gene induction and thereby resistance.

Studies on PVA-host interactions illuminate the dynamic process of virus-host co-evolution

Resistance is a manifestation of the co-evolution of viruses and their hosts. The co-evolution is driven by recombination and mutation of viruses and evolution of plant resistance genes, including genes involved in innate immunity and RNA silencing. Virus evolution, innate immunity and silencing are highly connected. The outcome of each specific host-virus interaction is determined by the properties of both the virus and the host. In this thesis PVA-host interactions studied have included alterations due to PVA recombination, molecular studies of a non-necrotic resistance to PVA, and RNA silencing induced by PVA. Our results clearly show how rather small changes in the virus genome can give rise to new virus-host interactions (**I**; **II**) and the importance of host factors for the virus-host interaction both between different species and within the same species (**I**; **III**).

The infectious clone pPVA-B11 did not infect PVA-susceptible potatoes systemically (Puurand *et al.,* 1996). Therefore, a new clone based on PVA-U (pUFL) that infects potato systemically was constructed (**I**). The chimeras produced during the construction of pUFL were used to study how recombination of two closely related viral strains might alter virus-host interactions. Some of the recombinant viral chimeras had completely new phenotypes compared to the parental phenotypes when they interacted with the host, showing the importance of small changes in the virus genome for the virus-host interaction. However, the different chimeras' interactions with potato and *N. benthamiana* did however not correlate at all. The chimera that had high viral titers in potato could have low viral titers in *N. benthamiana*, indicating the importance of host factors for virus-host interactions. The new clone pUFL infected susceptible potato systemically and could be further used for studying nnr resistance in potato (**III**). To study PVA movement in nnr plants *gfp* was inserted in P1 of pUFL. However, PVA-GFP could not infect any potato genotypes, but was still highly infectious in *N. benthamiana* (**II**). In *gfp*-transgenic *N. benthamiana,* PVA-GFP was a successful VIGS vector despite HC-Pro, probably due to the *gfp* insert in P1 decreasing the silencing suppressor activity of HC-Pro and alleviating virus symptoms. The decreased silencing suppression activity could also be one of the reasons that PVA-GFP was not able to infect potato. Somehow, the potato host factors are able to stop the weakened virus, but this is not possible for the host factors of *N. benthamiana*.

A cross of two diploid potato plants gave rise to a completely new resistance phenotype, nnr, which probably resulted from combination of new alleles (**III**;

Hämäläinen *et al.*, 2000). In the nnr plants unknown host factors inhibit vascular movement of PVA. In this study we have used functional genomics to prove that this is a new resistance phenotype also on the molecular level. Compared to S plants there is a slightly higher induction of defense genes in nnr plants, which might be basal resistance and there is also significant induction of *Pin2*. We have also confirmed that this is not an HR without cell death since no *PR-1* induction or SAR is induced in upper non-inoculated leaves.

In conclusion this thesis has illuminated new examples of the complex interactions between host and virus and the evolution of these interactions. Studies on these interactions are important aspects of plant pathology and highly relevant to the development of strategies for control of virus.

Conclusions

The main conclusions drawn from this study are:

- The infectious clone based on PVA-U, pUFL, was able to infect the potato population v2 systemically and it was not restricted to inoculated leaves as pPVA-B11.

- Recombination between closely related viral genomes of potyviruses can result in new viral strains with novel phenotypic traits.

- PVA-GFP can be used as a VIGS vector in *N. benthamiana* despite HC-Pro.

- In the model system including *N. benthamiana* (line 16c) and PVA-GFP, systemic progression of gene silencing and antiviral defense could be analyzed in a novel manner and DGIs could be detected by a visual marker.

- According to SSH 645 genes are induced in nnr plants and many of those are putative defense-related genes.

- According to microarrays and real-time PCR *Pin 2* is the only gene that is significantly higher induced in nnr than S plants.

- *PR-1* and SAR are not induced in systemic leaves of nnr plants.

- PVA can be detected in all types of leaf cells in nnr and S plants, i.e., PVA movement is not stopped until somewhere in the phloem transport.

Future perspectives

- Gene expression studies comparing DGIs with surrounding tissues might give some more insights to the spatial induction of the antiviral defense and also on the silencing mechanism in general.

- Other target genes than GFP should be tested in PVA VIGS.

- Silencing of *Pin2* by VIGS or through amiRNA would help to elucidate the importance of *Pin2* for nnr resistance. Silencing of other possibly interesting genes revealed by SSH would also help to identify the resistance mechanism.

- Gene expression studies on resistances similar to nnr, for example *sbm1* (Keller *et al.*, 1998) and *pvr1* (Murphy, 1998) could provide further insights into resistance mechanisms similar to *nnr*.

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