Molecular Studies on a Complex of Potyviruses Infecting Solanaceous Crops, and Some Specific Virus-Host Interactions

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Abstract

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This thesis constitutes a comprehensive analysis of the molecular and biological characteristics of three potyviruses (genus *Potyvirus*, family *Potyviridae*) naturally occurring in cultivated and wild species of family Solanaceae: *Peru tomato virus* (PTV), *Potato virus V* (PVV) and *Wild potato mosaic virus* (WPMV). In addition, the studies presented in this thesis focus on the genetic variability of isolates of PTV and PVV and on the role of the *Potato virus A* (PVA) 6K2 protein as a host-specific determinant of virus movement and symptom induction.

Determination of the complete genomic sequences of PVV, PTV and WPMV demonstrated that these viruses are typical members of the genus *Potyvirus*. Furthermore, comparison of the polyprotein amino acid sequences and the biological and serological characteristics of these three viruses supported their current taxonomic position as independent species of the genus *Potyvirus*.

The nucleotide sequences of the P1 protein, coat protein and non-translated regions of European and South American PVV isolates were determined and compared. Results showed limited genetic variability among the European isolates, in contrast to the higher variability found among the South American isolates of PVV. Phylogenetic analysis defined two distinct clusters, grouping the European isolates together but placing two South American isolates to a different group; these two isolates of PVV did not induce a hypersensitive response in an *Nv* gene-carrying potato cultivar in contrast to the European PVV isolates. Thus, European and South American PVV isolates belong to different strain groups. In addition, great genetic variability was detected among PTV isolates.

Analysis of phylogenetic relationships among PTV, PVV, WPMV and other members of the genus *Potyvirus* commonly found infecting solanaceous crop plants showed that PTV, PVV and WPMV are the most closely related viruses which together with *Potato virus Y*, *Pepper mottle virus*, *Pepper severe mosaic virus* and *Pepper yellow mosaic virus* constitute a group distinguishable from other potyviruses. Thus, members of this group seem to share a common ancestor.

The 6K2 protein of PVA was modified by deleting various portions or by introducing six histidine residues (6xHis) into various positions of this protein. These modifications disturbed functions required for viral infection in *Nicotiana tabacum*. Furthermore, inoculation of the insertion constructs to *N. benthamiana* plants did not result in systemic infection with the exception of one plant. This plant lacked typical PVA symptoms but had virus titers similar to the plants infected with the wild type virus: a single point mutation (Gly2 $\rightarrow$ Cys2) in the 6xHis-containing 6K2 had restored the viral movement functions. However, partial deletion of the 6xHis-tag to gain the original size of the 6K2 protein was required to restore the induction of symptoms in *N. benthamiana* and to enable systemic infection of *N. tabacum*. Taken together, these results indicate the 6K2 is a host-specific determinant for long-distance movement and exemplify that mutations that arise during viral propagation represent a mechanisms by which viruses can evolve and adapt to different hosts.

Keywords: *Potato virus V*, *Peru tomato virus*, *Wild potato mosaic virus*, genetic variability, strain groups, phylogenetic analysis, *Potato virus A*, 6K2 protein, viral movement, symptom induction, host-adaptation.

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A mis queridos padres, Carl-Ivar y Carmen,
a quienes les debo todo en la vida

Contents

Appendix

Papers I-IV

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

I. Oruetxebarria, I., Kekarainen, T., Spetz, C. and Valkonen, J.P.T. (2000). Molecular characterization of *Potato virus V* genomes from Europe indicates limited spatio-temporal strain differentiation. *Phytopathology* 90:437-444.

II. Spetz, C. and Valkonen, J.P.T (2003). Genomic sequence of *Wild potato mosaic virus* as compared to the genomes of other potyviruses. *Archives of Virology* 148:373-380.

III. Spetz, C., Taboada, A.M., Darwich, S., Ramsell, J., Salazar, L.F. and Valkonen, J.P.T. (2003). Molecular resolution of a complex of potyviruses infecting solanaceous crops at the center of origin in Peru. *Journal of General Virology*, 84:2565-2578.

IV. Spetz, C. and Valkonen, J.P.T. (2003). Functions of the potyviral 6K2 protein in long-distance movement and symptom induction are independent and host-specific. *Molecular Plant-Microbe Interactions,* accepted pending revision.

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Abbreviations

6K1 and 6K2	6 kDa proteins 1 and 2
6xHis	Six histidine residues
aa	Amino acid
BCMNV	*Bean common mosaic necrosis virus*
cDNA	Complementary DNA
CI	Cylindrical inclusion protein
CIRE	Cap-independent regulatory element
CMV	*Cucumber mosaic virus*
CP	Coat protein
eIF4E, eIF(iso)4E	Eukaryotic translation initiation factors 4E, (iso) 4E
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
HC-Pro	Helper component proteinase
HR	Hypersensitive response
IRES	Internal ribosome entry sites
kDa	Kilodalton
LMV	*Lettuce mosaic virus*
MAb	Monoclonal antibody
MP	Movement protein
NIa	Nuclear inclusion protein a
NIa-Pro	C-terminal proteinase domain of NIa
NIb	Nuclear inclusion protein b, RNA polymerase
nt	Nucleotide
NTR	Non-translated region
ORF	Open reading frame
ORSV	*Odontoglossum ringspot virus*
P1	P1 protein
P3	P3 protein
PAb	Polyclonal antibodies
PCR	Polymerase chain reaction
PepMoV	*Pepper mottle virus*
PepSMV	*Pepper severe mosaic virus*
PepYMV	*Pepper yellow mosaic virus*
PK	Protein kinase
PPV	*Plum pox virus*
PSbMV	*Pea seed-borne mosaic virus*
PTNRD	Potato tuber necrotic disease
PTV	*Peru tomato virus*
PVA	*Potato virus A*
PVV	*Potato virus V*
PVY	*Potato virus Y*
RdRp	RNA-dependent RNA polymerase
RCNMV	*Red clover necrotic mosaic virus*
RNP	Ribonucleoprotein
RT-PCR	Reverse transcription polymerase chain reaction

SE	Sieve element
TBSV	*Tomato bushy stunt virus*
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*
TCV	*Turnip crinkle virus*
TuMV	*Turnip mosaic virus*
TVMV	*Tobacco vein mottling virus*
WPMV	*Wild potato mosaic virus*
YMV	*Yam mosaic virus*
VPg	Viral genome-linked protein
ZYMV	*Zucchini yellow mosaic virus*

Introduction

Viruses are sub-microscopic, obligate intracellular parasites that are not functionally active outside their host cells. Like all biological entities, viruses posses genes, reproduce themselves and have the ability to adapt to changing environments. A virus is a form of life with very simple requirements (Diener, 1972). Its basic needs are a nucleic acid to be transmitted from generation to generation and a messenger RNA to direct the synthesis of viral proteins (Baltimore, 1992). Late Nobel Prize laureate Salvador Luria and pioneering researcher in the field of gene regulation James Darnell defined viruses as "entities whose genome is an element of nucleic acid, either DNA or RNA, which reproduce inside living cells and use their synthetic machinery to direct the synthesis of specialized particles, the virions, which contain the viral genome and transfer it to other cells".

Throughout history, viral diseases have left an indelible impression on humankind. For example, the Spanish flue pandemic during 1918 to 1919 was to blame for the death of approximately 21 million individuals around the globe, whereas smallpox killed an estimated 300 million people during the twentieth century. Other infectious diseases such as AIDS, yellow fever, polio are responsible for the death of millions of people whereas the recently described "ebola hemorrhagic fever" and "severe acute respiratory syndrome" indicate that viruses will continue to cause human suffering throughout the world.

To date, the total number of viruses is known to exceed 4000, and new viruses are continuously described. About one fourth of all known viruses have been described from plants (Agrios, 1997). They, also, have a great impact on us humans. Indeed, plant viruses cause important diseases in crop plants resulting in enormous economic losses. Development of strategies to control and/or eliminate viral diseases in plants requires knowledge on the molecular characteristics of viruses, their interactions with hosts and the different mechanisms by which viruses evolve and adapt to different hosts.

The studies presented in this thesis contribute to the understanding of the molecular aspects, genetic variability and virus-host interactions of some members of the largest family of plant-infecting viruses, the *Potyviridae*.

Family *Potyviridae*

The family *Potyviridae*, along with the *Picornaviridae*, *Sequiviridae*, *Comoviridae* and *Caliciviridae* families, form the "picorna-like group" of positive-sense, single-stranded RNA viruses defined on the similarities in the genome organization and expression strategy (Goldbach, 1986). Within this group, the family *Potyviridae* contains six established genera: *Potyvirus*, *Macluravirus*, *Bymovirus*, *Rymovirus*, *Tritimovirus*, and *Ipomovirus* (van Regenmortel *et al.*, 2000). Potyviruses and macluraviruses are aphid-transmitted, whereas mites transmit rymoviruses and

tritimoviruses. Whiteflies and fungi transmit ipomoviruses and bymoviruses, respectively. The genomes are usually monopartite, with the exception of the bipartite genome of bymoviruses. The virions are flexuous filaments without an envelope and have a diameter of 11 to 15 nm and a length of 650 to 900 nm (250–300 and 500–600 for the bymoviruses).

Potyviruses are the largest and economically most important group of plant-infecting viruses (Shukla *et al.,* 1994; Fauquet and Mayo, 1999). Currently, the genus *Potyvirus* contains 118 definite and 82 tentative species (van Regenmortel *et al.,* 2000), which represent more than 30% of all known plant viruses. Potyviruses are transmitted by aphids in a non-persistent manner (Shukla *et al.,* 1994). Some of them can also be transmitted through the seed of some of their hosts (Johansen *et al.,* 1994). Furthermore, they are transmitted in infected plant material such as cuttings and tubers (Shukla *et al.*, 1994).

Potyvirus taxonomy

Species demarcation in the family *Potyviridae* has always been problematic (van Regenmortel *et al.,* 2000). Traditional criteria to discriminate between species and isolates are predominantly based on serology and biological criteria such as host range, cross-protection and symptomatology (Shukla *et al.,* 1994). Current approaches have adopted the use of the genomic composition of the virus, mainly the coat protein (CP) and the 3' non-translated region (NTR) sequences (van Regenmortel *et al.,* 2000).

Members of the potyvirus group tend to have a narrow host range, confined to closely related genera. However, *Potato virus Y* (PVY), the type species of the genus can infect species within Chenopodiaceae, Comelinaceae and Solanaceae families. Members with even a more extensive host range exist, such as *Lettuce mosaic virus* (LMV) and *Bean yellow mosaic virus* that can infect plant species of eight and thirteen different families, respectively. Although host range assays and symptomatology are valuable tools in the initial description of viruses, conflicting results have been reported presumably due to differences in the environmental conditions affecting test plant growth or the use of different genotypes of plant species (Bos, 1999).

The use of antibodies raised against the viral CP (serology) is widely applied for detection of plant viruses. It has also been used to identify new viruses and to estimate how closely related they are as compared to the previously described viruses. When dealing with potyviruses, the use of serological methods has sometimes been hampered by the serological relationships between virus species (cross-reactions) or the lack of detection of all strains of a given virus. Cross-reactions are probably due to the recognition by antibodies of the highly conserved core of the CP. Lack of detection of some strains is probably due to the variability of the N-terminus of the CP, which also may be lost due to proteolysis during virus extraction from plant tissues (Shukla *et al.,* 1994).

Coat protein amino acid (aa) sequence comparisons are increasingly used as a criterion for virus classification. Three patterns of CP aa identities can be found: different potyvirus species have identities that range from 55 to 75%; closely related species have identities of 74 to 88%, and isolates of the same species have identities ranging from 90 to 99% (Shukla *et al.,* 1994). Beside the CP aa sequence, the nucleotide (nt) sequence of the 3'NTR can also be used as a marker for genetic relatedness. Isolates of the same species show a high degree of nt identity (83-99%), whereas the identities between different species are rather low (39-53%) (Lain *et* al., 1988; Frenkle *et al.,* 1989; Turpen, 1989; Bousalem *et al.,* 2000). Thus, the CP and the 3'NTR sequences have in many cases been very informative in determination of the taxonomic position of a virus isolate.

Cross-protection is a phenomenon in which systemic infection of a plant with one virus, "the protector", prevents subsequent infection with another virus, "the challenger" (McKinney, 1929). Cross-protection has been used as a criterion to indicate the relatedness of viruses including members of the genus *Potyvirus* (Bos, 1999; Matthews, 1991; Shukla *et al.,* 1994; Fribourg and Nakashima, 1984). Several mechanisms have been proposed to be involved in this phenomenon such as the competition for the replication sites in a cell (Bawden and Kassanis, 1945) and the hybridization of the protecting RNA with the challenging RNA (Palukaitis and Zaitlin, 1984). It has been demonstrated that the cross-protection conferred by *Tobacco mosaic virus* (TMV, genus *Tobamovirus*) can be overcome by challenge inoculation with infectious uncoated RNA (Sherwood and Fulton, 1982), and that a TMV mutant defective in the ability to produce CP did not confer protection against challenge inoculations (Sherwood, 1987). These data, combined with the findings that CP-RNA interactions are required for conferring cross-protection in TMV (Lu *et al.,* 1998), suggest that cross-protection is a result of the CP from the protecting virus inhibiting disassembly of the challenging virus as previously suggested (De Zoeten and Fulton, 1975; Sherwood and Fulton, 1982). Furthermore, studies on *Potato virus A* (PVA) isolates show that CP nt sequences over approximately 88% seem to be required for cross-protection in tobacco plants (Valkonen *et al.,* 2002), indicating that a high nucleotide sequence identity between the "protector" and the "challenger" are most probably required. This is supported by the findings that viruses, which normally co-infect a given host, do cross-protect when identical RNA sequences are introduced to the viral genome (Ratcliff *et al.,* 1999). Thus, RNA silencing, a mechanism by which cells recognize double-stranded RNA and activate specific degradation of it, seems to be involved in cross-protection, e.g., between potyvirus isolates (Valkonen *et al*., 2002).

Genome structure of potyviruses

The potyviral genome consists of a single-stranded (ss) positive-sense RNA molecule of about 10 kilobases. It is encapsidated by approximately 2000 copies of the CP (Matthews, 1991). The genome contains a single open reading frame (ORF), flanked by a NTR at both (5' and 3') ends (Fig. 1). The ORF encodes a large polyprotein (3000 to 3500 aa) that is co- and/or post-translationally cleaved by three viral-encoded proteinases (P1, HC-Pro and NIa-Pro) (Riechmann *et al*.,

1992). The P1 and HC-Pro mediate their own cleavage from the polyprotein (Carrington *et al*., 1989a; Verchot *et al*., 1991) whereas the NIa-Pro is responsible for the cleavage of the C-terminal two-thirds of the polyprotein (Dougherty and Carrington, 1988). As a result of the proteolytical events, ten mature proteins are produced (Table 1).

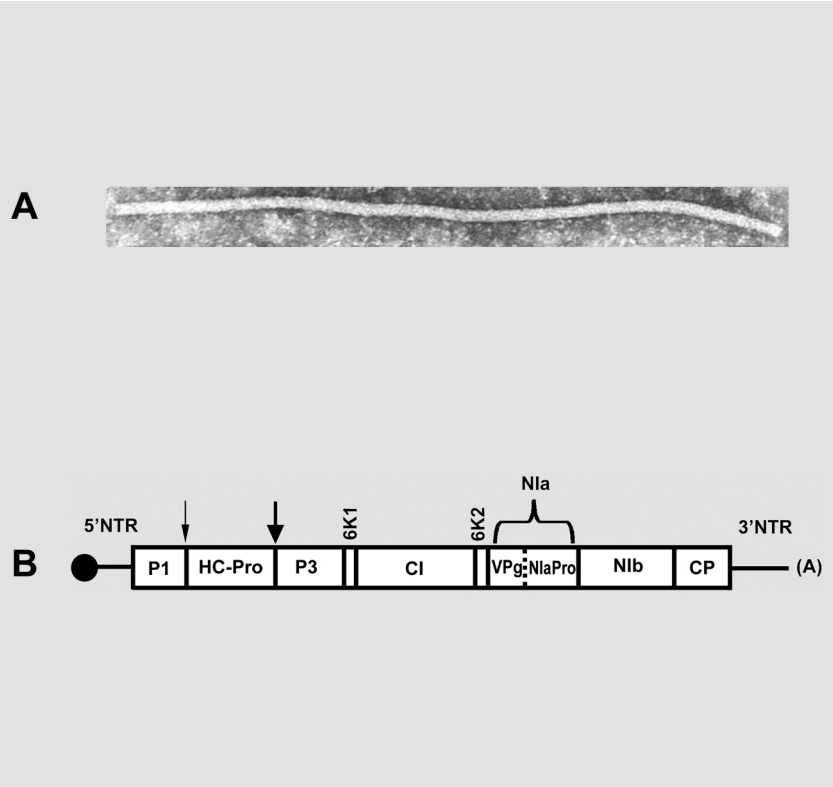

Figure 1. Particle morphology (A) and genome organization (B) of potyviruses. The proteinase cleavage sites in the polyprotein are presented by vertical lines. The P1 and HC-Pro cleavage sites are indicated by thin and thick arrows, respectively. Other cleavage sites are processed by the NIa-Pro. Horizontal lines at both ends indicate non-translated regions (NTR) of which the 3'NTR terminates with a poly (A) tail. The VPg, presented as a black circle is covalently attached to the 5'end of the viral genome. The mature potyviral proteins are: the first protein (P1), helper component proteinase (HC-Pro); the third protein (P3), 6 kDa protein 1 (6K1), cylindrical inclusion protein (CI), 6 kDa protein 2 (6K2), nuclear inclusion protein a (NIa) [which is further processed into the viral genome-linked protein (VPg) and the main viral proteinase (NIaPro)], Nuclear inclusion protein b (NIb) and the coat protein (CP). The known functions of the various proteins are presented in Table 1.

Table 1. Known functions of the mature potyviral proteins*

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

* All coding regions as well as the 5' and 3' NTR are essential for virus propagation (Klein *et al*., 1994; Kekarainen *et al*., 2002). Most viral proteins bind RNA in a sequence-unspecific manner (Merits *et al*., 1998). Genomic regions that are involved in symptom induction are described in the text. References: 1) Anandalakshmi *et al*., 1998; 2) Atreya *et al.,* 1992; 3) Atreya *et al.,* 1990; 4) Brantley *et al.,* 1993; 5) Brigneti *et al.,* 1998; 6) Carrington *et al.,* 1989a; 7) Carrington *et al.,* 1990; 8) Carrington *et al.,* 1989b; 9) Carrington *et al.,* 1998; 10) Dolja *et al.,* 1994; 11) Dolja *et al.,* 1995; 12) Dougherty *et al.,* 1989; 13) Eagles *et al.,* 1994; 14) Hong *et al.,* 1996; 15) Jenner *et al.,* 2000; 16) Kasschau and Carrington, 1998; 17) Klein *et al.,* 1994; 18) Lain *et al.,* 1990; 19) Leonard *et al.,* 2000; 20) Mahajan *et al.,* 1996; 21) Merits *et al.,* 1999; 22) Merits *et al.,* 1998; 23) Nicolas *et al.,* 1997; 24) Pirone *et al.,* 1984; 25) Rajamäki *et al.,* 1999; 26) Restrepo-Hartwig *et al.,* 1994; 27) Riechmann *et al*., 1992; 28) Rodriguez-Cerezo *et al.,* 1993; 29) Rojas *et al.,* 1997; 30) Schaad *et al.,* 1996; 31) Shukla *et al.,* 1989; 32) Verchot and Carrington, 1995; 33) Wittmann *et al.,* 1997.

Virus Propagation

The infection cycle of a potyvirus begins when the viral particle enters the cell via a wound (or during feeding by its vector aphid). Once inside the cell, the viral particle is disassembled, releasing its RNA to the cytoplasm. The potyviral RNA is subsequently translated by the host ribosomes. For most eukaryotic cellular mRNAs the process of translation is initiated by the recognition of the 5'cap structure (m7GpppN) by the eukaryotic initiation factor eIF4E [eIF4E and eIF(iso)4E in plants] (Browning, 1996; Bailey-Serres, 1999). In contrast to the cap structure present in cellular mRNA, potyviruses contain a viral-encoded protein (VPg) covalently linked to the 5'NTR. However, it has been shown that an intact VPg at the 5'end of the potyviral genome is not required for achieving infection (Riechmann *et al.,* 1989). Thus, it has been proposed that the initiation of translation of potyviruses might be similar to the one present in some other members of the *Picornaviridae* family, where the ribosome recruitment is mediated by stable RNA secondary structures (internal ribosome entry sites, IRES) in the 5'NTR (Pelletier and Sonenberg, 1988). Although no IRES-like structure has been found in potyviruses, cap-independent regulatory elements (CIREs) that promote cap independent translation have been identified in *Tobacco etch virus* (TEV), indicating that this region can regulate the translation efficiency (Niepel and Gallie, 1999).

Recent studies indicate that the VPg might actually be involved in the initiation of translation. *Arabidopsis thaliana* is a susceptible host for *Turnip mosaic virus* (TuMV) and TEV. Furthermore, the VPg of these two viruses has been shown to interact with the eIF(iso)4E factor of this host (Wittmann *et al*., 1997; Lellis *et al*., 2002). Arabidopsis mutants in which the expression of the eIF(iso)4E factor was compromised lost susceptibility to TuMV and TEV (Lellis *et al*., 2002). Consequently, it has been suggested that the role of the VPg in the initiation of translation could be comparable to that of 5'cap structure, serving as an assembly site for the translation initiation factor complex and subsequent recruitment of 40S ribosomal subunits (Lellis *et al*., 2002).

Similar to other positive-sense ssRNA viruses, potyviruses replicate through a complementary negative strand RNA molecule generated by the viral-encoded RNA-dependent RNA polymerase (RdRp); the nuclear inclusion protein b (NIb). Generation of the negative strand RNA molecule is mediated by the recognition of the 3'end of the positive strand RNA molecule by the RdRp, which probably requires the presence of specific secondary structures in the 3'NTR (Teycheney *et al.,* 2000; Haldeman-Cahill *et al.,* 1998). Once the negative strand RNA molecule has been synthesized, it is used as a template for the generation of subsequent positive-stranded RNA molecules.

Viral replication occurs in tight association with cellular membranes (Schaad *et al.,* 1997; Bienz, 1992). Various viral-encoded proteins are involved. The RNA polymerase activity of the NIb (Hong and Hunt, 1996) as well as the helicase activity of the CI (Laín *et al.,* 1990) indicate that these proteins are involved in the replication of potyviruses. Also, the *cis*-replicative function of the NIa (Murphy *et*

al., 1996; Schaad *et al.,* 1996) and the physical interactions of the NIa and NIb (Fellers *et al.,* 1998; Hong *et al.,* 1995; Li *et al.,* 1997) indicate that the NIa protein is also involved in viral replication, probably recruiting the NIb to the site of replication through protein-protein interactions (Daròs *et al.,* 1999). Furthermore, studies on the 6K2 protein of TEV suggest that the 6K2 protein forms part of the replication complex, most likely anchoring the replication complex to cellular membranes (Restrepo-Hartwig and Carrington, 1994; Schaad *et al.,* 1997).

Viral movement

To infect systemically a given host, the virus must move between adjacent cells through the plasmodesmata (cell-to-cell movement) and in the vascular tissue via the phloem (long-distance movement). Cell-to-cell as well as long-distance movement require specific interactions between viral movement proteins (MP) and host factors.

Prior to cell-to-cell movement, the virus must be transported from the site of replication to the plasmodesmata. Intracellular trafficking of cellular proteins and mRNAs is facilitated by their interactions with cytoskeletal elements such as microtubules and microfilaments (Langford, 1995; St Johnston, 1995). Thus, it is conceivable that viruses exploit the normal intracellular trafficking system of the host. For example, experimental evidence suggests that the TMV MP may facilitate intracellular movement of viral RNA through its interactions with microtubules and plasmodesmata. (Heinlein *et al*., 1995; McLean *et al*., 1995; Boyko *et al*., 2000).

Several potyviral proteins such as the CP and the CI have been proposed to be involved in cell-to-cell movement. Mutations within these proteins altered cell-to-cell movement without compromising replication of TEV (Dolja *et al.,* 1994; Carrington *et al.,* 1998). Furthermore, subcellular localization of these proteins in TEV-infected *Nicotiana tabacum* (tobacco) plants showed that both proteins are found in close proximity to the plasmodesmata (Rodriguez-Cerezo *et al.,* 1997). They might increase the size exclusion limit of plasmodesmata as shown for CP and HC-Pro of *Bean common mosaic necrosis virus* (BCMNV) and LMV (Rojas *et al.,* 1997). In addition, recent studies suggest that phosphorylation of viral MPs by host protein kinases (PK) can regulate intercellular trafficking of viruses (reviewed by Lee and Lucas, 2001). In the case of PVA it has been shown that the PKs of tobacco and a wild potato species (*Solanum commersonnii*) can differentially recognize the VPg protein, which correlates with the different abilities of this protein to support viral movement and accumulation of PVA in these two hosts (Puustinen *et al*., 2002; Rajamäki and Valkonen, 2002).

Long-distance movement of the virus means that the virus is able to move from the initially infected cell into the vascular tissue, where it is transported through the sieve elements (SE) to other parts of the plant. The study of long-distance movement is not as advanced as the cell-to-cell movement due to the complexity of the various cellular structures involved. Several potyviral proteins are involved in long-distance movement. The central region of the HC-Pro is essential for long-

distance movement, whereas it only debilitates mildly the cell-to-cell movement of TEV (Cronin *et al.,* 1995). This deficiency was complemented in *trans* by transgenic plants expressing the P1 and HC-Pro of TEV (Cronin *et al.,* 1995). Grafting experiments demonstrated that the HC-Pro must be present in inoculated as well as non-inoculated leaves in order for the virus to achieve systemic infection (Kasschau *et al.,* 1997). The CP is also needed for long-distance movement which is demonstrated by the fact that the core domain of the CP is indispensable for cell-to-cell movement while both the N- as well as the C-termini are required for the vascular transport of TEV (Dolja *et al.,* 1994, Dolja *et al.,* 1995).

Further evidence indicates that the 6K2 and VPg are involved in long-distance movement. PVA Isolates M and B11 infect systemically tobacco plants (Rajamäki *et al.,* 1998). Isolate B11 is able to infect systemically *Nicandra physaloides*, whereas isolate M is confined to the inoculated leaves. Substitution of the 6K2-VPg encoding region of isolate B11 with the corresponding one from isolate M affected systemic infection in *N. physaloides*, although no difference was observed in the inoculated leaves as compared to the wild type B11 (Rajamäki and Valkonen, 1999). Furthermore, mutational studies on this chimeric construct revealed that specific aa in the VPg and 6K2 proteins are determinants for systemic infection in *N. physaloides* (Rajamäki and Valkonen, 1999).

Host factors play a crucial role in viral movement. For example, TEV isolate Oxnard is able to systemically infect *N. tabacum* cv. V20, whereas isolate HAT is deficient in vascular movement in this host. However, substitution of the VPg-encoding region of TEV-HAT with the corresponding region of TEV-Oxnard alleviated restricted long-distance movement in this host resulting in a systemic spread of the virus (Schaad *et al*., 1997b). In addition, substitution of four aa in the VPg of *Tobacco vein mottling virus* (TVMV) isolate WT with those present in the corresponding region of isolate S restored deficiency of cell-to-cell movement in *N. tabacum* cv. TN 86 enabling the virus to systemically infect this host (Nicolas *et al*., 1997). Both findings indicate that host factors are involved in long-distance and cell-to-cell movement of viruses.

Symptom induction

Viral infections can result in the induction of a phenotypic response (symptoms), which in turn might lead to great economic loss due to the damage they cause in the crop they infect (Matthews, 1991). It has been estimated that the economic losses in the UK due to infection of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) by *Barley yellow dwarf virus* (genus *Luteovirus*) can be up to 5 million pounds per year (Hull and Davis, 1992). Furthermore, infection of citrus trees by *Citrus tristeza virus* (genus *Closterovirus*) in different parts of the world has been reported to cause losses of up to 24 million pounds per year (Hull and Davis, 1992).

Induction of symptoms is due to the direct or indirect interaction of the virus and the host. Several regions of the potyvirus genome have been shown to be involved in the generation of symptoms. The P3-6K1 region of *Plum pox virus* (PPV) is

involved in the severity of chlorotic mottle symptoms in *Nicotiana clevelandii* (Riechmann *et al.,* 1995) as well as in the ability to induce different symptoms in *Pisum sativum* (Sáenz *et al.,* 2000). In addition, studies on chimeric constructs between a nonwilting TEV strain (TEV-NW) and a wilting strain (TEV-HAT) indicated that two separate regions (the 3'end of the P3 and a fragment encompassing the CI-6K2-VPg) are necessary for the nonwilting response of TEV-NW in Tabasco pepper (Chu *et al.,* 1997). The region encompassing the NIa-NIb of *Pea seed-borne mosaic virus* (PSbMV) is involved in the induction of vein clearing symptoms in *P. sativum*, where the lack of symptoms is not correlated with low levels of viral accumulation, but probably due to an interference with host gene regulation (Johansen *et al.,* 1996). Several mutations in the HC-Pro of TVMV have an impact on the vein clearing symptoms generated in *N. tabacum*, although these mutations also affected the virus accumulation in the infected plants (Atreya *et al.,* 1992).

Non-coding regions also seem to play a role in symptom induction. In the case of TVMV, a segment of four adjacent direct repeats of a 14-nucleotide sequence in the 3'NTR was directly involved in the attenuation of typical TVMV symptoms (vein mottling and blotch symptoms) in *N. tabacum* without affecting the amount of virus produced in infected plants (Rodríguez-Cerezo *et al.,* 1991). Also, a deletion mutant lacking nucleotides 127 through 145 of the 5'NTR of PPV induced a milder chlorotic mottle than the wild type virus in *N. clevelandii*. No significant difference was observed in western analysis indicating that the mutated virus accumulates to levels similar to those of the wild type virus (Simón-Buela *et al.,* 1997).

The precise way by which viral proteins are involved in the development of symptoms is not fully known. However, experimental evidence suggests that the association of viral protein with cellular membranes might result in a phenotypic response. For example, the PVY CP has been found to associate with thylakoid membranes of chloroplasts (Gunasinghe and Berger, 1991) and transgenic *N. tabacum* plants expressing the PVY CP developed chlorosis and mosaic symptoms similar to the ones induced by PVY infection (Naderi and Berger, 1997). In the case of TMV, there is a positive correlation between the concentration of CPs associated to chloroplasts and the severity of chlorosis produced in *N. tabacum* (Reinero and Beachy, 1989). Thus, it has been proposed that the association of viral CPs with membranes in chloroplasts may contribute to the development of chlorosis, probably by disrupting functions required for chloroplast maintenance and/or development (Naderi and Berger, 1997; Culver 2002).

Variability of plant RNA viruses

For a virus to infect a given host, it must be able to spread from the initial infection foci to the rest of the plant. Evidently, this implies that the diverse viral processes such as replication, cell-to-cell and long-distance movement must be accomplished. These processes are mediated by viral-encoded proteins which require specific interactions with the host. Alterations within these proteins that

result in incompatible interaction with the host are deleterious. Consequently, the genetic composition of a virus is constrained by its host. However, a virus species is not constituted by a unique genomic sequence, but rather by a pool of variants and mutants "termed quasispecies" (Eigen, 1996) which are centered on a master sequence (Roossinck, 1997). Notably, the existence of genetic variants within a virus population increases the probability of survival and the ability of viruses to adapt to different hosts. Therefore, variability is one of the major elements of evolution. The process of evolution and/or adaptation is mainly based on mutations, recombination, reassortment and the interactions with the host.

Mutations, i.e. nucleotide substitutions within the viral genome, are common. RNA viruses rely on their own RdRp rather than the host polymerase for viral RNA synthesis. The mutation rates of RNA viruses are high, which is thought to be due to the low efficiency or absence of proofreading activity of the viral RdRp (Steinhauer *et al.,* 1992). It has been estimated that the error rates of the viral RdRp are approximately 10^{-4} (Domingo and Holland 1997), whereas the error rates of cellular DNA-dependent RNA polymerases are much lower (about 10^{-9}) (Roossinck, 1997).

Genetic exchange (reassortment) among viruses with segmented genomes has been previously documented (Fraile *et al.,* 1997; White *et al.,* 1995; Webster *et al.,* 1995). Within the plant viruses studied, it is thought that the broad host range and worldwide distribution of *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) is in part due to reassortment events, contributing to its enormous evolutionary success (Roossinck, 2002). Recombination events, which involve the exchange of genetic material between two nearly identical RNAs (homologous recombination) or between two different RNAs (non-homologous recombination), have also been reported in various plant viruses (Chenault and Melcher, 1994; Moonan *et al.,* 2000; Padidam *et al.,* 1999). Evidence of recombination has been reported in some members of the genus *Potyvirus*, including PPV (Cervera *et al.,* 1993) and *Yam mosaic virus* (YMV) (Bousalem *et al.,* 2000). In the case of TuMV, it has been suggested that recombination events that occurred in one host may have enabled some recombinants to infect new hosts (Ohshima *et al.,* 2002).

Many studies have focused on the genetic variability among isolates of the same species (intra-species variability). These studies indicate that certain genomic regions of the potyviral genome are flexible, permitting a high degree of molecular diversity. Examples include parts of the 5'NTR, P1 protein and the N-terminus of the CP (Aleman-Verdaguer *et al.,* 1997; Kekarainen *et al.,* 1999; Tordo *et al.,* 1995; Wisler *et al.,* 1995). Other genomic regions are conserved, such as the CI, NIa and NIb (Kekarainen *et al.,* 1999; Fuji and Nakamae, 2000) indicating that the variability differs according to the region analyzed.

As mentioned previously, a pool of genetic variants around a master sequence constitutes a viral population. However, the size of this population is not unlimited and it is usually constrained by various factors. For example, Schneider and Roossinck (2000) showed that the percentage of mutants after a single passage in *N. benthamiana* was higher for CMV (43%) than for TMV (29%), remaining

constant during serial propagations. CMV accumulated to lower levels than TMV in this host, indicating that the difference in the genetic diversity was not due to difference in the degree of viral replication (Schneider and Roossinck, 2000). Thus, the genetic diversity can vary among different virus species infecting a common host. In addition, it has also been shown that the genetic diversity of a virus can differ among different host species. For example, the percentage of CMV mutants found in *N. benthamiana* was lower than that found in *N. tabacum* (43% vs. 70%, respectively). Furthermore, TMV showed a higher percentage of mutations in *N. tabacum* (46%) than in *N. benthamiana* (29%) (Schneider and Roossinck, 2001). These findings indicate that the host plays a crucial role in the generation, but also limitation of viral diversity.

Vectors can also influence the genetic structure of a viral population. Experimental evidence indicates that there is a preferential selection of certain variants within a viral population for vector transmission (Perry and Francki, 1992; Suga *et al*., 1995; Hall *et al*., 2001). This vector-based selection (genetic bottleneck), results in propagation of a limited number of variants, which in turn decreases the genetic variability of the virus in the newly infected host (genetic drift). A particularly interesting consequence of bottlenecks is the founder effect (defined as the establishment of a new population by a small number of genetic variants from the mother population), which has been attributed to the relatively small genetic variability of *Zucchini yellow mosaic virus* (ZYMV) and *Tomato yellow curl Sardinia virus* (genus *Begomovirus*) in the island of Martinique and southern Spain, respectively (Desbiez *et al*., 1996; Sanchez-Campos *et al*., 2002).

Potyviruses infecting solanaceous crops

The plant family Solanaceae is compromised by ca 75 genera with more than 2,000 species. It is thought to have originated approximately 65 million years ago, having its major center of diversification in Central and South America (D'Arcy, 1991; Symon, 1991). Many species of Solanaceae are of high economic importance. Among these, the potato (*Solanum tuberosum*), is currently the fourth most important food crop in the world with an annual production of about 300 million tons (FAO, 2002). Other species belonging to the Solanaceae family with economic importance as edible crops are tomato (*Lycopersicon esculentum*) and pepper (*Capsicum* spp.). Occurrence of potyviruses in these crops is common (Table 2), resulting in great economic losses. Thus, the study of potyviruses infecting these crops is of great importance.

PVY is the most important and best-described potato-infecting potyvirus. It has a worldwide distribution, occurring in many potato cultivars and causes up to 80% yield reduction (Hooker, 1981). PVY is also a very important pathogen in pepper and tomato (Marte *et al.,* 1991; Zitter, 1993) causing up to 50% of yield loss (Thomas and McGrath, 1988). Potato isolates of PVY have been originally subdivided into three strain groups (De Bokx and Huttinga, 1981). The common strain (PVY^O) as well as the C strain (PVY^C) can be distinguished by hypersensitive response (HR) induced in standard potato cultivars carrying the *Ny* and *Nc* resistance genes, respectively (Jones, 1990; Valkonen *et al.,* 1996),

whereas the N strain (PVY^N) is characterized by induction of veinal necrosis in tobacco plants (De Bokx and Huttinga, 1981). PVY^N isolates that are involved in the potato tuber necrotic disease (PTNRD) have been assigned to the PVY^{NTN} subgroup (Le Romancer *et al.,* 1994). Biological as well as molecular characterization has been extensively done on various PVY isolates (Blanco-Urgoiti *et al.,* 1998; Boonham *et al.,* 1999; Tordo *et al.,* 1995; Robaglia *et al.,* 1989).

PVA is widely distributed in potato-growing areas (Hooker, 1981) and can decrease the yield by up to 40% (Bartels, 1971). PVA is uncommon in other solanaceous crops. However, it has been found in tamarillo (*Solanum betaceae*) plants (Bryan *et al*., 1992). Based on the symptoms induced in the potato cultivar King Edward, four different strain groups have been identified. PVA strain group 1 triggers a HR conferred by the Na_{ke} resistance gene, whereas members of the strain group 2 infect this cultivar without generating necrosis (Valkonen *et al*., 1995). Strain group 4 members cause stunning and yellowing in "King Edward" (Rajamäki *et al.,* 1998). Members of the strain group 3 do not infect systemically this potato cultivar (Valkonen *et al.,* 1995). The complete nucleotide sequence of isolates belonging to the strain groups 1, 2 and 3 (Puurand *et al.,* 1994; Kekarainen *et al.,* 1999) as well as the nucleotide sequence of the CP and 3'NTR region of strain group 4 isolates have been determined (Rajamäki *et al.,* 1998). Furthermore, a comprehensive biological and molecular characterization of 21 different isolates belonging to the four strain groups has been carried out (Rajamäki *et al.,* 1998).

Potato virus V (PVV) (Fribourg and Nakashima, 1984) is known to occur only in a limited number of potato cultivars grown in the Andean region of South America and Western Europe (Jeffries, 1998). No estimates on the yield reduction caused by PVV have been reported, although it has been suggested that they are similar to the ones caused by PVA (Oruetxebarria, 2001). PVV can be distinguished from PVY and PVA because it does not activate the genes for HR to PVA and PVY, but activates other PVV-specific (*Nv*) HR genes (Fribourg and Nakashima, 1984; Jones, 1990; Valkonen *et al.,* 1996). Furthermore, PVV has a narrower host range than PVY and PVA and does not cross-protect against them (Fribourg and Nakashima, 1984). No natural host other than potato has been reported for PVV. PVV has not been characterized at the molecular level and no strains have been described. However, serological relationship of PVV with PVY and two other potyviruses, *Wild potato mosaic virus* (WPMV) (Jones and Fribourg, 1979) and *Peru tomato virus* (PTV) (Fribourg, 1979; Fernandez-Northcote and Fulton, 1980), has been reported (Fribourg and Nakashima, 1984; Adam *et al.,* 1995).

Table 2. Potyviruses that have been reported in some solanaceous crops

Virus	Crop Species[a]				Reference
	Pepper	Potato	Tomato	Tobacco[b]	
Chilli vein-banding mottle virus [c] (CVbMV)	X			X	3
Chilli veinal mottle virus (ChiVMV)	X			X	15
Eggplant green mosaic virus (EGMV)			X	X	12
Eggplant severe mottle virus[c] (ESMV)			X	X	12
Indian pepper mottle virus (IPMV)	X			X	17
Pepper mild mosaic virus[c] (PMMsV, PMMV)	X			X	13
Pepper mottle virus (PepMoV)	X			X	14
Pepper severe mosaic virus (PepSMV)	X			X	4
Pepper vein banding virus [c] (PVBV)	X				11
Pepper veinal mottle virus (PVMV)	X		X	X	2
Pepper yellow mosaic virus (PepYMV)	X			X	9
Peru tomato virus (PTV)	X		X	X	5, 6
Potato virus A (PVA)		X		X	1
Potato virus V (PVV)		X		X	7
Potato virus Y (PVY)	X	X	X	X	8, 10, 18
Tobacco etch virus (TEV)	X		X	X	16

[a] Eggplant (*Solanum melongena*) and tamarillo (*Solanum betaceae*) are two additional crops that are commonly infected by potyviruses (Eagle and Gardner, 1994; Ladipo *et al*., 1988)
[b] Indicates viruses that are known to experimentally and/or naturally infect tobacco
[c] Viruses not recognized as species by ICTV
Reference: 1) Bartels, 1971; 2) Brunt and Kenten, 1971; 3) Chiemsombat *et al*., 1998; 4) Feldman and Garcia, 1977; 5) Fernandez-Northcote and Fulton, 1980; 6) Fribourg, 1979; 7) Fribourg and Nakashima, 1984; 8) Hooker, 1981; 9) Inoue-Nagata *et al*., 2002; 10) Jones *et al*., 1997; 11) Joseph and Savithri, 1999; 12) Ladipo *et al*., 1988; 13) Ladera *et al*., 1982; 14) Nelson and Wheeler, 1978; 15) Ong *et al*., 1979; 16) Purcifull and Hiebert, 1982; 17) Sandhu and Chohan, 1979; 18) Simons, 1956.

Aims of the Study

This study is part of a larger research program in the SLU Plant Virology Group that aims to study the variability in the genus *Potyvirus* as well as the molecular interactions between potyviruses and their host plants. This thesis focuses on the study of three potyviruses naturally occurring in cultivated and wild species of the Solanaceae (PTV, PVV and WPMV) and on the 6K2 protein of PVA.

The specific aims of this study were:

- To characterize the genomes of PVV, WPMV and PTV and study some biological properties of the viruses
- To define the relationship between WPMV, PVV and PTV and other potyviruses
- Elucidate the variability of PVV and PTV
- Study the role of the PVA 6K2 protein as a host range and virulence determinant in PVA

Results and Discussion

Previous to these studies only a limited number of studies on PVV, PTV and WPMV had been carried out. Little or no sequence data was publicly available for these viruses. In this study the complete genomic sequences of PVV, PTV and WPMV were determined. Various isolates of PVV and PTV were characterized at a biological and molecular level and strain groups were identified. These data provided novel information on the variability of PVV and PTV. Furthermore, the PVA 6K2 protein was studied as an example of a potyviral protein that plays a role in host-specificity and virulence, factors that are driving forces in virus speciation which gain its basic potential from the variability born during virus replication and subsequent selection of competent genome variants in different hosts.

Molecular and biological characterization of PVV, WPMV and PTV (I, II, III)

PVV

In order to characterize PVV at the molecular level, we sequenced the complete genome of the European isolate Dv-42 (**I**), previously identified as PVV (Valkonen, 1997). Sequence analysis revealed that the length of the single stranded RNA genome of PVV isolate Dv-42 was 9848 nt excluding the poly (A) tail. A single ORF, beginning at nucleotide 205 and ending at nucleotide 9406, was found to encode a putative polyprotein of 3066 aa. The length of the 5'NTR and the 3'NTR were 204 nt and 446 nt, respectively.

The polyprotein aa sequence of PVV was compared to some other potyviruses that are commonly found in solanaceous crops, such as PVY, PVA and *Pepper mottle virus* (PepMoV). PVV shared the highest similarity with PepMoV (64.2%) and some PVY isolates (~ 58%), whereas it only shared 44% similarity with PVA. At the CP aa level, similarities were within a similar range (67.5 % to PepMoV, ~65% to the different PVY isolates and 52.8 % to PVA). Phylogenetic analysis also indicated that PVV was closely related to PVY and PepMoV. Thus, our molecular characterization combined with the previous biological characterization of PVV (Fribourg and Nakashima, 1984) clearly demonstrated that PVV is a typical species of the genus *Potyvirus*.

In order to study the variability of PVV, seven European isolates collected during the last 35 years were analyzed (**I**). No biological or serological differences were observed among the PVV isolates. Furthermore, sequence comparisons of the 5'NTR and 3'NTR (nt) and the P1 and CP (nt and aa) revealed values of identities over 94% (**I**). Thus, in contrast to other members of the genus *Potyvirus*, such as PVA (Kekarainen *et al.,* 1999) and YMV (Aleman-Verdaguer *et al.,* 1997), the variability within the European PVV isolates was low. Hence, the low molecular variability as well as the lack of biological or serological differences suggested that the European isolates of PVV probably belong to a single strain (**I**). However,

since only European isolates were analyzed, further studies on PVV isolates from other parts of the world were expected to reveal higher variability.

Subsequently, three South American isolates and one additional European isolate of PVV were obtained from potatoes grown in Peru and Sweden, respectively (**III**). They reacted with the different PVV antibodies used (**III**). However, prior to including these isolates in PVV, molecular characterization of the 5' and 3'NTR, as well as the P1 and CP sequences, were carried out, which confirmed their identity as PVV (**III**).

A higher variability within PVV was found when the South American isolates were included in our study. The sequence identities of the 5'NTR (nt level) and the P1 protein (nt and aa level) could be as low as 78.9% (in the 5'NTR) and 83.7% (in the P1) (**III**), in contrast to our previous study including only European PVV isolates (**I**) and in which the sequence identity regardless of the region analyzed was above 94%. In addition, comparisons of the genetic variability between the South American and European isolates revealed a greater variability among the South American isolates (Table 3). Phylogenetic analysis using the CP aa sequence defined two distinct clusters. All of the European isolates (including the Swedish and Finnish isolates), and one South American isolate grouped together, whereas two of the South American isolates were placed to a different group (Fig. 2A, **III**).

Table 3. Percentage of nucleotide (nt) and amino acid (aa) sequence identity among South American and European PVV isolates

Genomic[a] region		**South American PVV isolates**	**European PVV isolates**
5'NTR	nt	78.9 – 100%	94.1 - 99.5%
P1	nt	83.7 – 99.4%	96.3 - 98.8%
	aa	84.1 – 99.0%	95.8 – 99.0%
CP	nt	90.0 – 99.8%	96.4 – 98.7%
	aa	95.2 – 99.3%	96.0 – 98.5 %
3'NTR	nt	93.9 – 99.8%	96.3 – 99.6%

[a] 5'NTR and 3'NTR, 5'-terminal and 3'-terminal non-translated regions, respectively; P1, first protein; CP, coat protein.

A higher resolution of the genetic variability was obtained when the P1-encoding aa sequence was analyzed. The South American isolate (PA13) that grouped with the European isolates based on the CP aa sequence was now separated from the European isolates (Fig. 2B, **III**). In addition, phylogenetic analysis of the P1 protein (Fig. 2B, **III**) revealed systematic grouping of the isolates from the United Kingdom and the Netherlands, consistent with our previous findings (**I**). [Interestingly, the Scandinavian isolates grouped with the isolates from the United Kingdom, which is similar to a previous study where the

Finnish isolate was analyzed (Oruetxebarria and Valkonen, 2001)]. These data indicated that the analysis of the P1 protein in many instances might be necessary to fully resolve strain diversification. It is worthy to mention that although the geographic grouping of the European isolates suggested a possible adaptation to the different potato cultivars grown in the different countries (**I**), further studies revealed no evidence for a selective pressure driving the nt sequence variability in the P1 sequences (Oruetxebarria and Valkonen, 2001). Hence, it seems that the variability within the P1-encoding region of the European PVV isolates is due to a random genetic drift rather than an adaptation to their respective hosts (Oruetxebarria, 2001).

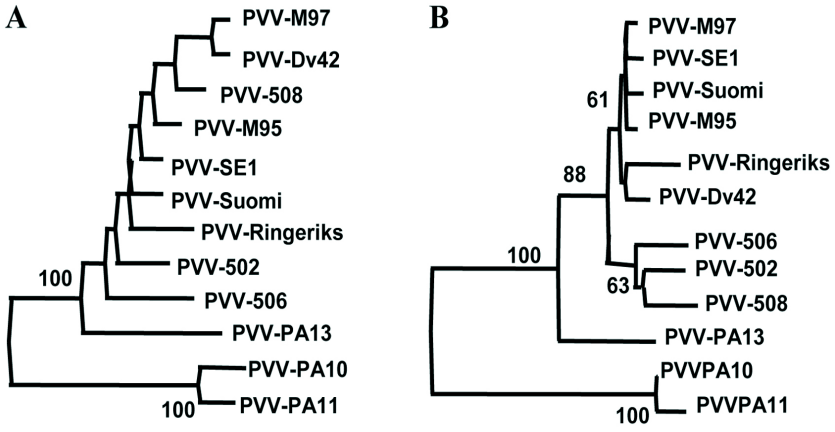

Figure 2. Phylogenetic trees based on the CP (A) and P1 protein (B) aa sequences of various PVV isolates. Bootstrap values exceeding 60 are presented. Origin of the isolates is the following: The Netherlands (502, 508, 506); United Kingdom (M95, M97, Dv42); Sweden (SE1); Finland (Suomi); Norway (Ringeriks); Peru (PA10, PA11, PA13).

Our previous biological characterization of the European isolates of PVV revealed no biological variability among them (**I**), although it was intriguing that no vein necrosis was observed in the potato hybrid A6 (*Solanum demissum* x *S. tuberosum* cv. Aquila) in contrast to what was originally described for this virus in Peru (Fribourg and Nakashima, 1984). Host plant responses were tested with two South American isolates and one European isolate (**III**). Both South American isolates induced vein necrosis in A6, while the European isolate infected systemically A6 without inducing any vein necrosis. Further differences were observed in the potato cultivar Pentland Ivory (which carries the *Nv* gene). Neither

of the South American isolates induced HR in contrast to the European isolate (Fig. 2D in **III**). These data indicated that the European and South American isolates belong to different strain groups according to strain group concept, which is based on the induction of a HR in potato cultivars that carry specific HR genes (Jones, 1990; Valkonen *et al.,* 1995).

In this study we accomplished a comprehensive molecular analysis of PVV. We determined the complete genomic sequence of one isolate and showed the existence of two different strain groups. Due to the fact that PVV is serologically related to WPMV and PTV (Fribourg and Nakashima, 1984; Adam *et al.,* 1995), and the taxonomic status of WPMV and PTV as independent species was not fully resolved (Salazar, 1995), we decided to characterize the two last mentioned viruses as well.

WPMV

When WPMV was first described (Jones and Fribourg, 1979), it was classified as a distinct potyvirus, but a possibility remained that it could be a distantly related strain of PTV or TVMV (Jones and Fribourg, 1979). Furthermore, WPMV was also placed as an intermediate species between PVV and PVY (Fribourg and Nakashima, 1984), creating doubts about the taxonomic position of this virus.

For the reasons given above, we determined the complete genomic sequence of the WPMV isolate described by Jones and Fribourg (1979) (**II**). Due to the fact that no previous sequence was publicly available, a strategy to amplify and clone the genome of WPMV was designed. This strategy used the previous knowledge on conserved amino acids motifs present in various potyviruses, based on which degenerate primers were designed. This strategy allowed us to amplify and clone different potyviruses without prior knowledge of the exact nucleotide sequence (**II, III**).

Four fragments, covering the complete genome of WPMV were amplified, cloned and sequenced (**II**). The genome of WPMV was found to consist of 9853 nucleotides excluding the poly (A) tail. A single ORF encoding for a putative polyprotein of 3065 aa starting at position 183 and ending at position 9378 was identified. The 5' and 3'NTR were 182 nt and 472 nt long, respectively.

Sequence comparison revealed that WPMV shared the highest polyprotein aa identity with PVV (77.4%) followed by PepMoV (58.9%) and PVY (52.5%). Phylogenetic analysis of the CP as well as the polyprotein aa sequence placed WPMV, PVV, PVY and PepMoV in the same group (**II**). Thus, the complete nucleotide sequence as well as the phylogenetic analysis showed that WPMV is a typical member of the genus *Potyvirus*. Furthermore, our molecular data indicate that WPMV is more closely related to PVV than to any other potyvirus from which sequence data are available. However, WPMV CP aa sequence identity with PVV (84.9%) is below the values observed among strains of the same virus (90-99%, Shukla *et al.,* 1994) and combined with the extensive host range and

serological studies previously carried out (Jones and Fribourg, 1979; Fribourg and Nakashima, 1984; Adam *et al.,* 1995) it seems justified to consider WPMV as a distinct species of the genus *Potyvirus*.

Although the WPMV isolate we characterized was the same as the one described by Jones and Fribourg (1979), we did not observe the same symptoms in *N. tabacum* as previously described. They included chlorotic blotches and necrotic broken rings, line patterns and spotting in lower systemically infected leaves and symptomless infection in the uppermost systemically infected leaves. In contrast, we observed vein clearing and mild mosaic symptoms (Fig. 3B, **II**). This difference suggests that while differences in plant growth conditions could play a role, it is also possible that the isolate might have undergone genetic changes during propagation in *N. tabacum* instead of its natural host (*Solanum chancayense*). Previous studies indicate that repeated passages of viruses by mechanical inoculation can result in symptom variants (Chen *et al*., 1994; Cabauatan *et al.,* 1995; Cabauatan *et al.,* 1999; Sugiyama *et al*., 2000). Also, it has been shown that maintenance of virus isolates by repeated mechanical inoculation rather than transmission by the natural vector may result in the loss of aphid transmissibility (Shukla 1994, Legavre *et al.,* 1996) and be associated with higher accumulation of the virus in infected tissues (Adrejeva *et al.,* 1999).

In 1989 an incidence of a viral-induced disease in tomato was reported in Germany. The initial studies carried out on this isolate suggested that it was a strain of PVV (Vetten *et al.,* 1992). However, further studies identified the virus as an isolate of WPMV rather than PVV (Adam *et al.,* 1995). Once the identity of the isolate was resolved, Adam and collaborators (1995) were able to trace back how the virus was introduced into Germany. It was concluded that WPMV had probably been introduced to Germany in pepino plants (*Solanum muricatum*) imported from South America, which were grown in close proximity of tomato plants. Thus, the study of virus isolates from wild species [such as WPMV (**II**)] is important and can be applied to predict emerging viral outbreaks in cultivated crops.

PTV

PTV has originally been described as a distinct member of the genus *Potyvirus* (Fribourg 1979; Fernandez-Northcote and Fulton, 1980). However, there is still some debate on whether PVV and PTV are different virus species (Salazar, 1995; Alvarez and Fernandez-Northcote, 1996; Stevenson *et al*., 2001). To address this issue, various criteria were used to determine the taxonomic position of PTV with respect to PVV and WPMV. The genome structure as well as the genetic variability of some PTV and PVV isolates was analyzed. Furthermore, some biological and serological properties of PVV, WPMV and PTV were studied and compared (**III**).

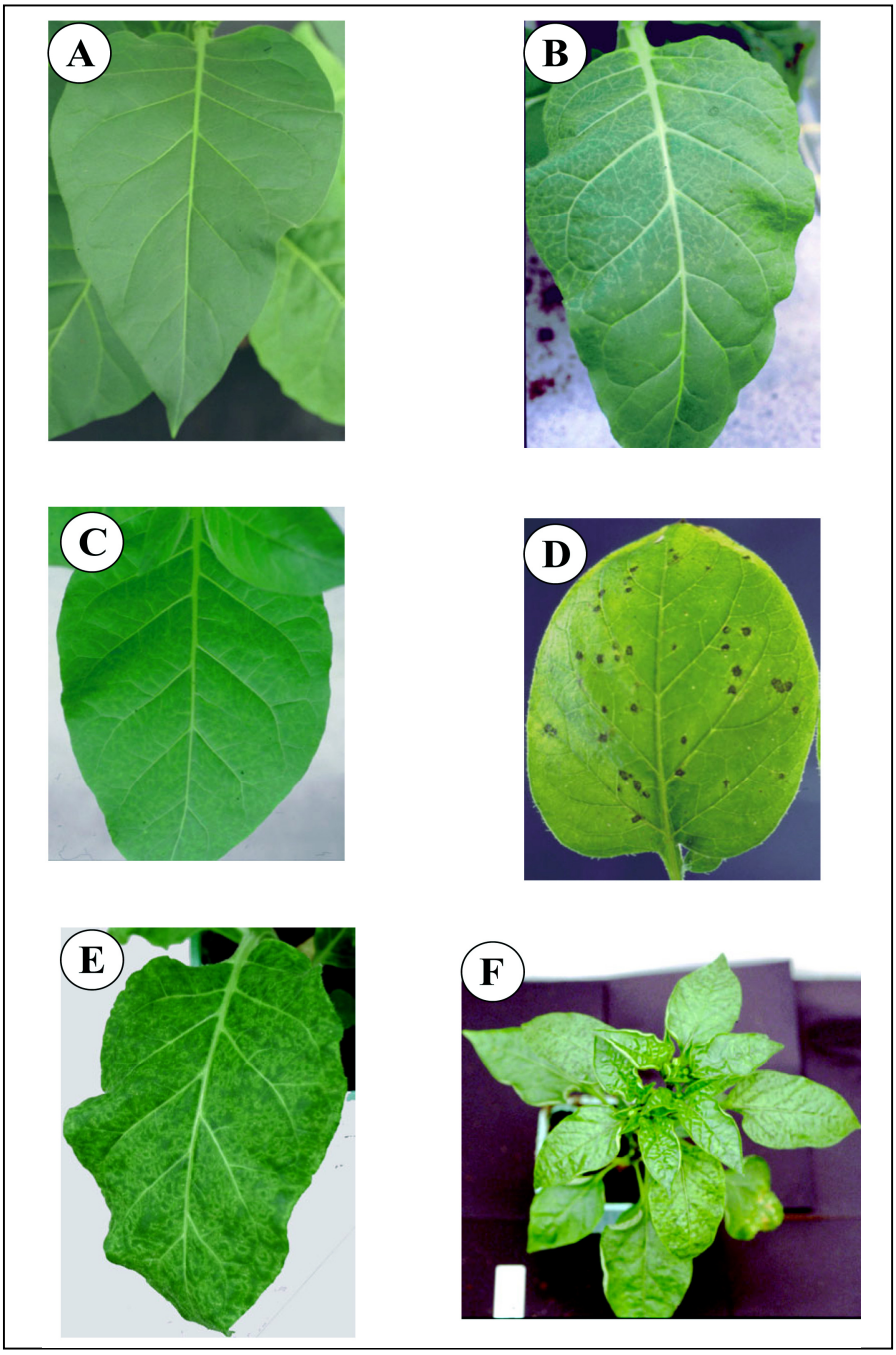

Figure 3. Some host responses to WPMV, PVV and PTV. (A) Healthy *Nicotiana tabacum*. (B) Vein clearing and mild mosaic symptoms observed in *N. tabacum* systemically infected with WPMV. (C) Mild vein clearing in *N. tabacum* and (D) localized necrotic lesions in the potato cv. Pentland Ivory induced by PVV (isolate Dv-42). (E) Severe mosaic and ringspot symptoms in *N. tabacum* and (F) severe mosaic symptoms in *Capsicum annuum* cv. RNaky as a result of systemic infection with PTV (isolate PPK13).

The complete nucleotide sequence of two PTV isolates (PPK11 and PPK13) was determined (**III**) using the same strategy as used for sequencing WPMV (**II**). Also, about 75% of the genome of two other isolates including the PTV isolate M4 described by Fribourg (1979) was determined. PTV isolates PPK11 and PPK13 were found to consist of 9892 and 9881 nucleotides, respectively, excluding the poly (A) tail. The length of the 5'NTR and 3'NTR of PPK13 were found to be 213 and 473 nt, respectively. The 5'NTR and 3'NTR of isolate PPK11 were longer, being 223 and 474 nt, respectively (**III**). However, one single ORF predicted to encode a polyprotein of 3065 aa was found in both viruses.

Sequence comparison among the PTV isolates as well as the phylogenetic analysis indicated that the isolates described in our study belong to more than one strain group (**III**). Phylogenetic analysis using the CP aa sequence clustered isolates PPK13, M4 and V2 in one group whereas isolate PPK11 was placed in a different branch (Fig. 3B in **III**). Interestingly this grouping correlated with the different symptoms observed in *N. tabacum* in which isolate PPK13 and M4 caused severe mosaic and ringspot symptoms (Fig. 3E) whereas isolated PPK11 induced a mild mosaic (**III**). Furthermore, as in the case of PVV, a higher resolution of the genetic variability was obtained when the P1 protein aa sequence was analyzed due to the separation of isolate M4 from the other isolates (Fig. 3A in **III**). Despite the fact that PTV is commonly found infecting tomatoes (Fribourg, 1979), no isolate from this host was analyzed in our study. However, molecular and biological characterizations of two tomato isolates as well as an isolate from a tropical fruit (*Solanum sessiliflorum*) grown in the Peruvian rain forest are being carried out. Whether these isolates belong to any of the PTV strain groups defined in our study (**III**) or belong to new ones is yet to be resolved.

Comparison of PTV with PVV and WPMV

Based on the comparison with previously described potyviruses, nine putative cleavage sites were found in the polyprotein of PTV (**III**). As in the case of PVV (**I**) and WPMV (**II**), most of the conserved amino acid motifs of potyviruses (Shukla *et al.,* 1994) were present in the polyprotein of PTV (**III**). For example the CCC and LAIGN motifs in the HC-Pro, which have been shown to be involved in cell-to-cell and long-distance movement (Cronin *et al.,* 1995), were present in the HC-Pro of PTV. The aphid-transmissibility motifs DAG (Atreya et al., 1995; Andrejeva *et al*., 1999) and PTK (Huet *et al*., 1994) were found in the CP and HC-Pro, respectively, in PTV (**III**). Interestingly, the KITC motif in the HC-Pro, shown to be involved in aphid transmission (Atreya and Pirone, 1993) was found as KLTC (identical to that of PVV and WPMV; **I**, **II**). It has been shown that mutations in the KITC motif can compromise aphid transmission of TVMV (Atreya and Pirone, 1993). However, the KLTC was found to be functional for aphid transmission of PVV (**I**). Thus, it seems possible that this motif is also compatible for aphid transmissibility of PTV.

Comparisons of the CP aa sequences [commonly used as genetic markers for species demarcation (Shukla *et al.,* 1994)] between PTV, PVV and WPMV were

carried out (**III**). CP aa sequence identities among the four PTV isolates (including the reference strain M4) were above 94%, whereas they shared ~ 86% and ~ 88% identity with WPMV and the various PVV isolates, respectively. In addition, the polyprotein aa sequence of PTV was compared to that of PVV and WPMV resulting in identities of ~74% (between PTV and PVV) and ~75% (between PTV and WPMV). These values were below the ones observed among different strains of potyviruses such as PSbMV (85.5%) and ZYMV (87.5%) that, on the other hand, were comparable to the identity of the two PTV isolates (88.6%) from which the complete genome was determined (**III**). Thus, although the CP aa sequence similarity might suggest that PTV is conspecific with PVV and WPMV, comparison of the entire polyprotein sequence clearly defined each of these viruses as independent species.

The serological relationships between WPMV, PTV and PVV were analyzed using PVV antibodies (**III**). DAS-ELISA results showed that the PVV PAb (polyclonal antibodies) as well as the PVV MAb (monoclonal antibody) 53/8 readily detected the PVV isolates, whereas the PTV isolates tested reacted only weakly. Neither the PVV PAb nor the PVV MAb detected WPMV. Western blot analysis using the PVV MAb confirmed the results obtained in the DAS-ELISA (**III**), showing a strong detection to the CP of PVV, weak detection to the CP of PTV and no detection of the WPMV CP (Fig. 4). These data are consistent with a previous study that indicates cross-reaction of PVV antibodies with PTV (Adam *et al.,* 1995).

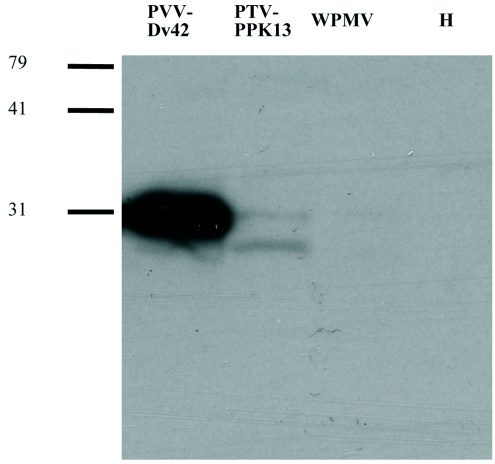

Figure 4. Western blot analysis of viral coat proteins (CP) using monoclonal antibody 53/8 prepared against PVV CP. Similar amounts of soluble protein (~) extracted from symptomatic tobacco leaves (*N. tabacum*) systemically infected with three potyviruses were analyzed. PVV-Dv42, *Potato virus V* isolate Dv42; PTV-PPK13, *Peru tomato virus* isolate PPK13; WPMV, *Wild potato mosaic virus*; H, healthy tobacco. Molecular size markers (M, in kilodaltons) are shown to the left. Strong and weak signals were observed for PVV- and PTV-infected leaves, respectively. No signal was observed for WPMV or healthy tobacco leaves. The two bands observed for PTV may be indicative of phosphorylated and non-phosphorylated forms of CP (Ivanov *et al*., 2001).

Cross-protection assays were also carried out to determine if PTV could prevent the systemic infection of PVV and WPMV. Considering the fact that strains of the same virus species usually cross-protect against each other (Matthews, 1991; Shukla *et al*., 1994; Bos, 1999), this assay could reveal a relationship of PTV with PVV or WPMV. Therefore, it was crucial to design tools to detect each of these viruses specifically in a mixed infection with others. We were able to achieve this by designing specific primer combinations that only amplified each of the corresponding viruses by reverse transcription PCR (RT-PCR). The results indicated that PTV does not provide cross-protection against WPMV or PVV (Fig. 5, **III**). These data support the taxonomic status of PTV as a distinct species. Indeed, the lack of cross-protection between PVV and PVY originally contributed to the assignment of PVV as distinct species of the genus *Potyvirus* (Fribourg and Nakashima, 1984).

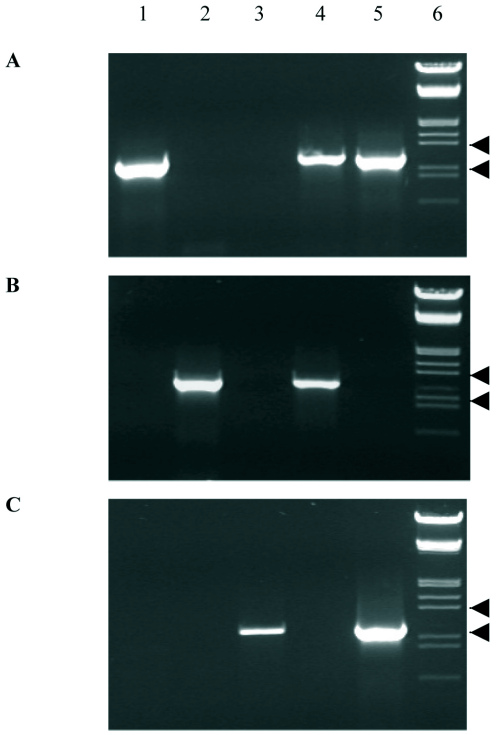

Figure 5. Virus-specific detection of *Peru tomato virus* (PTV, isolate PPK13) (A), *Potato virus V* (PVV, isolate Dv42) (B), and *Wild potato mosaic virus* (WPMV) (C) by RT-PCR. The forward and reverse primers were designed for the P1 and HC-Pro regions of the viral genome, respectively: Panel A, primers PPK13-5NF1 and PPK13-5NR1; Panel B, primers PVV-FT and PVV-RT; Panel C, primers WPMV-5NF1 and WPMV-5NR1. The same cDNA samples were amplified with the three primer pairs. Amplification products were analyzed by electrophoresis through a 1% agarose gel. Lane 1, PTV isolate PPK13; lane 2, PVV isolate Dv42; lane 3, WPMV; lane 4, leaf co-infected with PTV-PPK13 and PVV-Dv42; lane 5, leaf co-infected with PTV-PPK13 and WPMV; lane 6, lambda *Eco*RI/*Hin*dIII DNA size marker. Arrowheads in each panel indicate the positions of the 1375-bp and 947-bp markers. The nucleotide sequences of the various primers used are presented in **III**.

Host plant responses to PTV, WPMV and PVV were studied and compared (**III**). PTV was not able to infect systemically any of the potato cultivars tested, whereas systemic infection of pepper plants (*Capsicum annuum* cv. RNaky) was readily observed (Fig. 3D). In contrast, no systemic infection was detected in pepper plants inoculated with various PVV isolates (**I, III**). PVV is known to naturally occur only in some potato cultivars (Jones and Fuller, 1984; Jones, 1987; Jefffries, 1998) and no reports of PVV infecting pepper plants in the field exist to date. On the other hand, PTV is widely distributed in tomato and pepper fields grown along the Peruvian coast (Raymer *et al.,* 1972; Fribourg, 1979; Fernandez-Northcote and Fulton, 1980; Pernezny *et al.,* 2003) and has not been found to infect (experimentally) cultivated potatoes systemically (Fribourg, 1979; Fernandez-Northcote and Fulton, 1980; **III**).

While carrying out a survey on the natural occurrence of viruses in native Bolivian potato cultivars, Alvarez and Fernandez-Northcote (1996) detected the presence of PVV in seven out of fifteen native cultivars. In all cases where samples tested positive for PVV, they also tested positive for PTV. Furthermore, a higher number of samples were positive in DAS-ELISA for PTV than for PVV. Therefore, Alvarez and Fernandez-Northcote (1996) suggested that PVV should be considered as a potato-infecting strain of PTV (PTV-p). However, our studies on PVV and PTV (**I, III**) suggest that the proposed occurrence of PTV in potato might be due to a misidentification of the virus, caused by the cross-reaction of the antibodies, which could be verified by characterization of the potato isolates at a molecular level.

In this study we have applied conventional as well as more recent molecular approaches to resolve the taxonomic relationships among closely related potyviruses. Our studies indicate that a single criterion used for virus identification in many instances is not sufficient to clearly elucidate the actual causal agents of viral diseases and might, on the other hand, lead to confusions and misconceptions on the taxonomic position of potyviruses. Hence, a comprehensive study of the biological and molecular characteristics of members of the genus *Potyvirus* (as in our studies **I**, **II**, **III**) might result in reliable methods to identify and distinguish virus strains, which would be of considerable practical importance.

The "PVY group" of potyviruses

One of the most interesting outcomes of this study was obtained when the phylogenetic relationships among PTV, PVV, WPMV and other members of the genus *Potyvirus* commonly found infecting solanaceous crop plants were analyzed (**III**). The phylogenetic analysis using the polyprotein aa sequence showed that PTV, PVV and WPMV are the most closely related viruses and group with PepMoV and PVY to form a phylogenetically defined "PVY group" (Fig. 3C in **III**). Furthermore, based on the CP sequence data available, PepSMV (Rabinowicz *et al.,* 1993) and PepYMV (Inoue-Nagata *et al.,* 2002) (two potyviruses isolated from pepper in Argentina and Brazil, respectively) also belong to the phylogenetically defined "PVY group".

All isolates identified as PTV, PVV, PVY or PepMoV based on various criteria were placed in virus-specific groups, regardless of the genomic region used for the phylogenetic analysis (Fig. 3A and 3B in **III**). Therefore, this study (**III**) clearly shows that PTV, PVV, PVY, PepMoV and also WPMV, PepSMV and PepYMV each represent a different species, albeit very closely related, within the "PVY group".

Members of the phylogenetically defined "PVY group" are commonly found infecting species of Solanaceae, such as pepper, potato and tomato (Brunt *et al.,* 1996) which all have their center of origin or domestication in South America (Hawkes, 1990; Hancock, 1993; Smartt and Simmonds, 1995; Ochoa, 1999). Thus, it is likely that the potyviruses infecting these crops also originated in the same region. It has been suggested that at the center of origin of a crop, a great diversity might be expected not only in the total number of viruses affecting the crop, but also among the isolates of each virus (Jones, 1981). Our data on PVV and PTV support this presumption.

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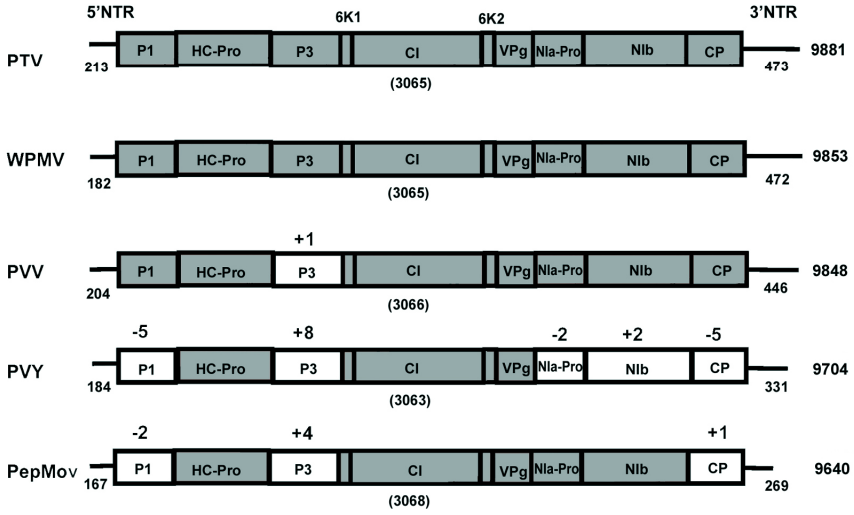

Figure 6. Schematic presentation (drawn to scale) of the genomic organization of the members of the "PVY group" from which the complete nucleotide sequences are available. The lengths of the viral genomes (nucleotides) are shown to the right. The polyprotein sizes (no. of amino acids) are presented below each polyprotein. The sizes of the 5´NTR and 3'NTR are positioned below the corresponding region. Proteins with identical sizes are shaded, whereas the size differences (number of amino acids) between viral proteins are indicated above the corresponding protein, as compared to PTV (isolate PPK13) and WPMV.

PTV, WPMV, PVV, PVY and PepMoV share striking similarities in their genome composition (Fig. 6). Several mature proteins have an identical size in these viruses, and other proteins differ only slightly in size. These slight differences have probably resulted from limited numbers of mutations, insertions and deletions within the viral genomes during the speciation of these viruses. Evolution is the process by which the genetic structure of a population changes by time, and in virus evolution, a strong factor contributing to such changes is host adaptation (Roossnick, 1997). Taken together, it seems likely that viruses belonging to the "PVY group" share a common ancestor, and subsequent speciation might have taken place in different hosts. Indeed, it has been suggested that also tobamoviruses occurring in brassicas, legumes and solanaceous plants have co-evolved with their respective hosts (Lartey *et al*., 1996; Gibbs *et al*., 1999).

Studies on the 6K2 protein of PVA (IV)

Subtle differences in specific viral proteins might result in an ability or inability of some isolates of the virus to infect a plant (Nicolas *et al*., 1997; Keller *et al*., 1998; Masuta *et al*., 1999; Borgstrom and Johansen, 2001). As mentioned previously, PVA strains B11 and M are both virulent in *Nicotiana tabacum* (Rajamäki *et al.,* 1998), although they differ in their ability to systemically infect *Nicandra physaloides*. Inoculation of PVA-B11 to *N. physaloides* results in the induction of severe vein chlorosis throughout the plant, whereas PVA-M is restricted to the inoculated leaves (Rajamäki and Valkonen, 1999). Experiments carried out with chimeric constructs between these two isolates revealed that a single aa substitution (Met5Val) in the 6K2 protein of PVA-M enhanced its virulence in *N. physaloides* (Rajamäki and Valkonen, 1999) indicating that the 6K2 protein is a determinant for systemic infection in *N. physaloides*, playing an important role in viral movement. Therefore, we were interested in further investigating the role of the 6K2 protein as a host-specific determinant.

To this end, deletion of various portions of the PVA-B11 6K2-encoding region, as well as insertions of six histidine residues (6xHis) into various positions of this protein were carried out (**IV**). This was accomplished by site-directed mutagenesis on the infectious cDNA of PVA-B11.

Deletions within the 6K2 protein of PVA resulted in an inability of the virus to systemically infect *N. tabacum* (**IV**). Insertions of a 6xHis into various positions of the 6K2 proteins also prevented systemic infection of PVA in *N. tabacum*, and also in *N. benthamiana* (**IV**). A spontaneous mutation which resulted in the substitution of Gly2 for Cys2 in an insertion construct that contained the 6xHis insert between Ser1 and Gly2 of 6K2 (p6K2A, Fig.1 in **IV**) enabled systemic infection in *N. benthamiana*. However, this mutation did not restore vascular movement in *N. tabacum* (**IV**). Subsequent passages of this mutant in *N. benthamiana* resulted in a deletion mutant, in which the last four His residues as well as the Cys2 and Thr3 of 6K2 were missing and which restored the 6K2 protein to its original size. This

mutant was able to systemically infect also *N. tabacum* (**IV**). These findings indicated that the role of the 6K2 in long-distance movement is host specific.

Occurrence of mutations is common during viral propagation (Shintaku *et al*., 1992; Schneider and Roossinck, 2000, Schneider and Roossinck, 2001; Liang *et al*., 2002). However, only those mutations that are advantageous for the virus will survive. Mutations that alter the conformation of a protein can have a great impact on the biological function(s) of the protein (Alberts *et al*., 1994; Ruas *et al*., 2002; Chen and Lee, 2003). Thus, it is conceivable that the mutation Gly2Cys in the N-terminus of the 6xHis-containing PVA 6K2 (**IV**) might have resulted in a structural modification that was compatible for long-distance movement in *N. benthamiana*, which on the other hand only partially restored the corresponding functions required for infection of *N. tabacum*. Furthermore, the spontaneous deletion that restored 6K2 to its original size (**IV**) might have restored the native structural conformation of this protein, thereby allowing the virus to infect both hosts. Hence, it is likely that the N-terminal part of the 6K2 is important in viral movement. Indeed, previously it has been suggested that conformational changes in viral proteins can affect the ability of viruses to systemically infect their hosts (Deom and He, 1997; Heaton *et al.,* 1991; Lin and Heaton, 1999).

As mentioned before, a single aa mutation in the PVA 6K2 protein enabled long-distance movement functions in *N. benthamiana,* but did not restore vascular movement in *N. tabacum* (**IV**). This is similar to a previous study showing that mutations in the movement protein of *Red clover necrotic mosaic virus* (RCNMV, genus *Dianthovirus*) abolish systemic infection in *N. edwarsonii*, but do not compromise systemic infection in *N. benthamiana* (Wang *et al.,* 1998). Additionally, replacement of the CP encoding region of TMV with the corresponding region of *Odontoglossum ringspot virus* (ORSV, genus *Tobamovirus*) compromises long-distance movement in *N. tabacum* but not *N. benthamiana* (Hilf and Dawson, 1992). Our findings (**IV**), combined with the previous studies, suggest that host factors involved in viral movement can differ between *Nicotiana* species.

The 6K2 protein exhibits viral movement functions in a host-dependent manner (Rajamäki and Valkonen, 1999; **IV**), similar to some other viral encoded proteins. However, the mechanism by which they regulate viral movement can be different. For example, the p19 and 2b proteins of *Tomato bushy stunt virus* (TBSV, genus *Tombusvirus*) and CMV, respectively, are host-specific long-distance movement determinants (Scholthof *et al*., 1995; Ding *et al*., 1995). In addition, both proteins are suppressors of RNA silencing (Beclin *et al*., 1998; Brigneti *et al*., 1998; Voinnet *et al*., 1999). Thus, it has been proposed that the TBSV p19 and the CMV 2b proteins can mediate viral movement by suppressing the host defense responses (Carrington, 1999; Hull *et al*., 2002). On the other hand, the TMV MP and the RCNMV 35 kDa protein, which also control viral movement in a host-dependent manner (Wang *et al.,* 1998, Waigmann *et al*., 2000), bind to single-stranded RNA molecules (Citovsky *et al*., 1990; Citovsky *et al*., 1992; Fujiwara *et al*., 1993) and can increase the size exclusion limit of plasmodesmata (Waigmann *et al*., 1994; Osman *et al*., 1992; Osman and Buck, 1992). These proteins are thought to

mediate viral movement in ribonucleoprotein (RNP) complexes with viral RNA, such that the RNP is transported from the membranous site of replication to the plasmodesmata for cell-to-cell translocation (Heinlein *et al*., 1998; Reichel and Beachy, 1998; Hull *et al*., 2002). It has been suggested that the functions of 6K2 in viral movement could be comparable to those of the TMV MP (Rajamäki and Valkonen, 1999). However, further studies that involve localization and RNA-binding properties of 6K2 are required to elucidate the mechanism by which this protein accomplishes its functions in viral movement.

Another interesting finding in our study was that although the mutation Gly2Cys in the PVA 6K2 enabled systemic infection in *N. benthamiana* it did not restore typical PVA symptoms (vein chlorosis and mild yellowing symptoms) in this host (Fig. 4 in **IV**). However, viral titers were similar to those observed in the wt PVA-B11 infected plants, as determined by DAS-ELISA (**IV**). Therefore, the lack of symptoms was not due a difference of virus accumulation, indicating that 6K2 has independent roles in viral movement and symptom induction. These results differ from a previous study in which the lack of symptoms conferred by the substitution of Lys307 for Glu307 in the HC-Pro of TVMV correlated with low viral accumulation in the infected tissue (Atreya *et al*., 1992). On the other hand, our results are similar to a previous study in which single aa substitutions (Asp157Asn and Asp199Gln) in the CP of *Turnip crinkle virus* (TCV, genus *Carmovirus*) resulted in the induction of milder symptoms (as compared to those induced by the wild type virus) without decreasing viral accumulation (Lin and Heaton, 1999). These mutations (Asp157Asn and Asp199Gln) also resulted in conformational changes of the CP (Lin and Heaton, 1999), which suggests that subtle changes in viral proteins (as those in our study **IV**) may disrupt virus-host interactions required for symptom expression.

A single mutation (Gly2Cys) in the 6K2 protein enabled systemic infection of a movement deficient construct in *N. benthamiana* but not *N. tabacum* (**IV**). However, during subsequent passages of this mutant in *N. benthamiana* new mutations occurred, resulting in a sequence deletion that allowed systemic infection in *N. tabacum* (**IV**). These findings exemplify that mutations arising during viral propagation represent a mechanism by which viruses can evolve and adapt to different hosts. As mentioned previously, the ability of some viruses to infect a given host in contrast to others is determined by the genetic composition of the viruses and their interactions with the hosts. Changes (such as mutations) within the viral-encoded proteins can result in incompatible interactions leading to disruption of essential processes such as viral replication, cell-to-cell and long distance movement. On the other hand, mutations can also be advantageous for a virus, enabling it to accomplish viral multiplication or movement functions in a non-host or avoid activation of a defense response in the host.

The 6K2 protein (**IV**), as well as other potyviral-encoded proteins (see Introduction), plays a role in the induction of symptoms. Whether or not the ability of some viral proteins to induce a phenotypic response is advantageous for a virus is questionable. Many plant viruses rely on vector transmission as a means of survival and spread. Various studies on insect behavior have revealed a strong

influence of physical plant properties (e.g. leaf color and shape) on the host-selection behavior (Prokopy *et al*., 1983; Harris and Rose, 1990; Roessingh and Städler, 1990; Bernays and Chapman, 1994). For example, the silverleaf whitefly (*Bemisia argentifolli*) shows a preference for feeding and oviposition on the light green leaves of *Hibiscus rosa-sinensis* L. cv. "Pink Versicolor" rather than on the darker leaves of cv. "Brilliant Red" (Liu and Stansly, 1998). In addition, oviposition of the carrot fly (*Psila rosae*) is higher on yellow and light green leaves than on dark green ones (Degen and Städler, 1997). Interestingly, the aphid *(Myzus persicae*) preferentially selects to feed on *Beet yellows virus* (genus *Closterovirus*)-infected sugarbeet (*Beta vulgaris*) plants showing vein clearing and yellowing symptoms [(similar to those observed in our study (**IV**)] rather than on healthy ones (Baker, 1960; Macias and Mink, 1969). Therefore, it is possible that viruses that induce symptoms could have an advantage over those that do not, increasing the attraction of the vector to their host and thereby favoring their dispersion rather than those that do not induce symptoms. However, viral-induced symptoms can also result in a severe disease, which might lead to death of the host plant. In this scenario, viruses that do not induce a phenotypic response in the host would have an advantage over those that do, allowing their hosts to survive and reproduce, thereby increasing the probability of the virus to survive.

The PVA 6K2 protein is membrane-bound (Merits *et al.,* 2002) and probably targets the replication complexes to the sites of viral replication in a way similar to the 6K2 protein of TEV (Restrepo-Hartwig and Carrington, 1994; Schaad *et al.,* 1997). Deletion of the 6K2-encoding region of PVA as well as insertions in the 6K2 of PVA and TEV inhibit viral replication, probably by disturbing the interactions between the 6K2 and cellular membranes required for a successful infection (Restrepo-Hartwig, 1994; Kekarainen *et al*., 2002; Merits *et al*., 2002). On the other hand, mutations within the CI/6K2 and 6K2/VPg cleavage sites of PVA affect polyprotein processing but do not inhibit viral replication (Merits *et al*., 2002). However, these mutations drastically reduce the infectivity of PVA in *N. tabacum* protoplast (Merits *et al*., 2002) and abolish systemic infection of PVA and TEV (Restrepo-Hartwig, 1994; Merits *et al*., 2002). Whether the lack of systemic infection of the deletion mutants and most of the insertion constructs (**IV**) were due to a deficiency in replication or cell-to-cell movement remains to be resolved. Further studies on replication at the protoplast level will be required to address this issue.

Conclusions

- Based on the RNA sequences and genome structures, PTV, PVV and WPMV are typical members of the genus *Potyvirus*. In addition, comparisons of the polyprotein aa sequences as well as the difference in the serological and biological properties of PTV, PVV and WPMV confirm their status as independent species of the genus *Potyvirus*.

- South American PVV isolates show a greater genetic variability than the European isolates of PVV. European and South American PVV isolates belong to two different strain groups based on the differential HR in the potato cultivar "Pentland Ivory" carrying the *Nv* gene. Division to the two strain groups is consistent with the grouping based on the phylogenetic analysis of the coat protein (CP) gene.

- The PTV isolates analyzed in this study belong to more than one genetic strain group. As in the case of PVV, a higher genetic resolution was obtained when the P1 protein sequence was analyzed in addition to the CP.

- Among Solanaceae-infecting potyviruses from which the complete nucleotide sequences are available, PTV, PVV and WPMV are the most closely related viruses. With PepMoV and PVY they form a group that is genetically distinct from other potyviruses. These viruses seem to share a common ancestor and have co-evolved with their respective hosts.

- The 6K2 protein of PVA is a host-specific determinant for long-distance movement in *N. tabacum* and *N. benthamiana*. In addition, the roles of 6K2 in viral movement and symptom induction can be independent in *N. benthamiana*. It seems likely that the N-terminal portion of this protein is important for these two functions.

- Spontaneous mutations that occur during virus propagation can result in the ability of viruses to systemically infect a non-host. This mechanism is likely to have played a crucial role in the evolution and speciation of plant viruses.

Future perspectives

- Currently, most of the Solanaceae-infecting potyviruses characterized at the molecular level have been isolated from crop species with economic importance. Little information is available, with the exception of WPMV, on potyvirus occurring in wild species of the Solanaceae. Further studies on the occurrence of potyviruses in wild species of the Solanaceae family as well as molecular characterization of these viruses would increase our knowledge on the variability and evolution of this group of viruses.

- Construction of infectious cDNA clones of PVV, PTV and WPMV would greatly advance the studied on replication and movement of these viruses. Mutational analysis of the infectious cDNA clones as well as chimeric constructs between these viruses could be used to map genomic regions involved in virus-host interactions in these viruses.

- Studies on the role of the 6K2 protein in viral movement should be continued. Localization studies as well as studies on the biochemical properties (such as RNA binding, phosphorylation and three-dimensional crystallography) of the 6K2 would provide further insights on the roles of this protein in viral movement.

- Identification of host factors involved in viral movement would be of great value to understand the process by which viruses accomplish intra- and inter-cellular movement.

- Currently, only limited numbers of studies have focused on the roles of viral proteins in the induction of symptoms. Studies on the mechanisms by which potyviral proteins (such as the 6K2) can induce a phenotypic response in their hosts would strengthen the knowledge on how this process is mediated.

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