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FACTORS AFFECTING INFECTION AND DETECTION OF THE SOILBORNE *POTATO MOP-TOP VIRUS*

DOCTORAL THESIS IN PLANT VIROLOGY

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ACADEMIC DISSERTATION

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Cover: The underground parts of a potato plant (roots, stolons and tubers).

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CONTENT

ABBREVIATIONS

+ssRNA	Positive-sense single-stranded ribonucleic acid
8K	8 kilodalton protein
aa	Amino acid
BNYVV	*Beet necrotic yellow vein virus*
BSMV	*Barley stripe mosaic virus*
CaMV	*Cauliflower mosaic virus*
CP	Coat protein
CRP	Cysteine-rich protein
cv.	Cultivar
DAS-ELISA	Double sandwich enzyme-linked immunosorbent assay
DCL	Dicer-like
DI RNA	Defective interfering ribonucleic acid
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplastic reticulum
ET	Ethylene
ETI	Effector-triggered immunity
FLASH-PCR	Fluorescent amplification-based specific hybridization
GFP	Green fluorescent protein
HC-Pro	Helper component proteinase
IC-RT-PCR	Immunocapture reverse transcriptase polymerase chain reaction
JA	Jasmonic acid
kDa	Kilodalton
myc	A transcription factor similar to myelocytomatosis viral oncogene
nm	Nanometer
nt	Nucleotide
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PLRV	*Potato leaf roll virus*
PMTV	*Potato mop-top virus*
PTI	PAMP-triggered immunity
PVY	*Potato virus Y*
PVX	*Potato virus X*
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT	Read-through

RTM	Restricted *Tobacco etch virus* movement – related gene group
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Salicylic acid
SEL	Size exclusion limit
siRNA	Small interfering ribonucleic acid molecule
TGB	Triple gene block
TGBp	Triple gene block protein
TMV	*Tobacco mosaic virus*
tRNA	transfer ribonucleic acid
TRV	*Tobacco rattle virus*
UTR	Untranslated region
vRNA	Viral ribonucleic acid

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. They are referred to in the text by their Roman numerals.

I. Latvala-Kilby S*, Aura JM[1]*, Pupola N, Hannukkala A, Valkonen JPT. (2009). Detection of *Potato mop-top virus* in potato tubers and sprouts: combinations of RNA2 and RNA3 variants and incidence of symptomless infections. Phytopathology 99: 519-531.
[1]currently Santala J.; *Equal contribution.

II. Santala J, Valkonen JPT. (2015). Light-induced partial recovery of potato sprouts from *Potato mop-top virus* is associated with RNA silencing. Submitted.

III. Samuilova O*, Santala J*, Valkonen JPT. (2013). Tyrosine phosphorylation of the triple gene block protein 3 regulates cell-to-cell movement and protein interactions of *Potato mop-top virus*. Journal of Virology 87: 4313-4321.
*Equal contribution.

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AUTHOR'S CONTRIBUTION

I. Johanna Santala prepared the PCR fragments for sequencing, conducted the sequence analysis, planned and conducted the experiments related to detection of PMTV from potato sprouts, and designed the RFLP analysis of PMTV isolates from potato tubers. She was responsible for the manuscript preparation and obtaining the contributions from co-authors together with S. Latvala-Kilby.

II. Johanna Santala planned and conducted all the experiments, and was responsible for writing the manuscript and obtaining contributions from the co-author.

III. Johanna Santala planned the mutations and cloned the constructs used in the study together with O. Samuilova. She planned the in vivo phosphorylation experiments including the agroinfiltration experiments and conducted them together with O. Samuilova. She planned and conducted the yeast two hybrid experiments and the infectivity experiments for mutant viruses. She was responsible for writing the manuscript together with O. Samuilova and obtaining contribution from the co-author.

ABSTRACT

The spraing symptoms on potato tubers caused by *Potato mop-top virus* (PMTV) are considered one of the most important quality problems in potato production in Nordic countries. PMTV is widely distributed in the potato growing areas of Nordic countries, but the main seed potato production areas in northern Sweden and the High Grade seed potato production zone in Finland are still free of the virus. Once introduced to a field, PMTV can remain infective within resting spores of its vector *Spongospora subterranea* f.sp*. subterranea* for several years even if potato is not grown in the field. No means are available to control the virus nor its vector in an infested field and therefore preventing the virus from spreading to new areas is essential.

PMTV has a tripartite single-stranded positive-sense RNA genome. RNA-RdRp encodes the viral replicase. PMTV-CP codes for a coat protein (CP) and a read through-part of CP that has been associated with the vector transmission. RNA-TGB encodes three triple gene block proteins (TGBp) and an 8 kDa cysteine-rich protein (8K). TGBp1, TGBp2 and TGBp3 are involved in cell-to-cell movement of the virus and 8K has been implicated as a suppressor of RNA silencing.

Prior to this study, only a limited number of PMTV sequences were available in databanks. To gain more information about genetic variability of the virus, 23 PMTV isolates obtained by immunocapture reverse transcriptase PCR (IC-RT-PCR) from symptomatic potato tubers from Finland and 5 isolates from asymptomatic minitubers from Latvia were partially sequenced. Phylogenetic analysis of the sequences revealed two distinguishable types of RNA-CP and RNA-TGB, each showing only a little genetic variability.

Currently, scoring of seed tubers for PMTV infection is based on the occurrence of spraing on tubers. However, IC-RT-PCR analysis of asymptomatic tubers grown in PMTV-infested fields revealed a high amount of symptomless infections in some cultivars. Thus, reliance on symptoms in seed potato inspections causes a risk of spreading the virus to new fields. The limited genetic variability of PMTV implicates that the currently used virus-specific serological and molecular methods can be reliably used to detect the virus. However, PMTV can’t normally be detected from shoots grown from infected tubers, as is done for many other potato viruses, and preparing samples from tubers is laborious. Therefore, a method was developed to test PMTV from sprouts grown in the dark by double sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

Although PMTV infection was readily detected by DAS-ELISA from the dark-grown sprouts, the virus was no longer detectable in sprouts exposed to light for a few days. Quantitative reverse transcriptase PCR targeting RNA-TGB confirmed that the viral titers decreased rapidly following light exposure. Northern blot analysis of low molecular-weight RNA extracted from both dark-grown and light-exposed sprouts showed accumulation of PMTV-specific small interfering RNA (siRNA) indicating that RNA silencing was acting against the virus in the potato sprouts. Moreover, the amount of siRNA did not decrease in the light-exposed sprouts although the viral titers were much lower than in the dark-grown sprouts. This indicated that RNA silencing was enhanced in the light-exposed sprouts, thus implicating for the first time that light affects antiviral silencing. Overexpression studies on *gfp*-transgenic *Nicotiana benthamiana* revealed PMTV TGBp1 and confirmed PMTV 8K to act as weak suppressors of silencing.

TGBp3 has an important role in the PMTV infection cycle as it interacts with TGBp2 to target viral ribonucleoprotein complex to plasmodesmata and assists the movement of the complex through the plasmodesmata to the adjacent cell. TGBp2 and TGBp3 themselves do not move cell-to-cell but are recycled back through endocytosis. Western blot analysis of TGBp3 showed that the protein was tyrosine phosphorylated by a plant kinase both *in vitro* and *in vivo*. Mapping and mutation of the putative phosphorylation sites revealed that TGBp3 was phosphorylated on two tyrosine residues, both of which are located within tyrosine-based sorting motifs implicated in endocytosis. Indeed, mutation of the first phosphorylation site restricted the virus to the originally infected cell, whereas mutation of the second site completely abolished viral infectivity. In yeast two hybrid assay mutation of the first phosphorylation site led to the strengthening of the previously reported TGBp2-TGBp3 interaction. Therefore, it was hypothesized that phosphorylation of TGBp3 affects PMTV infection by regulating the interaction between TGBp2 and TGBp3.

1 INTRODUCTION

1.1 *Potato mop-top virus*

Potato mop-top virus (PMTV) is the type species of genus *Pomovirus* (family *Virgaviridae*) (King et al., 2012). PMTV causes necrotic arcs and rings in the flesh and on the surface of potato tubers (*Solanum tuberosum* L.) (Calvert and Harrison, 1966; Kurppa, 1989b). Although some reduction in tuber yield has been reported (Kurppa, 1989b), the reduction of tuber quality by spraing symptoms is the main problem caused by PMTV. Indeed, PMTV is considered one of the most important quality problems in potato production in Nordic countries as occurrence of spraing symptoms makes the tubers unsuitable for French fry and chip production and symptomatic tuber lots are rejected in the fresh potato market.

1.1.1 Vector

PMTV is transmitted by *Spongospora subterranea* f. sp. *subterranea* (Wallr.) Lagerh. a soil-borne protoctist that causes powdery scab on potato (Arif et al., 1995; Harrison and Jones, 1970; Jones and Harrison, 1969). *S. subterranea* is an obligate parasite of plant roots. It can survive in the soil as resting spores even if suitable host plants are not available (Campbell, 1996). Primary zoospores are released from a resting spore by an unknown stimuli. The zoospores move toward the host plant, probably by chemotaxis. A zoospore then penetrates the host cell and forms multinucleate plasmodia that can form either secondary zoospores or resting spores (Merz, 2008). The zoospores can survive for only a couple of hours without a host plant, whereas the resting spore balls, each consisting of many thick-walled spores, can remain infective in the soil for several years (Jones and Harrison, 1969; Merz, 1995; 2008). Zoospores of *S. subterranea* aquire PMTV from an infected root or tuber tissue and transmit the virus to new host plants (Arif et al., 1995). The virus can remain infective inside the resting spores for many years (Jones and Harrison, 1969; Merz, 1995).

The incidence of powdery scab and the amount of *S. subterranea* DNA are higher on potato tubers grown in constantly wet soil than in soil with fluctuating moisture. The difference in the amount of DNA is more pronounced than the difference in the severity of powdery scab symptoms

(van der Graaf et al., 2005). These symptoms are most frequent at 12˚C (de Broer et al., 1985; van der Graaf et al., 2005), but there is a higher amount of *S. subterranea* DNA on tubers grown at 9˚C than at 12˚C (van der Graaf et al., 2005). In contrast, potato roots show more symptoms and contain higher levels of *S. subterranea* DNA at 17˚C than at 9˚C or 12˚C (van der Graaf et al., 2007). All root infections at 9˚C and most root infections at 12˚C are symptomless, and only a very small amount of *S. subterranea* DNA is detected (van der Graaf et al., 2007). As wet soil combined with low temperatures seems to favor infection of potato tubers with *S. subterranea*, it can be speculated that tuber infections with PMTV are also more frequent under these conditions. Indeed, there are indications that the PMTV infection rate in tubers is higher when the conditions are wet and cool from tuber initiation to harvest (Cooper and Harrison, 1973; Sandgren et al., 2002). However, a more recent study suggests that although higher temperatures favor *S. subterranea* root infections over tuber infections, the incidence of PMTV and spraing symptoms remain similar (Carnegie et al., 2010a). This indicates that both tuber and root infections are effective in transmitting the virus into tubers and temperature fluctuations between 12˚C and 20˚C do not affect the infection (Carnegie et al., 2010a).

Although PMTV infection in the field occurs via infection of plants by viruliferous *S. subterranea*, there is a poor correlation between susceptibility to powdery scab and incidence of PMTV in potato (Sandgren et al., 2002; Tenorio et al., 2006). These results suggest that PMTV can't be controlled based on *S. subterranea* resistant potato cultivars, because no cultivar is completely immune to *S. subterranean.* Chemical soil treatments for controlling *S. subterranea* are prohibited in Europe due to their negative environmental effects and high toxicity. Once PMTV has been introduced to a field with *S. subterranea*, it remains infective in the resting spores in the soil for at least a decade (Jones and Harrison, 1969; Merz, 1995; Jones and Harrison, 1972). This together with the lack of PMTV-resistant potato cultivars (Nielsen and Nicolaisen, 2003; Sandgren, 1995; Sandgren et al., 2002), makes the virus difficult to control.

1.1.2 Geographical distribution

PMTV was first detected in Ireland, England, and Scotland (Calvert and Harrison, 1966). It most likely originates in the Andean region of South America (Hinostroza and French, 1972; Salazar and Jones, 1975; Tenorio et al., 2006). An outbreak of spraing symptoms in North America in 2002 led to the first detection of PMTV in the USA (Lambert et al., 2003) and Canada (Xu et al., 2004). PMTV has also been detected in Costa Rica in Central America (Montero-Astúa et al., 2008). In Asia, PMTV has been

reported to in Japan (Imoto et al., 1981; Nakayama et al., 2010), China (Hu et al., 2013), and Pakistan (Arif et al., 2013). After the initial report from the British Isles, the virus has been detected in several European countries, including the Netherlands (van Hoof and Rozendaal, 1969), the Czech Republic (Novak et al., 1983), and Switzerland (Schwärzel, 2002).

In Nordic countries, PMTV was already detected in Norway in the late 1960's (Björnstad, 1969). PMTV has also been detected in Sweden, Finland, and Denmark (Ryden et al., 1986; Kurppa, 1989a; Nielsen and Mølgaard, 1997). In a survey made in Finland in 1987, soil samples and tubers from fields of the main potato production areas were examined for PMTV and the virus was found to be widely spread (Kurppa, 1989a). The virus was, however, not found in the main seed potato production area in Northern Ostrobothnia (Kurppa, 1989a). In Sweden, a survey was carried out in 1989-1991 using soil samples from the fields and soil falling off the tubers during grading. The results showed that PMTV was spread to the southern and middle parts of Sweden but not to the seed potato production areas in the North (Sandgren, 1995). Distribution of PMTV was surveyed in Jutland in Denmark, based on the occurrence of spraing symptoms in the highly susceptible cultivar Saturna and the virus was found widely spread in the potato growing areas (Nielsen and Mølgaard, 1997).

In 2005-2008, an extensive study in countries around the Baltic Sea gave a more comprehensive picture about the distribution of PMTV in the region (Santala et al., 2010). In Finland, virus-specific detection of PMTV from seed lots revealed the virus in all other potato production areas except the High Grade seed potato production zone. Examination of soil samples and tubers from potato production areas in Sweden showed that the virus had spread further north since the previous survey, but the virus was not found in the main seed potato production area. A recent study also found that PMTV was present throughout Sweden except in the two most northern counties, including the seed potato production area where the virus was not found (Beuch et al., 2014). Inspections in Norway and Denmark (Santala et al., 2010), revealed that PMTV can be found in all parts of these countries, although not all the fields used for potato production are infested. The virus was not found in the Baltic countries or northwestern Russia. One isolated incidence of PMTV occurred in 2005 in Latvia, where minitubers produced in a screenhouse tested positive for the virus by double sandwich enzyme-linked immunosorbent assay (DAS-ELISA), and the result was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing. However, the source of the infection could not be determined and no further infections occurred. PMTV-infected tubers were found in central Poland in 2008 and 2009, indicating that the virus is present in the country (Budziszewska et al., 2010).

1.1.3 Host range

Besides potato, *S. subterranea* has been found to transmit PMTV to other members of the *Solanaceae* family, although there is one report indicating infection of PMTV in sugar beet (*Beta vulgaris* L.) and spinach (*Spinacia oleracea* L.), belonging to the family *Chenopodiaceae* (Jones and Harrison, 1972). The solanaceous plants that can obtain PMTV naturally through vector transmission include tomato (*Lycopersicon esculentum* Mill.), some *Nicotiana* species (Jones and Harrison, 1969) and black nightshade (*Solanum nigrum* L.) (Andersen et al., 2002). Black nightshade is a common weed throughout the world (Holm et al., 1991) and could maintain PMTV infestation in a field during the years when potato is not grown. However, the weed is not very common in most areas of northern Europe. It occurs mainly at the southern coast in Finland (Lampinen and Lahti, 2011) and in southern and central parts of Sweden (Håkansson, 2003).

Some species of the families *Chenopodiaceae* and *Aizoaceae* are infected with PMTV through sap inoculation, but they have not been found to be infected through *S. subterranea* transmission (Jones and Harrison, 1969). For example, the common weed plant white goosefoot (*Chenopodium album* L.) can be infected with PMTV through sap inoculation but not by vector transmission (Jones and Harrison, 1972; Andersen et al., 2002), and is therefore not an alternative host of PMTV in the field.

1.1.4 Symptoms

Typical PMTV symptoms on potato tubers consist of brown arcs and lines, called spraing, on the surface and/or in the flesh of the tuber (Fig.1; Calvert and Harrison, 1966; Harrison and Jones, 1971). These necrotic arcs do not, however, limit the spread of the virus in the tuber (Harrison and Jones, 1971; Germundsson et al., 2002). Tuber symptoms have been reported to occur both in primary infection (i.e., infection of the tuber via the vector) and in secondary infection (i.e., infection of progeny tubers from the infected seed tuber) (Calvert and Harrison, 1966; Kurppa, 1989a). In secondary infection, tuber symptoms can also include cracking and deformation of the tubers (Calvert and Harrison, 1966; Kurppa, 1989a; Tenorio et al., 2006). Primary infection does not seem to have much effect on the tuber yield (Kurppa, 1989b; Nielsen and Molgaard, 1997), but secondary infection can cause yield reduction of up to 63 % in some cultivars (Kurppa 1989b; Carnegie et al., 2010b). The yield losses are probably a consequence of the haulm symptoms reported in secondary infections (Kurppa, 1989b; Nielsen and Molgaard, 1997). Also, Carnegie et al. (2010b) concluded that the reduction in yield correlated with the severity of foliar symptoms in Scotland.

Figure 1 PMTV symptoms on potato. Left panel shows two potato plants (cv. Tanu) grown from PMTV-infected seed tubers. The plant on the left is asymptomatic whereas the plant on the right shows symptoms of PMTV secondary infection including stunting and yellow patches on the leaves. The right panel shows mild spraing symptoms in the flesh and on the surface of tubers (cv. Annabelle).

Although spraing symptoms can sometimes be detected in the tubers right after harvest, they can be enhanced by storing the tubers in a fluctuating temperature and by cutting the tubers (Harrison and Jones, 1971; Kurppa, 1989b; Sandgren, 1995; Nielsen and Molgaard, 1997). The occurrence of symptoms in tubers varies greatly depending on the potato cultivar, growth conditions, and the treatment of the tubers after harvest (Kurppa, 1989b; Sandgren, 1995; Nielsen and Molgaard, 1997), and therefore can't be used as a reliable indicator of PMTV infection. Serological detection of PMTV by ELISA has revealed that many PMTV-positive tubers are symptomless (Nielsen and Molgaard, 1997; Sokmen et al., 1998; Sandgren et al., 2002).

The reliance on spraing symptoms in tubers as an indicator of PMTV infections is also diminished by the fact that these symptoms can be easily confused with those of *Tobacco rattle virus* (TRV, genus *Tobravirus*; *Virgaviridae*), which causes necrotic arcs in tuber flesh (Engsbro, 1984; Molgaard and Nielsen, 1996). Also other viruses, such as *Potato leaf roll virus* (PLRV, genus *Polerovirus*; *Luteoviridae*) and the NTN strain of *Potato virus Y* (PVY, genus *Potyvirus*; *Potyviridae*), and physiological disorders can cause necrotic symptoms in tubers (Manzer et al., 1982; Beczner et al., 1984; Le Romancer et al., 1994; Davies, 1998).

Symptoms caused by PMTV in the above-ground (green) parts of the plant have been reported to occur following secondary infection. They include uneven and delayed emergence of plants, mop-top symptoms (stunting) and yellow chlorotic patterns on the leaves (Fig.1) (Calvert and Harrison, 1966; Jones and Harrison, 1972; Kurppa, 1989a). However, the foliar symptoms are affected by environmental conditions such as temperature, light (shown on tobacco), and rainfall, such that their amount decreases in warm and dry weather (Carnegie et al., 2010a; Harrison and Jones, 1971b; Cooper and Harrison, 1973; Kurppa, 1989b). For example, the yellow patterns on leaves have not been reported to be related to PMTV infection in Finland (Kurppa, 1989b).

1.1.5 Methods used to detect PMTV

As tuber and foliar symptoms of PMTV depend on many factors related to the potato cultivar and environmental conditions, they can't be used for reliable detection of the virus. Therefore, PMTV detection should be carried out using virus-specific methods, such as DAS-ELISA, using PMTV coat protein (CP)-specific antibodies, immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR), or RNA extraction followed by RT-PCR using PMTV-specific primers (Sokmen et al., 1998; Sandgren et al., 2001; Germundsson et al., 2002). Additionally, other RT-PCR-based methods, such as RT-PCR-microplate hybridization and fluorescent amplification-based specific hybridization (FLASH-PCR), have been applied to further improve PMTV detection (Ryazantsev and Zavriev, 2009; Nakayama et al., 2010). Also, macroarray and RT-PCR-based methods for simultaneous detection of several potato viruses, including PMTV and some other viruses causing tuber necrosis, have been developed (Agindotan and Perry, 2008; Crosslin and Hamlin, 2011).

Sampling is probably the most critical step in PMTV detection, as distribution of the virus in the tuber can be uneven (Sokmen et al., 1998). Chances for detecting the virus can be elevated by taking samples from the stolon end of the tuber or from tissue with spraing symptoms, although the virus is not always present in high titers in or restricted to either tissue (Sokmen et al., 1998; Germundsson et al., 2002). For many plant viruses, tubers are tested by planting them in soil and sampling the emerging leaves, but this approach is not suitable for detecting PMTV, as the virus is poorly present in the leaves grown from an infected tuber (Carnegie et al., 2010). Occurrence of PMTV in a field can be studied also by sampling the soil, although neither the virus nor its vector appears uniformly distributed in a field (Jones and Harrison, 1972). A more accurate estimation of PMTV occurrence in a field can be obtained by using soil adhered to tubers (Sandgren, 1995). In both cases, solanaceous bait plants such as *Nicotiana* species or tomato are grown with the collected soil and after an appropriate time the roots are washed and used either to inoculate the leaves of test plants to observe symptoms (such as some *Chenopodium* spp.) or to directly test PMTV by specific detection methods (Jones and Harrison, 1969; Arif et al., 1994; Sandgren, 1995; Nakayama et al., 2010).

1.1.6 Genome and genetic variability

PMTV has a tripartite single-stranded positive-sense ribonucleic acid [(+)ssRNA] genome encapsidated into separate rod-shaped particles (Harrison and Jones, 1970; Kallender et al., 1990) (Fig.2). The size of RNA 1 of the isolate PMTV-Sw is 6043 nucleotides (nt). It encodes the viral RNA-dependent RNA polymerase (RdRp) (Savenkov et al., 1999). PMTV RNA 2 (3134 nt in PMTV-Sw) encodes CP (20 kDa) and a 91-kDa protein by read-through (RT) of the amber termination codon of CP (Kashiwazaki et al., 1995; Sandgren et al., 2001). However, the size of the RT domain varies, being only 67 kDa in Scottish isolate PMTV-S (Reavy et al., 1998). The RT region of CP has been associated with vector transmission of the virus (Reavy et al., 1998). RNA 3 contains open reading frames (ORF) for triple gene block (TGB) proteins 1 (51 kDa), 2 (13 kDa) and 3 (21 kDa), and an 8 kDa cysteine-rich protein (CRP) (Scott et al., 1994). The TGB proteins (TGBp) are needed for viral movement (Zamyatnin et al., 2004; Haupt et al., 2005), whereas the 8 kDa protein is a weak suppressor of silencing (Lukhovitskaya et al., 2013). Here, to avoid size-related confusion, the RNA species are referred to as RNA-RdRp, RNA-CP, and RNA-TGB, according to their genetic content (Torrance et al., 2009).

The 5' untranslated regions (UTR) of the three PMTV RNAs show little sequence similarity, whereas the similarity in the 3' UTRs is high, including an identical 80 nt 3' terminus that can be folded into a tRNA-like structure (Savenkov et al., 1999). Recently, the 5' -terminal 479 nt containing 110 nt of the TGB1 ORF and 3' -terminal 372 nt containing 76 nt of 8K ORF of RNA-TGB have been shown to form an additional RNA molecule by template switching during virus replication (Torrance et al., 1999; Lukhovitskaya et al., 2013). This RNA molecule was named defective interfering RNA (DI RNA) as it was shown to interfere with the accumulation of PMTV independently from protein production from the ORF (Lukhovitskaya et al., 2013).

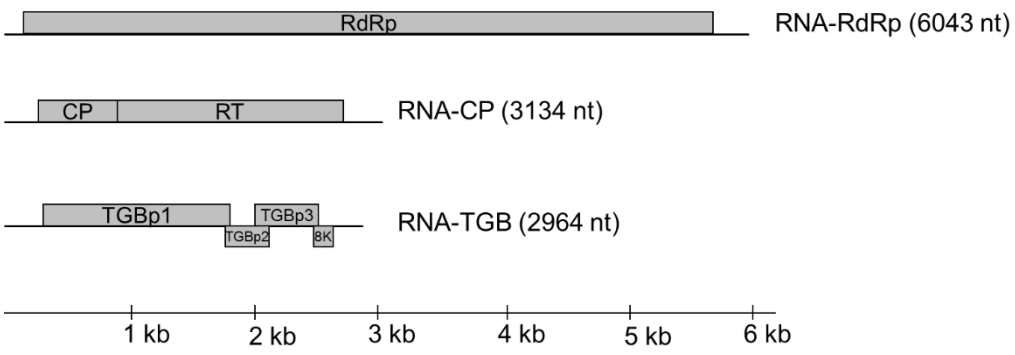

Figure 2 Schematic presentation of the PMTV genome according to isolate PMTV-Sw. RdRp: RNA dependent RNA polymerase; CP: coat protein; RT: read-through part of CP; TGBp: triple gene block protein; 8K: 8 kDa cysteine-rich protein.

Prior to 2009, only a limited number of PMTV sequences were available in databases. These included partial or complete sequences from three Danish isolates (Pecenkova et al., 2004), two Scottish isolates (Kashiwasaki et al., 1995; Reavy et al., 1998), and one isolate each from Sweden (Savenkov et al., 1999; Sandgren et al., 2001; Savenkov et al., 2003), Czech Republic (Cerovska et al., 2003), and Canada (Xu et al., 2004). Analysis of these and some other sequences not made available in the databases have shown that the RNA-RdRp and CP region of RNA-CP are highly conserved (Cerovska et al., 2003; Mayo et al., 1996; Nielsen and Nicolaisen, 2003; Pecenkova et al., 2004; Sandgren et al., 2001). However, analysis of two Swedish PMTV isolates revealed that the RT of RNA-CP is highly variable and contains many synonymous (resulting in no amino acid [aa] changes) and non-synonymous (resulting in aa changes) nt substitutions (Sandgren et al., 2001). RT-PCR restriction fragment length polymorphism (RT-PCR-RFLP) analysis of RNA-CP in 21 Danish isolates revealed two subgroups of PMTV, but the isolates were not sequences for further analysis (Nielsen and Nicolaisen, 2003). Moreover, the RT region of two Scottish isolates is shorter than that of Swedish or Danish isolates (Sandgren et al., 2001; Pecenkova et al., 2004). In contrast, PMTV RNA-TGB shows a high sequence similarity in the studied isolates. When RNA-TGB in three Danish, one Scottish, and one Swedish isolate were compared, the identity was found to be over 96% at nt level (Pecenkova et al., 2004). At aa level, TGB3 showed the lowest similarity (94% and 97%, when Danish isolates were aligned with a Scottish and a Swedish isolate, respectively). The 8K cysteine-rich protein of the Danish isolates had a less effective translation start codon (GUG) than the other isolates containing the more common codon AUG (Pecenkova et al., 2004).

There is only one indication on the biological significance of the observed sequence differences, as three partially sequenced Danish isolates with different symptom severity on test plants have been shown to differ from each other by only a few amino acids, two of which were found to correlate with groupings according to symptom severity (Pecenkova et al., 2004). These amino acids were located in the middle part of TGBp1 and the RT region of RNA-CP (Pecenkova et al., 2004). However, there is no further evidence on the importance of these amino acids in symptom development.

1.2 PMTV infection

As noted before, PMTV particles are transported to the host plant by the vector *Spongospora subterranea f.sp. subterranea*. For the successful infection of a host, the virus needs to utilize viral and host factors for replication and movement from the initially infected cell to adjacent cells. Plant viruses can't move to neighboring cells through the plant cell wall; instead they encode movement proteins that enable passage of the viral genome through intercellular communication channels (plasmodesmata) (Carrington et al., 1996). The three non-structural proteins encoded by RNA-TGB facilitate cell-to-cell movement of PMTV. Although TGB-containing viruses share many functional similarities, there are also notable differences, e.g., in the structures and functions of TGB proteins and the requirement for viral CP in movement, which have led to divergent models of movement for different groups of TGB-containing viruses (Morozov & Solovyev, 2003; Verchot-Lubicz et al., 2010). PMTV has been previously assigned to hordei-like TGB viruses that, unlike potex-like TGB viruses, require TGBp2 and TGBp3 for localization of TGBp1 to plasmodesmata (Zamyatnin et al., 2004; Lim et al., 2009). CP is not required for cell-to-cell movement of hordei-like viruses (Petty et al., 1990; Savenkov et al., 2003). A further division to Hordeivirus and Pomovirus models of transportation has also been suggested, as pomoviruses, unlike other studied hordeiviruses, utilize motile granules in the transportation of the TGBp1-ribonucleoprotein (TGBp1-RNP) complex to plasmodesmata, but further studies are needed to clarify the situation (Verchot-Lubicz et al., 2010). In the following, information is provided about PMTV replication, movement, and symptom induction, complemented with observations from other hordeiviruses where needed.

1.2.1 Intracellular localization, replication and accumulation of viral RNA

Studies on intracellular movement of PMTV proteins have mostly been done by overexpressing the proteins from gene expression vectors in plant cells rather than expressing tagged proteins from an infectious clone of the virus. Data from the overexpression experiments suggest that TGBp2 and TGBp3 are associated with the endoplastic reticulum (ER) network early in the infection, after which they are recruited to motile granules that move along the actin-ER network and are targeted to plasmodesmata by TGBp3 (Haupt et al., 2005). Targeting of TGBp2 to plasmodesmata depends not only on the presence of TGBp3, but also on the proper ratio of expression (10 to 1, respectively) of the two proteins (Tilsner et al., 2010). Later in infection, TGBp2 localizes to large vesicles (Haupt et al., 2005) that were recently found to be distorted chloroplasts (Cowan et al., 2012). TGBp2

interacts with specific lipids such as phosphatidic acid, a membrane lipid that is transported from the ER to chloroplast thylakoids (Awai et al., 2006; Cowan et al., 2012). Indeed, TGBp2 is detected on the chloroplast envelope and inside the chloroplast (Cowan et al., 2012). As viral RNA (vRNA) is also detected in the plastid preparations of infected cells, it might be that TGBp2 acts in the transportation of vRNA to/from chloroplast (Cowan et al., 2012).

When expressed alone, PMTV TGBp1 is distributed uniformly in the cell, whereas coexpression with TGBp2 and TGBp3 directs TGBp1 to the proximity of the plasma membrane and cell wall (Zamyatnin et al., 2004). The ability of PMTV TGBp1 to bind RNA (Cowan et al., 2002) and formation of the RNP -complex containing TGBp1 and vRNA in *Barley stripe mosaic virus* (BSMV, *hordeivirus*) infection (Lim et al., 2008) suggests that PMTV vRNA is transported intracellularly as a TGBp1-RNP-complex. After delivery of the TGBp1-RNP -complex to and through plasmodesmata, TGBp2 recruits TGBp3 to motile vesicles, possibly early endosomes, and the proteins are recycled through an endoplasmic pathway (Zamyatnin et al., 2004; Haupt et al., 2005).

Recently, PMTV negative-strand RNA, the viral replicative intermediate, was shown to associate with chloroplasts (Cowan et al., 2012). In the same study, CP and viral genomic RNA were found in the plastid preparations made from virus-infected leaves. These results implicate that viral replication and virion assembly of PMTV are associated with chloroplasts. Although TGBp2 is needed for movement of the virus, it is not needed for virus replication (Cowan et al., 2012). Accumulation of PMTV seems to be controlled antagonistically by two viral factors, 8K protein and DI RNA. The 8K protein acts as an RNA silencing suppressor enhancing the virus titers, whereas DI RNA is produced during replication and interferes with virus accumulation (Lukhovitskaya et al., 2013). This interplay between the two factors suggests that attenuated replication and low titers of vRNA may help the virus to avoid host defense mechanisms and would thereby be beneficial for the virus (Lukhovitskaya et al., 2013).

1.2.2 Cell-to-cell movement

The current model of PMTV cell-to-cell movement suggests that TGBp1 and vRNA associate in an RNP–complex, moved to and through plasmodesmata assisted by TGBp2 and TGBp3 that are not transported to the adjacent cell themselves (Cowan et al., 2002; Zamyatnin et al., 2004; Haupt et al., 2005; Lim et al., 2008; Tilsner et al., 2010). When coexpressed with other TGB proteins, TGBp1 moves from the originally infected cell to the adjacent cells but not further, indicating that TGBp2 and

3 facilitate the movement of TGBp1 but do not move through plasmodesmata themselves (Zamyatnin et al., 2004). Indeed, TGBp2 and TGBp3 both have the ability to increase the size exclusion limit (SEL) of plasmodesmata and to enter the endocytic pathway following the delivery of TGBp1 to plasmodesmata (Haupt et al., 2005). Fluorescently marked TGBp1 also moves cell-to-cell in the presence of vRNA, indicating that TGBp1 and vRNA, possibly as an RNP-complex, pass through the plasmodesmata (Zamyatnin et al., 2004; Lim et al., 2008). One study also suggests that, in the presence of PMTV CP, the RNA-CP can move cell-to-cell as particles (Torrance et al., 2009).

1.2.3 Long-distance (systemic) movement and symptom induction

Long-distance movement of viruses in plants commonly utilizes the plant vascular system and requires loading of the viral genome into phloem for transportation to the developing parts of the plant (Vuorinen et al., 2011). It seems that PMTV RNA-RdRp and RNA-TGB move systemically as a TGBp1-containing RNP-complex (McGreachy and Barker, 2000; Savenkov et al., 2003; Lim et al., 2008). Inoculation of *N. benthamiana* with only RNA-RdRp and RNA-TGB leads to systemic infection (Savenkov et al., 2003), indicating that CP encoded by the RNA-CP is not needed for long-distance movement of the virus. In contrast to RNA-RdRp and RNA-TGB, it has been suggested that RNA-CP moves systemically only in association with CP, possibly as particles (Cowan et al., 1997; Torrance et al., 2009). The systemic movement of RNA-CP requires the presence of CP-RT capable of interacting with TGBp1, whereas expression of CP alone inhibits systemic movement of RNA-CP (Torrance et al., 2009). The result suggests that RNA-CP does not bind TGBp1 directly. Instead, RNA-CP binds to CP and forms a virion. Incorporation of CP-RT into the virion is needed as CP-RT, but not CP alone, can form the TGBp1-interaction required for the systemic movement. It is not, however, clear whether the other viral RNAs can move systemically in association with CP (i.e., in virions) (Torrance et al., 2009).

While RNA-CP is not needed for systemic infection of PMTV RNA-RdRp and RNA-TGB in plants, virus particles are required for the transmission of PMTV by its vector *S. subterranea.* The RT domain of CP confers the virus-vector interactions needed for transmission (Reavy et al., 1998) and therefore RNA-CP is not dispensable for the natural infection of the virus. Also, development of symptoms (chlorosis) on *N. benthamiana* leaves requires the presence of RNA-CP (Savenkov et al., 2003). Indeed, PMTV infection causes abnormalities in chloroplasts and CP seems to associate with chloroplasts in infected cells (Cowan et al., 2012). These results suggest that the ability of PMTV to move systemically in the absence of

RNA-CP may partly explain why symptoms in the haulms of potato plants are rare. Indeed, PMTV virions are present in leaves only in low titers (Cowan et al., 2012).

1.3 Plant defense against viruses

The successful infection of a plant requires that the virus enters the plant cell, the virion is disassembled, viral proteins are translated, the nucleic acid is replicated, the virus is transported cell-to-cell and systemically, new virions are formed, and the virus is transmitted to new plants, as discussed in relation to PMTV in the previous chapters of this work. To defend against viruses, plants have evolved multiple mechanisms affecting different stages of the viral infection cycle.

1.3.1 Defense and counter-defense

A “zig-zag model” of defense and counter-defense for plant–pathogen interaction has been presented by Jones and Dangl (2006) and it divides plant immunity into two separate classes, namely pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). According to the model, PTI is based on the recognition of molecular patterns related to a wide range of pathogens and leads to an initial increase in plant defense. Pathogens may, however, become adapted to these initial defenses and overcome PTI by delivering special effector proteins into plant cells, which interferes with host defense signaling. Subsequently, plants have evolved specific systems involving receptor-like proteins to directly or indirectly detect the effector proteins thus leading to ETI. Although much of the early discoveries related to plant defense have been done with plant viruses, the zig-zag model was originally developed for bacterial and fungal pathogens and has only later been adapted for plant viruses by Mandadi and Scholthof (2013). They conclude that although viruses do not use microbial secretion systems to deliver proteins into host cells (as defined for classical effector proteins), they do encode proteins that, after translation in the host cell, promote virulence by interfering with host defense pathways.

Most plant-virus interactions are inhibited at a basal resistance level as the virus is recognized through general PAMPs, such as components of the virion or double-stranded RNA (dsRNA) (Frazer, 1986; Nürnberger & Lipka, 2005; da Cunha et al., 2006; Carr et al., 2010). For example, purified CP of the *Tobacco mosaic virus* (TMV) is recognized extracellularly similar to bacterial flagellin, the classical PAMP (Allan et al., 2001; Carr et al., 2010). The recognition leads to a burst of reactive oxygen species in TMV-susceptible tobacco, although the response is not effective enough to stop the infection (Allan et al., 2001). If the virus is able to infect a plant cell, viral dsRNA, such as secondary structures of viral ssRNA or replicative intermediates of the virus, can trigger the RNA silencing machinery of the plant. To overcome this PTI, many viruses encode proteins that suppress RNA silencing. These suppressor proteins can in turn be recognized by plant proteins, thus triggering ETI. One example of such interplay between a virus and a plant is the *Ny*-triggered resistance to PVY in potato (Tian and Valkonen, 2013). The dsRNA structures formed during PVY-infection trigger RNA silencing, which is overcome by PVY-encoded helper component proteinase (HC-Pro) that acts as a silencing suppressor. However, recognition of the HC-Pro of the PVY-O strain group by the dominant potato gene *Ny* leads to hypersensitive response followed by cell death, thus limiting the spread of the virus to a few cells. Viral infection can also be restricted to the initially infected leaves, as in the case of RTM–mediated resistance to the *Tobacco etch virus*, which does not limit virus accumulation or cell-to-cell movement nor causes cell death, but blocks vascular transport of the virus (Mahajan et al., 1998; Whitham et al., 2000; Chisholm et al., 2001) Also, recessive resistance limiting the movement of the virus has been found. For example, certain mutations of eukaryotic translation initiation factor 4E (eIF4E), that participates in the translation of viral proteins as well as in virus multiplication and cell-to-cell movement, have been shown to cause recessive resistance against potyviruses (Wittmann et al., 1997; Gao et al., 2004; Khan et al., 2009). Even in susceptible plants, in which the virus is able to overcome the different lines of plant defenses, viral infection causes transcription of genes related to defense and salicylic acid (SA), ethylene (ET), or jasmonic acid (JA) signaling (Whitham et al., 2003; Love et al., 2005).

1.3.2 RNA-silencing

Antiviral RNA silencing is triggered by dsRNA molecules, such as viral replicative intermediates and secondary structures of viral ssRNA genomes that act as substrates to Dicer-like (DCL) enzymes. DCL cleaves the dsRNA into short 20-25 nt duplexes. These duplexes are unwound, yielding small interfering RNAs (siRNA). The siRNA molecules are incorporated into an RNA-induced silencing complex (RISC) and direct the

endoribonuclease activity of the complex to any homologous long RNA molecule. The formed cleavage products can be generated into dsRNA through the activity of host-encoded RdRps and act as targets to DCL, thus yielding secondary siRNA. These secondary siRNAs target homologous RNA molecules and amplify silencing (Voinnet, 2001; Baulcombe, 2007; Carr et al., 2010).

As mentioned previously, RNA silencing is triggered by dsRNA, a molecular pattern associated with plant viruses, and silencing can be considered as a form of basal defense. However, RNA silencing does not completely block viral infection. The efficiency of antiviral silencing is also affected by environmental factors, such as temperature (Szittya et al., 2003). RNA silencing seems to exhibit tissue specificity as higher levels of BNYVV RNA and lower levels of viral siRNA accumulate in roots of *N. benthamiana*, compared with leaves of the same plants (Andika et al., 2005). Additionally, transgene-derived RNA silencing against soilborne (+)ssRNA viruses functions more efficiently in leaves than in roots (Germundsson et al., 2002; Andika et al., 2005).

1.3.3 Suppression of RNA silencing

As a counter-defense against RNA silencing by the host plant, viruses encode specific proteins that suppress silencing (Li and Ding, 2006). These suppressor proteins function by preventing generation or stabilization of siRNAs, inhibiting incorporation of siRNA molecules to RISC, or interfering with RISC (Pumplin and Voinnet, 2013).

TGBp1 proteins of potexlike TGB-containing viruses can function as silencing suppressors. For example, *Potato virus X* (PVX) TGBp1 has been indicated as a weak suppressor of silencing. *Agrobacterium* co-infiltration of PVX TGBp1 and GFP into *gfp*-transgenic *N. benthamiana* slightly increases the levels of GFP-derived mRNA and fluorescens but does not affect the accumulation of GFP specific siRNA, compared with infiltration of GFP alone (Senshu et al., 2009). The suppressor activity of PVX TGBp1 is also needed for the cell-to-cell movement of the virus, but has no effect on accumulation of the virus in the initially infected cells (Voinnet et al., 2000; Bayne et al., 2005). However, despite their ability to bind dsRNA, TGBp1 proteins of hordei-like viruses have not been reported as silencing suppressors. Instead, hordeiviruses commonly encode other silencing suppressors that are not needed for cell-to-cell movement (Jackson et al., 2009; Verchot-Lubicz et al., 2010). CRPs of many soilborne, multipartite (+)ssRNA viruses show weak silencing suppressor activity in the *Agrobacterium* co-infiltration assay (Andika et al., 2012).

Recently, silencing suppression activity of PMTV 8K was implicated, as the protein could compensate for the silencing suppression activity of a heterologous viral protein. Silencing suppression is needed for efficient cell-to-cell movement of the *Turnip crinkle virus* (TCV) and PMTV 8K was able to partially restore the movement of the suppression-deficient TCV-mutant (Lukhovitskaya et al., 2013).

2. AIMS OF THE STUDY

The overall aim of the study was to identify new viral and host factors affecting PMTV infection. Also, the study aimed to enhance the control of PMTV through improved detection of the virus from seed tubers.

Specific aims for individual publications were:

I. To determine genetic variability of PMTV in Finland, study the occurrence of symptomless infections in potato tubers, and develop a less laborious method for serological detection of PMTV in potato tubers.

II. To examine the role of light in limiting PMTV titers in the aboveground parts of a potato plant, study RNA silencing acting against PMTV, and test silencing suppression activities of specific PMTV proteins.

III. To study whether the viral movement protein TGBp3 is phosphorylated during PMTV infection, and investigate the influence of the phosphorylated amino acids on protein-protein interactions and virus movement.

3. MATERIALS AND METHODS

The materials and methods used in this thesis are described here only briefly. More detailed descriptions of the materials and methods are presented in publications **I**-**III** as listed in Table 1.

Potato tubers were obtained from fields heavily infested with PMTV (**I**, **II**). Additionally, PMTV infected minitubers were obtained from Latvia and symptomatic potato tubers were collected from the main potato production areas of Finland (**I**). *N. benthamiana* plants were grown from seed in a growth chamber (**I**, **II**, **III**).

Sequences of PMTV isolates were obtained from symptomatic tubers from Finland and minitubers from Latvia by IC-RT-PCR and subsequent sequencing of the amplicons. Phylogenetic analysis was conducted with a neighbor joining method. An RFLP analysis was designed and utilized to analyze additional PMTV isolates from symptomatic and symptomless tubers. The incidence of symptomatic and symptomless PMTV infections in different potato cultivars was studied by indexing tubers for symptoms and testing them for PMTV by DAS-ELISA (**I**).

To study PMTV detection from potato sprouts, PMTV-infected tubers were sprouted in darkness and tested by DAS-ELISA (**I**). Some sprouted potatoes were planted in pots and grown at 20˚C in a 16h/8h (light/dark) regime. The leaves and roots were tested for PMTV 30 days post-planting by DAS-ELISA. Progeny tubers were harvested 60 days post-planting, stored in the dark at 5˚C for 6 weeks and tested for PMTV by IC-RT-PCR.
Effect of light exposure on PMTV titers in potato sprouts was studied by exposing tubers sprouted in darkness to white light while leaving some of the sprouted tubers to dark as controls. Sprouts were collected at certain time points after beginning of the treatment and subjected to DAS-ELISA and/or RNA extraction followed by qRT-PCR or northern blotting to study the viral titers and accumulation of virus derived siRNA (**II**).

Mutations were introduced into infectious clone of PMTV (Savenkov et al., 2003) to study the effect of the mutations to PMTV infection in *N. benthamiana* by PCR (**III**). Additionally, wt and mutated infectious clones of PMTV were tagged with GFP and the infections were followed under UV-light (**III**). The infectious clone was also utilized to clone PMTV proteins into yeast two hybrid vectors to study protein-protein interactions (**III**) and into binary vectors for the agroinfiltration studies (**II**, **III**). Mutations were introduced into the cloned proteins (**III**). Wt and mutant PMTV TGBp3 were agroinfiltrated into fully expanded *N. benthamina* leaves and the

overexpressed proteins were subsequently subjected to western blotting to study phosphorylation of the proteins (**III**).

To study silencing suppression activity of PMTV TGBp1 and 8K, leaves of GFP-trangenic *N. bentamiana* 16C were co-infiltrated with either TGBp1 or 8K and GFP, and analyzed 3 dpi under UV-light and by western and northern blotting (**II**).

Table 1 Methods used in this thesis.

Method	Publication
Agroinfiltration	II, III
Cloning and sequencing	I, II, III
Biolistic inoculation of viral RNA with a gene gun	III
DAS-ELISA	I, II
Detection of GFP fluorescens under UV-light	II, III
Expression of recombinant proteins	I, II
IC-RT-PCR	I
Measurement of total chlorophyll content	II
Mutation of genes and infectious clones of viruses	III
Northern blotting	II
Phylogenetic analysis	I
PCR	III
Quantitative RT-PCR	II, III
RFLP analysis	I
Reverse transcription	II, III
RNA extraction	II, III
Western blotting	II, III
Yeast two-hybrid assay	III

4. RESULTS AND DISCUSSION

4.1 Two types of PMTV RNA-CP and RNA-TGB are detected from both symptomatic and symptomless tubers (I)

Previous studies on a few PMTV sequences (some of which are not deposited to the databank) indicate that although PMTV isolates are mostly highly conserved, there are also regions, such as RT of RNA-CP, that are more variable (Cerovska et al., 2003; Mayo et al., 1996; Nielsen and Nicolaisen, 2003; Pecenkova et al., 2004; Sandgren et al., 2001). The genetic variability of PMTV was further studied (**I**) by sequencing and analysing PCR amplicons from 18 spraing symptom-expressing tubers from fields in Finland and 5 asymptomatic tubers from a screenhouse in Latvia. Phylogenetic analysis of the obtained sequences and the sequences available in the databank showed that there are two types of RNA-CP and RNA-TGB in PMTV. CP sequences of RNA-CP were found to be nearly identical at both nt (98-100%) and aa (97-100%) levels but they formed two distinguishable clusters in the analysis. Similar but more prominent clustering was detected when RT sequences (97-100% and 98-100% identical at nt and aa levels, respectively) were used in the analysis. The two clusters, designated as RNA-CP-I and RNA-CP-II (RNA2-I and RNA2-II in paper **I**, respectively), differed at 21 nt positions within the RT domain. PCR amplicons containing the 8K gene and the 3'UTR were analyzed for RNA-TGB. The 3'UTR was almost identical in all the analyzed sequences, whereas the 8K gene sequences formed two groups distinguished from each other by nt substitutions resulting in aa substitutions in 8 positions. RNA-TGB-A (RNA3-A in paper **I**) consisted of 7 identical sequences and RNA-TGB-B (RNA3-B in paper **I**) included 21 sequences with additional point mutations in 3 of the studied sequences.

Existence of the two types of RNA-CP and RNA-TGB implicates that there might be biological differences associated with them in the respective PMTV isolates. One of the differences between the types of RNA-TGB is substitution of the common translation start codon AUG for a putatively less effective GUG codon for the 8K protein. As heterologously expressed 8K protein (RNA-TGB-A, start codon AUG) of PMTV has been shown to enhance necrotic symptoms caused by other viruses in *Nicotiana* spp. (Lukhovitskaya et al., 2005), association of the two types of RNA-CP and RNA-TGB with spraing symptoms was studied. RFLP analysis of PCR amplicons obtained from symptomatic and symptomless tubers revealed no correlation between the types of RNA or their combinations and the occurrence of symptoms (Table 4 in **I**). The most common combination (RNA-CP-II + RNA-TGB-B) was found equally as often in symptomatic and

symptomless tubers. Recent evidence shows that 8K is a suppressor of RNA silencing (Lukhovitskaya et al., 2013), which implicates that the differences observed in the 8K coding sequence might be relevant for virus infection. However, the possible biological significance of the two types of RNA-CP and RNA-TGB remains to be studied.

4.2 Variable incidence of symptomless PMTV infections in tubers and partial recovery of potato sprouts following exposure to light: implications on detection (I, II)

According to the findings presented in this work, the occurrence of PMTV symptoms in potato tubers is not related to a particular RNA-TGB or RNA-CP variant of the virus. Long-term field trials have, however, shown that different potato cultivars grown in the same field express different amounts of spraing symptoms (**I**, Santala et al., 2010). Moreover, when studied by IC-RT-PCR, it was found that some cultivars with a 10-fold difference in spraing incidence did not differ from each other in the total incidence of PMTV when the symptomless infections were taken into account (**I**). Indeed, in some cultivars, up to 100 % of the infections were found to be symptomless whereas in others almost all infected tubers expressed symptoms. There was also a small but significant difference in spraing incidence between two consecutive years when the cultivars were grown in the same field. Therefore, the results suggested that spraing expression depends on the genetic background of the potato cultivar and on the environmental conditions. Most importantly, the large and varying incidence of symptomless infections underlines the importance of testing the tubers for PMTV infection with virus-specific molecular and serological methods, as reliance on symptoms in detection of the virus causes a risk of spreading PMTV to new fields via infected, albeit symptomless, seed tubers.

Preparing samples from tubers is laborious and therefore the possibility of detecting PMTV from potato sprouts was investigated in this study (**I**). The results showed that PMTV can be easily detected by DAS–ELISA in sprouts that have developed in cold storage in darkness. Additionally, when recombinant PMTV CP from bacterial lysate was mixed with healthy sprout sap or tuber sap, the same amount of viral coat protein was more readily detected by DAS-ELISA from sprout sap than tuber sap. It is also possible to pool sprouts from many tubers and detect a single infected sprout among 50-100 non-infected sprouts, which makes testing of seed potato lots practically and economically feasible.

Table 2 Detection of PMTV in different parts of ten potato plants grown from infected tubers. Three cultivars were tested.

	Potato cultivar		
	Tanu	Seresta	Annabelle
PMTV detected in roots[a] (ELISA)	7/10	4/10	3/10
PMTV detected in leaves[a] (ELISA)	0/10	1/10	1/10
Plants with PMTV-infected progeny tubers[b] (IC-RT-PCR)	6/10	8/9[c]	9/10
% of progeny tubers[b] infected with PMTV (IC-RT-PCR)	24 %	48%	50%

[a] Roots and leaves were tested 30 days after planting

[b] Tubers were collected 60 days after planting and tested following storage at +5°C for 6 weeks

[c] One plant did not produce progeny tubers

In addition to potato sprouts grown in darkness, PMTV could be readily detected in the belowground parts of potato plants by antibody-based methods detecting CP (**II** and Table 2.). However, the virus was rarely detected by DAS-ELISA in the leaves of these potato plants. As the results implicated that PMTV can be detected in the parts of the potato plant growing in the dark but not in the light-exposed parts, a study was conducted to find out whether exposure to light limits PMTV infection. The results showed that exposure of sprouts to light for a few days decreased the virus amounts to a borderline or below the detection level of DAS-ELISA (Fig. 3). Also, samples were tested by qRT-PCR using primers targeting the TGBp1 ORF. Results showed that the amount of viral RNA decreases in sprouts exposed to light (**II**). Additionally, qRT-PCR analysis with primers targeting the RNA-CP showed that small amounts of PMTV RNA were present in leaves grown from infected tubers 4, 8, 15, 25, and 30 days post-planting (**II**). These results show that PMTV can move to the aerial parts of the plant, but rarely accumulates to detectable level. Results also suggest that exposure to light has a role in limiting the virus titers.

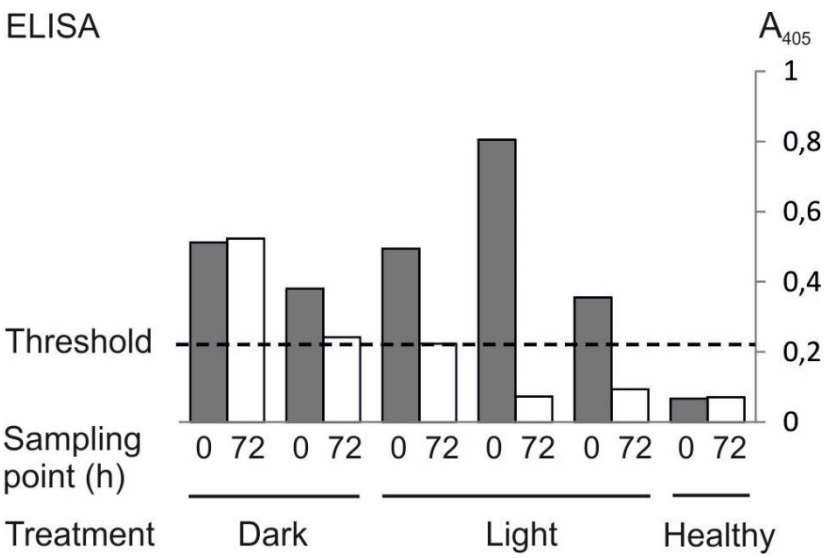

Figure 3 Decrease of virus titers in potato sprouts (cv. Victoria) upon exposure to light. Five PMTV-infected tubers and one healthy tuber were analyzed by DAS-ELISA in the experiment. The tubers were carefully kept in the dark prior to the experiment and one sprout from each tuber was collected as a sample prior to the treatments (grey bars). Subsequently, two of the PMTV-infected tubers were left in the dark and three PMTV -infected and one healthy tuber were exposed to light for 72 hours (h), after which another sprout from each tuber was analyzed (white bars). Absorbance measured by ELISA at the 405 nm wavelength is shown on the right. The dotted line indicates the threshold level of ELISA absorbance above which the sprout was considered positive for PMTV (i.e., three times the A_{405} value detected in the healthy sprout).

Taken together, the unreliable detection of PMTV based on observation of symptoms suggests a need for virus-specific molecular or serological detection method. The rare detection the virus in the aerial parts of potato plants and detection of the virus in dark-grown, but not light-exposed, sprouts by ELISA underlines the importance of proper sampling in seed potato inspections, as testing the light-exposed parts of potato plants is likely to lead to failure in detecting the infected seed tubers.

4.3 RNA silencing against PMTV may be accelerated in light and is suppressed by two viral proteins (II)

Exposure to high light intensity can favor induction and the systemic spread of silencing of the *gfp* gene in transgenic *N. benthamiana* (Kotakis et al., 2010). Therefore, experiments were conducted to study whether RNA silencing could explain the detected decrease in the amount of PMTV

in potato sprouts exposed to light. Indeed, the results showed that RNA silencing was acting against PMTV in sprouts kept both in the dark and the light, as indicated by the presence of PMTV-derived siRNA (**II**). Moreover, although the amount of viral RNA was lower in the light-exposed sprouts than in the sprouts kept in the dark, the abundance of the viral siRNA remained at about the same level, indicating that RNA silencing was acting more efficiently in the light-exposed sprouts (**II**). Previously, RNA silencing against soilborne (+)ssRNA viruses has been shown to function more efficiently in leaves than in roots (Germundsson et al., 2002; Andika et al., 2005). A recent study further shows that sense-mediated silencing (co-suppression) does not function in roots (Horn et al., 2014). Although other factors, such as tissue type (Andika et al., 2006; Horn et al., 2014) can be involved, the results (**II**) indicate that exposure to light and the subsequently more efficient RNA silencing at least partly explains the lower PMTV titers observed in the aboveground parts of potato plants. Indeed, changes in the wavelength and intensity of light regulate the transcriptome during many developmental processes in plants (Jiao et al., 2007). When an etiolated plant is exposed to light, many genes are upregulated, especially those encoding chloroplast-targeted proteins (Pogson and Albrecht, 2011). The potato sprouts developing in the dark are rich in amyloplasts formed mainly through the generation of proplastids from 'mother' amyloplasts (Sagisaka, 2008). When exposed to white light, these proplastids transform into chloroplasts rather than amyloplasts (Zu et al., 1984; Pogson and Albrecht, 2011). As PMTV TGBp2 and RNA are detected from plastid preparations made from PMTV-infected leaves and TGBp2 associates with distorted chloroplasts (Haupt et al., 2005; Cowan et al., 2012), changes in the plastids upon light exposure can also affect the virus concentrations. Additionally, high light conditions favor upregulation of several silencing-related genes (Kotakis et al., 2010).

Plant viruses encode suppressor proteins acting against RNA silencing in their hosts (Li and Ding 2006; Ding and Voinnet 2007). However, *Agrobacterium* co-infiltration commonly used to investigate suppression activities of proteins has not indicated such activity for any PMTV proteins, although such a role has been implicated for PMTV 8K in a complementation study (Lukhovitskaya et al., 2013). TGBp1 acts as a silencing suppressor in potexlike TGB-containing viruses (Senshu et al., 2009) and small CRPs (such as PMTV 8K) of hordeilike viruses have been shown to be weak suppressors of silencing (Andika et al., 2012). PMTV TGBp1 and 8K were studied for their silencing suppression activities by co-infiltration of an *Agrobacterium* strain expressing one of the two proteins, with another strain expressing the *gfp* gene (silencer of endogenous GFP) into leaves of a GFP-transgenic line of *N. benthamiana*. The results indicated that PMTV TGBp1 and 8K both act as silencing suppressors (**II**). The suppression activity of these proteins was found to be weak. Silencing suppression was consistently found only when the *Agrobacterium* strains

expressing TGBp1 or 8K were infiltrated in the ratio of 6:1 to the *Agrobacterium* strain expressing *gfp*. Andika et al. (2012) have shown that CRPs encoded by two soilborne viruses, BNYVV and TRV, suppress silencing more efficiently in roots than in leaves. Therefore, the silencing suppression activity of PMTV TGBp1 and 8K may be significant in sprouts grown in the dark because RNA silencing in such sprouts is less efficient.

4.4 Phosphorylated tyrosine residues of PMTV TGBp3 are important for viral infection and TGBp2-TGBp3 interaction (III)

Despite the important role of TGBp3 in targeting the viral RNP-complex to plasmodesmata for cell-to-cell movement (Verchot-Lubicz et al., 2010), PMTV TGBp3 is present at a very low concentration in an infected cell and is therefore difficult to detect (Donald et al., 1993; Zhou and Jackson, 1996; Gorshkova et al., 2003; Lim, et al., 2009; Shemyakina et al., 2011). In fact, PMTV TGBp3 was detected in a PMTV-infected plant for the first time in this study (**III**). Western blot analysis using an anti-myc antibody revealed the protein from leaf tissue infected with a PMTV mutant containing myc-tagged TGBp3. Furthermore, western blot analysis using an anti-phosphotyrosine antibody revealed that TGBp3 was tyrosine-phosphorylated by plant kinase activity both *in vitro* and during viral infection in *N. benthamiana*. Mapping of the possible phosphorylation sites revealed two putatively phosphorylated tyrosine (Y) residues located in the central ($_{87}$YY<u>Y</u>QDLN$_{93}$) and C-proximal ($_{116}$QEFP<u>Y</u>GNI$_{123}$) part of TGBp3. Western blot analysis of overexpressed mutant TGBp3s with alanine substitutions introduced to tyrosine residues $_{87}$YYY$_{89}$ and/or Y$_{120}$ indicated that both sites were phosphorylated in the leaves of *N. benthamiana*, because only substitution of all these residues prohibited phosphorylation of TGBp3.

Alanine substitutions of the tyrosine residues in the phosphorylation sites of TGBp3 led to the loss of PMTV infectivity (**III**). Further investigations with confocal microscopy using GFP-tagged infectious PMTV clones revealed that alanine substitutions of $_{87}$YYY$_{89}$ resulted in the loss of virus movement, confining the virus to the initially infected cells, whereas substitution of Y$_{120}$ seemed to abolish the infectivity of PMTV. The result with $_{87}$YYY$_{89}$ is further supported by a previous study showing that tyrosine 89 has a crucial role in PMTV cell-to-cell movement (Tilsner et al., 2010). Also, mutation of $_{89}$YQDLN to $_{89}$GQDGN impairs plasmodesmatal targeting of PMTV TGBp3 and its ability to gate plasmodesmata open (Haupt et al., 2005).

The current model of PMTV movement suggests that TGBp3 and TGBp2, which do not move to adjacent cells but are recycled via an endocytotic pathway (Haupt et al., 2005; Tilsner et al., 2010), interact with each other to mediate TGBp1-RNP-complex transportation to and through plasmodesmata (Cowan et al., 2002; Zamyatnin et al., 2004). Therefore, the effect of the above-mentioned mutations on TGBp3-TGBp2 interaction was studied. Substitution of tyrosine 89 to glycine had shown no alteration in PMTV TGBp3-TGBp2 interaction in a yeast two-hybrid assay of a previous study (Tilsner et al., 2010). In our study, however, β-galactosidase activity assay of yeast cells cotransformed with TGBp3 and TGBp2 constructs revealed that mutation of $_{87}$YYY$_{89}$ increased the strength of TGBp3-TGBp2 interaction (**III**). Substitution of tyrosine 120 to alanine in TGBp3 didn't alter its interaction with TGBp2 compared with the wild type protein.

Taken together, the findings suggest that the phosphorylated tyrosine residue(s) at positions 87-89 of TGBp3 play a role in PMTV cell-to-cell movement by regulating TGBp3-TGBp2 interaction. As phosphorylation is a reversible mechanism (Bond et al., 2011), it can participate in recycling these proteins during viral infection. Further evidence on a regulative role of the observed tyrosine phosphorylation is provided by the location of tyrosines 89 and 120 within the putative tyrosine-based sorting motifs $_{89}$YQDL, which is conserved among hordei-like viruses (Solovyev et al., 1996), and the motif $_{120}$YGNI, respectively. Tyrosine phosphorylation of this type of sorting motifs has been shown to regulate endocytosis in animal cells (Shiratori et al., 1997; Paolo et al., 1999; Schaefer et al., 2002).

5. CONCLUSIONS AND FUTURE PROSPECTS

The main findings reported in this thesis include, firstly, the high incidence of symptomless PMTV infections in potato tubers, highlighting the need for virus-specific serological or molecular detection of the virus, especially in the inspection of seed potatoes. The limited genetic variability of CP genes in PMTV isolates suggests that the currently used monoclonal antibodies and PCR primers can be used to reliably detect the virus. However, detection of PMTV can't be reliably done from light-exposed plant parts, such as emerging stems and leaves, in contrast to many other potato viruses. Therefore, a method for testing dark-grown sprouts was developed to provide a less laborious testing method of tubers. To prevent further spread of PMTV to new fields, such as the High Grade seed potato growing area in northern Finland, it would be important to adopt virus - specific detection of PMTV as part of seed potato certification.

Secondly, this study implicates that light accelerates antiviral RNA silencing and thus provides at least a partial explanation for the lower efficiency of RNA silencing against viruses in roots than in leaves of plants, a phenomenon also reported by other authors. Silencing suppression activities of PMTV TGBp1 and 8K were found and confirmed, respectively, in this study. Some silencing suppressors encoded by soilborne viruses show higher efficiency in roots than in leaves. Whether the silencing suppressors TGBp1 and 8K of PMTV also exhibit such preferences remains a subject for future studies. As viruses are dependent on living hosts, this kind of adaptation to underground parts of the plant would seem advantageous for a soilborne virus, such as PMTV, because it enables accumulation of high virus titers without directly affecting the growth of, and flow of photosynthesis products from, the aboveground parts.

The finding that both PMTV TGBp1 and 8K CRP act as silencing suppressors indicates that PMTV combines features of both potex-like and hordei-like viruses, and further supports the previously suggested division of TGB-containing viruses into *Potexvirus*, *Hordeivirus* and *Pomovirus* groups. Whether the other species of genus *Pomovirus* also encode two silencing suppressors remains to be studied in the future.

Thirdly, TGBp3 phosphorylation by plant tyrosine kinase activity seems necessary for PMTV infection. It seems like an interesting project for future to identify and isolate the kinase responsible for phosphorylation of TGBp3, as closer knowledge on its functions may be useful to design plants that are resistant to PMTV. In such experiments it is important to take into account the fact that gene silencing functions differently in the underground

and aboveground parts of the potato plant. Recently, a collaborative project has established an experimental system based on composite potatoes, with transgenic roots on non-transgenic shoots, for studying host genes related to interactions with soilborne pathogens in roots (Horn et al., 2014). The system allows root infection of PMTV via the vector *S. subterranea* and can thus be used to study the PMTV infection in more natural conditions.

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7. REFERENCES

Agindotan B, Perry KL. 2008. Macroarray detection of eleven potato-infecting viruses and *Potato spindle tuber viroid*. Plant Disease 92: 730-740.

Allan AC, Lapidot M, Culver JN, Fluhr R. 2001. An early tobacco mosaic virus – induced oxidative burst in tobacco indicates extracellular perception of the virus coat protein. Plant Physiology 126: 97-108.

Andika IB, Kondo H, Nishiguchi M, Tamada T. 2012. The cysteine-rich proteins of beet necrotic yellow vein vírus and tobacco rattle vírus contribute to efficient suppression of silencing in roots. Journal of General Virology 93: 1841-1850.

Andika IB, Kondo H, Rahim MD, Tamada T. 2006. Lower levels of transgene silencing in roots is associated with reduced DNA methylation levels at non-symmetrical sites but not at symmetrical sites. Plant Molecular Biology 60: 423-435.

Andika IB, Kondo H, Tamada T. 2005. Evidence that RNA silencing-mediated resistance to *Beet necrotic yellow vein virus* is less effective in roots than in leaves. Molecular Plant-Microbe Interactions 18 (3):194-204.

Andersen BAB, Nicolaisen M, Nielsen SL. 2002. Alternative hosts for potato mop-top virus, genus Pomovirus and its vector *Spongospora subterranea* f.sp. *subterranea*. Potato Research 45: 37-45.

Arif M, Torrance L, Reavy B. 1994. Improved efficiency of detection of potato mop-top furovirus in potato tubers and in the roots and leaves of soil-bait plants. Potato Research 37: 373-381.

Arif M, Torrance L, Reavy B. 1995. Acquisition and transmission of potato mop-top furovirus by a culture of *Spongospora subterranea* f. sp. *subterranea* derived from a single cystosorus. Annals of Applied Biology 126, 493-503.

Arif M, Ali M, Rehman A, Fahim M. 2013. Occurrence of potato mop-top virus in Northwest of Pakistan. European Journal of Plant Pathology 137: 787-796.

Awai K, Xu C, Tamot B, Benning C. 2006. A phosphatidic acid-binding protein of the chloroplast inner envelope membrane involved in lipid trafficking. Proceedings of the National Academy of Sciences 103: 10817-10822.

Baulcombe DC. 2007. Amplified silencing. Science 315: 199-200.

Bayne EH, Rakitina DV, Morozov SY, Baulcombe DC. 2005. Cell-to-cell movement of potato potexvirus X is dependent on suppression of RNA silencing. Plant Journal 44: 471–482.

Beuch U, Persson P, Edin E, Kvarnheden A. 2014. Necrotic diseases caused by viruses in Swedish potato tubers. Plant Pathology 63: 667-674.

Beczner L, Horvath J, Romhanyi I, Förster H. 1984. Studies on the etiology of tuber necrotic ringspot disease in potato. Potato Research 27: 339-352.

Björnstad A. 1969. Spredning av potet-mop-topp-virus (PMTV) med settepotater. Jord og avling 2: 2–4. (In Norwegian)

Bond AE, Row PE, Dudley E. 2011. Post-translational modification of proteins; methodologies and applications in plant sciences. Phytochemistry 72:975–996.

Budziszewska M, Wieczorek P, Nowaczyk K, Borodynko N, Pospieszny H, Obrepalska-Steplowska A. 2010. First report of *Potato mop-top virus* on potato in Poland. Plant Disease 94: 920.

Calvert EL, Harrison BD. 1966. Potato mop-top a soil-borne virus. Plant Pathology 15: 134–139.

Campbell RN. 1996. Fungal transmission of plant viruses. Annual Review of Phytopathology 34: 87-108.

Carnegie SF, Davey T, Saddler GS. 2010a. Effect of temperature on the transmission of *Potato mop-top virus* from seed tuber and by its vector, *Spongospora subterranea*. Plant Pathology 59: 22-30.

Carnegie SF, Cameron AM, McCreath M. 2010b. Foliar symptoms caused by *Potato mop-top virus* on potato plants during vegetative propagation in Scotland and their association with tuber yield, spraing and tuber infection. Potato research 53: 83-93.

Carr JP, Lewsey MG, Palukaitis P. 2010. Signalling in induced resistance. In: Advances in Virus Research. Vol. 76. JP Carr and G Loebenstein (eds). Elsevier.

Carrington JC, Kasschau KD, Mahajan SK, Schaad MC. 1996. Cell-to cell and long-distance transport of viruses in plants. Plant Cell 8: 1669-1681.

Cerovska N, Moravec T, Rosecka P, Filigarova M, Pecenkova T. 2003. Nucleotide sequences of coat protein coding regions of six potato mop-top virus isolates. Acta Virologica 47: 37–40.

Chisholm ST, Parra MA, Anderberg RJ, Carrington JC. 2001. Arabidopsis RTM1 and RTM2 genes function in phloem to restrict long-distance movement of tobacco etch virus. Plant Physiology 127: 1667-1675.

Cooper JI, Harrison BD. 1973. Distribution of potato mop-top virus in Scotland in relation to soil and climate. Plant Pathology 22, 73-78.

Cowan GH, Torrance L, Reavy B. 1997. Detection of potato mop-top virus capsid readthrough protein in virus particles. Journal of General Virology 78: 1779-1783.

Cowan GH, Lioliopoulou F, Ziegler A, Torrance L. 2002. Subcellular localization, protein interactions, and RNA binding of *Potato mop-top virus* Triple gene block proteins. Virology 298:106–115.

Cowan GH, Roberts AG, Chapman SN, Ziegler A, Savenkov EI, Torrance L. 2012. The potato mop-top virus TGB2 protein and viral RNA associate with chloroplasts and viral infection induces inclusions in the plastids. Frontiers in Plant Science 3: article 290.

Crosslin JM, Hamlin LL. 2011. Standardized RT-PCR conditions for detection and identification of eleven viruses of potato and *Potato spindle tuber viroid*. American Journal of Potato Research 88: 333-338.

Da Cunha L, McFall AJ, Mackey D. 2006. Innate immunity in plants: a continuum of layered defenses. Microbes and infection 8: 1372-1381.

Davies HV. 1998. Physiological mechanisms associated with the development of internal necrotic disorders in potato. American Journal of Potato Research 75: 37-44.

De Boer RF, Taylor PA, Flett SP, Merriman PR. 1985. Effects of soil temperature, moisture, and timing of irrigation on powdery scab of potatoes. In: Parker CA, Rovira AD, Moore KJ, Wong PTW, eds. Ecology and Management of Soilborne Plant Pathogens. St. Paul, MN, USA: APS Press, 197–8.

Ding SW, Voinnet O. 2007. Antiviral immunity directed by small RNAs. Cell 10: 413-426.

Donald RGK, Zhou H, Jackson AO. 1993. Serological analysis of barley stripe mosaic virus-encoded proteins in infected barley. Virology 195: 659–668.

Engsbro B. 1984. Susceptibility of potato varieties to spraing (tobacco rattle virus). Tidsskrift for Planteavl 88, 311-315. (In Danish)

Frazer RSS. 1989. Genes for resistance to plant viruses. CRC Critical Reviewes in Plant Science 3: 257-294.

Gao Z, Johansen E, Eyers S, Thomas CL, Ellis THN, Maule AJ. 2004. The potyvirus recessive resistance gene, sbm1, identifies novel rol for translation initiation factor eIF4E in cell-to-cell traficking. The Plant Journal 40: 376-385.

Germundsson A, Sandgren M, Barker H, Savenkov EI, Valkonen JPT. 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.

Gorshkova EN, Erokhina TN, Stroganova TA, Yelina NE, Zamyatnin AA Jr, Kalinina NO, Schiemann J, Solovyev AG, Morozov SY. 2003. Immunodetection and fluorescent microscopy of transgenically expressed hordeivirus TGBp3 movement protein reveals its association with endoplasmic reticulum elements in close proximity to plasmodesmata. Journal of General Virology 84: 985–994.

Haupt S, Cowan GH, Ziegler A, Roberts AG, Oparka KJ, Torrance L. 2005. Two plant-viral movement proteins traffic in the endocytic recycling pathway. Plant Cell 17: 164–81.

Harrison BD, Jones, RAC. 1970. Host range and some properties of potato mop-top virus. Annals of Applied Biology 65: 393–402.

Harrison BD, Jones RAC. 1971a. Factors affecting the development of spraing in potato tubers infected with potato mop-top virus. Annals of Applied Biology 68: 281-289.

Harrison BD, Jones RAC. 1971b. Effects of light and temperature on symptom development and virus content of tobacco leaves inoculated with potato mop-top virus. Annals of Applied Biology 67: 377-387.

Haupt, S, Cowan GH, Ziegler A, Roberts AG, Oparka KJ, Torrance L. 2005. Two plant-viral movement proteins traffic in the endocytic recycling pathway. Plant Cell 17: 164-81.

Hinostroza AM, French ER. 1972. Potato mop-top virus in cork-diseased Peruvian potatoes. American Potato Journal 49: 234-239.

Holm LG, Plucknett DL, Pancho JV, Herberger JP. 1991. The worlds worst weeds: Distribution and Biology. Pp 430-434. United press of Hawaii.

Horn P, Santala J, Nielsen SL, Hühns M, Broer I, Valkonen JPT. 2014. Composite potato plants with transgenic roots on non-transgenic shoots: A model system for studying gene silencing in roots. Plant cell reports 33: 1977-1992.

Hu X, Lei Y, Xiong X, He C, Liu M, Nie X. 2013. Identification of *Potato mop-top virus* (PMTV) in potatoes in China. Canadian Journal of Plant Pathology 35: 402-406.

Håkansson S. 2003. Weeds and weed management on arable land. An ecological approach. CABI Publishing, Cambridge,USA, 274 s.

Imoto M, Toclfihara H, Iwaki M, Nakamura H. 1981. (Occurrence of potato mop-top virus in Japan). Annals of the Phytopathological Society of Japan 47: 409. (In Japanese)

Jackson AO, Lim HS, Bragg J, Ganesan U, Lee MY. 2009. Hordeivirus replication, movement, and pathogenesis. Annual Review of Phytopathology 47: 385-422.

Jiao Y, Lau OS, Deng XW. 2007. Light-regulated transcriptional networks in higher plants. Nature review 8: 217-230.

Jones JDG, Dangl JL. 2006. The plant immune system. Nature 444: 323-329.

Jones RAC, Harrison BD. 1969. Behaviour of potato mop-top virus in soil and evidence for its transmission by Spongospora subterranea (Wallr) Lagerh. Annals of Applied Biology 63: 1-8.

Jones RAC, Harrison BD. 1972. Ecological studies on potato mop-top virus in Scotland. Annals of Applied Biology 71: 47-57.

Kallender H, Buck KW, Brunt AA. 1990. Association of three RNA molecules with potato mop-top virus. Netherland Journal of Plant Pathology 96: 47-50.

Kashiwazaki S, Scott KP, Reavy B, Harrison BD. 1995. Sequence analysis and gene content of potato mop-top virus RNA 3: Further evidence of heterogeneity in the genome organization of furoviruses. Virology 206: 701–706.

Khan MA, Yumak H, Goss D. 2009. Kinetic mechanism for the binding of eIF4F and Tobacco etch virus internal ribosome entry site RNA. The Journal of Biological Chemistry 284: 35461-35470.

King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds.). 2012. Virus Taxonomy, Classification and Nomenclature of Viruses, Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, USA.

Kotakis C, Vrettos N, Kotsis D, Tsagris M, Kotzabasis K, Kalantidis K. 2010. Light intensity affects RNA silencing of a transgene in *Nicotiana benthamiana* plants. BMC Plant Biology 10: 220-231.

Kurppa A. 1989a. The distribution and incidence of mop-top virus in Finland as determined in 1987 and on the variation of disease symptoms in infected potatoes. Annales Agriculturae Fenniae 28: 285-295.

Kurppa A. 1989b. Reaction of potato cultivars to primary and secondary infection by potato mop-top furovirus and strategies for virus detection. EPPO Bulletin 19: 593-598.

Lambert DH, Levy L, Mavrodieva VA, Johnson SB, Babcock MJ, Vayda ME. 2003. First report of *Potato mop-top virus* on potato from the United States. Plant Disease 87: 872.

Lampinen R, Lahti T. 2011. Kasviatlas 2010. Helsingin Yliopisto, Luonnontieteellinen keskusmuseo, Kasvimuseo, Helsinki. (In Finnish)

Le Romancer M, Kerlan C, Nedellec M. 1994. Biological characterization of various geographical isolates of potato virus Y inducing superficial necrosis on potato tubers. Plant Pathology 43: 138-144.

Li F, Ding SW. 2006. Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. Annual Review in Microbiology 43:191-204.

Lim HS, Bragg JN, Ganesan U, Lawrence DM, Yu J, Isogai M, Hammond J, Jackson AO. 2008. Triple gene block protein interactions involved in movement of *Barley stripe mosaic virus*. Journal of Virology 82: 4991-5006.

Lim HS, Bragg JN, Ganesan U, Ruzin S, Schichnes D, Lee MY, Vaira AM, Ryu KH, Hammond J, Jackson AO. 2009. Subcellular localization of the *Barley Stripe Mosaic Virus* Triple Gene Block proteins. Journal of Virology 83: 9432–9448.

Love AJ, Yun BW, Laval V, Loake GJ, Milner JJ. 2005. *Cauliflower mosaic virus*, a compatible pathogen of Arabidopsis, engages three distinct defence-signalling pathways and activates rapid systemic generation of reactive oxygen species. Plant Physiology 139: 935-948.

Lukhovitskaya NI, Thaduri S, Garushyants SK, Torrance L, Savenkov EI. 2013. Deciphering the mechanism of defective interfering RNA (DI RNA) biogenesis reveals that a viral protein and the DI RNA act antagonistically in virus infection. Journal of Virology 87: 6091-6103.

Lukhovistkaya NI, Yelina NE, Zamyatnin AA Jr, Schepetilnikov MV, Solovyev AG, Sandgren M, Morozov SYu, Valkonen JPT and Savenkov EI. 2005. Expression, localization and effects on virulence of the cystein-rich 8-kDa protein of Potato mop-top virus. Journal of General Virology 86: 2879-2889.

Mahajan SK, Chisholm ST, Whitham SA, Carrington JC. 1998. Identification and characterization of a locus (RTM1) that restricts long-distance movement of *Tobacco etch virus* in *Arabidopsis thaliana*. The Plant Journal 14: 177-186.

Mandadi KK, Scholthof KBG. 2013. Plant immune responses against viruses: how does a virus cause disease? The Plant Cell 25: 1489-1505.

Manzer FE, Merriam DC, Storch, RH, Simpson GW. 1982. Effect of time of inoculation with potato leafroll virus on development of net necrosis and stem-end browning in potato tubers. American Journal of Potato Research 59: 337-349.

Mayo MA, Torrance L, Cowan G, Jolly CA, Macintosh SM, Orrega R, Barrera C, Salazar LF. 1996. Conservation of coat protein sequence among isolates of potato mop-top virus from Scotland and Peru. Archives of Virology 141: 1115–1121.

McGreachy KD, Barker H. 2000. Potato mop-top RNA can move long distance in the absence of the coat protein: Evidence from resistant, transgenic plants. Molecular plant-microbe interactions 13: 125-128.

Merz U. 1995. PMTV-like particles inside resting spores of *Spongospora subterranea*. Journal of Phytopathology 143: 731-733.

Merz U. 2008. Powdery scab of potato – occurrence, life cycle and epidemiology. American Journal of Potato Research 85: 241-246.

Mølgaard JP, Nielsen SL. 1996. Influences of post harvest temperature treatments, storage period and harvest data on the development of spraing caused by rattle virus and mop-top virus. Potato Research 39: 571-579.

Montero-Astúa M, Vasquéz V, Turecheck WW, Mertz U, Rivera C. 2008. Incidence, distribution, and association of Spongospora subterranea and Potato mop-top virus in Costa Rica. Plant Disease 92: 1171-1176.

Morozov SY, Solovyev AG. 2003. Triple gene block: modular design of a multifunctional machine for plant virus movement. Journal of General Virology 84: 1351-1366.

Nakayama T, Maoka T, Hataya T, Shimizu M, Fuwa H, Tsuda S, Motoyuki M. 2010. Diagnosis of *Potato mop-top virus* in soil using bait plant bioassay and RT-PCR-microplate hybridization. American Journal of Potato Research 87: 218-225.

Nielsen SL, Mølgaard JP. 1997. Incidence, appearance and development of potato mop-top furovirus-induced spraing in potato cultivars and the influence on yield, distribution in Denmark and detection of the virus in tubers by ELISA. Potato Research 40, 101-110.

Nielsen SL, Nicolaisen M 2003. Identification of two nucleotide sequence sub-groups within *Potato mop-top virus*. Archives of Virology 148: 381–388.

Novak JB, Rasocha V, Lanzova J. 1983. (Demonstration of potato mop-top virus in Czechoslovak Socialist Republic.). Sbornik UVTIZ Ochrana Rostlin 19: 161-167. (In Czech)

Nürnberger T, Lipka V. 2005. Non-host resistance in plants: new insights into an old phenomenon. Molecular Plant Pathology 6: 335-345.

Paolo GP, Nucifora PGP, Fox AP. 1999. Tyrosine phosphorylation regulates rapid endocytosis in adrenal chromaffin cells. Journal of Neuroscience 19: 9739–9746.

Pecenkova T, Moravec T, Filigarova M, Rosecka P, Cerovska N. 2004. Extended sequence analysis of three Danish *Potato mop-top virus* (PMTV) isolates. Virus Genes 29: 249–255.

Petty ITD, French R, Jones RW, Jackson AO. 1990. Identification of Barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. The EMBO Journal 9(11): 3453-3457.

Pogson BJ, Albrecht V. 2011. Genetic dissection of chloroplast biogenesis and development: an overview. Plant Physiology 155: 1545-1551.

Pumplin N, Voinnet O. 2013. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. Nature Reviews Microbiology 11: 745-760.

Reavy B, Arif M, Cowan GH, Torrance L. 1998. Association of sequences in the coat protein/readthrough domain of potato mop-top virus with transmission by Spongosspora subterranea. Journal of General Virology 79: 2343–2347.

Rydén K, Eriksson B, Insunza V. 1986. Rostringar hos potatis orsakade av potatismopptoppvirus (PMTV). Växtskyddsnotiser 50: 97-102. (In Swedish)

Sagisaka S. 2008. The proliferation of amyloplasts in meristematic cells of developing stolons of potato and apple callus: progenitors of proplastids. Journal of Plant Physiology 165: 1678-1690.

Salazar LF, Jones RAC. 1975. Some studies on the distribution of potato mop-top virus in Peru. American Potato Journal 52: 143-150.

Sandgren M.1995. Potato mo-top virus (PMTV): distribution in Sweden, development of symptoms during storage and cultivar trials in field and glasshouse. Potato Research 38: 387–397.

Sandgren M, Plaisted RL, Watanabe KN, Olsson S, Valkonen JPT. 2002. Evaluation of some North and South American potato breeding lines for resistance to *Potato mop-top virus* in Sweden. American Journal of Potato Research 79: 205–210.

Sandgren M, Savenkov EI, Valkonen JPT. 2001. The readthrough region of *Potato mop-top virus* (PMTV) coat protein encoding RNA, the second largest RNA of PMTV genome, undergoes structural changes in naturally infected and experimentally inoculated plants. Archives of Virology 146: 467-477.

Santala J, Samuilova O, Hannukkala A, Latvala S, Kortemaa H, Beuch U, Kvarnheden A, Persson P, Topp K, Orstad K, Spetz C, Nielsen SL, Kirk HG, Budziszewska M, Wieczorek P, Obrepalska-Steplowska A, Pospieszny H, Kryszczuk A, Sztangret-Wisniewska J, Yin Z, Chrzanowska M, Zimnoch-Guzowska E, Jackeviciene E, Taluntyte L, Pupola N, Mihailova J, Lielmane I, Jarvekulg L, Kotkas K, Rogozina E, Sozonov A, Tikhonovich I, Horn P, Broer I, Kuusiene S, Staniulis J, Uth JG, Adam G. Valkonen JPT. 2010. Detection, distribution and control of *Potato mop-top virus*, a soil-borne virus, in northern Europe. Annals of Applied Biology. 157: 163-178.

Savenkov EI, Germundsson A, Zamyatnin AA Jr, Sandgren M, Valkonen JPT. 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.

Savenkov EI, Sandgren M and Valkonen JPT. 1999. Complete sequence of RNA 1 and the presence of tRNA-like structures in all RNAs of Potato mop-top virus, genus Pomovirus. Journal of General Virology 80: 2779-2784.

Schaefer AW, Kamei Y, Kamiguchi H, Wong EV, Rapoport I, Kirchhausen T, Beach CM, Landreht G, Lemmon SK, Lemmon V. 2002. L1 endocytosis is controlled by a phosphorylation-dephosphorylation cycle stimulated by outside-in signalling by L1. Journal of Cell Biology 157: 1223–1232.

Schwärzel R. 2002. Sensibilité des raciness et tubercules des variétés de pommes de terre à la gale poudreuse et qualques résultats de lutte chimique. Revue Suisse d'Agriculture 34 : 261-266.

Senshu H, Ozeki J, Komatsu K, Hashimoto M, Hatada K, Aoyama M, Kagiwada S, Yamaji Y, Namba S. 2009. Variability in the level of RNA silencing suppression caused by triple gene block protein 1 (TGBp1) from various potexviruses during infection. Journal of General Virology 90: 1014-1024.

Shiratori T, Miyatake S, Ohno H, Nakaseko C, Isono K, Bonifacino JS, Saito T. 1997. Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. Immunity. 6: 583–589.

Scott KP, Kashiwazaki S, Reavy B, Harrison BD. 1994. The nucleotide-sequence of potato mop-top virus RNA2 –a novel type of genome organization for a furovirus. Journal of General Virology 75: 3561–3568.

Shemyakina EA, Erokhina TN, Gorshkova EN, Schiemann J, Solovyev AG, Morozov SY. 2011. Formation of protein complexes containing plant virus movement protein TGBp3 is necessary for its intracellular trafficking. Biochemie 93: 742–748.

Sokmen MA., Barker H, Torrance L. 1998. Factors affecting the detection of potato mop-top virus in potato tubers and improvement of test procedures for more reliable assays. Annals of Applied Biology 133: 55–63.

Solovyev AG, Savenkov EI, Agranovsky AA, Morozov SY. 1996. Comparison of the genomic cis-elements and coding regions in RNAβ components of the hordeiviruses Barley stripe mosaic virus, Lychnis ringspot virus and Poa semilatent virus. Virology 219: 9–18.

Szittya G, Silhavy D, Molnár A, Havelda Z, Lovas A, Lakatos L, Banfalvi Z, Burgyan J. 2003. Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. The EMBO Journal 22: 633–40.

Tenorio J, Franco Y, Chuquillanqui C, Owens RA, Salazar LF. 2006. Reaction of potato varieties to *Potato mop-top virus* infection in the Andes. American Journal of Potato Research 83: 423-431.

Tian YP, Valkonen JPT. 2013. Genetic determinants of *Potato virus Y* required to overcome or trigger hypersensitive resistance to PVY strain group O controlled by the gene *Ny* in potato. Molecular plant-microbe interactions 26: 297-305.

Tilsner J, Cowan GH, Roberts AG, Chapman SN, Ziegler A, Savenkov E, Torrance L. 2010. Plasmodesmal targeting and intercellular movement of potato mop-top pomovirus is mediated by a membrane anchored tyrosine-based motif on the luminal side of the endoplasmic reticulum and the C-terminal transmembrane domain in the TGB3 movement protein. Virology 402: 41–51.

Torrance L, Cowan GH, Sokmen MA, Reavy B. 1999. A naturally occurring deleted form of RNA2 of Potato mop-top virus. Journal of General Virology 80: 2211-2215.

Torrance L, Lukhovitskaya NI, Schepetilnikov MV, Cowan GH, Ziegler A, Savenkov EI. 2009. Unusual long-distance movement strategies of *Potato mop-top virus* RNAs in *Nicotiana benthamiana*. Molecular plant-microbe interactions 22: 381-390.

van der Graaf P, Wale SJ, Lees AK. 2007. Factors affecting the incidence and severity of *Spongospora subterranea* infection and galling in potato roots. Plant Pathology 56: 1005-1013.

van der Graaf P, Lees AK, Wale SJ, Duncan JM. 2005. Effect of soil inoculums level and environmental factors on potato powdery scab caused by *Spongospora subterranea*. Plant Pathology 54: 22-28.

van Hoof AA., Rozandaal A. 1969. Het voorkomen van 'potato mop-top virus' in Nederland. Neth. J. Plant Pathol. 75, 275.

Verchot-Lubicz, J, Torrance L, Solovyev AG, Morozov SY, Jackson AO, Gilmer D. 2010. Varied movement strategies employed by triple gene block-encoding viruses. Molecular plant-microbe interactions 23: 1231–1247.

Voinnet O. 2001. RNA silencing as a plant immune system against viruses. Trends in Genetics 17: 449-459.

Voinnet O, Lederer C, Baulcombe DC. 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell 103, 157–167.

Vuorinen AL, Kelloniemi J, Valkonen JPT. 2011. Why do viruses need phloem for systemic invasion of plants? Plant Science 181: 355-363.

Whitham SA, Anderberg RJ, Chisholm ST, Carrington JC. 2000. Arabidopsis RTM2 gene is necessary for specific restriction of tobacco etch virus and encodes unusual small heat shock-like protein. The Plant Cell 12: 569-582.

Whitham SA, Quan S, Chang HS, Coper B, Eates B, Zhu T, Wang X, Hou YM. 2003. Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. The Plant Journal 33: 271-283.

Wittmann S, Chatel H, Fortin MG, Laliberte JF. 1997. Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of Arabidopsis thaliana using the yeast two-hybrid system. Virology 234: 84-92.

Xu H, DeHaan TL, De Boer SH. 2004. Detection and confirmation of *Potato mop-top virus* in potatoes produced in the United States and Canada. Plant Disease 88: 363-367.

Zamyatnin AA, Solovyev AG, Savenkov EI, Germundsson A, Sandgren M, Valkonen JPT, Morozov SY. 2004. Transient coexpression of individual genes encoded by the triple gene block (TGB) of *Potato mop-top virus* reveals requirements for TGBp1 trafficking. Molecular Plant-Microbe Interactions 17: 921-930.

Zhou, H, Jackson AO. 1996. Expression of the barley stripe mosaic virus RNA β "triple gene block". Virology 216: 367–79.

Zu YS, Merkle-Lehman DL, Kung SD. 1984. Light-induced transformation of amyloplasts into chloroplasts in potato tubers. Plant Physiology 75: 142-145.