Transgenic Resistance to PMTV and PVA Provides Novel Insights to Viral Long-Distance Movement

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Abstract

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The studies in this thesis describe forms of transgenic resistance to plant viruses and how they can be used for studying viral infection cycle.

S. tuberosum cv. Saturna expressing the *CP* gene of *Potato mop-top virus* (PMTV) was grown in a field infested with the viruliferous vector of PMTV, *S. subterranea*. The incidence of PMTV-infected tubers was lower in the CP-transgenic potato than in non-transgenic potato. RNA dot-blot analysis revealed that in tubers infected with PMTV, all three RNAs were present. *N. benthamiana* plants expressing the *CP* gene of PMTV were inoculated by two different methods i) mechanical inoculation to leaves and ii) growing plants in soil infested with viruliferous *S. subterranea.* Results showed that the expression of the transgene-derived RNA (or CP) inhibits replication of homologous RNA 2 in transgenic *N. benthamiana*. Furthermore, the results showed that transgene-mediated resistance to PMTV differs in roots and leaves.

Mechanical inoculation with PMTV on CP-transgenic *N. benthamiana* resulted in symptomless, systemic movement of RNA 1 and RNA 3, but not the CP-encoding RNA (RNA 2). These findings show that PMTV RNA 1 and RNA 3 can infect and move systemically in *N. benthamiana* without the CP and RNA 2.

N. benthamiana transformed with the P1 and VPg cistron, respectively, of *Potato virus A* (PVA) displayed: i) resistance to PVA infection, ii) susceptibility, or iii) systemic infection followed by recovery from PVA infection of new leaves. Long-distance transport of PVA from lower, infected parts of recovered plants was compromised in the recovered tissue. This result suggests that PVA is moving as ribonucleoprotein complex other than virus particles.

N. benthamiana transformed with a polycistronic transgene encoding the CI-NIa-CP cistrons of PVA was susceptible to PVA infection. VPg (the N-proximal part of NIa) is a well-known virulence factor of potyviruses and its possible role in suppression of RNA silencing was studied. PVA VPg was found to increase the severity of disease symptoms when expressed from a *Potato virus X* vector in *N. benthamiana*. However, PVA VPg did not show apparent RNA silencing suppression activity. The reason why the polycistronic transgene did not provide resistance could not be resolved.

Keywords: Transgenic resistance, *Potato mop-top virus,* PMTV, *Potato virus A*, PVA, *Nicotiana benthamiana*, viral movement, pathogenesis, RNA silencing

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Appendix

Papers I-IV

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

I. Germundsson, A., Sandgren, M., Barker, H., Savenkov, E.I. & Valkonen, J.P.T. 2002. Initial infection of roots and leaves reveals different resistance phenotypes associated with coat protein gene-mediated resistance to *Potato mop-top virus*. *Journal of General Virology 83*, 1201-1209.

II. Savenkov, E.I., Germundsson, A., Zamyatnin, A.A. Jr., Sandgren, M. & Valkonen, J.P.T. 2003. *Potato mop-top virus*: the coat protein-encoding RNA and the gene for cysteine-rich protein are dispensable for systemic virus movement in *Nicotiana benthamiana*. *Journal of General Virology 84,* 1001-1005.

III. Germundsson, A. & Valkonen, J.P.T. 2005. P1- and VPg-transgenic plants show similar resistance to *Potato virus A* and may compromise long distance movement of the virus in plant sections expressing RNA silencing-based resistance. *Virus Research, accepted for publication*

IV. Germundsson, A., Savenkov, E.I. & Valkonen, J.P.T. 2005. VPg of *Potato virus A* alone does not suppress RNA silencing but affects virulence of a heterologous virus. *Manuscript*

Abbreviation

BNYVV	*Beet necrotic yellow vein virus*
BS	bundle sheath cells
BSMV	*Barley stripe mosaic virus*
CC	companion cells
CI	cylindrical inclusion
CMV	*Cucumber mosaic virus*
CNV	*Cucumber necrosis virus*
CP	coat protein
CRP	cysteine rich protein
CymRSV	*Cymbidium ringspot virus*
eIF	eukaryotic translation initiation factor
EP	epidermal cells
ER	endoplasmatic reticulum
f. sp.	*formae specialis*
GFP	green fluorescent protein
GUS	β-glucoronidase
HC-Pro	helper-component proteinase
LMV	*Lettuce mosaic virus*
miRNA	microRNA
MP	movement protein
MS	mesophyll cells
NIa	nuclear inclusion protein a
NIb	nuclear inclusion protein b
ORF	open reading frame
PCV	*Peanut clump virus*
PD	plasmodesmata
PDR	pathogen-derived resistance
PMTV	*Potato mop-top virus*
PP	phloem parenchyma cells
PPV	*Plum pox virus*
PSbMV	*Pea seed-borne mosaic virus*
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
RNAi	RNA interference
SE	sieve element
SEL	size exclusion limit
siRNA	short interference RNA
SMV	*Soybean mosaic virus*
TEV	*Tobacco etch virus*
TGB	triple gene block
TMV	*Tobacco mosaic virus*

ToMV	*Tomato mosaic virus*
TRV	*Tobacco rattle virus*
TuMV	*Turnip mosaic virus*
TVMV	*Tobacco vein mottling virus*
UTR	untranslated region
VPg	viral genome linked protein

Introduction

Potato (*Solanum tuberosum* L.) and other tuber bearing *Solanum* species originate in the highland regions of the Andes in Peru and Bolivia (Burton, 1966). Potato is the fourth most important crop grown world wide, being appreciated for its nutritional value as well as its uses in the starch and food processing industry.

Potato-infecting viruses are among the most important group of disease agents with potato late blight pathogen (*Phytophtora infestans*) and the bacterial wilt agent (*Ralstonia solanacearum*). Viruses are obligate intracellular parasites that can only multiply in living cells. They cause a multitude of diseases in micro-organisms, plants, animals and humans. There are about 1000 plant-infecting viruses known today (Hull, 2002) and most plant viruses have a broad host-range. Plant viruses also have a great impact on humans. They cause important diseases in crop plants resulting in yield losses as well as enormous economic losses. As viruses are intracellular parasites, chemical treatments cannot be applied against them. Consequently, viral diseases must be prevented by using virus-free planting materials, virus-resistant cultivars and appropriate crop rotation in the field. Virus-resistant cultivars can be generated through traditional breeding or modern biotechnology by developing transgenic plants. For developing resistant cultivars, it is important to understand the molecular biology of the virus and its interaction with the particular host plant.

The studies presented in this thesis contribute to the understanding of transgenic resistance to plant viruses. In addition, they show how plants transformed with viral genes can be used for studying the infection cycle of a virus.

Viral infection cycle

The genome of plant viruses may consist of DNA (single- or double-stranded) or RNA (positive-, negative-, or double-stranded). Most plant viruses have a positive-strand RNA genome that is encapsidated into viral particles by the viral coat protein (Hull, 2002). Viruses are unable to replicate on their own. Instead they must enter a host cell and utilize the host machinery for nucleic acid and protein synthesis to be able to replicate and accumulate to high levels in the host. Successful infection requires compatible interactions between the virus and host-encoded factors and evasion from host defence mechanisms.

Plant viruses enter the cell through wounds made mechanically or by vectors. Positive-strand RNA viruses can be transmitted by aphids, thrips, mites, nematodes or protoctista. When the virus has entered the host cell it starts to disassemble, releasing its RNA to the cytoplasma. Subsequently, the viral RNA is translated on ribosomes, resulting in viral proteins required for viral replication. The virus-encoded replicase (RNA-dependent RNA polymerase [RdRp]) synthesizes a negative strand using the positive strand as a template. The negative strand is then used as a template for generation of positive-stranded RNA molecules (Hull, 2002).

Following replication, viruses must move intracellularly from the site of replication to the plasmodesmata (PD). Intracellular transport of cellular macromolecules is facilitated by association with endoplasmatic reticulum (ER) and probably the cytoskeletal element such as microtubules and microfilaments (Langford, 1995; St Jonston, 1995). It is likely that plant viruses utilize the normal intracellular trafficking system of the host to move to the PD (McLean *et al.,* 1995). The openings of PD have a diameter of about 2.5 nm, limiting molecular transport between plant cells to small molecules and metabolites with a molecular mass of up to 1 kilodalton (kDa) (Lucas *et al.,* 1993). The plasmodesmal size exclusion limit (SEL) can be increased to allow intercellular movement of large endogenous protein or invading viruses (Lucas, 1995). Several types of viral movement proteins (MPs) have been identified, promoting cell-to-cell spread of virus through PD (Lucas, 1995; Carrington *et al.,* 1996). Some viruses, such as *Tobacco mosaic virus* (TMV, genus *Tobamovirus*), encode a single MP that is able to modify SEL of PD and facilitate transport of itself and nucleic acids (reviewed by Ding, 1998). Others like *Potato virus X* (PVX, genus *Potexvirus*) and *Potato mop-top virus* (PMTV, genus *Pomovirus*) contain a set of movement genes called triple gene block (TGB), which encodes three proteins that are involved in transport of the viral RNA through PD (Petty & Jackson, 1990; Beck *et al.,* 1991; Herzog *et al.,* 1998). Viral cell-to-cell movement occurs through PD and the virus has to move through several cell types (epidermal (EP), mesophyll (MS), bundle sheath (BS), phloem parenchyma (PP) and companion cells (CC)) until they are loaded into the sieve element (SE) (Lucas & Lee, 2004). Once the virus has entered the SE it can be transported over long distances to other parts of the plant following the source-sink transition. Finally, viruses must exit the SE and re-establish replication and cell-to-cell movement in tissue distant from initial infection site (Cronin *et al,* 1995). It is not yet well known how viruses enter, move through, or exit the vascular system.

Resistance to plant viruses

Transgenic resistance

The first time the concept pathogen-derived resistance (PDR) was mentioned was in 1985 (Sanford & Johnston). It was suggested that a host expressing genetic material from the pathogen would interfere with important processes in the life cycle of the pathogen and result in resistance to the pathogen (Sanford & Johnston, 1985). The approach was first demonstrated with viruses namely CP gene-mediated resistance to TMV in tobacco plants (Powell *et al.,* 1986). Transgenic expression of the CP of TMV provided effective levels of resistance to closely related strains of TMV. Later, many plant species have been transformed with the *CP* gene of different viruses, conferring resistance to the homologous virus. Apart from the *CP* gene, other genes have also been used. For example the replicase gene (Golemboski *et al.,* 1990; Zaitlin *et al.,* 1994; Audy *et al.,* 1994; Brederode *et al.,* 1995) and defective forms of genes encoding movement proteins (Lapidot *et al.,* 1993; Cooper *et al.,* 1995; Beck *et al.,* 1994; Melander *et al.,* 2001) have been used to obtain plants resistant to viruses.

Initially, it was thought that pathogen-derived resistance was operating through the expressed viral protein (Powell-Abel *et al.,* 1986). It was proposed that the presence of transgene-derived CP blocked disassembly of the virus particle and thereby prevented release and translation of the viral genome. However, it was later shown that transgenic plants expressing an untranslatable form of *Tobacco etch virus* (TEV, genus *Potyvirus*, family *Potyviridae*) CP were able to confer resistance to TEV (Lindbo & Doughert, 1992a). This type of resistance was based on RNA and became known as RNA-mediated resistance. Consequently, PDR was divided into two groups; 1) protein-mediated resistance which requires expression of the protein and gives low level but relatively broad spectrum resistance and 2) RNA-mediated resistance which does not require expression of the protein, provides high-level and specific resistance and is not easily overcome by high inoculum doses (Lomonosoff, 1995).

Protein-mediated resistance

There are at least some cases where the accumulation of CP itself rather than CP mRNA is important for resistance. The CP gene-mediated resistance to TMV is effective against virus particles (Wu, 1990). However, when TMV virions are treated so that a few molecules of coat protein are removed from the 5´ terminus of the viral RNA, allowing ribosomes to bind to the 5´noncoding region of the RNA, they are able to infect TMV CP transgenic plants (Register *et al.,* 1988). In plants transformed with the *CP* gene of TMV or *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family *Bromoviridae*) respectively, decreased levels of CP correlate with a decrease in resistance (Neijdat & Beachy*,* 1989; Okuno *et al.,* 1993). Furthermore, transgenic plants expressing a defective form of CP in which the CP mRNA is expressed but no protein is translated are susceptible to TMV. These findings indicate that expression of protein is needed for resistance (Powell *et al.,* 1990). Overall these findings indicate that the resistance operates by inhibiting a step in the viral replication prior to the release of viral RNA.

Two models have been proposed to explain CP gene-mediated resistance (Register *et al.,* 1989): *i*) presence of transgene-derived coat protein in the cytoplasm of a cell may favour assembly instead of disassembly and thereby inhibit uncoating of the incoming virus particles, *ii*) there might be some kind of receptor or uncoating site within the cell responsible for disassembly of virus particles which is blocked by the transgene-derived coat protein preventing the incoming virus to attach.

RNA-mediated resistance

RNA-mediated resistance does not require the translation of the transgene. Furthermore, transformed plants may be initially susceptible to the virus, although 3-5 weeks after inoculation, symptomless leaves develop from the infected plants (Lindbo & Dougherty, 1992a, b; Swaney *et al.,* 1995; Guo & García, 1997). These symptomless leaves contain undetectable amounts of virus and are resistant to new infection with the same virus; a phenomenon known as “recovery”.

The “recovery” has been analysed in detail. Tobacco plants transformed with the CP-encoding region (cistron) of TEV are initially susceptible to TEV. However, 3-5 week post inoculation new developing leaves have recovered from infection (Lindbo & Dougherty, 1992a). No virus is detected in the recovered tissue and recovered plants are resistant to subsequent infection with TEV. Only those plants previously inoculated with TEV show resistance, indicating that an initial infection of the virus is required to induce the virus-resistant state. No viral protein is detected in the recovered tissue and a drastic decrease (12-22 fold) of the transgene-derived mRNA is observed. However, run-off assay from isolated nuclei from the transgenic plants reveals that the transcription rate is not affected (Lindbo *et al.,* 1993). In addition, transgenic plants expressing an untranslatable CP RNA can also confer resistance (Lindbo & Dougherty, 1992b). These findings indicated that the decrease in the mRNA expression must be due to a RNA degradation mechanism acting in the cytoplasm. Thus, it was suggested that the presence of “unacceptably” high levels of a particular transcript would trigger a sequence-specific post-transcriptional RNA degradation mechanism resulting in silencing of the gene (post-transcriptional gene silencing, PTGS, or RNA silencing). Most of the transgenic virus resistance is probably based on RNA silencing.

RNA silencing

Post-transcriptional gene silencing (PTGS) or RNA silencing was first described in plants. However, related processes have later been found in *Neurospora crassa*, in which it is called quelling, and in different animal systems, in which it is referred to as RNA interference (RNAi) (Fire *et al.,* 1998; Vauchert *et al.,* 1998; Kooter *et al.,* 1999; Matzke *et al.,* 2001).

In the beginning of 1990’s petunia plants were transformed with a gene encoding one of the key enzymes (chalcone synthase) in the synthesis of floral pigments. Rather than obtaining an intensive violet colour the flowers became white i.e., they lost the pigment synthesis (Mol *et al.,* 1989; van der Krol *et al.,* 1990). The lack of pigment correlated with low levels of both the transgene transcript and the endogenous transcript.

RNA silencing is a natural defence mechanism protecting the plant against virus infection (Figure 1). It is triggered by double stranded RNA (dsRNA) formed during replication of RNA viruses. Furthermore, RNA silencing can also target self-complementary single-stranded ‘hairpin’ RNAs that are introduced in the plant (Llave *et al.*, 2000). The dsRNA molecule is recognized and cleaved by a host-encoded ribonuclease III (RNAse III)-like enzyme termed Dicer (Berstein *et al.,* 2001) into small RNA molecules (21-26 nucleotides), so called short interfering RNA (siRNA) (Hamilton & Baulcombe, 1999; Zamore *et al.,* 2000; Berstein *et al.,* 2001; Johansen & Carrington, 2001). The production of siRNA by Dicer requires ATP and other host encoded proteins (Zamore *et al.,* 2000; Bernstein *et al.,* 2001). The small RNAs are incorporated into a RNA-induced silencing complex called RISC that target mRNA with homologous sequence for degradation (Hammond *et al.,* 2000; Bernstein *et al.,* 2001; Tabara *et al.,* 2002). In

plants siRNAs are divided into short (21-22 nt) and long (24-26 nt) size classes (Hamilton *et al*., 2002). The two different size classes of siRNA may play different roles in the RNA silencing pathway. The short siRNA class is involved in the degradation of mRNA, while the long siRNA class correlates with systemic silencing and methylation of DNA (Hamilton *et al*., 2002), which would ensure maintenance of silencing (Jones *et al.,* 1999). Methylation has been suggested to be important for controlling gene expression in many organisms (reviewed by Martienssen & Richards, 1995; Selker, 1997; Finnegan *et al.,* 1998). Methylation of transgene could cause production of aberrant transcripts, important for triggering the RNA silencing (English *et al.,* 1996; Jones *et al.,* 1999). Only DNA sequences complementary to siRNA become methylated, suggesting direct RNA-DNA interaction (Matzke *et al.,* 2001). The exact mechanism of RNA-directed DNA methylation is not yet known (Jones *et al.,* 1999, Matzke *et al.,* 2001) but many studies suggest small RNAs to play a crucial role (Llave *et al.,* 2000; Mette *et al.,* 2001).

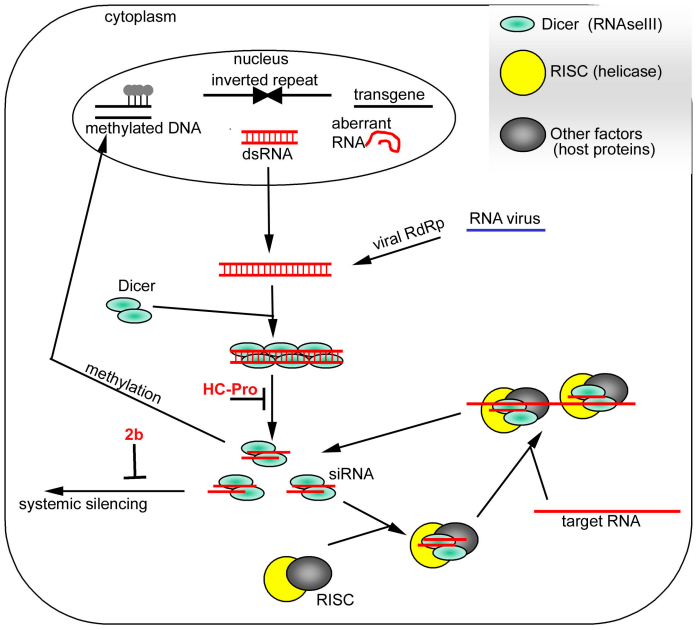

Figure 1. A simplified model of RNA silencing in plants. RNA silencing is triggered by dsRNA originating from replicating RNA viruses, DNA viruses or transgene mRNA. dsRNA is cleaved by RNAse III-like enzyme (Dicer) into small interfering RNAs (siRNA) that are incorporated into the RNA-induced silencing complex (RISC) and guide it to RNA molecules with homologous sequence, which is degraded, generating more siRNA. siRNA can move systemically in the plants and cause systemic silencing. In addition siRNA are thought to be important for methylation of transgene DNA. Viral-encoded RNA silencing suppressors can interfere with different steps in the RNA silencing pathway.

RNA silencing is an important defence mechanism that has also been shown to play a crucial role in endogenous developmental regulation in plants and animals (Llave *et al.,* 2002). The small endogenous RNAs (~21nt in length), called micro RNAs (miRNAs) interact with their targets through near-perfect complementarity and direct mRNA degradation and translational repression (Llave *et al.,* 2002; Rhoades *et al.,* 2002; Carrington & Ambros, 2003). miRNAs were first discovered in *Caenorhabditis elegans* when screening for mutants with developmental defects (Lee *et al.,* 1993; Reinhardt *et al.,* 2000) and have since then been identified in *Drosophila*, humans and plants (Voinnet, 2002). In contrast to siRNA that are generated by cleavage of dsRNA, miRNA arise from a precursor molecule of about 70 nt that are transcribed from non-protein-coding genes. The precursor molecule is processed by Dicer and the mature miRNAs act as negative regulator for specific target mRNAs (Carrington & Ambros, 2003). Despite differences in origin, siRNAs and miRNAs are chemically similar and functionally interchangeable, guiding specific RNA degradation. Indeed, a few of the viral RNA silencing suppressors (see below) such as the potyviral HC-Pro, P15 of pecluvirus and P19 of tombusvirus have been shown to interfere with the miRNA activity (Zilberman *et al*, 2003; Dunoyer *et al.,* 2004).

Suppression of RNA silencing

RNA viruses replicate via the formation of a dsRNA molecule. As mentioned previously, dsRNA molecules trigger RNA silencing. Therefore, to be able to infect plants, plant viruses have evolved strategies to overcome RNA silencing (Voinnet *et al*., 1999): they encode proteins that can suppress RNA silencing. Studies on viral synergism gave a hint that viruses might encode silencing suppressors (Vance *et al.,* 1995; Pruss *et al.,* 1997). Synergism refers to a situation in which two viruses co-infect a plant, and one of them might assist the other virus, leading to increase in the accumulation of virus and enhanced symptom development. Co-infection of plants with PVX and *Potato virus Y* (PVY, genus *Potyvirus*, family *Potyviridae*) can result in a three to ten fold increase in the levels of PVX as compared to single infected plants (Vance, 1991). This synergistic effect can also be observed in coinfection of plants with PVX and *Tobacco vein mottling virus* (TVMV, genus *Potyvirus*, family *Potyviridae*) and TEV, respectively (Shi *et al.,* 1997). Experiments utilizing chimeric viruses and transgenic plants identified the potyviral HC-Pro as the synergism determinant in potex-potyvirus interaction (Vanc *et al.,* 1995; Pruss *et al.,* 1997). It was suggested that the potyviral HC-Pro might suppress a host defence mechanism that would normally restrict accumulation of potexvirus. Later, HC-Pro was shown to be able to suppress RNA silencing and was the first silencing suppressor to be identified (Anandalakshmi *et al.,* 1998; Brigneti *et al.,* 1998; Kasschau & Carrington, 1998). Expression of HC-Pro can reverse established silencing (Brigneti *et al.,* 1998). The exact mechanism of how HC-Pro interferes with the RNA silencing pathway is not yet elucidated but it has been shown to work downstream of the systemic silencing signal (Mallory *et al.,* 2001).

Another viral protein that was identified as a silencing suppressor is the 2b protein of CMV (Brigneti *et al.,* 1998). CMV 2b has been shown to prevent transmission of the systemic silencing signal (Guo & Ding, 2002). It prevents initiation of silencing but cannot reverse already established silencing. One possibility is that CMV 2b binds directly to the signal. However, CMV 2b localizes to nucleus and this localization has been shown to be important for suppression (Lucy *et al.,* 2000). It has therefore been suggested that CMV 2b can induce or enhance transcription of endogenous silencing suppressors or repress transcription of positive regulators of RNA silencing (Moissiard & Voinnet, 2004).

Both HC-Pro and 2b had earlier been characterized as virulence factors. Pathogenicity factors from many other viruses have therefore been studied and many of them are able to suppress RNA silencing (Voinnet *et al.,* 1999) (Table 1). However, besides being virulence factors there are few similarities between these proteins suggesting that the ability to suppress RNA silencing evolved as additional features of proteins that already had divers functions (Moissiard & Voinnet, 2004). So far, many suppressors of RNA silencing have been characterized by different methods.

Cross-protection

A plant infected with one virus might be protected against other strains of the same virus species. This is called cross-protection and was first described by McKinney (1929). He showed that infection of a host plant with a mild strain of a TMV protected the plant against subsequent superinfection by severe strains of the same virus. Soon it was suggested that prior infection with a mild virus could reduce or eliminate damage due to infection by a severe strain of the same virus (Salaman, 1937). One proposed mechanism of cross-protection was that there were limited virus-specific multiplication sites in the cell. If one virus occupied all these sites, an incoming related strain would not be able to multiply (Bawden & Kassanis, 1945). Another theory was that the first strain used all essential metabolites required by the second strain to multiply (Matthew, 1991). Nowadays it is known that cross-protection is based on RNA silencing (Ratcliff *et al.,* 1999). In a series of cross-protection experiments, it was shown that PVX carrying the reporter gene β-glucuronidase (GUS) plus part of the green fluorescent protein (GFP, PVX-GUS.GF) were not able to infect TMV-GFP infected plants.

Table 1. Examples of RNA silencing suppressors found in plant viruses.

Virus group	Example of virus	Protein	Possible interference	References
Begomovirus	ACMV, TYLCV	AC2, C2	DNA binding, transcriptional activator?	15
Carmovirus	TCV	P38	?	14, 10
Closterovirus	BYV	P21	?	11
Crinivirus	SPCSV	P22	?	6
Cucumovirus	CMV	2b	Nuclear localization, necessary for suppression and prevent systemic spread of silencing?	15
Hordeivirus	BSMV	γβ	?	17
Luteovirus	BMYV	P0	?	9
Nodavirus	FHV	B2	?	8
Pecluvirus	PCV	P15	?	4
Potexvirus	PVX	P25	?	16
Potyvirus	TuMV, PVY, TEV	HC-Pro	Prevents accumulation of siRNA	1, 2, 5
Sobemovirus	RYMV	P1	?	15
Tenuivirus	RHBV	NS3	?	3
Tobamovirus	ToMV	P130	?	7
Tombusvirus	TBSV	P19	Binds siRNA, preventing incorporation in RISC?	15, 12
Tospovirus	TSWV	NSS	?	3, 13

ACMV: *African cassava mosaic virus*; BSMV: *Barley stripe mosaic virus*; BWYV: *Beet western yellows virus*; BYV: *Beet yellows virus*; CMV: *Cucumber mosaic virus*; FHV: *Flock house virus*; PCV: *Peanut clump virus*; PVY: *Potato virus Y*; PVX: *Potato virus X*; RHBV: *Rice hoja blanca virus*; RYMV: *Rice yellow mottle virus*; SPCSV: *Sweet potato chlorotic stunt virus*; TBSV: *Tomato bushy stunt virus*; TCV: *Turnip crinkle virus*; TEV: *Tobacco etch virus*; ToMV: *Tomato mosaic virus*; *TSWV*: *Tomato spotted wilt virus*; TuMV: *Turnip mosaic virus*; TYLCV: *Tomato yellow leaf curl virus*.
Anandalakshmi *et al.,* 1998; 2) Brigneti *et al.,* 1998; 3) Bucher *et al.,* 2003; 4) Dunoyer *et al.,* 2002; 5) Kasschau & Carrington, 1998; 6) Kreuze *et al.,* 2005; 7) Kubota *et al.,* 2003; 8) Li *et al.,* 2002; 9) Pfeffer *et al.,* 2002; 10) Qu *et al.,* 2003; 11) Reed *et al.,* 2003; 12) Shilhavy *et al.,* 2002; 13) Takeda *et al.,* 2002; 14) Thomas *et al.,* 2003; 15) Voinnet *et al.,* 1999; 16) Voinnet *et al.,* 2000; 17) Yelina *et al.,* 2002.

Potato mop-top virus

Potato mop-top virus (PMTV) is the type member of the genus *Pomovirus*. It is economically one of the most important plant viruses in the Nordic countries. Virus infection causes some quantitative yield loss, but the qualitative losses are more important. PMTV causes symptoms known as "spraing" (brown arcs and circles) in the potato tuber flesh. These symptoms make them unsuitable for the processing-industry. Depending on the cultivar, other symptoms such as stunting of shoots and yellowing of leaves may occur (Sandgren, 1996). Infection may also spread throughout the plant if an infected mother tuber is planted (secondary infection). Plants originating from primary-infected tubers often show symptoms in the foliage (Björnstad, 1969). Secondary infection can result in a decrease of up to 26% of the tuber weight (Calvert, 1968). There are differences in how potato cultivars respond to PMTV infection. For example, *S. tuberosum* cv. Saturna,

widely used in the Scandinavian potato processing industry, is very sensitive to PMTV infection (Barker *et al.,* 1998a). Cultivars Apell and Desirée, on the other hand, are more resistant. Cultivar Bintje is tolerant to PMTV and infected tubers are symptomless. PMTV is known to occur in northern Europe, Canada, North America, Japan and the Andean region of South America (Reavy *et al.,* 1997). Besides infecting *S. tuberosum*, PMTV also infects other species of *Solanaceae* and *Chenopodiaceae* (Harrison *et al.,* 1997).

The genome of PMTV consists of three single stranded (ss) positive sense RNA molecules, RNA 1, RNA 2 and RNA 3 (Scott *et al.,* 1994), which are separately encapsidated within rod-shaped virions (Harrison & Jones, 1970) (Figure 2). The nucleotide sequence of RNA 2 and RNA 3 of the Scottish PMTV isolate T (Scott *et al.,* 1994; Kashiwazaki *et al.,* 1995) and the complete nucleotide sequence of RNA 1 of the Swedish PMTV isolate Sw (Savenkov *et al.,* 1999) has been determined. The sizes of the RNA components of PMTV-Sw are 6.035 kilobases (kb) (RNA 1), 3.134 kb (RNA 2) and 2.964 kb (RNA 3) (Savenkov *et al.,* 1999; Sandgren *et al.,* 2001; Savenkov, 2002). The tRNA-like structure in the 3´-end is identical in all the RNA components (Savenkov *et al.,* 1999). Many RNA viruses contain more than one gene on a single genomic RNA, whereas in an eukaryotic mRNA only the first open reading frame (ORF) is translated on a normal eukaryotic mRNA (Miller & Koev, 2000). Viruses have solved this problem by using different expression strategies. PMTV uses translational readthrough to express the RdRp encoded by RNA 1 and the read-through domain encoded by RNA 2 (Savenkov *et al.,* 1999). The first gene of the three *TGB* genes (*TGBp1*) is expressed directly from RNA 3 whereas the other two TGB proteins, TGBp2 and TGBp3, are translated from a single subgenomic RNA by leaky scanning mechanism (Lukhovitskaya *et al.,* 2005).

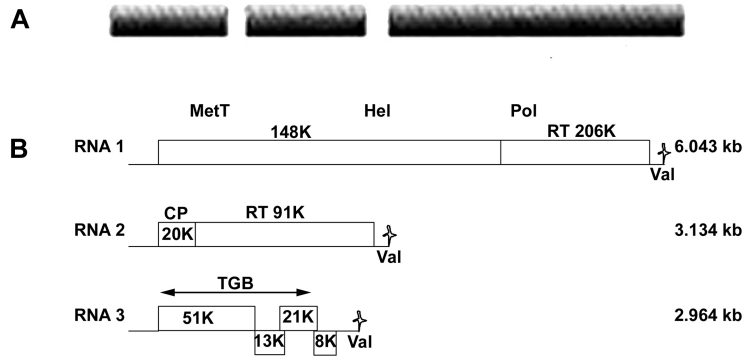

Figure 2. Particle morphology (A) and genome organization (B) of *Potato mop-top virus* (PMTV-Sw). The viral genome consists of three RNA molecules (RNA 1, 2 and 3), which are separately encapsidated into rod-shaped virus particles. The large rectangles represent the ORFs. In the 3´end of each RNAs is a tRNA-like structure. The size of the individual RNA molecules are indicated to the right.

RNA 1 contains two ORFs for proteins with a molecular mass of 148 kDa and 206 kDa, respectively. Analysis of the coding capacity of the sequence of RNA 1 predicts the presence of a methyltransferase, a helicase and a polymerase domain. The 206 kDa protein is a read through protein and the GDD motif, characteristic for RNA-dependent RNA polymerases (RdRp), is located in the C-terminal part of the read-through domain. The 5´-end contains an untranslated region (UTR), with some similarity to the 5´-UTR of RNA 2 but not to the 5´-UTR of RNA 3 (Savenkov *et al.,* 1999).

RNA 2 encodes two proteins, a 20 kDa CP and a 91 kDa read-through protein (Kashiwazaki *et al.,* 1995; Sandgren *et al.,* 2001). The latter might be involved in vector transmission (Tamada *et al.,* 1996; Reavy *et al.,* 1998). The best-studied read-through protein is that of *Beet necrotic yellow vein virus* (BNYVV, genus *Benyvirus*) transmitted by *Polymyxa betaea*. Isolates of BNYVV that have been propagated through several passages in test plants by mechanical transmission generate deletions in the RNA sequence encoding the read-through protein. These deletions are associated with loss of BNYVV-transmissibility by the vector (Tamada *et al.,* 1996; Reavy *et al.,* 1998). In the case of PMTV, deletions in the read-through protein are also associated with the loss of transmission by the vector *Spongospora subterranea* (Reavy *et al.,* 1998). Thus, it has been suggested that the read-through domain encoded by RNA 2 of PMTV contain specific determinants important for acquisition and transmission of the virus by its natural vector (Reavy *et al.,* 1998).

RNA 3 contains four ORFs (Scott *et al.,* 1994) encoding four putative proteins of 51 kDa, 13 kDa, 21 kDa and 8 kDa. The 51 kDa, 13 kDa and 21 kDa proteins which are similar to the TGB proteins found in several other virus genera such as *Potexvirus*, *Carlavirus*, *Allexivirus*, *Foveavirus*, *Hordeivirus*, *Benyvirus* and *Pecluvirus* (Morozov & Solovyev, 2003). TGBp1 of PMTV has an RNA binding capacity, which is thought to be necessary for the formation of complexes with viral genomic RNAs, and is important for cell-to-cell movement of viruses (Morozov & Solovyev, 2003; Oparka, 2004). Localization studies on *N. benthamiana* infected with *Peanut clump virus* (PCV, genus *Pecluvirus*) revealed that TGBp1 localizes to the PD. However, there is no association of the PCV TGBp1 to the PD when TGBp1 is expressed by a transgene (Erhardt *et al.,* 1999a). The transgenically expressed TGBp1 is localized to PD following infection of the transgenic plant with a PCV mutant, where *TGBp1* is deleted. In addition, systemic movement of the virus is restored (Erhardt *et al.,* 1999a). Interestingly, the transgenic PCV TGBp1 does not support systemic infection of TGBp1-deficient BNYVV (Erhardt *et al.,* 1999b), indicating that TGBp1 requires homologous TGBp2/TGBp3 for movement of the virus (Lauber *et al.,* 1998; Erhardt *et al.,* 1999b; Solovyev *et al.,* 1999). Furthermore, TGBp1 of BNYVV and *Barley stripe mosaic virus* (BSMV, genus *Hordeivirus*) localizes to punctuate bodies at the cell periphery in infected leaf cells (Erhardt *et al.,* 2000; Lawrence & Jackson, 2001). In leaves expressing TGBp1 of PMTV fused with the green fluorescent protein (GFP-TGBp1) in the presence of TGBp2 and TGBp3 of PMTV, GFP-TGBp1 localizes to intermediated bodies and in cell wall-embedded punctuate bodies (Zamyatnin *et al.,* 2004). These intermediate bodies originate

from the cortical ER. Furthermore, TGBp1 are able to move to the neighboring cells immediately adjacent to the transfected cell when co-expressed with TGBp2 and TGBp3. Thus, it is likely that viruses encoding TGB proteins utilize the same strategy for cell-to-cell movement.

PMTV is transmitted by the protoctista *Spongospora subterranea* f.sp. *subterranea*, which is also the causing agent of powdery scab on potato tubers. *S. subterranea* belongs to the family *Plasmodiophoracea*, kingdom Protoctista (Agrios, 1997). Some other economically important members of this family are *P. betae* (vector of BNYVV) and *Plasmodiophora brassica* (casual agent of clubroot on *Brassicaceae*) (Agrios, 1997). Plasmodiophorids are obligate parasites. They do not form hyphae but a plasmodium. Plasmodiophorids produce biflagellate zoospores from resting spores. The symptoms on potato tubers, caused by *S. subterranea*, begin as purple brown tumour-like swelling at the site of infection, which during the maturation become hollow filled with fine brown powder consisting of resting spores (Harrison *et al.,* 1997). Powdery scab normally affects only the outer tissue, but may sometimes penetrate more deeply and destroy the whole tuber (Harrison *et al.,* 1997). *S. subterranea* forms resistant spores capable of surviving in soil for more than 10 years (Harrison *et al.,* 1997). Under favourable conditions, such as in the presence of water, the resting spores germinate and release zoospores. When reaching the host plant, either by swimming or carried by soil water, zoospores attach, encyst and penetrate the host. After penetration, the pathogen becomes a multinucleate plasmodium. As more nuclei are formed, the plasmodium increases in size. Eventually, the plasmodium is cleaved into segments to form zoosporangia. Each zoosporangia contains four to eight uninucleate zoospores. The zoospores are released from the zoosporangia into the soil and can re-infect roots and tubers (Agrios, 1997).

Development of powdery scab disease is associated with cool weather conditions and high humidity in soil. The optimal temperature range for *S. subterranea* infection is 14-20°C (Teakle, 1988). One of the most important factors affecting development of powdery scab is soil water. The zoospores need a certain level of moisture to be able to move. Therefore, high soil water content, which increases the release and movement of the zoospore, are required (Harrison *et al.,* 1997, Teakle 1988). Resting spores seem to germinate more readily when air-dried before re-moistening (Teakle, 1988).

The zoospores of *S. subterranea* can acquire PMTV virions while they develop in a virus infected host cell. There are two different virus-fungal vector relationships characterized (Campbell, 1996). The *in vitro* transmission involves adsorbance of virions onto the surface of zoospores. In this case the virions are not present inside the resting spores. The other mechanism is *in vivo* transmission, in which the virions enter the zoospores when the fungus grows in an infected host. In this case the resting spores do contain the virus. PMTV is transmitted by *in vivo* mechanism and the virus is located in the zoospores emerging from vegetative sporangia or resting spores. The details of the mechanism of acquisition are still uncertain. PMTV does not multiply in the resting spores. Resting spore formation makes it possible for both *S. subterranea* and PMTV to survive for a long period

of time (>10 years) in the soil (Jones & Harrison, 1972; Harrison *et al.,* 1997). There are some differences in the susceptibility of potato cultivars to *S. subterranea*, but all cultivars are affected (Harrison *et al.,* 1997).

Potato virus A

Potato virus A (PVA) belongs to the genus *Potyvirus* (family *Potyviridae*), which is the largest genus of plant infecting viruses (Shukla *et al.,* 1994). Diseases caused by potyviruses are common in agricultural crops, and PVA can decrease the potato yield by up to 40% (Bartels, 1971). Symptoms vary from mild mosaic to rugosity of the leaves. The virus particle is flexuous and rod-shaped, and contains a single stranded, messenger polarity RNA molecule of about 10 kb. It is encapsidated by approximately 2000 copies of the CP (Matthew, 1991). Covalentely bound to the 5´end of the genome is the VPg (Murphy *et al.,* 1991). The 3´end is polyadenylated (Shukla *et al.,* 1994). The viral RNA encodes one ORF that is translated by the host ribosomes into one single large polyprotein. This polyprotein is proteolytically cleaved into ten functional proteins by three proteinases: the C-proximal proteinase domain of the nuclear inclusion protein a (NIa-Pro), the P1 protein (P1) and the helper component proteinase (HC-Pro) (Revers *et al.,* 1999) (Figure 3; Table 2).

Recent studies shows that the VPg of TEV and *Turnip mosaic virus* (TuMV, genus *Potyvirus*, family *Potyviridae*) interact with eukaryotic translation initiation factor 4E (eIF4E and eIF(iso)4E in plants) of *Arabidopsis thaliana*. *Arabidopsis* mutants in which the expression of the eIF(iso)4E is blocked are not infected by these viruses (Wittman *et al.,* 1997; Lellis *et al.,* 2002). Furthermore, *Lettuce mosaic virus* (LMV, genus *Potyvirus*, family *Potyviridae*) is not able to replicate in eIF(iso)4E-deficient *Arabidopsis* plants (Duprat *et al.,* 2002). These findings indicate that the VPg might be involved in the initiation of translation. It has been suggested that the VPg could function like a typical 5´cap structure recruiting the translation initiation factor and the 40S ribosomal subunits (Lellis *et al.,* 2002).

Potyviruses do not have any specialized MPs. Instead, several potyviral proteins are involved in movement such as the cylindrical inclusion (CI) protein (Carrington *et al.,* 1998), the HC-Pro (Kassachau *et al.,* 1997), the VPg (Nicolas *et al.,* 1997; Schaad *et al.,* 1997), the CP (Dolja *et al.,* 1994, 1995) and the 6K2 protein (Rajamäki & Valkonen 1999, Spetz & Valkonen, 2004). The CI of TVMV localizes to the PD forming specific structures. These structures have central channels which are connected to the plasmodesmal openings (Rodriguez-Cerezo *et al.,* 1997). The CP can be detected inside these channels as well (Rodriguez-Cerezo *et al.,* 1997). One model of potyviral cell-to-cell movement might be that newly synthesized viral RNA molecules associate with the CP either as virion or as a ribonucleoprotein complex (Carrington *et al.,* 1998). These virions or ribonucleoprotein complexes are guided through the plasmodesma-associated CI structures into the plasmodesmata. Release of the virion or the RNP complex into the neighbouring cells would complete the transport process.

The central region of HC-Pro has been shown to be important for long-distance movement. Mutations in the HC-Pro of TEV prevent systemic movement of the virus, but only slow down cell-to-cell movement (Cronin *et al.,* 1995). Mutations within the CP encoding region of TEV alter the virion assembly, cell-to-cell and long-distance movement (Dolja *et al.,* 1994, 1995). Mutations in the N-terminal part of the CP cistron allow virion assembly but prevent long-distance movement. These results indicate that the potyviral CP is important for virion assembly, cell-to-cell and long-distance movement. However, the function of the CP in these processes is distinct and separable from each other (Dolja *et al.,* 1994)

6K2 and VPg of PVA also play a crucial role in long-distance movement of the virus. PVA isolate B11 is able to infect *Nicandra physaloides* systemically while isolate M is restricted to inoculated leaves. On the other hand, PVA B11 fails to systemically infect *Solanum commersonii* whereas PVA M infect this host systemically. Single amino acid substitutions within the 6K2 and VPg encoding regions result in the ability of PVA M to move systemically in *N. physaloides* and PVA-B11 to move systemically in *S. commersonii* (Rajamäki & Valkonen 1999, 2002). Thus, 6K2 and VPg of PVA are determinants of systemic movement.

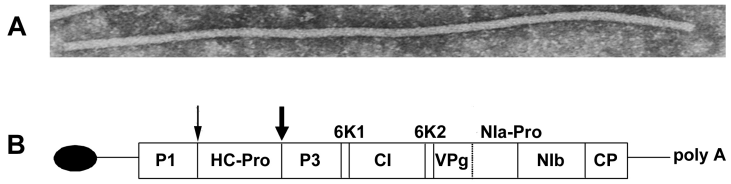

Figure 3. Particle morphology (A) and genome organization (B) of potyviruses. The viral RNA is encapsidated into filamentous particles. The genome contains one ORF, which is processed into ten mature proteins [protein 1 (P1), helper component proteinase (HC-Pro), protein 3 (P3), the 6 kDa protein 1 (6K1),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

Table 2. Known functions of the mature potyviral proteins.

Name	Function*	References
P1	Proteinase	7
	Genome amplification	21, 32
	Accessory factor for suppression of RNA silencing	1, 5, 16
HC-Pro	Proteinase	6, 8
	Cell-to-cell and long-distance movement	17, 29
	Genome amplification	2, 36
	Aphid transmission	24
	Suppressor of RNA silencing	1, 5, 16
P3	Genome amplification	21, 28, 36
6K1	RNA replication	27
CI	Helicase	13, 18
	Cell-to-cell movement	9
6K2	Virus replication	26
	Long-distance movement	25
VPg	RNA replication	22, 30
	Cell-to-cell and long-distance movement	23, 25, 30
	Forms complex with the eukaryotic translation initiation factor eIF4E and eIF(iso)4E	19, 33
NIa-Pro	Proteinase	12
NIb	RNA-dependent RNA polymerase	14
CP	Encapsidation	31
	Cell-to-cell and long-distance movement	10, 11
	Genome amplification	20
	Aphid transmission	3

*Potyviral proteins are multifunctional. All potyviral proteins are essential for virus propagation (Kekarainen *et al.,* 2002) and most of them have been implicated as symptom determinants, which are not specified here. References: 1) Anandalakshmi *et al.,* 1998; 2) Atreya *et al.,* 1992; 3) Atreya *et al.,* 1990; 4) Brantley & Hunt, 1993; 5) Brigneti *et al.,* 1998; 6) Carrington *et al.,* 1989a; 7) Carrington *et al.,* 1990; 8) Carrington *et al.,* 1989b; 9) Carrington *et al.,* 1998: 10) Dolja *et al.,* 1994; 11) Dolja *et al.,* 1995; 12) Dougherty *et al.,* 1989; 13) Eagles *et al.,* 1994; 14) Hong *et al.,* 1996; 15) Jenner *et al.,* 2000; 16) Kasschau & Carrington 1998; 17) Klein *et al.,* 1994; 18) Laín *et al.,* 1990; 19) Léonard *et al.,* 2000; 20) Mahajan *et al.,* 1996; 21) Merits *et al.,* 1999; 22) Merits *et al.,* 1998; 23) Nicolas *et al.,* 1997; 24) Pirone *et al.,* 1984; 25) Rajamäki & Valkonen, 1999; 26) Restrepo-Hartwig *et al.,* 1994; 27) Riechmann *et al.,* 1992; 28) Rodriguez-Cerezo *et al.,* 1993; 29) Rojas *et al.,* 1997; 30) Schaad *et al.,* 1996; 31) Shukla & Ward, 1989; 32) Verchot & Carrington, 1995; 33) Wittmann *et al.,* 1997.

Potyviruses are transmitted by aphids in a non-persistent manner. Aphids can aquire virus particles when feeding on an infected plant. 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 transmission of the virus must therefore be very fast. The acquisition takes only a couple of seconds and the aphid looses the ability to transmit the virus within a few minutes. It has been shown that the DAG aa motif (Asp-Ala-Gly) in the CP (Shukla *et al.,* 1994) as well as the KITC- (Lys-Ile-Thr-Cys) (Atreya *et al.,* 1992; Blanc *et al.,* 1998; Sasaya *et al.,* 2000) and the PTK-motif (Pro-Thr-Lys) (Peng *et al.,* 1998) in the HC-Pro are involved in potyviral transmission by aphids. Some potyvirus can also be transmitted through the seed of some of their hosts (Johansen *et al.,* 1994). Furthermore they are transmitted in infected plant material such as cuttings and tubers (Shukla *et al.,* 1994).

Aims of the study

The aim of this study was to obtain and characterize transgenic resistance to two viruses, PMTV and PVA, in plants. The transgenic plants resistant to the viruses were also used to study the viral infection cycle of PMTV and PVA. The more specific aims were to:

- study resistance to PMTV in transgenic potato and *N. benthamiana* expressing the *CP* gene of PMTV.
- study the long-distance movement of PMTV.
- study the types of resistance to PVA in *N. benthamiana* plants transformed with single cistrons or polycistronic transgene derived from PVA.
- study the function of VPg of PVA as a virulence determinant and its possible role in overcoming transgenic resistance.

Results and discussion

CP gene-mediated resistance to PMTV in *N. benthamiana* and *S. tuberosum*

In order to achieve resistance to PMTV, *N. benthamiana* has been transformed with the *CP* gene from the Scottish PMTV-T isolate (Reavy *et al.,* 1995). The expression level of PMTV CP varies in different transgenic lines, but all lines are immune or highly resistant to PMTV. In some transgenic lines, viral replication can be detected in a small proportion of plants but no symptoms develop (Barker *et al.,* 1998b). Strong resistance is only expressed if the *CP* gene is translated and all lines are resistant irrespective of the steady-state levels of transgene RNA transcript or protein (Barker *et al.,* 1998b). *S. tuberosum* cv. Saturna has also been transformed with the same construct as *N. benthamiana* (Barker *et al.,* 1998a). All Saturna lines are resistant to PMTV when grown in infested soil in the greenhouse.

In order to evaluate resistance to PMTV under natural conditions, transgenic lines of cv. Saturna were grown in a naturally PMTV-infested field in Halland, Sweden (**I**). A few infected tubers were found, but the incidence of infection was lower (9/132, 7%) compared to the non-transgenic lines (12/64, 20%). A dot-blot hybridization analysis was carried out to determine whether all three RNA molecules were present in infected tubers (**I**). RNA1 and TGB-encoding RNA (RNA 3) were detected in the presence of the CP-encoded RNA (RNA 2) in infected potato tubers (**I**). In some infected tubers no RNA 1 was detected. Since it encodes the RdRp it is likely that RNA 1 was present but in low concentrations and therefore not detectable.

Only RNA 1 and RNA 3 but not RNA 2 are detected in inoculated as well as systemically infected leaves of CP-transgenic *N. benthamiana* plants (McGeachy & Barker, 2000). These transgenic *N. benthamiana* plants were analysed in more detail in order to address the different results obtained from CP-transgenic potato plants grown in infested soil compared to mechanically inoculated CP-transgenic *N. benthamiana* plants (**I**). CP-transgenic *N. benthamiana* plants were inoculated with PMTV by two different methods; i) mechanical inoculation to leaves and ii) growing plants in soil infested with viruliferous *S. subterranea*. Leaf and root tissues were analysed. Neither RNA 2 nor RNA 3 were detected in leaves of CP-transgenic *N. bethmaniana* plants growing in infested soil, but in roots both RNAs could be detected (Figure 4). This indicates that there was no systemic movement of PMTV from infected roots to the above-ground parts. In transgenic *N. benthamiana* plants mechanically inoculated with PMTV, RNA 3 was detected in the leaves in five out of 10 plants but RNA 2 was not detected in any inoculated leaf. In roots, RNA 3 was detected in seven out of ten plants but RNA 2 was detected only in three out of ten plants in very low amounts. These results indicate that the transgene-derived RNA or CP inhibits replication of RNA 2 but does not inhibit the replication of RNA 1 and RNA 3 (**I**; McGeachy & Barker, 2000). The results also suggest that RNA 1 and 3 are able to replicate and move systemically

in the absence of the CP-encoding RNA in the CP-transgenic *N. benthamiana* plants (**I**; McGeachy & Barker, 2000). Finally, the results reveal that expression of transgenic resistance differs in roots and leaves (**I**).

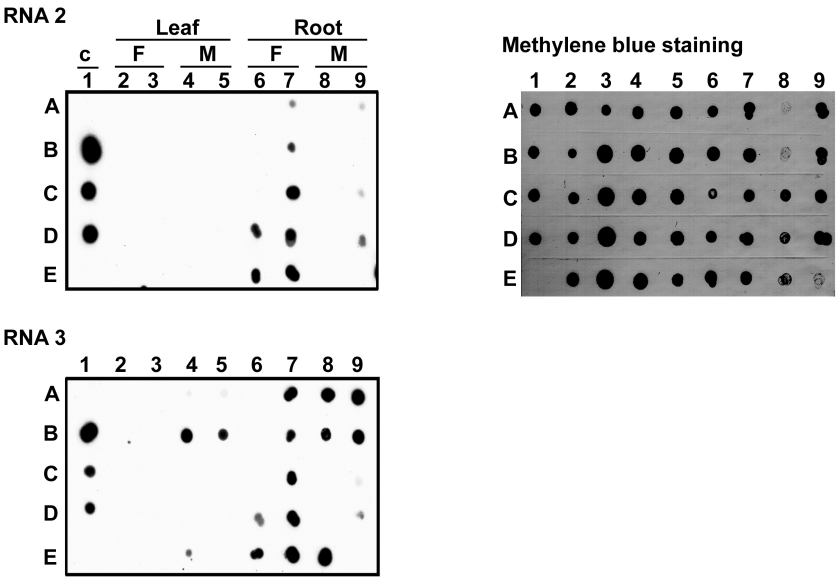

Figure 4. Detection of PMTV RNA 2 and RNA 3 in roots and leaves of *N. benthamiana*. PMTV CP-transgenic *N. benthamiana* plants were inoculated by two methods i) mechanical inoculation (M) and ii) growing in PMTV infested soil (F). Root as well as leaf tissue were analysed by dot-blot hybridization using probes specific for RNA 2 and RNA 3, respectively. RNA from healthy non-transgenic plants (A1) was dotted as a negative control. RNA from PMTV-infected roots (B1 and D1) as well as RNA from infected leaves (C1) was dotted as positive control. Methylene blue staining confirmed equal staining. Note changes in nomenclature of RNA 2 and RNA 3 as compared to paper I.

Recently, it has been shown that RNA silencing may not work efficiently in root tissue (Andika *et al.,* 2005). *N. benthamiana* plants transformed with the 54 kDa read through domain of BNYVV display either resistance or susceptibility to mechanical inoculation of the virus. However, consistent with our results (**I**), plants that are resistant to BNYVV upon mechanical inoculation are susceptible to BNYVV when grown in infested soil. Viruliferous *P. betae* is able to infect roots of the transgenic plants and BNYVV accumulates to lower levels as compared to non-transgenic plants, thus exhibiting a moderate level of resistance expressed as limited replication and/or movement in roots. Some of the transgenic BNYVV lines that are initially susceptible to the virus partially recover from infection, i.e, new developing leaves show reduced levels of BNYVV. In contrast, virus levels remain constant in new developing roots. In resistant plants there are low amounts

of transgene-derived mRNA in leaf tissue, in contrast to in root tissue, whereas the opposite results are formed with siRNA. These findings suggest that RNA silencing works inefficiently in roots, which may be the reason why also transgenic resistance to PMTV was not very efficiently expressed in *N. benthamiana* roots or the tubers grown in the field.

The field soil where the potato tubers and roots are growing does not reach high temperatures. It has been shown that RNA silencing may not work efficiently at lower temperatures (Szittaya *et al.,* 2003). Furthermore, it is likely that the resistance can differ when plants are mechanically inoculated with the virus and when they are infected by the natural vector (**I**; Andika *et al.,* 2005). The vector might suppress some general host defence mechanism making the plant more susceptible to virus infection. Our results supported by the more recent results from BNYVV-resistant transgenic plants (Andika *et al.,* 2005) show that there is a complex interaction between host, vector and virus. Designing resistance to soilborne viruses may need some novel approach. Also, it shows how important it is to test resistance to virus under natural condition with inoculation of the virus by its natural vector.

Studies on PMTV long-distance movement in *N. benthamiana*

The viral infection cycle consists of various processes such as: replication, intra-cellular movement, cell-to-cell movement, long-distance movement (vascular transport) and propagation in the systemically infected parts. The study of these processes can be approached by the generation of an infections viral cDNA clone, which permits genetic manipulation (e.g. mutations, insertions and deletions) of the viral genome. Genomic RNAs of PMTV isolated from experimental host plants as well as infected tubers in the field consist of both full-length copies and deletion derivatives of RNA 2 and RNA 3 (Torrance *et al.,* 1999; Sandgren *et al.,* 2001). Thus, it was important to produce a homogenous inoculum for functional studies of the PMTV genome. By generation of a full-length PMTV cDNA clone of a Swedish isolate (Sw) under bacteriophage T7 RNA polymerase promoter (**II**)**,** we obtained an important molecular tool for studying long-distance movement of PMTV.

Previous results have indicated that RNA 2 is eliminated in transgenic *N. benthamiana* plants expressing PMTV CP and that RNA 1 and 3 can replicate and move systemically in the absence of RNA 2 (**I**; McGeachy & Barker, 2000). *In vitro* transcripts of each genomic RNA of the infectious PMTV cDNA clone were inoculated to wt *N. benthamiana* plants (**II**). The infectivity of the cDNAs was high and PMTV accumulated to similar levels as in *N. benthamiana* plants infected by growing in PMTV infested soil.

In order to study whether PMTV can infect and move long-distances without the CP-encoding RNA 2, transcripts of RNA 1 and RNA 3 were inoculated to *N. benthamiana* plants. Results showed that RNA 1 and RNA 3 were sufficient for infection and that PMTV moved systemically (**II**). The results provide evidence that RNA 1 and RNA 3 can infect and move systemically in absence of RNA 2.

Furthermore, since RNA 2 encodes the viral CP it is likely that RNA 1 and RNA 3 of PMTV are able to move in a non-virion form. It has been shown that CP of some viruses, for example BSMV and *Tobacco rattle virus* (TRV, genus *Tobravirus*), are not needed for systemic virus movement (Harrison & Robinson, 1986; Petty & Jackson, 1990). Instead these viruses move as ribonucleoprotein complexes consisting of viral RNA and virus movement protein. In the case of CMV, CP is necessary for cell-to-cell movement while formation of virions is dispensable (Blackman *et al.,* 1998; Kaplan *et al.,* 1998; Schmitz & Rao, 1998). Furthermore, CMV has been suggested to enter the SE as a ribonucleoprotein complex consisting of viral RNA, MP and CP. In the SE, the virus particles are assembled before being transported with the translocation stream (Schmitz & Rao, 1998). It is possible that PMTV may also form virus particles in the SE, which could be important for efficient transport and thereby efficient systemic infection. Alternatively, interaction between the CP, the viral RNA and host factors may be required for efficient unloading from the SE and thereby efficient systemic infection. Thus, although CP is not necessary for systemic infection, CP may be required for optimisation of the viral infection cycle.

Infection with PMTV RNA 1 and RNA 3 in absence of RNA 2 did not cause any symptoms although the RNA amounts were no different from leaves infected with all three viral RNA (Figure 5; **II**; McGeachy & Barker, 2000). Thus, RNA 2 is necessary for symptom development in *N. benthamiana*. Induction of symptoms requires direct or indirect interaction between the virus and the host. The precise way by which symptoms are induced is poorly understood but it has been suggested that symptoms are caused by metabolic changes in the plant during multiplication of the virus. For example, yellow lesion in BNYVV-infected *Tetragonia expansa* and *Chenopodium quinoa* show reduced levels of chlorophyll (Jupin *et al.,* 1992) and transgenic *N. tabacum* plants expressing PVY CP developed chlorosis and mosaic symptoms similar to symptoms caused by PVY infection (Naderi & Berger, 1997). In the case of CMV, which usually causes yellow local lesion in *C. quinoa*, the sizes of the lesions are reduced when the CP is mutated (Schmitz & Rao, 1998). This indicates that lack of an interaction between the host and viral CP results in reduced symptom. Thus, the CP encoded by RNA 2 may interact with host factors, which results in development of symptoms. Further studies are required to reveal the function of PMTV CP both in viral movement and symptom development in *N. benthamiana*.

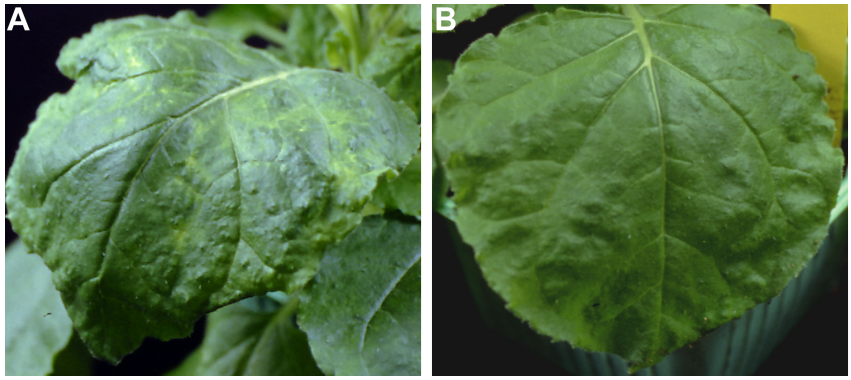

Figure 5. Infection with all three RNA molecules (RNA 1, 2 and 3) of PMTV causes mosaic symptom in *N. benthamiana* (A). Inoculation with RNA 1 and RNA 3 (TGB encoding RNA) causes symptomless systemic infection in the same host (B).

Characterization of *N. benthamiana* transformed with PVA encoding regions

Engineered resistance to potyviruses using viral sequences as transgenes has been reported in many studies (Lawson *et al*., 1990; Lindbo & Dougherty, 1992a, b; Lindbo *et al*., 1993; Vardi *et al*., 1993; Audy *et al*., 1994; Dougherty *et al*., 1994; Hammond & Kamo, 1995; Pehu *et al*., 1995). In order to achieve resistance to PVA, *N. benthamiana* was transformed with the VPg cistron of PVA, the P1 cistron of PVA and with a polycistronic transgene comprising the CI, NIa and CP cistrons of PVA, respectively (**III**; **IV**; Figure 6).

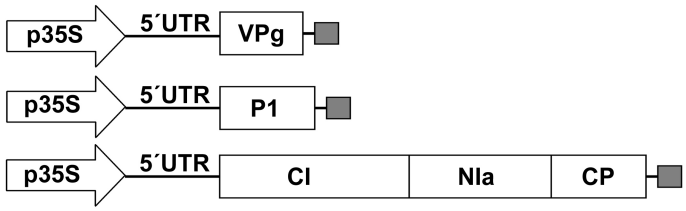

Figure 6. Schematic presentation of the transgene under 35S *Cauliflower mosaic virus* promoter introduced to *Nicotiana benthamiana* by *Agrobacterium*-mediated transformation. The transgene contain the 5´UTR of PVA in order to enhance expression of the transgene.

One line out of eight VPg-transgenic (tt6), and one line out of seven P1-transgenic (ac14) lines were resistant to PVA infection. Resistance correlated with no or very low expression of the transgene (**III**). All other lines were susceptible to infection. However, two VPg-transgenic lines (tt7 and tt9) and one line of the P1-transgenic lines (ac8) showed recovery phenotype starting 3 weeks post

inoculation. Expression of transgene in these plants was reduced and accumulation of siRNA was detected. Recovered tissue was resistant to subsequent inoculation of PVA (**III**). All other lines remained infected.

All *N. benthamiana* plants transformed with the CI-NIa-CP transgene were susceptible to PVA (**IV**). Northern analysis confirmed expression of the transgene. No line displayed the recovery phenotype.

Our results show that *N. benthamiana* plants transformed with the P1 and VPg cistron of PVA displayed resistance to PVA infection, susceptibility or systemic infection followed by recovery of new leaves from PVA infection (**II**). Similar resistance phenotypes in plants transformed with potyviral encoding region have been reported earlier. For example, *N. benthamiana* plants that have been transformed with the P1 gene of *Plum pox virus* (PPV, genus *Potyvirus*, family *Potyviridae*) show different levels of resistance (Tavert-Roudet *et al*., 1998). Some of the lines are immune to PPV infection, others show recovery phenotype whereas others continue to be infected by the virus. The P1 encoding region of PVY^O has been introduced to potato cv. Pito (Mäki-Valkama *et al*., 2000). Seven lines showed resistance to PVY^O while 18 lines were susceptible. The plants resistant to PVY^O are susceptible to PVY^N, PVX and PVA indicating that the resistance is virus-strain specific. Tobacco has been transformed with the NIa and NIb encoding region of PVY (Vardi *et al*., 1993). Some lines were susceptible to PVY while others showed a high degree of resistance to PVY infection. Tobacco plants transformed with 6K2 and VPg encoding region of TEV (Swaney *et al*., 1995) displayed phenotypes varying from resistant to susceptible upon TEV infection.

Previous studies have shown that PVA HC-Pro transgenic *N. benthamiana* plants accumulate high levels of HC-Pro (Savenkov & Valkonen, 2001). Furthermore, these plants are susceptible to PVA but 21-28 dpi the upper leaves recover from virus infection. Expression of a transgene encoding the TEV NIa, the TMV CP and the CP of *Soybean mosaic virus* (SMV, genus *Potyvirus*, family *Potyviridae*) confers resistance to PVY, TEV and TMV in tobacco plants, respectively (Marcos & Beachy, 1997). Furthermore, tobacco plants expressing two NIa encoding regions (TEV NIa-PVY NIa, TEV NIa-TVMV NIa and TVMV NIa-PVY-NIa) are resistant to infection when inoculated with the respective virus (Fellers *et al.,* 1998a). Thus, it was unexpected that none of the *N. benthamiana* lines transformed with the polycistronic transgene, despite expression of the transgene, display any resistance or recovery phenotype when inoculated with PVA. One explanation might be that any of the PVA proteins produced from the transgene can interfere with a host defence related mechanism.

Studies on the VPg of PVA as a putative suppressor of RNA silencing

VPg is known to play a crucial role in potyviral infection. A single aa substitution within the VPg allows PVA isolate B11 to move systemically in *S. commersonii*, a host where it usually is restricted to inoculated leaves (Rajamäki & Valkonen, 2002). Furthermore, VPg has been shown to be a host range determinant, since

PVA isolates carrying a certain form of VPg are compatible for efficient systemic infection in one host species but will be restricted to inoculated leaves in another species (Schaad & Carrington, 1996; Schaad *et al.,* 1997; Rajamäki & Valkonen, 1999, 2004). VPg has also been suggested to be a suppressor of host defence (Rajamäki & Valkonen, 2002, 2003). Therefore, we were interested in investigating the role of VPg as a suppressor of RNA silencing and whether this would explain the response of the CI-NIa-CP transgenic plants to PVA infection (**IV**).

Expression of VPg from a PVX-based expression vector in *N. benthamiana* caused necrotic lesion on inoculated leaves and necrotic lesions and streaks on petiole and veins in systemically infected leaves (**IV**). Thus, VPg increased virulence of PVX. RNA silencing suppressors often cause necrotisation of leaf tissue when expressed from a heterologous virus (Pruss *et al.,* 1997; Brigneti *et al.,* 1998; Burgyán *et al.,* 2000; Yelina *et al.,* 2002; Canto *et al.,* 2004; Kreuze *et al.,* 2005; Te *et al.,* 2005).

The first approach employed to test the RNA silencing suppressor activity of VPg was cross-protection assay (Figure 7; **IV**; Ratcliff *et al.,* 1999; Yelina *et al.,* 2002). Replication of two viruses, partially sharing the same sequence, will induce RNA silencing and prevent systemic infection of the secondly inoculated virus. A PVX-based vector containing VPg and partial GFP-encoding region was inoculated to *N. benthamiana* (**IV**). Three days post-inoculation a TMV expressing the entire GFP was inoculated to the same leaf. Absence of fluorescens in systemically infected leaves indicate that TMV.GFP is not able to infect the plants systemically due to induction of RNA silencing by PVX.VPg.GF (**IV**).

Agrobacterium infiltration assay is another test regularly used for studying RNA silencing suppression activity of a protein (Figure 7; Voinnet *et al.,* 1998, Voinnet *et al.,* 2000; Guo & Ding, 2002). RNA silencing of stably integrated and highly expressed GFP transgene can be initiated by leaf infiltration with an *Agrobacterium* culture carrying the same GFP construct (Voinnet *et al*., 1998). Local RNA silencing can be seen as a progressive loss of GFP in the infiltrated area. Silencing spreads systemically throughout the plant, starting along the veins in the systemically silenced leaves and spreading to the leaf lamina. Tissue co-infiltrated with GFP and a virus-encoded suppressor of RNA silencing will remain fluorescent and there will be no systemic silencing (Voinnet *et al*., 2000). *Agrobacterium* co-infiltration of GFP and VPg in GFP-expressing transgenic *N. benthamiana* (line 16c; Brigneti *et al.,* 1998) resulted in both local and systemic silencing seen as loss of fluorescence under UV-illumination (**IV**).

The findings of the aforementioned two assays indicate that VPg is not able to interfere with the initiation of silencing. Although systemic silencing of line 16c after co-infiltration with GFP and VPg suggests that VPg may not suppress the silencing signal we performed two additional tests in order to study it more closely. In the first test we created grafted plants with GFP-expressing *N. benthamiana* line 16c as root stock and top shoot, whereas the middle part was from a VPg-expessing plant (Figure 7; **IV**). Silencing was induced by infiltrating

gfp gene to the rootstock. In the second test we infiltrated GFP to the leaf tip of GFP expressing *N. benthamiana* line 16c and VPg to the leaf base of the same leaf (Figure 7; **IV**). In that way the silencing signal has to pass through the VPg expressing part. In both tests, systemic silencing was observed (**IV**). Taken together, our results provide no evidence that VPg is able to suppress RNA silencing. However, VPg might suppress or enhance the expression of host genes and thereby alter symptom severity when expressed from heterologous virus. Further studies are required to address this question.

Another mechanism by which VPg might interfere with transgenic resistance is via regulation of replication of viral RNA. Thereby, it might help the virus to avoid being recognized by the host defence system. Such a role has been suggested for p33 of *Cucumber necrosis virus* (CNV) (genus *Tombusvirus*, family *Tombusviridae*) (Panavas *et al.,* 2005). P33 of tombusviruses is anchored to the membranes of peroxisomes and mitochondria, the site of tombusvirus replication (Rubino & Russo, 1998; Panavas *et al.,* 2005). Recently, it was shown that p33 of CNV is located in multimolecular complexes consisting of p33, p32, viral RNA and unknown host factors (Panavas *et al.,* 2005). In contrast to plus-strand RNA, no or very little minus-strand RNA can be found in cytoplasm. One explanation to keep the minus-stranded RNA in these multimolecular complexes bound to replicase would be to decrease the detection of dsRNA by the RNA silencing apparatus (Panavas *et al.,* 2005). VPg is bound to the 5´end of the viral RNA and has been shown to be important for viral replication (Murphy *et al.,* 1996; Schaad *et al.,* 1996; Fellers *et al.,* 1998b; Lai, 1998). Thus, it is possible that VPg is controlling the replication of viral RNA in the same or similar way as p33 of CNV. If VPg can control the amount of dsRNA in the cytoplasm by regulating the replication of the virus, the virus could avoid being recognized by the RNA silencing apparatus.

VPg interacts with the eukaryotic translation initiation factor eIF(iso)4E (Wittmann *et al.,* 1997; Léonard *et al.,* 2000; Schaad *et al.,* 2000) and may interfere with functions of the host translation apparatus. Translation of viral RNAs by the host machinery is crucial for the virus infection cycle. *Arabidopsis* with mutations at the eIF(iso)4E locus show increased resistance to TuMV infection (Duprat *et al.,* 2002; Lellis *et al.,* 2002). Furthermore, recessive resistance genes against PVY in pepper and LMV in lettuce have been shown to encode for eIF4E (Ruffel *et al.,* 2002; Nicaise *et al.,* 2003). Besides the binding of TuMV-VPg to eIF(iso)4E, TuMV-VPg has been shown to interact with other components of the translation initiation machinery such as the eIF(iso)4E-eIF(iso)4G complex. Presence of a cap analogue interferes with the interaction between VPg and the eIF(iso)4E-eIF(iso)4G complex, suggesting that VPg can interfere with translation of capped cellular mRNA (Plante *et al.,* 2004). Hence, it is possible that PVA-VPg may suppress or enhance translation of host mRNA. These host proteins may be involved in viral replication, viral translation, symptom development or host defence.

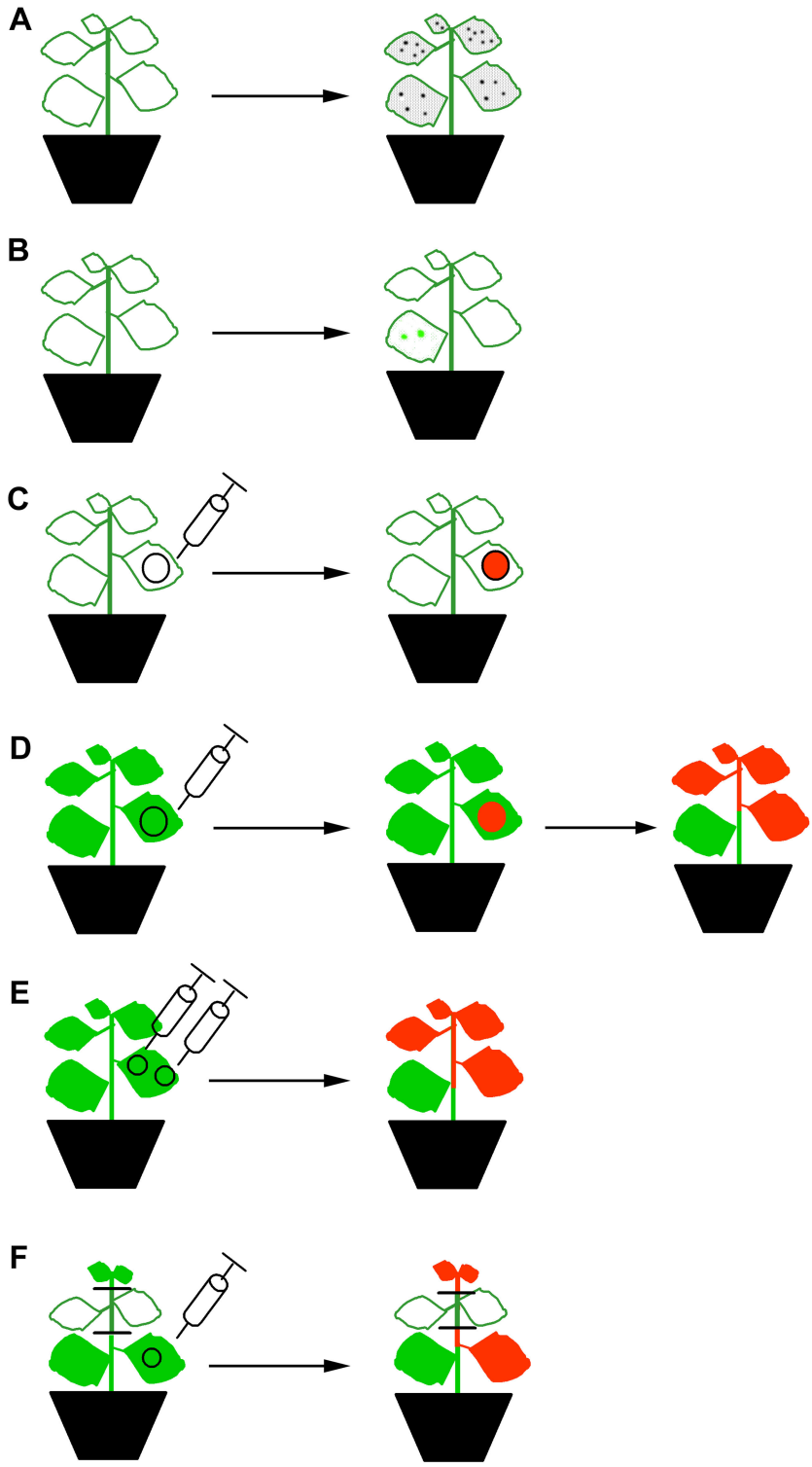
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Figure 7. Methods that were applied to study VPg as a possible suppressor of RNA silencing. Red colour indicates no GFP-fluorescence under UV-light. (A) Inoculation with *Potato virus X* (PVX) expressing VPg of PVA (PVX.VPg) to wt *Nicotiana benthamiana* causes necrosis on the stem, petioles and systemically infected leaves. (B) Cross-protection by PVX.VPg.GF against TMV.GFP restricted TMV.GFP infection to inoculated leaves. (C) Co-infiltration of *Agrobacterium tumefaciens* strains expressing GFP, hairpinGFP and VPg to a leaf of wt *N. benthamiana* did not result in green fluorescence. (D) Co-infiltration of *A. tumefaciens* strains expressing GFP and VPg to a leaf of GFP-transgenic *N. benthamiana* (line 16c) resulted in induction of *gfp* silencing observed as loss of fluorescence. Silencing of *gfp* started in infiltrated leaf and spread systemically in the plant. (F) Infiltration of GFP to the tip of a leaf of GFP-transgenic *N. benthamiana* (line 16c) and VPg to the base of the same leaf resulted in silencing of *gfp* that spread systemically. (E) Generation of *N. benthamiana* plants composed of three parts. VPg-transgenic *N. benthamiana* (line tt2 or tt7) was used as middle insert between GFP-expressing 16c rootstock and GFP-expressing 16c reporter scion. Infiltration of *A. tumefaciens* strain expressing GFP resulted in silencing of the infiltrated leaf as well as of the GFP-expressing reporter scion.

Recently, it was shown that although the cysteine rich protein (CRP) of PMTV causes severe symptoms and necrotisation in infected tissue of *N. benthamiana* when expressed from a PVX based vector, it cannot suppress RNA silencing (Lukhovitskaya *et al.,* 2005). Similarly, p33 of *Cymbidium ringspot virus* (CymRSV, genus *Tombusvirus*, family *Tombusviridae*) causes necrotic symptoms when expressed from heterologous virus although no silencing suppression activity has been shown (Burgyán *et al.,* 2000). ORF6 of TMV enhances virulence of PVX in *N. benthamiana* plants but not in *N. tabacum* plants (Canto *et al.,* 2004). However, when ORF6 is expressed from TRV vector it enhances virulence of TRV in both *N. benthamiana* and tobacco plants although it is not able to suppress RNA silencing (Canto *et al.,* 2004). The ORF6 of *Tomato mosaic virus* (ToMV, genus *Tobamovirus*) binds to eukaryotic elongation factor 1-α (EF−1−α) and has been suggested to have some effect on translation regulation or cellular gene expression (Morozov *et al.,* 1993). Thus, it is possible that PVA-VPg as well as CNV-p33, PMTV-CRP and ORF6 of TMV and ToMV interfere with cellular functions other than RNA silencing, which results in physiological disturbance observed as symptoms. Further studies are needed to reveal the function of VPg in symptom development.

Studies on PVA long-distance movement in P1- and VPg-transgenic *N. benthamiana*

N. benthamiana plants transformed with the P1- and VPg- cistron of PVA were used to study whether recovered tissue would support long-distance movement of PVA. Scions of wt, healthy plants were grafted onto P1- and VPg-transgenic plants that had recovered from PVA infection (**III**). At least two fully recovered leaves were retained on the root-stock. No PVA was detected in the grafted wt top scion. In contrast, PVA was detected in the wt top scions grafted onto plants that had not recovered from infection (**III**). These results indicate that PVA is not able to move through recovered tissue.

To date, it is not clear whether potyviruses move from cell-to-cell and over long distance in plants as virions or as other ribonucleoprotein complex. Mutations of the conserved residues Arg154 and Asp198 in the CP of TEV prevent the virus from forming virus particles (Dolja *et al.,* 1994). Furthermore, these mutants are arrested in cell-to-cell movement. Deletion of the N-terminal part of the CP allows formation of virus particles but cell-to-cell movement is arrested and systemic infection is prevented (Dolja *et al.,* 1994). These findings suggest that cell-to-cell movement requires assembly of virions or that the viral RNA moves as a ribonucleoprotein complex and the CP interacts with host factors to facilitate movement. It has been shown that the CI and CP of TVMV associate into plasmodesma-like structure and RNA can be found inside these structures (Rodriguez-Cerezo *et al.,* 1997). This suggests CP to have a direct role in potyviral movement. However, it is unknown whether the virus moves as virions or as a complex involving RNA, CP and other unidentified proteins.

Our results (**III**) suggest that PVA moves as a ribonucleoprotein complex other than virions or that long-distance movement of viruses in the plant is more complex than earlier thought. It is known that virus moves cell-to-cell in the inoculated leaf until it reaches the SE. In the SE it can be transported long distances, until it reaches an upper, non-infected leaf where it will be unloaded to the CC and move cell-to-cell to the BS, MS and EP. If it is transported long distances without need of loading and unloading it would be possible for some virus particles to escape the RNA silencing apparatus and move through the recovered part into the unsilenced wt scion. Another potyvirus, *Pea seed-borne mosaic virus* (PSbMV), is able to move through recovered tissue in recovered PSbMV NIb transgenic pea lines (Van den Boogaart *et al.,* 2004). Different result may be due to different viruses, host and/or experimental conditions and different efficiency in expression of the transgenic resistance.

Our results can be explained if PVA was unloaded from SE into CC and other phloem cells for replication when moving systemically in the phloem. During replication the virus is exposed to RNA silencing. If RNA silencing is efficient, no virus would be able to escape the RNA degradation and move through recovered tissue. In order to follow systemic infection of PVA we inoculated a PVA construct carrying GFP into *N. benthamiana* (**III**). It has been shown that 48h post inoculation the PVA-GFP has exited the inoculated leaf in 67% of the plants inoculated. After 72h post inoculation the virus has exited the inoculated leaf in all plants tested (Rajamäki *et al.,* 2005). However, we were not able to see any systemic infection until 8 dpi (**III**). This result suggests that PVA is loaded into SE for transport over short distances, unloaded to CC and other phloem cells for replication and loaded to SE again. This would be consistent with the long time needed for systemic transport. Further studies are required to reveal the precise mechanism of PVA movement in plants.

Transgenic resistance to PMTV and PVA provides novel insights to viral long-distance movement

Host factors play a crucial role in viral movement and symptom development. Lack of compatible interaction between host factors and viral proteins results in ineffective or deficient infection observed as resistance. By studying resistance to plant viruses the knowledge of the virus infection cycle can be increased.

One example where studies on resistance have resulted in increased knowledge of a step in the virus infection cycle is the study on TEV infection in *N. tabacum.* TEV isolate HAT is able to systemically infect *N. tabacum* cv. V20, whereas isolate Oxnard is restricted to inoculated leaves in this host (Schaad *et al.,* 1997). However, substitution of the VPg-encoding region of TEV-HAT with the corresponding region of TEV-Oxnard results in the ability of TEV-Oxnard to move systemically in *N. tabacum* cv. V20 (Schaad *et al*., 1997). It has been shown that VPg of TEV isolate HAT interacts with eIF4E of tobacco cv. V20 (Schaad *et al.,* 2000), whereas the VPg of TEV isolate Oxnard does not. This shows (as mentioned earlier) that interaction between the potyviral VPg and eIF4E and eIF(iso)4E is important for virus infection and suggests a role for VPg in initiation of translation (Lellis *et al.,* 2002; Duprat *et al.,* 2002).

Introduction of viral genes to the plant genome (transgenic resistance) often results in resistance based on RNA silencing to the corresponding virus. However, transgenic resistance can also be used to study the infection cycle of plant viruses.

For example, RNA silencing suppressors encoded by plant viruses were first discovered in transgenic plants. As previously mentioned, RNA viruses replicate via the formation of a dsRNA molecule which can induce RNA silencing. To be able to infect plants, viruses must be able to avoid or escape this defence mechanism. GUS transgenic tobacco plants, silenced for GUS, are susceptible to wt PVX. However, they are resistant to infection with a PVX construct carrying the GUS sequence, due to homology dependent RNA degradation. Crosses between the tobacco plants silenced for GUS and tobacco plants expressing P1/HC-Pro of TEV result in restoration of the GUS expression. Furthermore, these transgenic plants are susceptible to the PVX construct carrying the GUS sequence (Anandalakshmi *et al.,* 1998). These findings show that plant viruses encode proteins, such as the P1/HC-Pro of TEV, that are able to suppress RNA silencing.

In this thesis we describe transgenic resistance to two different viruses (PMTV and PVA) belonging to different genera, *Pomovirus* and *Potyvirus*, respectively. Transgenic resistance achieved by expressing viral genes is probably in most cases based on RNA silencing (Van den Boogart *et al.,* 2004; Lindbo & Dougherty, 2005). Our results show that resistance to PVA by expressing P1 or VPg cistron of PVA in *N. benthamiana* is based on RNA silencing (**III**). Furthermore, the results suggest that resistance to PMTV in *N. benthamiana* expressing the PMTV CP is also based on RNA silencing (**I**).

This thesis also provides examples on how transgene-derived resistance can be used to study long-distance movement of viruses. Infection with PMTV in CP-transgenic *N. benthamiana* suggested that RNA 1 and the TGB-encoded RNA (RNA 3) could move in the absence of the CP-encoded RNA (RNA 2) (**I**; McGeachy & Barker, 2000). Inoculation of RNA transcript of RNA 1 and RNA 3 in wt *N. benthamiana* resulted in systemic, symptomless infection (**II**). These findings provide evidence that RNA 1 and RNA 3 of PMTV can infect and move systemically without the formation of virus particles.

Recovered sections of VPg- and P1- transgenic *N. benthamiana* did not allow transit of PVA from the lower, infected parts of the plants (**III**). It has not been elucidated whether potyviruses move as virus particles or viral RNA. Our results suggest that PVA is moving as viral RNA not protected in the virion for all the time. The step-wise systemic movement model proposed could accommodate both evidence for need of CP for potyviral movement and vulnerability of potyviral RNA to RNA silencing during the long-distance movement.

Conclusions

The main conclusions from the results presented in this thesis are:

- The incidence of PMTV infection is lower in PMTV CP-transgenic *S. tuberosum* cv. Saturna compared to non-transgenic potato when grown in PMTV-infested field.

- Expression of resistance in PMTV CP-transgenic *N. benthamiana* is different depending on whether the plant is inoculated mechanically or by *S. subterranea*, the natural vector of PMTV. Resistance to PMTV in *N. benthamiana* is more effective in leaves than in roots.

- The CP-encoding RNA (RNA 2) of PMTV is needed for symptom induction in *N. benthamiana* but is dispensable for systemic movement, indicating that virus particles are not needed for cell-to-cell and long-distance movement of RNA 1 and RNA 3 of PMTV in the plant.

- *N. benthamiana* expressing P1 and VPg encoding regions of PVA display *i*) resistance to PVA infection *ii*) susceptibility, or *iii*) systemic infection followed by recovery from PVA infection. In contrast, all *N. benthamiana* lines transformed with a polycistronic transgene consisting of CI, NIa and CP of PVA are susceptible to PVA.

- PVA VPg does not have detectable RNA silencing suppressor activity but increases the severity of symptoms when expressed from a heterologous virus.

- PVA is not able to move through recovered tissue in P1- and VPg-transgenic *N. benthamiana*.

- While pathogen-derived resistance provides efficient protection against PMTV and PVA in *N. benthamiana*, the transgenic plants also help to elucidate important aspects in long-distance movement of the viruses.

Future perspective

- Studies on host factors involved in cell-to-cell movement and long-distance movement of both PMTV and PVA would provide further insights into these processes. A better understanding of these processes could be helpful in obtaining resistant plants.

- The precise role of PMTV RNA 2 in symptom development is not yet known. Identification of host factors interacting with the CP or the read-through domain would be of a great value to understand these processes.

- VPg of many potyviruses have been shown to interact with the eIF4E and/or eIF(iso)4E. This interaction is required for systemic infection. Studies on host gene expression during this interaction could provide further insights on the role of VPg in the infection cycle.

- Studies on host factors (others than eIF4E and eIF(iso)4E) interacting with VPg would be necessary to reveal the exact role of VPg in symptom development and host defence mechanisms.

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